Probiotic Properties and Bile Salt Hydrolase Activity of Some Isolated Lactic Acid Bacteria

Asmaa Negm El-Dein[#], Azza M. Nour El-Deen, Sahar M. Tolba[*], Einas H. El-Shatoury[*], Ghada A. Awad, Mohamed Khaled Ibrahim[*] and Mohamed A. Farid

Chemistry of Natural & Microbial Products Department, National Research Center, Giza and

[*]Microbiology Department, Faculty of Science, Ain Shams University, Cairo, Egypt.

EIGHT lactic acid bacterial (LAB) isolates were obtained from food and non-food sources and identified by 16S rRNA gene sequence analysis. Based on the sequencing results, the isolates belong to two species of lactobacilli, *Lactobacillus plantarum* and *L. rhamnosus*. These strains were then compared with a reference strain, *Lactobacillus casei*, to assess various probiotic properties, such as haemolytic activity, histamine formation ability, stress tolerance under certain stress conditions, antibiotic susceptibility and *in vitro* adhesion ability. Moreover, bile salt hydrolase (BSH) activity was evaluated both qualitatively and quantitatively. The results showed that none of the isolates demonstrated any haemolytic activity or histamine formation. The isolated strains were also tolerant to acidic and alkaline conditions (pH 2.5, 3.5 and 9) for 3 and 6 h, as well as osmotic (3 M NaCl) and heat (55 and 70°C) stress, but were more responsive to oxidative and bile stress. The bacterial isolates also expressed high amounts of BSH, ranging from 90 to 142 U/mg in active cells, compared to *L. casei* (74 U/mg), which may be useful in cholesterol reduction. All bacterial isolates were resistant to vancomycin and susceptible to amoxicillin, cloxacillin and penicillin. All isolates were also highly hydrophobic (>70%), indicating that they are not easily flushed from the intestines.

Key words: LAB, Probiotic properties, Stress tolerance, Bile salt hydrolase.

Introduction

Probiotics are defined as "live microorganisms which, when administrated in adequate amounts, confer health benefits to their hosts" (FAO/ WHO, 2002). *Lactobacillus* and *Bifidobacterium* species are the most highly studied probiotics and are usually commensal and non-pathogenic inhabitants of the human and animal intestines (Pereira & Gibson, 2002a, b). A potential probiotic should possess several desirable properties to exert its beneficial effects, the most important of which are the absence of haemolytic activity, resistance to acid and bile salts to facilitate survival during passage through the gastrointestinal tract (GIT), adherence to the intestinal mucosa of the host, viability during processing and storage and resistance to heat and osmotic stresses (FAO/WHO, 2002 and Kumar & Kumar, 2015). Bacterial survival is also challenged by the residual presence of antibiotics after antibiotic therapy. The sensitivity and tolerance of probiotics to these challenges are key parameters for their application in food (Ruiz et al., 2011). Therefore, these properties were taken into consideration during bacterial isolation to determine the maximum health benefits of probiotic bacteria.

Lactobacillus bacteria are considered to be valuable probiotics because they contribute to many health-stimulating effects, such as cholesterol-lowering ability, prevention of osteoporosis and food allergy, alleviation of constipation, protection against urogenital diseases, protection against both colon and bladder cancer (Lourens-Hattingh & Viljoen,

2001), increased resistance to malignancy and infectious illness (Roller et al., 2004 and Nomoto, 2005), and immune system modulation (Isolauri et al., 2001 and Bove et al., 2012).

Although cholesterol is an essential component of mammalian cell membranes and is the precursor of steroid hormones, vitamin D and primary bile acids (Tabas, 2002), elevated serum and dietary cholesterol levels are correlated with increased risks of both coronary heart disease and colon cancer. Lowering serum cholesterol by approximately 1% results in a 2-3% reduced risk of coronary heart disease (Manson et al., 1992).

