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Full Length Research Paper

Functional characteristics of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* from ikii, a Kenyan traditional fermented maize porridge

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Fermented foods have been associated with probiotics: microorganisms that are useful to the host. The potential probiotic characteristics of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* strains isolated from *ikii*, a traditional fermented maize porridge, were studied and with regard to acid/bile tolerance, antimicrobial activity, utilisation of fructooligosaccharide, production of exopolysaccharides and safety attributes. Out of 19 strains of *L. plantarum* and one *L. rhamnosus*, 18 strains of *L. plantarum* were able to tolerate pH 2 for a period of 3 h. All the *L. plantarum* and *L. rhamnosus* strains assayed tolerated pH 2.5 for a period of 3 h, population counts remaining at levels of between 5 to7 log cfu/ml. Following 3 h exposure to pH 2, 18 *L. plantarum* strains were able to tolerate and grow to levels between 5 and 6 log cfu/ml in MRS supplemented with 0.3% bile salts within a period of 24 to 48 h. Sixteen (16) strains of *L. plantarum* exposed to pH 2.5, were able to grow to levels of 5 and 7 log cfu/ml in MRS broth supplemented with 0.3% bile within a period of 24 to 48 h. The assayed strains showed antimicrobial activity against *E. faecalis, S. aureus* and *E. coli*. Two (2) strains of *L. plantarum* were able to utilise a fructooligosaccharide. All 20 strains were able to produce exopolysaccharides. None of the assayed strains showed haemolytic and gelatinase activity. It is therefore possible that *L. plantarum* involved in production of *ikii* do have potential probiotic attributes.

Key words: Probiotics, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, acid/bile tolerance, antimicrobial activity, exoplysaccharides, fructooligosacharides.

INTRODUCTION

Fermentation is one of the oldest technologies used for food preservation. The fermentation process results in the production of acids and probable bacteriocins that prevent growth of microorganisms hence increasing the shelf life of fermented products (Chen and Hoover, 2003; Corsetti, 2004). This is a very valuable attribute especially in rural areas where advanced food preservation technologies such as refrigeration are not affordable, also people have began to appreciate more of naturally preserved than chemically preserved foods (Rolle and Satin, 2000).

Fermented foods are associated with 'good bacteria' referred to as probiotics (Patricia et al., 2002; Helland et al., 2004). Probiotics, as defined in a FAO/WHO (2002) report, are live 'microorganisms which when administered in adequate amounts confer a health benefit on the host'. Probiotics are beneficial bacteria in that they favourably alter the intestinal microflora balance such as reconstruction of normal intestinal microflora after disorders caused by diarrhoea, antibiotic therapy and radiotherapy), inhibit the growth of harmful bacterial, promote good digestion, boost immune function and increase resistance to infection (Patricia et al., 2002; Helland et al., 2004). Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients (Grajek et al., 2005; Parvez et al., 2006). People with flourishing intestinal colonies of beneficial bacteria are better equipped to

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fight the growth of disease causing bacteria (Holzapfel and Schillinger, 2001; Helland et al., 2004). It is imperative therefore that research and technology is now focusing a lot of attention on fermentation technologies and their products with an aim of tapping into the possible associated health benefits.

Ikii is a fermented porridge produced by people of the Kamba community mainly found in the eastern province of Kenya. The product is as a result of fermentation of dry maize grits (Kalui et al., 2008). The porridge thus produced can store for as long as a week under ambient temperatures (20 - 30°C) without spoiling. The product is a favorite amongst children, breastfeeding mothers and the aged. Despite ikii being a popular traditional product, the microorganisms involved in its production have not been assessed to determine their potential for probiotic attributes as well as their ability to produce bacteriocins and other inhibitory substances. There is therefore no available information that can be utilized to explore and exploit this traditional technology with an aim of producing a consistent, safe, long storing and health value added product. The purpose of this study was to characterize *L. plantarum* and *L. rhamnosus* strains isolated from ikii for potential probiotic attributes and the ability to produce antimicrobial substances.

MATERIALS AND METHODS

Samples

20 *L. plantarum* and *L. rhamnosus* strains characterized for potential probiotic attributes in this study were isolates obtained from ikii as described earlier (Kalui et al., 2008).

Chemicals

Chemicals and reagents were purchased from Sigma, Fischer Scientific and BDH laboratory supplies. Microbial culture media were purchased from Oxoid. All the reagents were of analytical grade.

Safety attributes

The safety attributes studied were gelatinase and haemolysis activities.

Gelatinase activity

Gelatinase activity was investigated as described by Harrigan and McCance (1990). A 16 h old culture was streaked into nutrient gelatin agar (Oxoid). The plates were incubated anaerobically in anaerobic jars containing wet sachets (Merck) for 48 h at 37°C after which they were flooded with $HgCl_2$ solution (15% $HgCl_2$ in 20% v/v HCl) and observed for clear zones surrounding colonies (positive reaction for gelatin hydrolysis).

Haemolysis activity

Hemolytic activity was investigated as described by Gerhardt et al. (1981). A 16 h old culture broth was streaked into sterile blood agar. The blood agar was prepared by adding 7% oxblood, that had been preserved in ethylenediaminetetraacetic acid (EDTA), into sterile blood agar base at 45°C. Plates were incubated anaerobically at 37°C for 48 h after which they were observed for β and ά haemolysis indicated by a clear zone around colonies with a greenish colour for β-haemolysis but none for ά -haemolysis.

