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Bile and Acid Tolerance of *Lactobacillus plantarum* KCA-1: A Potential Probiotical Agent

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Abstract: A previously characterized biosurfactant-producing *Lactobacillus plantarum* KCA-1 isolated from the vagina of a healthy premenopausal woman was used in this study. *Lactobacillus plantarum* KCA-1 was tested for survival in different concentrations (0, 0.05, 0. 1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0) of porcine and oxgall bile extract, incubated at 37°C for 48 h. Acid tolerance of the culture was studied by inoculating the organism (10^5CFU mL^{-1}) in de Man Rogas Sharpe (MRS) broth at different pH values adjusted with HCl and incubated at 37°C for 48 h. *Lactobacillus plantarum* strain KCA-1 that produced biosurfactant needed for preventing adhesion of pathogens was tolerant to oxgall bile. There was an increase of 5 log cycles from the initial bacterial inocula (1×10^5cfu mL^{-1}) to 1×10^{10}cfu mL^{-1} at 0.05 and 0.1% concentrations of oxgall bile and 4 log cycle increase (1×10^9cfu mL^{-1}) at 0.5% oxgall concentration. There was a consistent reduction of 1 log cycle of every 0.5% increase of the oxgall for tested concentrations (0.5 to 4.0%). In addition, *Lactobacillus plantarum* KCA-1 was tolerant at pH 2.5 for 48 h of incubation as there was 2.3 to 3.8 increase in the log cycles. There was a corresponding increase in the number of cells from 1×10^5 CFU to 1×10^9 CFU mL^{-1} at pH 4.5. The tested potential probiotic strain *Lactobacillus plantarum* KCA-1 exhibited a satisfactory degree of acid and bile tolerance. The findings suggests that the strain may be a promising candidate for use as a dietary adjunct, but more studies are needed.

Key words: *Lactobacillus plantarum*, probiotics, bile acids, tolerance

INTRODUCTION

As the use of probiotics, propelled by both basic and clinical research become more visible in the western world, Africa that is in dire need of the health benefits associated with it, is yet to wake up from slumber and embrace the concept. Probiotics is defined as live micro-organisms which when administered in adequate amounts, confer a health benefit on the host (Reid *et al*., 2003). Two genera, Lactobacillus and Bifidobacterium, have been selected as probiotic agents, both of which predominantly form part of the normal human intestinal microbiota.

It is now in the public domain of the enormous benefits of using probiotics for the prevention and treatment of various disorders. As far as reported clinical studies are available, coupled with large experimental and therapeutic evidence (Limdi *et al*., 2006), probiotics now do have a significant role in the prevention and treatment of various gastrointestinal maladies. In a review by

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Reid and Bruce (2006) substantial clinical evidence exist for the application of probiotics against urogenital tract infections and a recent study has shown that certain strain of lactobacilli can augment antimicrobial treatment for bacterial vaginosis (Anukam *et al*., 2006). When selecting lactic acid bacteria for use as probiotics, especially Lactobacillus and Bifidobacterium, considerations are always given for conditions that mimic the gastrointestinal tract. To provide health benefits, probiotics must overcome physical and chemical barriers such as acid and bile in the small intestine. These bile acids are products of cholesterol metabolism in the liver and they play role in the digestive process. Some strains of *Lactobacillus acidophilus* were found to secrete bile salt hydrolase, which catalyzes the hydrolysis of glycine or taurine-conjugated bile salts into amino acid residues and free bile salts (Corzo and Gilliland, 1999). However in Gram-positive bacteria, the toxicity pattern of bile acids resembles that of detergents such as sodium dodecylsulfate (Flahaut *et al*., 1996), therefore it becomes necessary to test the bile acid tolerance of potential lactobacilli to be used as probiotics. The aim of the this study is to determine bile and acid tolerance of *Lactobacillus plantarum* KCA-1

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

Lactobacillus plantarum KCA-1 a previously characterized (Anukam *et al*., 2005) vaginal isolate from a healthy premenopausal woman was employed in this study. Isolation and molecular characterization was done by using Polymerase Chains Reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) at the Canadian Research and Development Centre for Probiotics (CRDC) Gene sequencing was done in the summer of 2004 at Robart's sequencing facility, University of Western Ontario. The isolate was prepared from the stock culture preserved in glycerol stored at -80°C and inoculated in MRS broth/agar. The preparation was incubated anaerobically using BBL™ GasPack system (Becton Dickinson, NY, USA).