Cholesterol levels are regulated by a number of drugs; however, these drugs have serious side effects and impose great financial burdens on patients (Ward et al., 2007). Sanders (2000) revealed the ability of lactic acid bacteria (LAB) to decrease total cholesterol and low-density lipoprotein levels. The mechanisms of LAB serum cholesterol reduction may be exerted through cholesterol and bile salt metabolism. These mechanisms include deconjugation and co-precipitation of bile via bile salt hydrolase (BSH) activity (Corzo & Gilliland, 1999a), binding of cholesterol to the probiotic cellular surface (Liong & Shah, 2005), integration of cholesterol molecules into the probiotic cellular membrane (Noh et al., 1997), assimilation of cholesterol during growth (Pigeon et al., 2002), disruption of cholesterol micelles (Araki et al., 1996), conversion of cholesterol to coprostanol (Lye et al., 2010) and production of short-chain fatty acids from oligosaccharides (Preter et al., 2007).

Therefore, the aim of the present study was to identify to LAB isolated from different sources at the species level and evaluate their probiotic properties. The LAB were characterized by phenotypic tests and identified by 16S rRNA gene sequencing. The probiotic properties evaluated included tolerance to low pH and bile and other desirable properties, such as haemolytic activity, histamine formation ability, hydrophobicity, antibiotic susceptibility and BSH activity.

Materials and Methods

Isolation and initial identification of LAB

LAB were isolated from food and non-food sources (cow butter, cheese, mother's milk, baby faeces, chicken manure and soil). Samples from mothers' milk and baby faeces were collected during the first three months of lactation. All samples were collected in sterile containers

and screened for LAB within a few hours of collection. Diluted samples were inoculated on De Man, Rogosa and Sharpe (MRS) agar medium, incubated at 37°C for 48 h under anaerobic conditions, and purified (according to their morphological characteristics) by re-plating onto the same medium. Eight isolates were recovered and initially identified as LAB based on their colony morphology, Gram staining, spore formation ability, motility, catalase reaction, lactose fermentation ability and growth on MRS medium at 10 and 45°C as described by Harrigan & McCance (1976), Wood & Holzapfel (1995) and Tanasupawat et al. (1998). *L. casei* (obtained from Chr. Hansen, Inc., Denmark) was used as a reference strain throughout this work.

Haemolytic activity

Blood haemolysis activity of the selected bacterial isolates was evaluated on Columbia agar plates (Oxoid) supplemented with 5% animal blood and incubated at 37°C for 24 h (Lombardi et al., 2004).

Histamine formation

The ability of the eight bacterial isolates to produce histamine on improved decarboxylase medium was qualitatively assayed according to the method of Bover-Cid & Holzapfel (1999). Pyridoxal-5-phosphate was added at 0.005% (w/v) as a cofactor for the decarboxylation reaction since it strongly promotes amino acid decarboxylase activity (Recsei et al., 1985). Bromocresol purple was used at 0.006% (w/v) as a pH indicator. All strains were streaked in duplicate with and without histidine (as a control) and incubated for 4 days at 37°C under anaerobic conditions.

Molecular characterization of the selected bacterial isolates

Amplification of the 16S rRNA gene

Genomic DNA of the eight bacterial isolates was extracted directly from colonies grown on solid MRS medium in accordance with the modified method of Ishikawa et al. (2000). A single isolated colony was suspended in 10 µl of distilled water and boiled in a thermocycler for 5 min. Then, the colony was centrifuged at 10,000 rpm for 3 min. One microliter of the supernatant was used as the RNA template. PCR amplification of the partial 16S rRNA gene was performed using a primer pair consisting of 16S-1F (5'-AGAGTTTGATCCTGGCTCAG-3') (Edwards et al.,1989) and 517R (5'-ATTACCGCGGCTGCTGG-3')

(Heuer et al., 1997). The PCR reaction mixture comprised 1 μl of RNA, 30 nM of each primer and 25 μl of Dream Taq master mix from Fermentas with water added to a total volume of 50 μl.

PCR was performed using an Applied Biosystems 2720 Thermo Cycler with the following program: 5 min for initial denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C; and finally 10 min at 72°C.

The purity and size of each PCR product were examined by gel electrophoresis on a 1% (w/v) agarose gel. The gel was stained with 0.05% (w/v) ethidium bromide dye, visualized under UV light and photographed using a gel documentation system (Gel Doc). The size of the obtained fragment was estimated by comparison with a standard 1-Kb ladder (Fermentas).