Acid tolerance

Acid tolerance was determined according to Lei and Jakobsen (2004) and Mishra and Prasad (2005). Test strain cultures (*L. plantarum* and *L. rhamnosus* strains) were grown for 16 h in MRS broth at 37°C. An aliquot of 0.1 ml of the 16 h old culture was inoculated into MRS broth whose pH had been adjusted to 2 and 2.5 using 1 N HCL. Samples were drawn after 0, 1, 2 and 3 h and immediately diluted 10 fold in phosphate buffer pH 7 to eliminate medium acidity. Decimal dilutions of samples were made using maximum recovery diluent (Oxoid) and 0.1 ml taken from 10^{-5}, 10^{-6} and 10^{-7} dilutions pour plated, in duplicates, in 20 ml of MRS agar. Plates were incubated at 37°C for 48 h anaerobically using anaerobic jars containing wet anaerucult sachets (Merck). Viable counts were determined by counting number of colonies from plates containing 10 to 100 colonies and logarithmic colony forming units per millilitre (log cfu/ml) determined from the average.

Bile tolerance

Acid and bile tolerances were determined according to modified methods of Lei and Jakobsen (2004) and Mishra and Prasad (2005. Following 3 h of exposure to pH 2 and 2.5, 1 ml of the acid stressed cultures were diluted in phosphate buffer at pH 7 to eliminate effects of the low pH medium acidity. Aliquots of 0.1 ml of each of the acid stressed neutralized cultures was thereafter transferred into 9 ml of MRS broth supplemented with 0.3% bile salts (Sigma) and incubated for 48 h at 37°C. Viable cell counts of acid stressed cultures inoculated in MRS-bile broth and sampled at 0, 3, 24 and 48 h were determined using pour plate method in MRS agar and incubated at 37°C in anaerobic jars containing wet anaerocult sachets (Merck).

A comparison of growth characteristics under bile and non-bile conditions was determined for one of the *L. plantarum* strains (code 63) showing tolerance to pH 2 as well as pH 2.5 and growth in 0.3% bile supplemented MRS broth. A 0.1 ml of 16 h old culture of *L. plantarum* 63 strain was acid stressed for 3 h and inoculated into MRS supplemented with 0.3% bile and MRS without bile.

Absorbance of the culture broths was determined every 3 h for 12 h using DR Lange CADAS 100 spectrophotometer at 600 nm. Growth curves of absorbance against time were then plotted for each of the growth conditions.

Antimicrobial activity

Antimicrobial activity was checked by using the agar-spot test (Ivanova et al., 2000). Isolates were screened for production of antimicrobial against gram positive strain, *Staphylococcus aureus* ATTC 25923 as the indicator microorganism. Isolates showing an inhibition zone were further characterized for antimicrobial activity against *Enterococcus faecalis* NCTC 775, *S. aureus* NCTC 6571 and *Escherichia coli* NCTC 10418. Strains showing antimicrobial characteristics were further characterized for production of bacteriocins.

Bacteriocin bioassay

Bacteriocin activity was detected according to Lei and Jakobsen

(2004). Test isolates were grown anaerobically in MRS broth (to prevent formation of H_2O_2) at 37°C for 24 h. Bacterial cells were removed by centrifugation (4193 x g, 4°C for 20 min) to obtain a cell free supernatant. The supernatant was neutralized using 1 N NaOH to exclude antimicrobial effect of organic acid, then filtered through a 0.2 um pore size filter. Nutrient agar seeded with overnight cultures of the indicator strains of *E. faecalis* NCTC 775, *E. coli* NCTC 10418 and *S. aureus* NCTC 6571, was pour plated and 10 mm wells made into the solidified agar. The wells were sealed at the bottom with sterile non-seeded agar. About 100 ul aliquots of sterile neutralized supernatant were placed into the agar wells in duplicates for each test isolate. The plates were kept at 4°C for 2 h to allow for diffusion of antimicrobial substances and then incubated for 48 h at 37°C.

For control, wells were made, in duplicates, for each of the test isolates into which the following combination of 100 ul of test solutions were added, sterile uninoculated MRS broth, non-neutralised supernatant, neutralised supernatant that had been incubated for 1 h at 37°C with pepsin, neutralised supernatant that had been incubated for an hour with α-amylase, neutralised supernatant that had been incubated at 37°C with lipase. The enzymes were prepared in potassium phosphate buffer pH 7 at a concentration of 1 mg/ml. The plates were kept at 4°C for 2 h followed by incubation at 37°C for 48 h then observed for zone of inhibition.

Following the method of Torkar and Matijasic (2003), non-neutralized supernatant was assayed for sensitivity of antimicrobial substance to proteolytic and non-proteolytic enzymes. This was done to eliminate doubt as to the possibility of production of bacteriocins by assayed strains isolated from ikii. 2 wells were made adjacent to each other in a seeded nutrient agar. For this assay, *E. faecalis* (NCTC775) was used as the indicator strain. Non-neutralized supernatant was put into one well and enzyme put into the adjacent well. As control, phosphate buffer, pH 7, used for making enzyme solutions was used in the place of non-neutralized supernatant. After incubation at 37°C for 48 h, plates were observed for a 'dimple' of growth/reduced inhibition zone on side of well containing supernatant and adjacent to well containing enzyme.

Exopolysaccharide production

Exopolysaccharide (EPS) production was determined according to the method of Savadogo et al. (2004). Isolates showing a slimy growth in MRS agar were selected for this test. Isolates were cultured in MRS broth and incubated aerobically at 37°C for 24 h. The cultured broth was treated with 17% v/v of 80% trichloroacetic acid (TCA) to precipitate proteins in the broth and centrifuged at 4193 x g for 20 min after which the supernatant was separated from the cells.

To obtain EPS, 10 ml of cold ethanol was added to 5 ml of supernatant and stored at 4°C overnight. The mixture was centrifuged at 4193 x g for 20 min at 4°C to collect the precipitated material. The precipitate was resuspended in 5 ml of distilled water, 10 ml of cold ethanol added, centrifuged and supernatant decanted out. The precipitate pellets were then dried at 100°C in a hot air oven. Non-cultured sterile MRS broth was used as control and treated similarly to the cultured MRS broth.