Bile Tolerance

The ability of *Lactabiclus plantarum* KCA-1 to grow in the presence of bile salts was examined at CRDC between 2006/2007. Two types of bile were used, namely; porcine bile extract containing glycine and taurine conjugates of hyodeoxycholic acid and other bile salts (Sodium cholate and sodium deoxycholate) and oxgall (BD Difco™, USA). Porcine bile salt tolerance was evaluated by preparing different concentrations of the bile (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0%) wt/vol in MRS broth. *Lactobacillus plantarum* KCA-1 strain (10^8 cfu mL^{-1}) that produces biosurfactants was inoculated and incubated anaerobically at 37°C for 48 h. The control was composed of MRS broth without bile salt. Bacterial survival was monitored by measuring the absorbance with a Multiskan Ascent V1.24 (Ascent software version 2.6) at 600 nm. The absorbance values obtained were plotted against the different bile concentrations (%) weight/volume.

For oxgall bile, concentrations ranging from 0, 0.05, 0.1. 0.5, 1.0, 1.5. 2.0, 2.5, 3.0, 3.5 and 4.0% wt/vol were prepared in MRS broth and 100 µL of (10^8 cfu mL^{-1}) *Lactobacillus plantarum* KCA-1 was inoculated and incubated at 37°C for 48 h and absorbance read with the Multiskan Ascent.

To determine the number of surviving cells in the different bile concentrations after 48 h of incubation, 10-fold serial dilutions were made using MRS broth. Ten micro-liter was taken out from each dilution and plated onto MRS agar in duplicates and the plates were incubated anaerobically by using the BBL™ GasPack system (Becton Dickinson, NY, USA). After incubation of plates at 37°C for 48 h, colonies were counted and results expressed as percentage inhibition with respect to the cell count in the absence of the bile salts. The growth inhibition was calculated as follows:

$$\text{Growth inhibition (\%)} = [(\log Nmc - \log Nmb)/\log Nmc] \times 100.$$

Where Nmc = No. of viable count in the control MRS broth after 48 h of incubation and Nmb = No. of viable count in MRS broth containing the bile salts after 48 h of incubation.

Acid Tolerance

Acid tolerance of the *Lactobacillus plantarum* KCA-1 cultures was studied by incubating the organisms in MRS broth at different pH values (2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0) adjusted with hydrochloric acid (HCl) and the value determined with digital pH-meter (Φ240 pH/Temp Meter, Beckman, USA) and cultures were incubated at 37°C for 48 h. Bacterial survival in the different pH values was monitored by measuring the absorbance with a Multiskan Ascent V1.24 (Ascent software version 2.6) at 600 nm. The absorbance values obtained were plotted against the different pH values. To determine the number of surviving cells in the different pH preparations after 48 h of incubation, 10-fold serial dilutions were made using MRS broth. Ten microliter was taken out from each dilution and plated onto MRS agar in duplicates and the plates were incubated anaerobically by using the BBL™ GasPack system (Becton Dickinson, NY, USA).

Acid tolerance was determined by comparing the colony forming unit (CFU mL^{-1}) on the plates from the absorbance values from the Multiskan.

RESULTS

The ability of *Lactobacillus plantarum* KCA-1 to grow and survive in the presence of porcine bile and oxgall was examined. After 48 h of incubation, the optical densities (OD) of the medium containing different concentrations of the bile salts were read. Table 1 shows an increase from 0.25 to 0.53 OD_{600} nm^{-1} within the porcine bile concentrations tested (0.1 to 1.0%) and there was a corresponding increase in the viable cell counts with a 3 log cycle increase (Data not shown). To determine whether the *Lactobacillus* strain can survive in high concentrations of bile, the organism was inoculated in oxgall with varying concentrations ranging from 0 to 4.0%. The result in Table 2 indicates that at high concentrations of oxgall bile, growth of the *Lactobacillus* strain was inhibited. Table 3

Table 1: Survival of Lactobacillus plantarum KCA-1 in porcine bile salt after 48 h of incubation