Partial 16S rRNA gene sequencing
PCR products were purified with a Qiagen extraction kit according to the manufacturer's instructions before injection into the DNA sequencer (ABI 3130 genetic sequence analyser). Sequencing was performed using PCR-amplified segments of approximately 500 bases covering the V3 region of the 16S rRNA gene sequence with the 1F primer using a Dynamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech).

Sequence analysis and phylogenetic tree construction
The 16S rRNA gene sequence of each isolate was compared with all accessible sequences in the BLAST database (www.ncbi.nlm.nih.gov/blastn). Multiple sequence alignment was conducted using Clustal W within the Bioedit program. A neighbour-joining phylogenetic tree was constructed using the Phylip package 3.69.

Screening for stress tolerance of viable cells
Stress tolerance tests were performed according to Parente et al. (2010) with minor modifications. Overnight cultures of the eight bacterial isolates grown in MRS broth at 37°C were adjusted to an OD_{600} of approximately 0.6. Bacterial cells were then harvested by centrifugation at 12,000 rpm for 10 min, washed twice with 20 ml of sterile 0.2 M phosphate buffer at pH 7, standardized to a final OD_{600} of 1 and re-suspended in different stress solutions: 0.1 M glycine-HCl buffer, pH 3.5, for 3 and 6 h and MRS broth, pH 2.5 using 1 N HCl, for 3 and 6 h (for acid stress); 0.1 M glycine-NaOH buffer,

pH 9 for 3 and 6 h (for alkaline stress); 3 M NaCl for 3 and 6 h (for osmotic stress); 0.05% H_2O_2 (v/v) for 30 min (for oxidative stress) and 0.1 M phosphate buffer, pH 7, at 55 and 70°C for 15 min (for heat stress). The treated bacterial cells were then sub-cultured in MRS broth and incubated at 37°C for 24 h. Tests for acid, alkaline, osmotic and oxidative stress were performed at room temperature. Bacterial cells re-suspended in 0.2 M phosphate buffer at pH 7 and stored for 1 h at 4°C were used as controls. For detergent stress, the bacterial cells were inoculated into MRS broths supplemented separately with 0.2% (v/v) Tween 80, 0.05 and 0.1% (w/v) bile salts and 0.15% (w/v) pancreatic enzymes and incubated at 37°C for 24 h. The percent viability was calculated as follows:

$$\% \text{ Viability} = (OD_{sample} / OD_{control}) * 100$$

BSH assays

Qualitative determination of BSH activity
BSH activity was assayed qualitatively by determining bile salt deconjugation ability according to the method of Taranto et al. (1995). Briefly, bile salt plates were prepared by adding 0.1% (w/v) bile salt to MRS agar. Ten microliters of overnight liquid cultures were spotted on agar plates and incubated for 72 h. The presence of precipitated bile acid around the bacterial colonies (opaque halo) was considered a positive result.

Quantitative determination of BSH activity
BSH activity was determined by a two-step procedure as described by Tanaka et al. (1999). A 10 mM concentration of dithiothreitol (DTT) was used to reduce enzyme oxidation. Samples were prepared as follows: cells of overnight cultures were centrifuged for 10 min at 10,000 rpm at 4°C, washed twice with 0.1 M sodium-phosphate buffer, pH 7.0, and re-suspended in the same buffer to give an optical density of approximately 1 at 600 nm. Three samples were then prepared. For sample 1, 10 ml of the cell suspension was sonicated for 1 min at level 3 with 50% duty cycle and constant cooling (Vibra-Cell™ Ultrasonic Liquid Processor). For sample 2, 10 ml of cell suspension was treated with 1% cetyltrimethyl ammonium bromide or cetrimonium bromide (CTAB, a chemical substance used to extract intracellular enzymes). The two mixtures were centrifuged separately for 10 min at 10,000 rpm at 4°C. The supernatant was stored as a cell-free extract. Sample 3 involved suspending 10 ml of

active cells in 0.1 M sodium-phosphate buffer, pH 7.0. BSH activity was then determined in three samples in a two-step procedure according to Tanaka et al. (1999) and Lee & Takahashi (1966). A standard curve was constructed with glycine, 10 mM DTT and 50 μl of 15% (w/v) trichloro acetic acid (TCA). One unit is defined as the enzyme activity that liberates 1μmol of amino acids from the substrate per min.