Total carbohydrates

Total carbohydrates of obtained exopolysaccharides were determined according to the phenol sulphuric acid method (Dubois et al., 1956). A portion of 1 ml of 5% phenol in distilled water was added to 1 g of the dried precipitate followed by addition of a fast running 5 ml of concentrated sulphuric acid to ensure immediate mixing. The solution was allowed to stand for 10 min, shaken and incubated at 30°C for 20 min and absorbance determined at 490 nm. Distilled water was used as control and treated similarly to the EPS containing solutions. Values obtained were correlated to those of a glucose solution standard with a linearity of 0.99 and results expressed as glucose equivalent mg/ml.

Carbohydrate monosaccharide composition

Monosaccharide composition of the harvested EPS was determined according to Savadogo et al. (2004). The EPS obtained from culture broth were hydrolysed into their constituent sugar monomers by adding 1 ml of 1 M H_2SO4 into 1 ml of EPS solution and incubating the mixture for 2 h at 100°C. The solution was then neutralised using 4 N NaOH and phenolphthalein as indicator. The sugar monomer composition was determined using HPLC (Shimadzu) with an RI detector, 75:25 acetyl nitrile mobile phase, NH2P5E column and a temperature of 30°C. A standard solution composed of known monomer sugars and in known quantities was used to determine identity of EPS monomer composition of sugars. The peak areas obtained were used to calculate the estimated percent composition of the monomers.

Fructooligosaccharide utilization

To modified MRS basal agar medium (MRS without carbohydrate and lab lemco), 2% of inulin was added to form an MRS-FOS agar medium (Kaplan and Hatkins, 2000), autoclaved and filter sterilised FOS was added to the liquid agar at 45°C followed by pour plating. The basal medium contained 0.05% L-cysteine (BDH), 1.5% agar (Oxoid), and 30 mg of bromocresol purple (BDH) as indictor.

Isolates that had been grown for 16 h in MRS broth were streaked into the MRS-FOS agar plates followed by anaerobic incubation in anaerobic jars containing anaerocult sachets (Merck) at 37°C for 48 h. The plates were then observed for a yellow hallo around the colonies - a positive indication of acid production from FOS fermentation. As control, 16 h old culture of isolated strains were streaked into MRS agar containing 2% glucose and 2% FOS.

In addition, following standard procedures for sugar fermentation profile tests, strain isolates were harvested from 16 h old culture broths by centrifugation at 4193 x g for 20 min at 4°C, washed with modified MRS broth (MRS broth with no carbohydrate and lab lemco) and inoculated into modified MRS broth containing 1% FOS and andrade's indicator. MRS broth containing glucose as carbon source was used as control. Mineral oil was added to create anaerobic conditions and the tubes were incubated at 37°C for 5 days and observed daily for red colour. The pH and OD (600 nm) for MRS-FOS as well as MRS-glucose broths were determined to compare the effect on growth by and ability to utilize the 2 types of carbon sources.

RESULTS AND DISCUSSION

Haemolysis and gelatinase activity

Safety is one of the recommended attributes in the FAO/WHO (2002) guidelines on evaluation for probitics. Haemolysis activity would break down the epithelial layer while gelatinase activity would derange the mucoid lining interfering with the normal functioning of these very important linings across which many physiological substances are exchanged and would cause pathways for infections. All the 20 assayed strains showed no positive haemolysis and gelatinase activity. All strains inoculated

Table 1. Survival (log_{10} cfu/ml) of *L. plantarum* and *L. rhamnosus* strains isolated from ikii under pH 2.0 for 3 h in MRS broth.

Strain code	0 h	1 h	2 h	3 h
L. plantarum 6	7.82	7.58	7.41	7.52
L. plantarum 8	8.32	7.36	7.25	7.10
L. rhamnosus 19	7.29	6.99	4.30	4.11
L. plantarum 20	7.10	6.49	6.48	6.52
L. plantarum 43	7.37	6.64	4.30	3.97
L. plantarum 63	7.03	7.21	7.05	7.13
L. plantarum 89	5.48	6.59	6.29	6.31
L. plantarum 101	7.13	7.18	7.03	6.60
L. plantarum 109	7.22	7.46	6.96	7.89
L. plantarum 155	7.16	7.23	6.98	7.01
L. plantarum 174	7.19	6.08	6.33	5.20
L. plantarum 182	7.35	7.74	7.26	6.59
L. plantarum 184	7.18	7.14	7.08	7.56
L. plantarum 185	7.10	6.29	6.79	6.33
L. plantarum 186	7.11	7.81	6.47	5.68
L. plantarum 187	7.42	7.40	7.11	6.77
L. plantarum 189	7.20	7.18	6.20	5.47
L. plantarum 190	7.12	7.00	6.63	6.54
L. plantarum 169	7.53	5.74	5.60	5.67
L. plantarum 183	7.06	6.53	6.17	7.58

into gelatine agar produced a mucoid substance around the colonies. This may be an indication that the strains may be able to produce a mucoid substance that enhances the mucosal lining of the gastrointestinal tract (GIT) hence adding on to the benefits of the mucoid layer of the GIT. *L. plantarum* and *L. rhamnosus* isolated from ikii may therefore be said to be safe with regard to haemolysis and gelatinase activity. To classify the isolates as safe, there may be a need to do further analysis such as determining the possibility of their production of biogenic amines.