Porcine bile salt (%)	Optical deensity (OD)/600 nm
0.1	0.254
0.2	0.290
0.3	0.281
0.4	0.258
0.5	0.338
0.6	0.290
0.7	0.406
0.8	0.422
0.9	0.434
1.0	0.533

Table 2: Survival of *Lactobacillus plantarum* KCA-1 in oxgall after 48 h of incubation

Oxgall (%)	Optical density (OD)/600 nm
0.0	0.445
0.05	0.530
0.1	0.610
0.5	0.348
1.0	0.451
1.5	0.348
2.0	0.330
2.5	0.254
3.0	0.231
3.5	0.186
4.0	0.142

Table 3: Acid tolerance of *Lactobacillus plantarum* KCA-1 after 24 h of incubation

pH	Optical density (OD)/600 nm
2.0	0.002
2.5	0.002
3.0	0.003
3.5	-0.002
4.0	0.001
4.5	0.066
5.0	0.226
5.5	0.245
6.0	0.349
6.5	0.457

Table 4: Percentage growth inhibition of *Lactobacillus plantarum* KCA-1 by Oxgall Bile

Oxgall bile concentration (%)	Growth inhibition (%)
0	0
0.05	0
0.1	0
0.5	8
1.0	12
1.5	22
2.0	25
2.5	43
3.0	47
3.5	59
4.0	68

shows the corresponding increase in growth inhibition (%) as the concentration of oxgall increases. At 2% oxgall concentration, there was a 25% growth inhibition while at a concentration of 4%, a corresponding 68% growth inhibition of the strain was observed.

The ability of *Lactobacillus plantarum* KCA-1 to survive at different pH values is shown in Table 4. At pH of 2 to 3, the strain exhibited resistance for 24 h (data not shown) with a reduction of only 2 log cycles but between 4 and 6.5 there was an increase in growth as shown by increase in the optical density at 600 nm.

DISCUSSION

The selection criteria for probiotcs have been highlighted by various guidelines including the World Health Organization and Food and Agricultural Organization (WHO/FAO, 2001). Previous study by Anukam and Reid (2007) on this strain presents properties that may allow its use as a biotherapeutic agent for infections of the urogenital tract. However, as the strain looks promising, oral administration portends that the organism will have to traverse the acidic and bile environment of the gastrointestinal tract. This is an important probiotic characteristic as the small intestine contain high concentrations of bile acids which is inhibitory to a large proportion of enteric bacteria (Fernandez *et al*., 2003). However, there is still no consensus about the precise concentration to which the selected strain should be tolerant. The physiological concentrations of bile acids in the small intestine have been shown to hover between 5000 and 20,000 μM by Hofmann (1991). In this study, there was a stimulatory effect of oxgall bile at 0.1% but the trend seems to decrease as the concentration increases above 1%. At a concentration of 0.5% bile, equivalent to 12,255 μM as shown by Kociubinsky *et al*. (1999), the lactobacillus strain was still resistant, contrary to 0.15-0.3% that have been used by other investigators (Golden and Gorbach, 1992; Fernandez *et al*., 2003) of bacterial probiotic characteristics. *Lactobacillus plantarum* KCA-1 exhibited a high level of tolerance against oxgall bile, thus suggesting that the strain could be a potential probiotic candidate for gastrointestinal disorders. However, at higher concentrations of oxgall bile, the strain started decreasing. This is in line with the recent study of

Taranto *et al*. (2006) demonstrating that at higher concentrations, bile salts induces complete permeabilization of cells, abolish glucose uptake and severely distorts the cell envelope, as shown by electron microscopy. Also detailed analytical studies revealed a change in the phospholipid to glycolipid ratio and also in lipid proportions of the cell membrane. In another study Kurdi *et al*. (2006) reported that free bile acids at the minimum inhibitory concentration, disturbance of the membrane integrity occurs and that this effect can lead to leakage of proton (membrane DeltapH and DeltaPsi dissipation), potassium ion and other cellular components and eventually cell death. This study has revealed that under high concentration of oxgall bile, *Lactobacillus plantarum* KCA-1 survives well and this findings supports previous work of Liong and Shah (2005) indicating the survival of *Lactobacillus acidophilus* at higher concentrations of bile salts.

