African Journal of Biotechnology Vol. 6 (7), pp. 939-949, 2 April 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Evaluation of the probiotic potential of lactic acid bacteria isolated from faeces of breast-fed infants in Egypt

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Accepted 16 January, 2007

The probiotic-related characteristics of 55 strains of lactic acid bacteria isolated from the faeces of 3 - 6 months old breast-fed infants were determined. The API 50 CH and SDS-PAGE techniques were employed to ascertain the identity of the isolated strains. The predominant species among the isolated strains were *Lactobacillus (Lb.) acidophilus*, *Lb. plantarum*, *Enterococcus (E.) faecium*, and *E. faecalis*. Probiotic properties such as bile resistance, acid tolerance, and adhesion to intestinal mucous were assessed. *In vitro* results obtained showed that five strains, *Lb. plantarum* (P1 and P164), *Lb. pentosus* (P191), and *Lb. fermentum* (P10, P193) were able to meet the basic requirements for probiotic functions as they demonstrated probiotic characteristics such as tolerance to pH 3, growth in 0.4% oxgall and adhesion to intestinal mucous. The results obtained in this investigation will be used to select potentially probiotic strains for *in vivo* study.

Key words: Probiotics, lactic acid bacteria, bile resistance, acid tolerance, adhesion.

INTRODUCTION

Probiotics are usually defined as microbial food supplements that when administered in adequate amounts exert beneficial effects on the host. Evidence for probiotic, health promoting effects for a few well-characterized LAB strains is increasing (Saarela et al., 2000). Recent scientific investigation has supported a role for probiotics as a part of a healthy diet for humans and animals and may be an avenue to provide a safe, cost effective, barrier against microbial infection (Parvez et al., 2006). On the industrial scale, a large number of dairy products are present on the market and are being promoted with health claims based on various characterristics (Succi et al., 2005). Strains of *Lactobacillus (Lb.) acidophilus*, *Lactobacillus paracasei*, and *Bifidobacterium* isolated from human or animal intestinal tracts have been the most extensively studied probiotics (Saito, 2004). They are increasingly incorporated into food as dietary adjuncts (Lourens-Hattingh and Viljoen, 2001; Bernet et al., 2004; Patrignani et al., 2006).

A number of requirements have been identified for strains to be effective probiotic microorganisms. They must simply be of human origin, and be able to survive through the gastrointestinal tract (Maruo et al., 2006). Required characteristics include resistance to gastric acid and physiological concentrations of bile and adherence to intestinal epithelial cells (Schillinger et al., 2005). Adherence of probiotic bacteria to intestinal mucosa is the first

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step in gut colonization. Gut colonization is important for a beneficial health effect as it prolongs the time the microorganism can influence the gastrointestinal immune system and microbiota of the host (Kirjavanainen et al., 1998; Forestier et al., 2000).

Feeding methods have a significant influence on the relative proportions of bacteria that establish in the infant gut (Vernaza et al., 2006). Breast-fed infants develop a probiotic-rich gut microflora with less pathogenic bacteria, compared with formula-fed individuals (Weizman et al., 2005). LAB and Bifidobacteria dominate the microbiota of the full-term neonates especially when breast-fed with a health promoting effect on the child (Arici et al., 2004), where human milk is a source of lactic acid bacteria for the infant gut (Martin et al., 2003). LAB constitutes an integral part of the healthy gastrointestinal (GI) microecology and influence host metabolism (Gibson and Fuller, 2000).

Isolation and study of new strains from the intestinal flora may lead to the development of novel probiotic products. The objective of the current study was to isolate identify and characterize a number of LAB isolated from infant faeces. The strains were further characterized by tolerance to low pH and bile, and adhesion to intestinal mucous. The competitiveness of selected strains with reference probiotic strains was also evaluated.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

Strains used in this study were isolated from faeces of healthy breast-fed infants born in Alexandria, Egypt, aged from 3 - 6 months. The cultures were preserved in reconstituted skim milk in eppindorf tubes, stored at -80°C with glycerol (20%, v/v). Prior to use, strains were subcultured (1%, v/v) twice in MRS broth (for lactobacilli strains) or M17 (for cocci strains), incubated aerobically overnight at 37°C to obtain a concentration of approximately 10^7 cfu/ml. A strain with known probiotic properties, *Bifidobacterium* (B1), was obtained from the culture collection at the Laboratory of Microbial Biochemistry, Faculty of Agriculture, Alexandria University.

Faecal samples preparation, isolation, and enumeration of LAB

Fresh faecal samples were obtained from two healthy breast-fed infants. Samples were collected twice (Table 1) at 3 and 5 months of age (sample A), and at 4 and 6 months of age (sample B). Samples were collected in sterile plastic bags early in the morning, transported to the laboratory at 4 ± 1°C and assayed within 1 h. A one gram was homogenized and then diluted with 0.85% NaCl, 0.1% peptone and 0.01% cysteine; pH 7.0. Serial dilutions were spread onto plate count agar (PCA) for total count, Rogosa agar for the isolation of lactobacilli, Rogosa and Sharpe (MRS) supplemented with 0.5% L-cysteine for the isolation of LAB and the growth of bifidobacteria (Haddadin et al., 2004), and MRS supplemented with 100 mg/l neomycin sulfate, 15 mg/l nalidixic acid and 3 g/l lithium chloride for the recovery of bifidobacteria (Laroie and Martin, 1991). Plates were incubated in BBL anaerobic jar (Becton Dickinson Microbiology Systems, Sparks, MD) provided with disposable BBL gas generating pack (CO_2 system envelopes, Oxoid, Ltd., West Heidelberg, Victoria) at 37°C for 48 h. The conventional pour plate method for the enumeration of microbes was employed. Well-isolated colonies selected on the basis of colony morphology and/or colony color were randomly picked, purified twice by streaking on the corresponding isolation medium plates. The purity was also checked by microscopic examination. This above procedure was repeated twice for each faecal sample.