Acid tolerance

Of the 20 *Lactobacillus* strain isolates from ikii assayed for acid tolerance, 18 (all *L. plantarum* strains) were viable to a level of between 5 and 7 log cfu/ml after 3 h exposure to pH 2 (Table 1). 2 strains *L. rhamnosus* code 19 and *L. plantarum* code 43 showed a count of 4 and 3 log cfu/ml, respectively after 3 h of exposure to the same pH. The viable counts for both these strains (*L. rhamnosus* 19 and *L. plantarum* 43) was at a level of log 6 after exposure to pH 2 for 1 h, but viable counts reduced thereafter to reach levels of 4 and 3 log cfu/ml, respectively. These results indicate that the strains were able to tolerate and survive low pH within the first 1 h but tolerance declined with further exposure to low pH. There was no observed increase in counts for all the isolate strains, even for the 18 *L. plantarum* strains that remained stable for 3 h in low pH 2, counts remaining within the same log cycle or increasing/decreasing by only one log cycle within the 3 h period of exposure to pH 2. This indicates that the 18 *L. plantarum* strains were stable and could tolerate a low pH 2 but they were not able to grow. Various other studies have reported no growth in strains exposed to a low pH within the period of exposure (Lei and Jakobsen, 2004; Mishra and Prasad, 2005; Liong and Shah, 2005). Liong and Shah (2005) while working with strains of *Lactobacillus acidophilus* and *Lactobacillus casei* observed that the viability of acid sensitive strains of *L. casei* decreased slowly during the first hour of exposure to low pH but this was followed by a rapid decrease at the second hour, an observation similar to the findings related to pH 2.0 sensitive strains in this study.

All 20 strains assayed were within a range of 5 and 7 log counts throughout the period of exposure to pH 2.5 for 3 h, some increasing or decreasing by one log cycle (Table 2). 2 isolated strains, *L. rhamnosus* code 19 and *L. plantarum* code 43, not tolerant to pH 2.0 (Table 1), were stable in pH 2.5 (Table 2) showing viable counts of 6 log cfu/ml by the end of 3 h exposure to pH 2.5 as compared to viable counts of 4 and 3 log cfu/ml, respectively on exposure to pH 2. For all isolated strains, there was no observed change in viable counts indicating that these isolates were able to tolerate and survive in a low pH of 2.5 but were not able to grow in this low pH. Lei and Jakobsen (2004) reported stability but non-growth of strains of strain isolates from koko (a millet fermented product) exposed to pH 2.5 for a period of 4 h, isolates surviving at a level of 5 log cfu/ml

The results (Table 1) indicate that those strains not able to tolerate a pH of 2 were able to tolerate a higher pH of 2.5 (Table 2). This shows that the best pH to select for strains with probiotic potential is pH 2 since it is at this level and not pH 2.5 that discrimination according to pH sensitivity could be achieved. 10% of isolates studied could not survive pH 2. One strain *L. plantarum* code 43 could not tolerate environmental conditions of pH 2. This shows that tolerance to acid and other gastrointestinal stresses is strain and not species specific. Similar results have been reported (Morelli, 2000; Huang and Adams, 2004). For strains to survive and colonise the gastrointestinal tract, microorganisms should express tolerance to acid and bile salts (Gibson, 1998). It has been suggested that food intake could protect bacteria during gastric passage (Morelli, 2000; Dunne et al., 2001; Charalampopoulos et al., 2002). The pH, physical and chemical characteristics of a food carrier in which potential probiotics are relayed into the gut may have a buffering effect and significantly influence survival of the microorganisms (Patel et al., 2004). There is therefore a need for consideration of inclusion of foods during in vitro characterisations for probiotic potential.

Bile tolerance

After exposure to pH 2.0 and 2.5 at 37°C for 3 h, *L. plan-*

Table 2. Survival (log $_{10}$ cfu/ml) of *L. plantarum* and *L. rhamnosus* strains isolated from ikii under pH 2.5 for 3 h in MRS broth.

Strain code	0 h	1 h	2 h	3 h
L. plantarum 6	7.02	6.90	6.67	6.89
L. plantarum 8	8.18	7.61	7.20	6.93
L. rhamnosus 19	7.32	7.44	7.22	7.26
L. plantarum 20	7.09	7.24	7.24	6.95
L. plantarum 43	4.36	6.37	6.75	6.10
L. plantarum 63	7.29	7.38	7.21	7.20
L. plantarum 89	5.30	6.43	5.65	6.63
L. plantarum 101	7.00	7.02	7.01	6.99
L. plantarum 109	7.41	7.33	7.23	7.21
L. plantarum 155	7.34	7.38	7.29	6.96
L. plantarum 174	7.20	7.22	7.11	6.92
L. plantarum 182	7.23	7.30	7.37	7.29
L. plantarum 184	7.84	7.04	6.89	6.80
L. plantarum 185	7.20	7.08	7.18	7.92
L. plantarum 186	7.21	6.07	7.09	7.18
L. plantarum 187	7.32	6.47	6.39	6.47
L. plantarum 189	6.56	7.50	7.40	7.29
L. plantarum 190	7.63	7.61	7.69	7.51
L. plantarum 169	7.41	7.42	7.30	7.49
L. plantarum 183	7.38	7.21	6.92	7.20

Table 3. Survival (log $_{10}$ cfu/ml) of *L. plantarum* and *L. rhamnosus* strains isolated from *ikii* in MRS broth supplemented with 0.3% bile salts, following a 3 h exposure to pH 2.