In addition to bile acid resistance, probiotic microbes are mostly delivered in a food matrix that must pass through the stomach with high pH between 1.5 and 3.0. In this study, *Lactobacillus plantarum* KCA-1 survived pH 2 and 3 and these values have been suggested to be optimal for probiotic strains (Usman, 1999). Hutt *et al*. (2006), recently observed the screening of *Lactobacillus* and *Bifidobacterium* sp. strains according to their activity in various acid conditions which could precede the clinical efficacy studies for adjunct treatment with probiotics in cure of different gastrointestinal and urinary tract infections. The present results from *Lactobacillus plantarum* KCA-1 have demonstrated a high degree of bile and acid tolerance. Further *in vivo* studies are now planned to establish whether this strain will be used as a probiotic in conferring health benefits on the host.

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REFERENCES

Anukam, K.C., E.O. Osazuwa, I. Ahonkhai and G. Reid, 2005. 16S rRNA gene sequence and phylogenetic tree of lactobacillus species from the vagina of healthy Nigerian women. Afr. J. Biotechnol., 4: 1222-1227.

Anukam, K.C., E.O. Osazuwa, I. Ahonkhai, M. Ngwu, G. Osemene, A.W. Bruce and G. Reid, 2006. Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: Randomized, double-blind, placebo controlled trial. Microbes Infect., 8: 1450-1454.

Anukam, K.C. and G. Reid, 2007. *Lactobacillus plantarum* and *Lactobacillus fermentum* with probiotic potentials isolated from the vagina of healthy Nigerian women. Res. J. Microbiol., 2: 81-87.

Corzo, G. and S.E. Gilliland, 1999. Measurement of bile salt hydrolase activity from *Lactobacillus acidophilus* based on disappearance of conjugated bile salts. J. Dairy Sci., 82: 466-471.

Fernandez, M.F., S. Boris and C. Barbes, 2003. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. J. Applied Microbiol., 94: 449-455.

Flahaut, S., J. Frere, P. Boutibonnes and Y. Auffray, 1996. Comparison of the bile salts and sodium dodecylsulfate stress responses in *Enterococcus faecalis*. Applied Environ. Microbiol., 62: 2416-2420.

Golden, B.R. and S.L. Gorbach, 1992. Probiotics for Humans. In: Probiotics-the Scientific Basis, Fuller, R. (Ed.), London: Chapman and Hall, pp: 355-376.

Hofmann, A., 1991. Enterohepatic Circulation of Bile Acids. In: Handbook of Physiology. Schultz, S.G., J.G. Forte and B.B. Rauner (Eds.), Section 6: The Gastrointestinal Syst., 3: 567-580.

Hutt, P., J. Shchepetova, K. Loivukene, T. Kullisaar and M. Mikelsaar, 2006. Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero-and uropathogens. J. Applied Microbiol., 100: 1324-1332.

Kociubinsky, G., P. Perez and G. De Antoni, 1999. Screening of bile resistance and bile precipitation in Lactic Acid Bacteria and Bifidobacteria. J Food Prot., 62: 905-912.

Kurdi, P., K. Kawanishi, K. Mizutani and A. Yokota, 2006. Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. J. Bacteriol., 188: 1979-1986.

Limdi, J.K., C. O'Neill and J. Mclaughlin, 2006. Do probiotics have a therapeutic role in gastroenterology?. World J. Gastroenterol., 12: 5447-5457.

Liong, M.T. and N.P. Shah, 2005. Acid and bile tolerance and cholesterol removal ability of Lactobacillus strains. J. Dairy Sci., 88: 55-66.

Reid, G., J. Jass and T. Sebulsky *et al*., 2003. Potential use of probiotics in clinical practice. Clin. Microbiol. Rev., 16: 658-672.

Reid, G. and A.W. Bruce, 2006. Probiotics to prevent urinary infections: The rationale and evidence. World J. Urol., 24: 28-32.

Taranto, M.P., G. Perez-Martinez and G. Font de Valdez, 2006. Effect of bile acid on the cell membrane functionality of lactic acid bacteria for oral administration. Res. Microbiol., 157: 720-725.

Usman, H.A., 1999. Bile tolerance, taurocholate deconjugation and binding of cholesterol by *Lactobacillus gasseri* strains. J. Diary Sci., 82: 243-248.

WHO/FAO., 2001. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Report 2001.http://www.fao.org/es/ESN/probio/probio.htm (accessed 26 January 2007).