Identification of recovered LAB strains

Isolates were identified to the genus level following the criteria of Sharpe, (1979) using the morphological, phenotypic and biochemical methods. Cultures were microscopically examined for Gram stain and catalase production (Harigon and McCane, 1976) and then tested for growth at 10°C for 10 days, growth at 45°C for 48 h, and the production of CO_2 from glucose (El Soda et al., 2003), Growth on SF broth medium and in the presence of 6.5% NaCl was also determined for isolates with a cocciod morphology.

Strain characterization using the API system

API 50 CH and API 20 STEP (Biomerieux, Marcy l'Etoile France) were used for the identification of lactobacilli, and for the study of carbohydrate fermentation profiles. Interpretation of the fermentation profiles were facilitated by systematically comparing the results obtained from the isolates studied with information from the computer-aided database APILAB plus V.3.2.2. in which the identification of a microorganism is accompanied by the following information:

(i) The percentage of identification (% id), which is an estimate of how closely the profile corresponds to the taxon relative to all the other taxa in the database.
(ii) The T-index, which represents an estimate of how closely the profile corresponds to the most typical set of reactions for each taxon. Its value varies between 0 and 1, and is inversely proportional to the number of atypical tests.
(iii) Comments on the quality of identification derived from the %id and the T-index of the selected taxon (excellent identification %id > 99.9 and T> 0.75).

SDS-PAGE of the whole- cell proteins

Isolates were characterized using sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the whole-cell proteins for comparison with the available protein pattern database of LAB. *Lb.* strains were inoculated with 2% of 24 h overnight culture and the cells were collected after 16 h incubation by centrifugation (7000 × g, 10 min, 4°C). The cell pellet was washed twice with sodium phosphate buffer (0.01 M; pH 7.3) containing 0.08% NaCl. Coccoid isolates were plated on the M17 agar and incubated for 48 h. Bacterial cells were collected and suspended in eppindorf tubes containing 1 ml phosphate buffer (0.01 M; pH 7.3). After centrifugation, the resulting pellet was washed with the same buffer. The cell pellet was resuspended in 0.9 ml sample treatment buffer (0.062 M Tris HCl buffer containing 5% v/v mercaptoethanol and 10% glycerol; pH 6.8). The ice cooled cell suspension was then treated with an Ultrasonic XL 2020 apparatus using a needle probe tip and mixed by vortex. 0.1 ml of SDS (20%) was added to this suspension. The mixture was heated at 95°C for 10 min and then ice-cooled. The whole cell protein

Table 1. Total microbial count (CFU/ml faecal homogenate) in samples obtained from two 3 - 6 months old healthy breast-fed infants using different selective media. Counts are averages of two independent experiments (n = 2).

Child	Age (Months)	PCA (Total bacterial count)	Rogosa (Lactobacilli)	MRS-C (LAB and Bifidobacteria)	MRS-NNL (Bifidobacteria)
A	3	3.85×10^{10}	3.57×10^{9}	1.64×10^{10}	2.59×10^{9}
	5	5.0×10^{6}	1.8×10^{6}	1.7×10^{6}	6.7×10^{5}
B	4	1.8×10^{8}	1.2×10^{7}	1.5×10^{8}	8.94×10^{7}
	6	1.51×10^{8}	3.11×10^{6}	1.11×10^{8}	2.57×10^{6}

extracts were obtained by centrifugation (7000 × g, 10 min, and 4°C) of the suspension. The ID electrophoresis of these protein extracts was run according to the method described by Pot et al. (1994). Subsequently, registration of the protein electrophoresis patterns, normalization of the densitometric traces, grouping of the strains by the Pearcon product moment correlation coefficient (r) and UPGMA (Unweighted Pair Group Method Using Averages Linkages) cluster analysis were performed by the techniques described by Pot et al. (1994) using the package Gel-Compar software (Applied Maths, Sint-Martens-Latem, Belgium; 4.0). Isolates were identified by comparison of their protein patterns to the fingerprints of reference strains obtained from the different culture collections of LAB present at the database of the Laboratory of Microbial Biochemistry at the faculty of Agriculture, Alexandria University. The accuracy of the electrophoretic analysis was determined by inclusion of reference bacterial protein extracts in each slab gel. Reproducibility was determined by repetition of the electrophoretic runs and the duplication of the injection of the same sample.

Treatment of bacteria prior to adhesion

The isolates were propagated in MRS broth overnight at 37°C. Bacteria were harvested by centrifugation (10,000 x g, for 10 min) at 4°C, and washed twice with phosphate buffer saline (PBS; 10 mM KH_2PO_4, 150 mM NaCl, pH 7.2). The optical density of the bacterial suspensions at 600 nm was adjusted with PBS to 0.5 ± 0.02 giving a count that varied between 10^6 and 10^8 CFU/ml

Preparation of intestinal mucous

Human intestinal mucous was obtained from faeces of healthy new-borns (15 - 36 months of age) according to the method of Ouwhand et al. (1999) with modification. Faecal samples were suspended in ice-cold PBS containing 0.5 g/l NaN_3 to prevent bacterial growth. The suspension was shaken for 1 h at 4°C and centrifuged for 30 min at 4°C at 15,000 × g. From the clear supernatant, the mucous was precipitated twice with ice-cold ethanol (final concentration of 60%), dissolved in ultra pure water, and then resuspended in HH buffer (HEPES HANKS buffer, pH 7.4) as 10 mg/ml.