Strain code	0 h	3 h	24 h	48 h
L. plantarum 6	6.17	1.60	1.30	2.85
L. plantarum 8	3.90	1.65	2.37	4.45
L. rhamnosus 19	3.85	1.35	2.51	3.85
L. plantarum 20	4.82	3.48	2.99	5.45
L. plantarum 43	2.84	2.00	4.49	5.44
L. plantarum 63	3.60	0.955	5.20	6.47
L. plantarum 89	3.70	0.15	0.85	Not detected
L. plantarum 101	4.51	0.86	0.40	Not detected
L. plantarum 109	4.00	0.62	5.36	6.03
L. plantarum 155	5.38	0.93	0.60	2.04
L. plantarum 169	5.58	1.85	1.90	6.04
L. plantarum 174	6.08	3.20	4.78	5.83
L. plantarum 182	5.81	2.60	2.58	2.60
L. plantarum 183	5.92	2.60	3.89	6.15
L. plantarum 184	6.08	3.20	3.72	6.58
L. plantarum 185	6.06	0.30	4.08	6.58
L. plantarum 186	6.07	2.62	3.53	6.26
L. plantarum 187	6.05	4.02	5.20	6.53
L. plantarum 189	5.86	3.00	3.83	6.09
L. plantarum 190	6.01	0.93	2.88	6.41

tarum and *L. rhamnosus* strain isolates from ikii were assayed for bile tolerance (Tables 3 and 4). 4 strains (*L. plantarum* 63, *L. plantarum* 109, *L. plantarum* 174, and *L. plantarum* 187) of the assayed isolates showed a viable count of 5 log cfu/ml after 24 h in MRS broth supplemented with 0.3% bile (Table 3) indicating that these strains were able to grow in the bile supplemented conditions. Viable counts increased to reach levels of 5 and 6 log cfu/ml for 13 isolates (65%) of the assayed strains after a 48 h period of incubation in MRS supplemented with 0.3% bile. Of these 13 isolates, *L. plantarum* coded 63, 109, 174, and 185, increased in viable counts from as low a viable count of 0 log cfu/ml after 3 h in MRS-bile broth reaching levels of 5 and 6 log cfu/ml after 24 and 48 h, respectively. 7 strain isolates (*L. plantarum* code 20, 169, 183, 184, 186, 189 and 190) showed a significant increase in viable counts between the 24 h and 48 h period of exposure to MRS-bile broth, increase ranging from 1 at 24 h and 6 log cfu/ml at 48 h. 2 of the isolate strains (*L. plantarum* code 43 and 187) showed a steady increase in viable counts ranging from 2 to 6 log cfu/ml at 3 and 48 h, respectively. These results show that these strains were able to grow in broth supplemented with 0.3% bile. 2 strains of *L. plantarum* coded 89 and 101 decreased to 0 log cfu/ml counts after 3 h exposure to 0.3% bile. These results show that the 2 *L. plantarum* strains were not able to tolerate and grow in conditions containing bile. Strain isolate *L. plantarum* code 187 decreased by only 2 log cycles within the first 3 h of exposure to 0.3% bile did not show further decrease and increased steadily within the period of exposure, reaching the initial level of 6 log cfu/ml at 48 h. *L. rhamnosus* code 19 did not show tolerance to 0.3% bile after exposure to pH 2.0. Initial viable counts immediately on exposure reached a level of 3 log cfu/ml reducing further to a count of1 log cfu/ml and increasing to reach a final level of 3 by the end of 48 h.

On exposure to 0.3% bile salts of isolates from pH 2.5, 19 of the 20 isolates showed a viable log cfu/ml count level of 5 and 6 (Table 4). Only 1 isolate, code 109, showed a less viable count of 3 log cfu/ml. After 3 h exposure to 0.3% bile, only 1 isolate, code 63 had a count of 6 log cfu/ml, all the other 19 isolates had a viable count of between 1 and 3 log cfu/ml as observed for isolates from pH 2.0 and exposed to similar conditions of 0.3% bile. 16 of the isolates showed count of 5 and 7 log cfu/ml after 24 h exposure to 0.3% bile salts. 3 of the 20 strains (6, 89 and 155) did not show a significant increase in cfu/ml counts at 3, 24 and 48 h. These same strains did not show recovery and growth either after exposure to pH 2.0 and 0.3% bile salts for 48 h, counts remaining at 10^2 for strains 6 and 155 while 89 was not detected at 48 h. Strain 19 showed recovery and growth between 3 and 24 h but declined again by 1 log cycle and was detected at level 4 log cfu/ml at 48 h. The same isolate, which is the only strain of *L. rhamnosus* isolated from ikii, did not show tolerance to pH 2, levels of viable counts reaching 4 log cfu/ml by the end of the 3 h exposure to this low pH.

Table 4. Survival (log $_{10}$ cfu/ml) of *L. plantarum* and *L. rhamnosus* strains isolated from ikii in MRS broth supplemented with 0.3% bile salts, following a 3 h exposure to pH 2.5.

Strain code	0 h	3 h	24 h	48 h
L. plantarum 6	6.11	2.79	3.00	2.99
L. plantarum 8	5.91	3.94	6.22	6.10
L. rhamnosus 19	5.84	2.09	5.26	4.30
L. plantarum 20	5.81	3.42	7.04	6.92
L. plantarum 43	5.11	3.73	5.06	6.19
L. plantarum 63	6.23	6.32	5.33	6.68
L. plantarum 89	5.71	3.06	2.00	3.00
L. plantarum 101	5.96	0.85	5.44	6.91
L. plantarum 109	3.99	3.99	5.76	6.58
L. plantarum 155	5.93	0.95	2.50	3.04
L. plantarum 169	5.63	2.90	5.53	6.98
L. plantarum 174	6.07	3.58	5.64	5.43
L. plantarum 182	5.93	3.36	6.37	6.03
L. plantarum 183	5.62	3.48	6.33	5.78
L. plantarum 184	6.10	3.48	6.06	6.26
L. plantarum 185	6.11	2.95	5.85	6.03
L. plantarum 186	6.09	3.43	6.16	5.81
L. plantarum 187	6.49	3.61	6.11	6.36
L. plantarum 189	6.46	3.08	6.20	5.99
L. plantarum 190	6.17	3.72	6.19	5.61

Immediately on exposure to 0.3% bile, 60% of strains from pH 2 showed a viable count of 5 log cfu/ml (Table 3) compared to 95% from pH 2.5 (Table 4). All strains had a viable count of less than 5 log cfu/ml after 3 h of incubation in bile.