In vitro adhesion assay

The crystal violet method was used to determine adhesion ability (Vesterlund et al., 2005). Mucous stock suspension was prepared by dissolving 10 mg/ml in HH buffer. The test cultures were added as a volume of 100 µl into microtiter polystyrene plate wells previously coated with 150 µl of human intestinal mucous. The greater volume of the mucous compared to the volume of the added bacteria was used to avoid the contact of the stain with the polystyrene. Bacteria were adhered at 37°C for 1 h, and the non-adherent bacteria were removed by washing the wells three times with 250 µl of PBS. The adherent bacteria were fixed at 60°C for 20 min and stained with crystal violet (100 µl/ well, 0.1 % solution) for 45 min. Wells were subsequently washed five times with PBS to remove excess stain. The stain bound to the bacteria was released by adding 100 µl of citrate buffer (20 $mmol^{-1}$; pH 4.3). After 45 min incubation at room temperature, the absorbance values at 640 nm were determined using the microtiter plate reader (Universal automated microplate reader EL_x 800, Germany). Stained mucous without added bacteria was used as negative control while mucous with added *Bifidobacterium longum* (B1) culture was used as positive control. Results were expressed by subtracting the absorbance value of this negative control from absorbance value recorded for all samples according to Vesterlund et al. (2005). Each determination was triplicate. The above protocol was repeated with a modified treatment that involved the pre-incubation of mucous into the microtiter polystyrene plate wells overnight at 4°C prior to the bacterial adhesion.

Acid tolerance

Overnight cultures of the test isolates were inoculated (1% v/v) in MRS broth (Oxoid) previously adjusted to pH values 2, 3, and 4 with 1 N NaOH or HCl. The cultures were incubated aerobically at 37°C for 6 h. Culture turbidity was monitored at 650 nm (Pharmacia LKB, Novaspec II, England) for growth at hourly intervals. The control comprised MRS broth adjusted to pH 6. The experiment was conducted in triplicate.

Bile salt tolerance

Bile-resistance was determined using the broth assay. Overnight cultures (1% v/v) were inoculated in MRS broth (control cultures) and MRS broth containing 0.2, 0.3 and 0.4 (w/v) oxgall (Bronadica, Hispan Lab, SA) and incubated aerobically at 37°C for 6 h. The pH of control and test cultures was adjusted to 6 with 1 N HCl or NaOH. Cultures turbidity was hourly monitored spectroscopically for growth at 650 nm. The control comprised MRS broth without bile. The experiment was conducted in triplicate

Statistical analysis of data

Data were analyzed using the one way ANOVA or the general linear models procedure of the Statistical Analysis Software (SAS institute, Cary, NY, Version 8.2, 2001). Significant differences between means were calculated by using Duncan's multiple range

Table 2. API identification results for LAB strains isolated from faecal samples obtained from two 3 - 6 months old healthy breast-fed infant

Strain No.	Species identity	Similarity %
P1	*Lb. plantarum*	99.9%
P162	*Lb. plantarum*	98.6
P164	*Lb. plantarum*	94.7%
P167	*Lb. plantarum*	1%
P171	*Lb. plantarum*	99.4
P172	*Lb. plantarum*	93.8
P173	*Lb. plantarum*	99.8
P186	*Lb. plantarum*	86.2%
P2	*Lb. acidophilus*	99.1%
P3	*Lb. acidophilus*	96.4%
P4	*Lb. acidophilus*	79
P5	*Lb. acidophilus*	87.0%
P6	*Lb. acidophilus*	93.9
P7	*Lb. acidophilus*	95
P8	*Lb. acidophilus*	96.6
P9	*Lb. acidophilus*	99.1
P103	*Lb. acidophilus*	99.1
P105	*Lb. acidophilus*	84.7
P106	*Lb. acidophilus*	95
P108	*Lb. acidophilus*	96.7
P109	*Lb. acidophilus*	99.8
P110	*Lb. acidophilus*	95
P111	*Lb. acidophilus*	96.7
P112	*Lb. acidophilus*	96.7
P145	*Lb. acidophilus*	99.3
P153	*Lb. acidophilus*	96.5
P169	*Lb. acidophilus*	98.9
P170	*Lb. acidophilus*	99.1
P181	*Lb. acidophilus*	99.1
P185	*Lb. acidophilus*	89.9
P189	*Lb. acidophilus*	89.9
P184	*Lb. delb. lactis*	76
P102	*Lb. brevis*	99.9
P161	*Lb. paracasei paracasei*	98.7
P163	*Lb. paracasei paracasei*	98.7
P160	*Lb. pentosus*	95.7
P191	*Lb. pentosus*	99.9
P192	*Lb. pentosus*	99.9
P116	*Lb. salivarius*	99.9
P10	*Lb. fermentum*	63
P193	*Lb. fermentum*	95
P107	*En. faecalis*	91.8
P113	*En. faecalis*	99.2
P132	*En. faecalis*	95.9
P142	*En. faecalis*	81.8
P143	*En. faecalis*	81.8
P115	*En. faecium*	85.5
P118	*En. faecium*	96.9
P155	*En. faecium*	65
P166	*En. faecium*	81.5
P187	*En. faecium*	95
P188	*En. faecium*	95
P174	*En. durans*	65
P176	*En. durans*	65
P165	*Aerococcus rividans*	89.1

tests, where means of triplicate values were declared significantly different when the probability level is ($p < 0.05$).

RESULTS

Isolation and identification of microorganisms isolated from faecal samples

Levels of lactic acid bacteria in the infant feces ranged from 2.5 x 10^6 to 3.8 x 10^{10} cfu/g and were higher in the younger infants (Table 1). The highest counts were obtained on PCA and MRS-C. Fifty five isolates, primarily lactobacilli were recovered from infant faecal samples (Table 2). Fifty six percent of the identified *Lb.* strains were *Lb. acidophilus*, which accounted for 42% of the total isolated strains. The most frequently recovered species from the faecal samples were *Lactobacillus plantarum*, *E. faecium*, and *E. faecalis*. Table 3 shows the phenotypic identification of the faecal isolates.

Whole cell protein fingerprints were used to construct the dendrogram presented in Figure 1 (Pot and Janssens, 1993). The protein analysis confirmed the phenotypic identification for most isolates. The taxonomic position of 15 isolates could not be elucidated by SDS-PAGE (data not shown). The results of the SDS-PAGE techniques confirmed almost 28% of the API results. On the other hand, a notable discrepancy occurred between API and SDS-PAGE profiles for some lactobacilli. For instance, cultures identified as *Lb. acidophilus* (P9) using the API system were classified by the SDS-PAGE as *Lactobacillus delbrueckii lactis* (similarity 63%).

In vitro adherence assay

Fifty five isolates were tested for their ability to adhere to intestinal mucous. Statistical analysis revealed differences in adhesion among the isolates ($p < 0.05$) (Figure 2). The strongest *in vitro* adhesion was observed for strains of *E. durans* (P174), *E. faecium* (P166), *Lb. plantarum* (P164), and *Lactobacillus pentosus* (P191). These isolates all competed and adhered more than the reference probiotic strain *B. longum* (B1). However, some

Table 3. Phenotypic characterization of 55 isolates recovered from faecal samples of two 3 - 6 months old healthy breast-fed infants. Cultures were differentiated according to their morphological and physiological characteristics into 4 categories: (a) 13 facultatively heterofermentative, (b) 25 obligatory homofermentative, (c) 3 obligatory heterofermentative, and (d) 14 enterococci. *Reference strain.

Strains	Strain no.	Gram staining	Catalase test	Growth at 45°C	Growth at 10°C	CO^2 production
Lb. plantarum [a]	P1	+	-	+	+	-
Lb. plantarum [a]	P162	+	-	+	+	-
Lb. plantarum [a]	P164	+	-	+	+	-
Lb. plantarum [a]	P167	+	-	+	-	-
Lb. plantarum [a]	P171	+	-	+	-	-
Lb. plantarum [a]	P172	+	-	+	-	-
Lb. plantarum [a]	P173	+	-	+	-	-
Lb. plantarum [a]	P186	+	-	+	-	-
Lb. acidophilus [b]	P2	+	-	+	-	-
Lb. acidophilus [b]	P3	+	-	+	-	-
Lb. acidophilus [b]	P4	+	-	+	-	-
Lb. acidophilus [b]	P5	+	-	+	-	-
Lb. acidophilus [b]	P6	+	-	+	-	-
Lb. acidophilus [b]	P7	+	-	+	-	-
Lb. acidophilus [b]	P8	+	-	+	-	-
Lb. acidophilus [b]	P9	+	-	+	-	-
Lb. acidophilus [b]	P103	+	-	+	-	-
Lb. acidophilus [b]	P105	+	-	+	-	-
Lb. acidophilus [b]	P106	+	-	+	-	-
Lb. acidophilus [b]	P108	+	-	+	-	-
Lb. acidophilus [b]	P109	+	-	+	-	-
Lb. acidophilus [b]	P110	+	-	+	-	-
Lb. acidophilus [b]	P111	+	-	+	-	-
Lb. acidophilus [b]	P112	+	-	+	-	-
Lb. acidophilus [b]	P145	+	-	+	-	-
Lb. acidophilus [b]	P153	+	-	+	-	-
Lb. acidophilus [b]	P169	+	-	+	-	-
Lb. acidophilus [b]	P170	+	-	+	-	-
Lb. acidophilus [b]	P181	+	-	+	-	-
Lb. acidophilus [b]	P185	+	-	+	-	-
Lb. acidophilus [b]	P189	+	-	+	-	-
Lb. delb. lactis [b]	P184	+	-	+	-	-
Lb. brevis [c]	P102	+	-	+	-	+
Lb. paracasei paracasei [a]	P161	+	-	+	+	-
Lb. paracasei paracasei [a]	P163	+	-	+	-	-
Lb. pentosus [a]	P160	+	-	+	+	-
Lb. pentosus [a]	P191	+	-	+	+	-
Lb. pentosus [a]	P192	+	-	+	+	-
Lb. salivarius [b]	P116	+	-	+	-	-
Lb. fermentum [c]	P10	+	-	+	-	+
Lb. fermentum [c]	P193	+	-	+	-	+
En.faecalis [d]	P107	+	-	+	+	-
En.faecalis [d]	P113	+	-	+	+	-

Table 3. Contd.

En.faecalis [d]	P132	+	-	+	+	-
En.faecalis [d]	P142	+	-	+	+	-
En.faecalis [d]	P143	+	-	+	+	-
En.faecium [d]	P115	+	-	+	+	-
En.faecium [d]	P118	+	-	+	+	-
En.faecium [d]	P155	+	-	+	+	-
En.faecium [d]	P166	+	-	+	+	-
En.faecium [d]	P187	+	-	+	+	-
En.faecium [d]	P188	+	-	+	+	-
En.durans [d]	P174	+	-	+	+	-
En.durans [d]	P176	+	-	+	+	-

members of those strains together with strains of *Lb. paracasei paracasei* failed or significantly showed poor adhesion to the intestinal mucous. There was a considerable variation in species of *Lb. acidophilus* which accounted for almost 42% of the total tested strains among which strains *Lb. acidophilus* strains P185, P112, P169, P170, P181, and P153 recorded high adhesion values, conversely, the strains P 109, and P 189 showed no marked adhesion to the intestinal mucous. Compared to the reference strain, there was a 0.01 absorbance difference between that of the reference strain and strains of *Lactobacillus fermentum* strains (P10, P193), where they showed relative moderate adhesion compared to the tested strains. Incubation of mucous overnight prior to the adhesion assay procedure insignificantly affected the adhesion capacities of all tested strains (data not shown).