20% of the strains prior exposed to pH 2 and 70% prior exposed to pH 2.5 had a viable count of 3 log cfu/ml or more but less than 5 log cfu/ml after 3 h of incubation in 0.3% bile. 85% of strains from pH 2.5 showed an increase in viable counts at 24 h while 20% responded similarly for strains from pH 2.0. At 48 h, 65% of strain isolates prior exposed to pH 2 and 80% of strains prior exposed to pH 2.5 had attained a count of 5 log cfu/ml and more. These results indicate that more strains from pH 2.5 were able to tolerate, survive, recover faster and grow in 0.3% bile than those exposed to pH 2.0.

Comparison of results for strains exposed to pH 2 and 2.5 also emphasize that pH 2 may be the best for assaying for potential probiotics as it is more discriminative in terms of acid as well as bile tolerance. One isolate (code 63) from pH 2 that showed a remarkable recovery from no count at 3 h, reaching levels of 10^5 and 10^6 at 24 and 48 h, respectively. It also showed remarkable tolerance to 0.3% bile salts after exposure to pH 2.5, being the only isolate that had a count level of more than 5 log cfu/ml at 3 h of inoculation in MRS containing 0.3% bile salt. All strains that showed survival and growth in 0.3% bile salts after exposure to pH 2 were also able to survive and grow under similar conditions after exposure to pH 2.5 indicating that these strains could survive low pH and actually grow in bile salts reaching 5 log cfu/ml and more, which is a good indicator for probiotic potential.

One isolate, *L. plantarum* code 63, that had shown tolerance to low pH and 0.3% bile salts was assayed to compare for growth in MRS containing 0.3 % bile salts and MRS broth without any bile, for a period of 12 h, following exposure to pH 2 and 2.5 for a period of 3 h. Increase in OD was observed for all broth combinations (Figure 1). However, growth was highest in pure MRS especially for culture from pH 2.5. These results indicate that the strain was able to tolerate low pH of 2 and 2.5 and that after exposure to low pH, it could survive and grow in MRS with or without 0.3% bile salts, though growth was observed to be lower in MRS containing 0.3% bile salts than without the bile salts. This shows that *L. plantarum* code 63 is able to survive low pH created by gastric juice in stomach and is also able to survive and grow in conditions of bile within the duodenum. Increase in OD in inoculated MRS containing 0.3% bile salts was more or less the same for both cultures from pH 2 and 2.5 indicating that exposure to either pH did not have a differing effect on the strain. Observations of faster growth in MRS broth without bile salts as compared to that with bile salts have also been reported (Lei and Jacobsen, 2004; Mishra and Prasad, 2005).

Antimicrobial activity

Results for antimicrobial activity of *L. plantarum and L. rhamnosus* isolates from ikii were as shown in Table 5. The highest observed growth inhibition zone in this study was 26 mm (against *E.faecalis)*, while the least was 12 mm (against *E. coli)*. 4 of the isolates (*L. plantarum* codes 186, 187, 189, 190) did not show any inhibition against *E. coli*. One *L. plantarum* code 186 showed an inhibition zone of 15 mm against *S. aureus* but did not show inhibition against *E. faecalis* and *E. coli*. All isolates (except for code 186) showed the highest growth inhibition against *E. faecalis*, followed by S. *aureus* and least inhibition was against *E. coli*, with some of the strains showing no inhibition at all against *E. coli* (Table 5). During a previous study on ikii, the fermentation process was observed to suppress coliforms (Kalui et al., 2008). Observed growth inhibition in this study indicate that the assayed *Lactobacilli* produced antimicrobial products such as organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins that were able to inhibit growth of *E. faecalis, S. aureus* and *E. coli*, all of which are food contaminants and pathogens. Lactic acid bacteria have been reported to exert a strong antagonistic activity against food contaminating microorganisms (De Martinis et al., 2001; Ogunbanwo et al., 2003; Hernandez et al., 2004). The assayed *L. plantarum and L. rhamnosus* showed highest antagonism against *E. faecalis*, which is a lactic acid bacteria and least against *E. coli*, which is gram

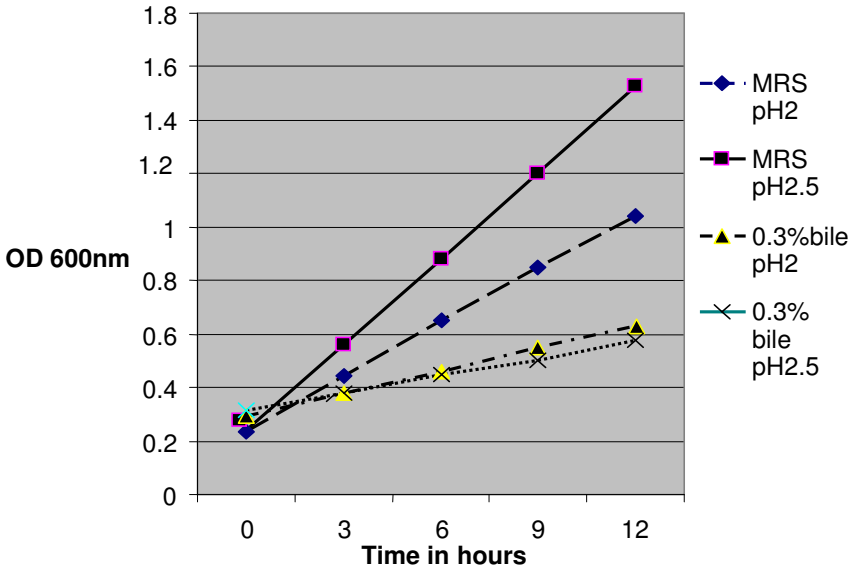

Figure 1. Changes in OD (600 nm) of isolate coded 63 (*L. plantarum*) after inoculation into MRS broth and MRS broth containing 0.3% bile salts following exposure to pH 2 and 2.5.