Growth in the presence of bile salts

All strains were tested for their ability to grow in presence of bile salts (0.2, 0.3, and 0.4%). The isolates differed in their ability to grow in MRS supplemented with 0.4% oxgall (Figure. 3). The highest reduction of bacterial survival occurred after 3 h of exposure to the stress factor. About 43% of the tested isolates showed survival after 0.4% bile treatment. Data presented in Figure 3 indicate that the most bile resistant strain was the reference strain *B. longum* (B1) followed by *Lb. plantarum* (P1, P164, and P167), and two isolates of *Lb. fermentum* (P193, 10). The viability of these isolates increased after 3 h of exposure to bile salts. (P106) was the only *Lb. acidophilus* isolate that was able to survive the bile salt concentration, where growth was significantly ($p < 0.001$) inhibited for the rest of their examined strains. In general, there was a marked delay to poor growth observed in the remaining *Lb. plantarum, Lb. paracasei paracasei,* and *Enterococcus* cultures.

Acid tolerance of cultures

The effect of acidity on the viability of the isolates was assessed by adjusting the growth medium to different pH values (2, 3, 4, and 5). At pH 2, the strains' viability was insignificantly affected ($p > 0.05$), where this pH value was considered as the lethal for all cultures (data not shown). Only 28 isolates out of the 55 tested were able to survive at pH 3. Data presented in Figure 4 shows that *Lactobacillus salivarius*, *Lb. pentosus*, and all enterococci strains were the acid sensitive, loosing their viability after 3 h. None of the *Lb. acidophilus* isolates replicated and some lost viability at this pH. An exception is isolate P3 which showed the greatest acid tolerance of the isolates ($p < 0.001$). Cultures of *E. faecalis* remained viable, but were unable to multiply.

One isolate of *Lb. plantarum* (P1) was able to survive at pH 3. Isolates of *Lb. paracasei paracasei* showed marked loss in viability. Cultures of *Lactobacillus*. *brevis* displayed insignificant survival at pH 3. On the other hand, strains of *Lb. fermentum* (P10, P193) demonstrated another interesting feature of acid tolerance, in addition to three cultures of *Lb. pentosus* that showed a significant increase in turbidity after 3 h of incubation.

DISCUSSION

Identification and characterization of a strain are important criteria for the selection of probiotics (van der Aa Kuhle et al., 2005). In this research, 55 isolates of lactic acid bacteria were recovered from faecal samples of infants of 3 - 6 old months. Isolates were identified using the API system for primary identification and SDS-PAGE protein patterns for confirmation. The phenotypic characterization by the API 50 CH was used to select strains for further specific characterization (Johansson et al., 1993). The taxonomic status of some strains was not clarified by the SDS-PAGE techniques. High strain dissimilarity cannot be attributed to a possible different origin of the refer-

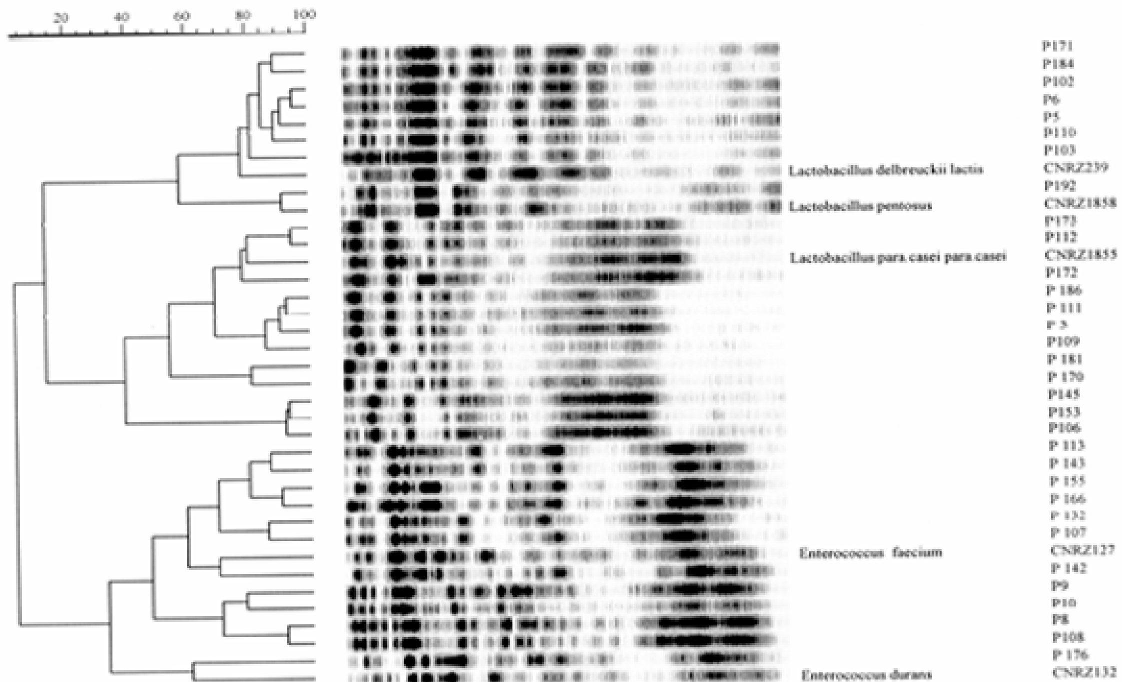

Figure 1. Dendrogram of digitized and normalized protein patterns showing the clustering of 32 LAB strains isolated from faecal samples obtained from two 3 - 6 months old healthy breast-fed infants, as determined by the SDS-PAGE and evaluated by the Pearson product moment correlation coefficient (r) and the unweighted pair group algorithm with arithmetic averages (UPGMA).

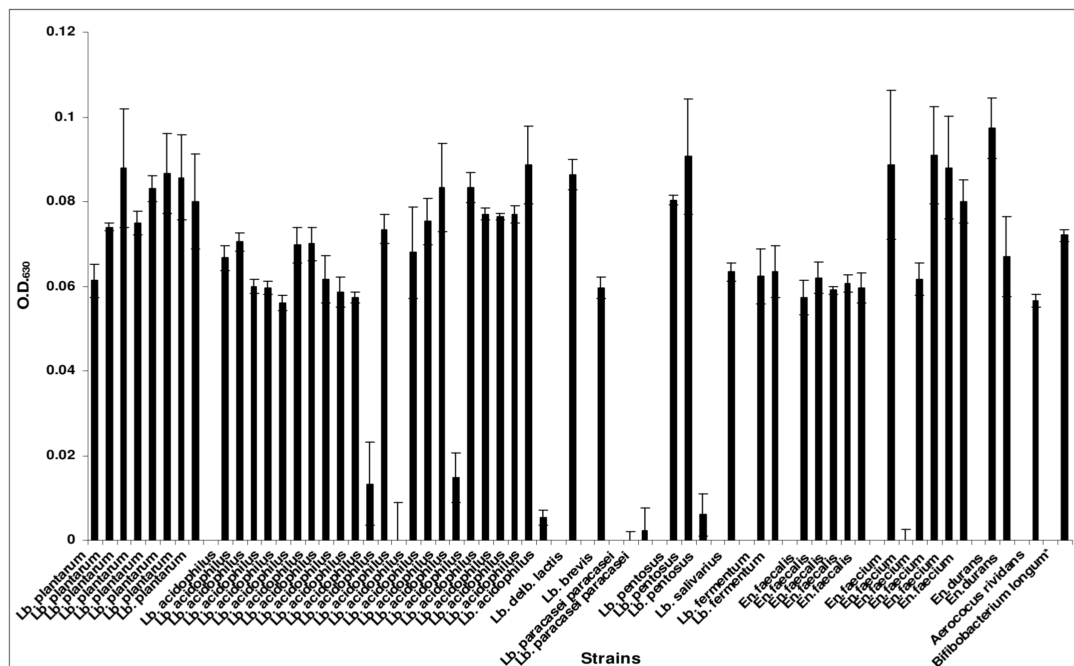

Figure 2. Adhesion of probiotic strains to intestinal mucous isolated from faecal samples of 15 - 36 months old infants. Adhesion is expressed as the turbidity caused by crystal violet stain bound to the adhering bacteria as released by 20 mmol^{-1} citrate buffer. Bars represent the mean ± standard deviation of triplicates O.D$_{640}$ values recorded for each strain. * Reference strain used.