Table 5. Antimicrobial activity of homofermentative *Lactobacillus* isolates from ikii against *E. faecalis, S. aureus* and *E. coli.*

Lactobacillus strain	Inhibition zone (mm)		
	E. faecalis **(NCTC 775)**	***S. aureus*** **(NCTC 6571)**	***E. coli*** **(NCTC 10418)**
L. plantarum (6)	23	16	17
L. plantarum (8)	20	20	17
L. rhamnosus (19)	20	18	14
L. plantarum (20)	25	17	16
L. plantarum (43)	24	16	16
L. plantarum (63)	25	20	16
L. plantarum (89)	25	18	12
L. plantarum (101)	20	18	18
L. plantarum (109)	27	20	17
L. plantarum (155)	26	16	13
L. plantarum (169)	28	18	17
L. plantarum (174)	22	18	16
L. plantarum (182)	22	16	12
L. plantarum (183)	22	16	12
L. plantarum (184)	25	18	15
L. plantarum (185)	22	20	16
L. plantarum (186)	no inhibition	15	no inhibition
L. plantarum (187)	26	14	no inhibition
L. plantarum (189)	24	15	no inhibition
L. plantarum (190)	24	16	no inhibition

negative and a non-lactic acid bacteria. Some strains did not show any antagonism against *E. coli. A*nti-microbial activity is reported to be highest against closely related species (De Martinis et al., 2001; Ogunbanwo et al., 2003; Hernandez et al., 2004), and this phenomena was observed in this study.

Table 6. Effect of enzymes on antimicrobial compounds produced in the supernatant by Lactobacillus isolates from ikii.

Lactobacillus strain	Diameter of growth 'dimple' (mm) on side with enzyme	
	Pepsin	Amylase
L. plantarum (6)	none	none
L. plantarum (8)	none	none
L. rhamnosus (19)	3	none
L. plantarum (20)	4	none
L. plantarum (43)	3	none
L. plantarum (63)	6	none
L. plantarum (89)	4	none
L. plantarum (101)	none	none
L. plantarum (109)	none	none
L. plantarum (155)	none	none
L. plantarum (169)	4	none
L. plantarum (174)	none	none
L. plantarum (182)	4	none
L. plantarum (183)	4	none
L. plantarum (184)	4	none
L. plantarum (185)	3	none
L. plantarum (186)	3	none
L. plantarum (187)	4	none
L. plantarum (189)	none	none
L. plantarum (190)	4	none

Bacteriocin bioassay

None of the neutralized supernatants from *L. plantarum and L. rhamnosus* isolates showed inhibition against indicator strains *E. faecalis, S. aureus* and *E. coli*. This indicates that observed inhibition exhibited by non-neutralized supernatant, whose pH was an average of 3.9, may have been as a result of organic acids and that there may have been no other antimicrobial factors such as bacteriocins. However, with the aim of eliminating any doubt as to the possibility of production of bacteriocins by the assayed *Lactobacillus* isolates from ikii, the non-neutralized supernatant was assayed further for sensitivity of antimicrobial substance to proteolytic and non-proteolytic enzymes. Supernatants from 13 strain isolates showed a growth dimple on the side of wells containing pepsin, a proteolytic enzyme, highest growth being 6 mm in diameter, for supernatant from strain isolate *L. plantarum* 63 (Table 6). All other growth 'dimples' were either 3 or 4 mm in diameter. There were no observed growth 'dimples' on side of wells containing α-amylase and control (phosphate buffer Ph 7).

These results show that there was an antimicrobial substance produced by some of the strains whose antimicrobial effect was being destroyed in the presence of pepsin a proteolytic enzyme, but not α-amylase. The results indicate that the antimicrobial substance in supernatant was proteinicious and there is a possibility of it being a bacteriocin. It is therefore possible that some *L. plantarum* strains isolated from ikii are able to produce bacteriocins. The activity of these bacteriocins may be pH dependent such that they are only able to show antimicrobial activity at low pH. Activity of bacteriocins has been shown to be pH dependent. Mensah (1990) reported the presence of an antimicrobial substance other than organic acids produced by isolates from fermented maize dough that was optimally active at a pH of 3. It is possible that the bacteriocins got adsorbed onto the surface of cell membranes of bacteriocin producer cells and were therefore not released into the supernatant in amounts and concentrations capable of producing observable inhibition of indicator microorganisms. Further evidence of bacteriocin presence is that inhibition zones in this study were observed to have a clear-cut boundary, an attribute associated with bacteriocins; inhibition due to acid is reported to be diffuse (De Martinis et al., 2001). Observations in this study therefore indicate that there is a possibility of antimicrobial activity observed in this study being partly as a result of bacteriocins and this necessitates for a more detailed study to establish the possibility of the isolates from *ikii* having the ability to produce bacteriocins.