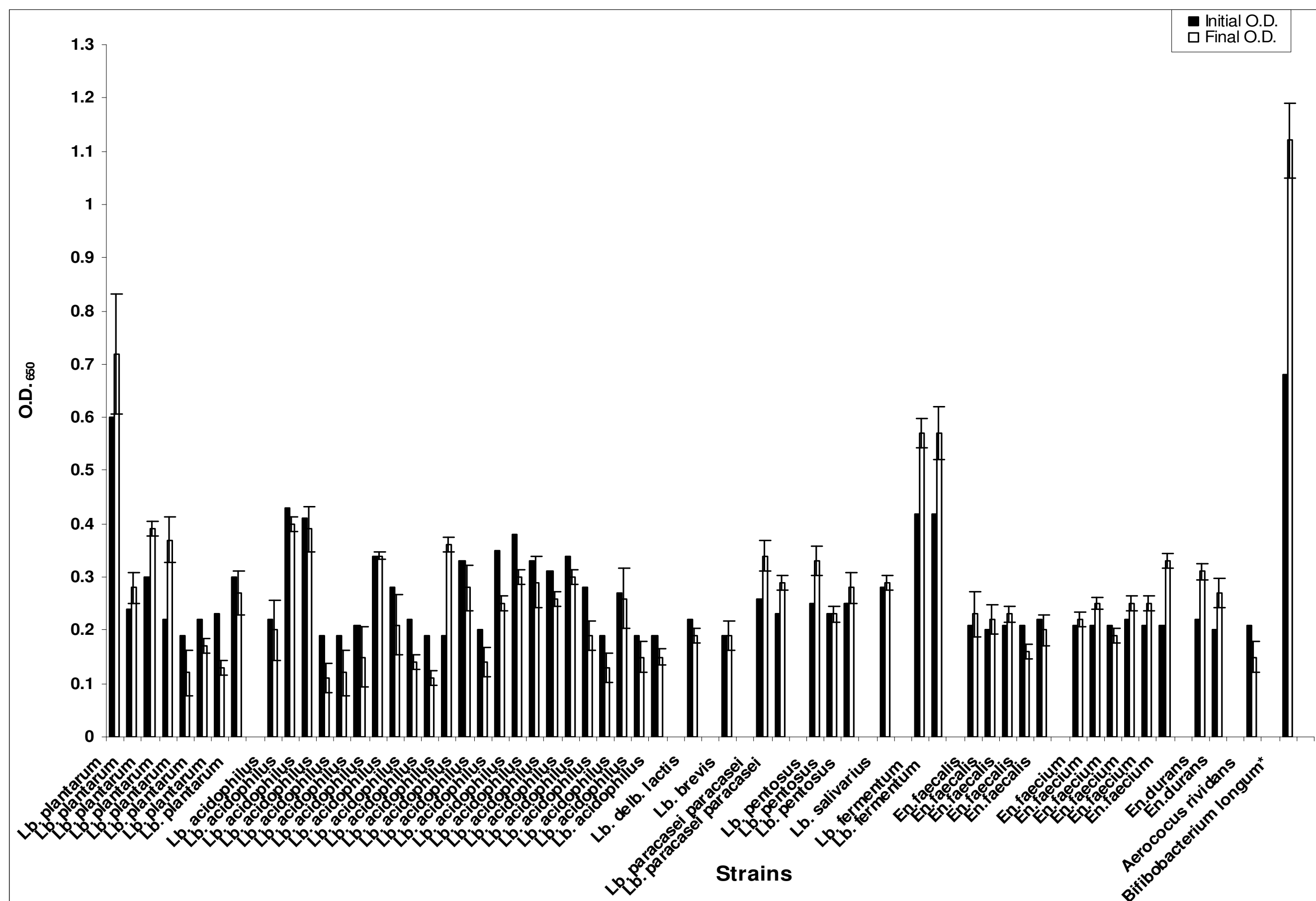

Figure 3. Survival of faecal isolates in MRS broth supplemented with 0.4% oxgall, as determined by the cultures turbidity after 3 h of exposure. Bars represent the standard error of the mean values of the O.D.$_{600}$ measurements of three independent experiments (n = 3).* Reference strain used. Boxed and empty bars represent initial and final O.D.$_{600}$, respectively.

ence strains (Xanthopoulos et al., 1999). However, the findings of our study generally suggested that the analysis of whole-cell protein profiles provides an effective method for distinguishing isolates to the species level.

Resistance to gastric acidity, bile salts, and adherence to intestinal mucous are among the *in vitro* tests that are frequently suggested for the evaluation of the probiotic potential of a bacterial strain (Schillinger et al., 2005). *In vitro* studies only partially mimic *in situ* conditions in the gut ecosystem (Dunne et al., 2001). Although, such *in vitro* systems are not fully adequate to predict the functionality of the strain in the human body, they are useful tools for screening numerous samples and the selection of LAB species for further testing as probiotics.

Selected probiotic strains used in our study have health effects documented in human studies (Salminen et al., 1998). Adhesiveness to the human intestine is one of the most important characteristics of probiotic LAB. Attachment of probiotic strains to the epithelial cells and intestinal mucosal is prerequisite for the intestine colonization, as it influence the time of bacteria retention in the intestine and the functional activity of bacteria (Lin et al., 2006). Bacterial adhesion to intestinal surfaces in vitro has been assessed using intestinal cell lines of human origin (Chauvière et al., 1992; Lehto and Salminen, 1997). In the current study, the human intestinal mucous isolated from faeces of healthy Egyptian infants of 36 months of age were used as a substratum for the adhesion of probiotic strains. A marked difference in adhesion could be observed between our tested strains. This is in agreement with earlier observations (Kirjavainen et al., 1998). Also within the same species large differences in the level of adhesion has been detected. This could be attributed to several factors such as the non-specific reaction by charge, non-specific reaction by hydrophobicity. Another suggested factor is the presence of the protenaceous components in the surface layered proteins of the strains that are involved in the adhesion process through their binding to carbohydrate portions of the colonic mucous layer (Saito, 2004). Therefore, the differences

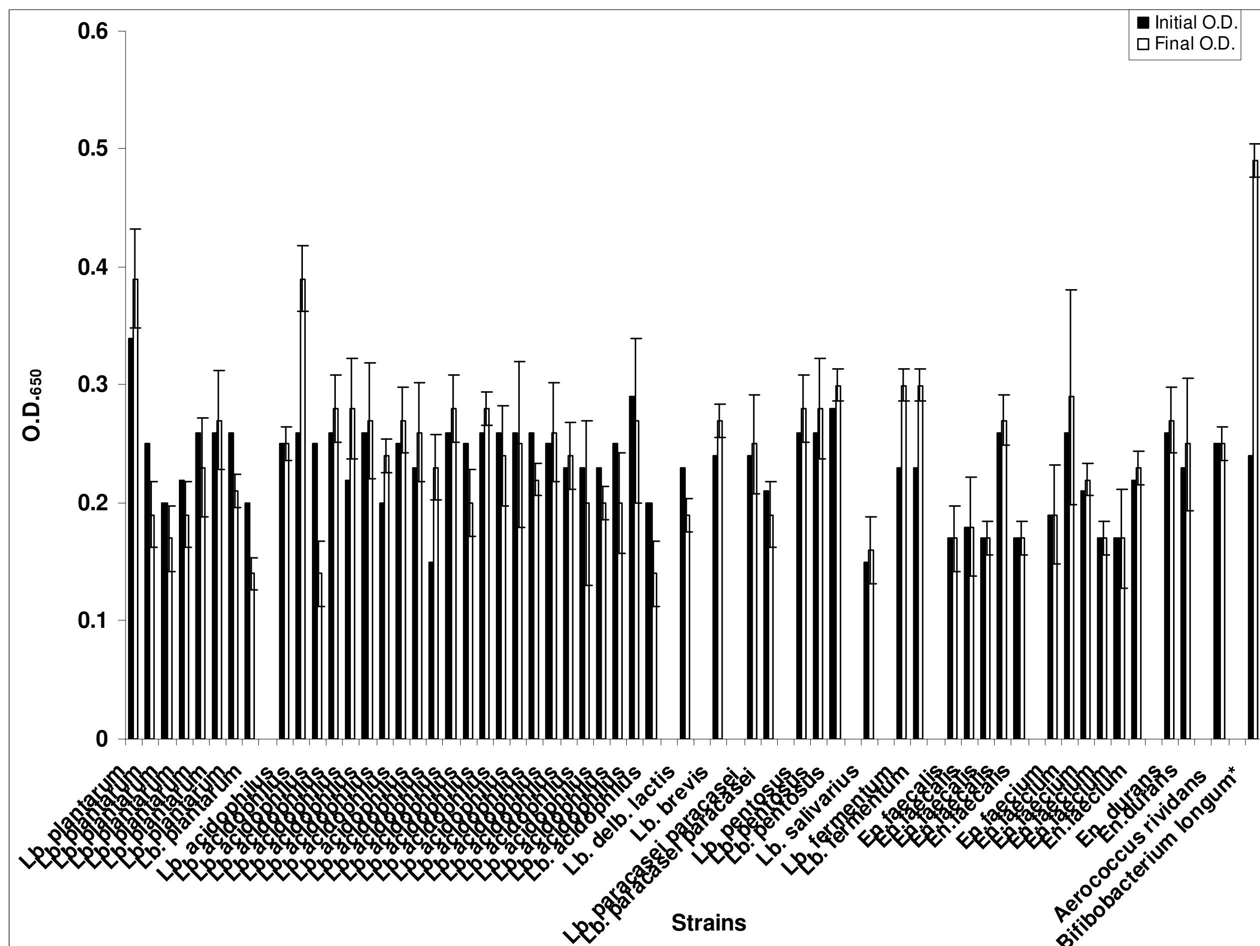

Figure 4. Acid tolerance of faecal isolates grown for 3 h at 37°C in MRS broth adjusted to pH 3. Bars represent the standard error of the mean values of the $O.D._{600}$ measurements of three independent experiments (n = 3). * Reference strain used.

observed among our strains shows that each probiotic strain should be judged by its own merits and that extrapolation from related strains is not acceptable. *Lb. acidophilus* was the most frequently recovered species from infant faecal samples. This finding indicates that this species survived better in the gastrointestinal tract than the other strains. Jacobsen et al. (1999) attributed the survival of lactobacilli in the intestinal tract to their adhesion ability, which was in good agreement with our results as most of our *Lb. acidophilus* isolates were highly to moderately adhesive to the intestinal mucous.

Different survival rates of *Lactobacillus* species have been observed in previous studies (Ronka et al., 2003). Probiotic tests conducted on *Lb. fermentum* indicated its potential as good probiotic candidate adapted to adhere to intestinal mucous, tolerate the gastrointestinal tract acid and bile conditions. These results were in accordance with those previously reported by Pereira et al. (2003) and Pereira and Gibson (2002). Moreover, they found that no undesirable microbial-metabolic characteristics have been caused which could hamper its use as a probiotic for human consumption.

Three hours at pH value of 3 was chosen to determine acid resistance, as this simulates residence time in the stomach (Olejnik et al., 2005). During passage through the upper alimentary tract, the microorganisms are subjected to several stress factors. Kim et al. (1999) defined for *Lb. lactis* pH 2.5 as lethal, and for *L.lactis* subsp. *cremoris* pH 3.0 as lethal. Our results fell close to the previous observations, where pH 2 and pH 3 were determined to be lethal and sublethal pH values respectively for the majority of the recovered strains.

Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. The presence of bile salts in the environment of bacteria cultures is much more detrimental than the effect of low pH. The choice of the bile concentration selected for our screening (0.4% Oxgall solution) was based on its being equivalent to the physiological concentration in the duodenum or the human bile

juice (Hofmann, 1991; Brashears et al., 2003). Many authors investigated the effect of bile on survival of LAB. Kim et al. (1999) examined the effect of bile concentration in the range of 0 - 0.4% on the *Lb. lactis* survival and they reported inhibiting effect of bile at concentration over 0.04%. They detected that all bacterial cells were killed at 0.2% and higher (Olejnik et al., 2005). Comparing to this study, our experiments showed much more resistance to detrimental actions of bile salts where the viability of strains of *Lb. plantarum* and *Lb. fermentum* seemed to improve when exposed to high levels of oxgall (0.4%).

Few of our *Lb. acidophilus* strains showed significant acid and bile tolerance. This result was contrary to the finding of Liong and Shah, (2005), where most strains of *Lb. acidophilus* showed greater acid tolerance, in addition to growth in the presence of bile salts that was ascribed to the high levels of secreted bile salt hydrolase. Bile salt activity has been detected in *Lactobacillus* and *Enterococcus* (Begley et al., 2006). Bile salt hydrolytic (BSH) activity may contribute to the resistance of LAB to the toxicity of conjugated bile salts in the duodenum and therefore is an important colonization factor (De Smet et al., 1995). This may explain the variation recorded among our tested strains. However, recent data indicate that relationship between bile salt hydrolase activity and resistance to bile salts in lactobacilli is still under debate (Moser and Savage, 2001; Pinto et al., 2006). Furthermore, other factors such as membrane characteristics and variation in surface properties may have influenced the bile tolerance of strains (Schär-Zammaretti and Ubbink, 2003; Begley et al., 2005)

In conclusion, not all desirable probiotic characteristics were present in a single isolate, where many isolates displayed varying individual but promising capabilities to adhere, survive the acidic conditions, and bile concentration. However, the results obtained in this *in vitro* investigation allow the selection of some potentially probiotic strains. Among the 55 investigated isolates, five strains of *Lb. plantarum*, *Lb. fermentum* and *Lb. pentosus* demonstrated abilities that are similar and or greater than the *B. longum* reference strain. Future research using *in vivo* studies should be undertaken on selected isolates.

ACKNOWLEDGMENTS

This research was supported by grant received from the US.-Egypt Board on Scientific and Technological Cooperation. The fund was established under an agreement between the government of the United States of America and the Government of the Arab Republic of Egypt on Science and Technology Cooperation. The authors wish to thank the research team involved with the project for their hard work and devotion.

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