Exopolysaccharides

Results for exopolysaccharide production were as shown in Table 7. All the assayed strains produced exopolysac-

Table 7. Carbohydrates concentration and monomer composition of exopolysaccharides produced by *L. plantarum and L. rhamnosus* strain isolates from *ikii.*

Isolate	Exopolysaccharides (mg/l)	Monomer composition (%)
6	407.23	80.02% glucose, 19.98% fructose
8	407.05	29.8% glucose, 69.7% fructose, 0.5% others
19	379.11	84.6% glucose, 15..3 %fructose, 0.09 others
20	379.29	67.02% glucose, 11.20% fructose, 7.18% 14.other,58% other
43	419.94	71.11% glucose, 11.34% sucrose
63	425.13	91.8% glucose, 8% fructose,
89	373.74	98.6% glucose 0.23% other, 1.16 other
101	298.53	100% glucose
109	402.93	95.4% glucose, 4.6 % fructose
155	407.94	94.7% glucose, 4..9% fructose, 0.3% other
169	419.58	94.4% glucose, 2% fructose, 3.6% other
174	419.05	72..29% glucose, 27.71% fructose
182	390.04	99.7% glucose, 0.14 fructose, 0.18 other
183	373.03	82.9% glucose, 17% sucrose, 0.07 other
184	431.04	75% glucose, 25% fructose,
185	425.09	83.29% glucose, 16.71% fructose
186	412.06	80.31% glucose, 18.70 fructose
187	426.39	100% glucose
189	416.72	97.2% glucose, 2.4 fructose. 0.4% other
190	404.36	63.47% glucose, 26.71% fructose,

charides ranging from 298.53 mg/l (*L. plantarum* strain code 101) to 431 mg/l (*L. plantarum* strain code 184). Monomers comprising the polysaccharides were glucose and fructose as well as a few not identified in this study but in very low concentrations of less than 1%. Glucose was the dominant monomer except for strain *L. plantarum* 8 where fructose was the dominant monomer. Savadogo et al. (2004) reported exopolysaccharide production by isolates from Burkina Faso fermented milk samples and in concentrations ranging from 181 to 814 mg/l. Frengova et al. (2002) reported ranges of 10.40 to 460.27 mg/l by isolates from kefir grains. In both studies, glucose was reported as the dominant monomer. Other studies have reported the presence of fructose, galactose, rhamnose, mannose (Frengova et al., 2002; Savadogo et al., 2004; Tallon et al., 2003). Absence of these monomers in this study may have been as a result of glucose being the carbon source, in the quoted reports above lactose was used as the carbon source. The carbon source type may have had an influence on the biosynthesis pathway of the polysaccharides. De Vuyst and Degeest (1999) reported that the carbon source may have influence on the type and amount of exopolysaccharide produced.

Fructooligosaccharide utilization

Two strains of *L. plantarum* coded 101 and 109 tested positive for fructooligosaccharide utilization. Colour change in both cases was observed after 24 h and became eminent after 36 and 48 h for *L. plantarum* 109 and 101 respectively. Acid production (carbohydrate utilisation) was observed for all 20 strains when glucose was used as the carbohydrate source. These results show that *L. plantarum* strain codes 101 and 109 are able to utilize inulin (FOS) and produce acid (Table 8). The results further indicate that colour change observed when FOS was used as carbohydrate source was as a result of utilization of inulin since the strains that did not show colour change were able to do so when glucose was used as the carbon source (Table 8).

The OD (600 nm) increased from 0.228 and 0.235 to 1.5 and 1.9 for *L. plantarum* strain codes 101 and 109 culture broths, respectively; pH reduced from 6.8 (at start of experiment) to 4.4 and 4.2 for *L. plantarum* strain codes 101 and 109 respectively after 48 h for inulin as source of carbon (Table 8). Optical density (600 nm) for culture in glucose MRS broth was found to be approximately 1.9 just like that of FOS- MRS broth indicating that FOS is equally as good a substrate as glucose in supporting and maintaining growth.

The ability of strains to ferment oligosaccharides is an important attribute for probiotic potential (Kaplan and Hutkins, 2000; Bengmark, 2003; Desai et al., 2004). This is because the availability of carbohydrates that escape the digestion and absorption in the small intestine have a major influence on the microbiota that become established in the colon (Kaplan and Hutkins, 2000). These carbohydrates, therefore, have the potential of being pre-

Table 8. Utilisation and effect on growth of *L .plantarum* 101 and *L. plantarum* 109 strains isolated from ikii on FOS with inulin or glucose.

Strain code	OD (600 nm)		pH	
	Inulin	Glucose	Inulin	Glucose
101	1.5	1.9	4.4	3.9
109	1.9	1.9	4.2	3.9

biotics and could be selective for probiotic micro-organisms in the colon. The two *L. plantarum* strains from *ikii*, due to their ability to ferment inulin which is a FOS, may therefore be able to utilize prebiotics bringing about the positive attributes associated with probiotics inhabiting the colon.

Conclusion

In conclusion, this study has shown that some *L. plantarum* strains isolated from ikii have potential probiotic attributes. Some of the assayed strains showed antimicrobial activity which may have been beyond the effect of organic acids, and had bacteriocin attributes. Some assayed *L. plantarum* strains showed tolerance to low pH 2 and growth in 0.3% bile conditions hence are able to survive and grow in physiological conditions of the GIT. The assayed strains showed safety with regard to haemolytic and gelatinase activity, were able to produce EPS and some were able to utilise FOS, all of which are characteristics attributed to probiotics. Further studies need be carried out on *L. plantarum* strains from ikii to ascertain their further probiotic attributes and their actual physiological probiotic effects. Such studies could include but not limited to: response to simulated stomach duodenum passage, susceptibility to antibiotics, adhesion to human cell linings, hydrophobicity attributes, binding characteristics to human extra-cellular matrix, cholesterol reduction ability.

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