Antibiotic susceptibility test

An antibiotic susceptibility test was performed using the disc diffusion method as recommended by the standard criteria (CLSI, 2006). Antibiotic discs (Bioanalyse limited, Turkey) were placed on inoculated MRS media and incubated at 37°C for 24 h. Based on the inhibition zone size, the results were interpreted as resistant (R), intermediately resistant (IR), or susceptible (S) to antimicrobial agents.

Cell surface hydrophobicity test

The ability of *Lactobacillus* active cells to adhere to the hydrocarbon n-hexadecane (Merck, Germany), which is a measure of their hydrophobicity, was determined according to Vinderola & Reinheimer (2003). Cell surface hydrophobicity (H%) was calculated with the formula H% = [(OD$_0$–OD) / OD$_0$] * 100, where OD$_0$ and OD are the optical densities before and after extraction with n-hexadecane, respectively.

Results and Discussion

Initial identification of LAB

Eight bacterial isolates (NMP4765, NMP4768, NMP47620, NMP47621, NMP47625, NMP47628, NMP47633 and NMP47634) were selected and tentatively affirmed as LAB based on morphological and physiological characteristics. All isolates were Gram-positive, non-spore-forming, non-motile, catalase-negative rods or cocci with circular, opaque, creamy white-coloured colonies. They were able to ferment glucose and lactose and grew well at 10°C and 45°C.

A blood haemolysis assay was also used to test haemolytic activity, which is another feature of probiosis. All isolates were γ-haemolytic, indicating that they possess a probiotic property. Histamine formation by the isolated Lactobacilli was also tested since histamine is one of the most important biogenic amines of bacterial origin in food. Additionally, histamine has a toxicological effect and is an indicator of poor hygiene in some food products (Marine´-Font et al., 1995). The results demonstrated in this study indicate that none of the bacteria tested were histamine producers, suggesting they are safe for human consumption. Although some LAB demonstrate substantial histidine decarboxylase activity, little histamine production was observed in the LAB isolated in this study (Joosten & Northold, 1989 and Straub et al., 1995).

Molecular characterization of the selected bacterial isolates

Nucleotide sequencing of 500 bases of the 16S rRNA gene for the eight bacterial isolates revealed that they belonged to two species of lactobacilli. Isolates NMP47620, NMP47621, NMP47625, NMP47628 and NMP47634 were identified as *L. rhamnosus*, while isolates NMP4765, NMP4768 and NMP47633 were identified as *L. plantarum*. The nucleotide sequences of the eight isolates were submitted to the BLASTN database (www.ncbi.nlm.nih.gov/blastn), whereas the isolates NMP4765, NMP4768 and NMP47633 were deposited in GenBank as *L. plantarum* under the accession numbers KU985432, KU985433 and KU985438, respectively. The other isolates, NMP47620, NMP47621, NMP47625, NMP47628 and NMP47634, were deposited in GenBank as *L. rhamnosus* under the accession numbers KU985434, KU985435, KU985436, KU985437 and KU985439, respectively. The phylogenetic tree of the isolates (Fig. 1) shows that they were separated into 2 clades. Isolates identified as *L. rhamnosus* were grouped together in one clade with a 100% bootstrap value, and those defined as *L. plantarum* were grouped together.

Since isolates NMP4765, NMP4768 and NMP47633 showed 99% blast sequence identity with *L. plantarum*, *L. pentosus* and *L. paraplantarum*, respectively, alignment of the first 350 nucleotides in the gamma region (V3) of the16S rRNA gene, which is the hypervariable region, is shown in Fig. 2. Point mutations present at positions 5, 24, 46 and 226 indicated that these isolates were more closely related to *L. plantarum* than to *L. pentosus* or *L. paraplantarum*. Closely related species of Eubacteria were identified using primers for the V3 region (Muyzer et al., 1993 and Heuer & Smalla, 1997). Simpson et al. (2002) examined a group of lactobacilli from the gastrointestinal tracts of animals and showed that the V3 region permitted discrimination between inter-species of lactobacilli.
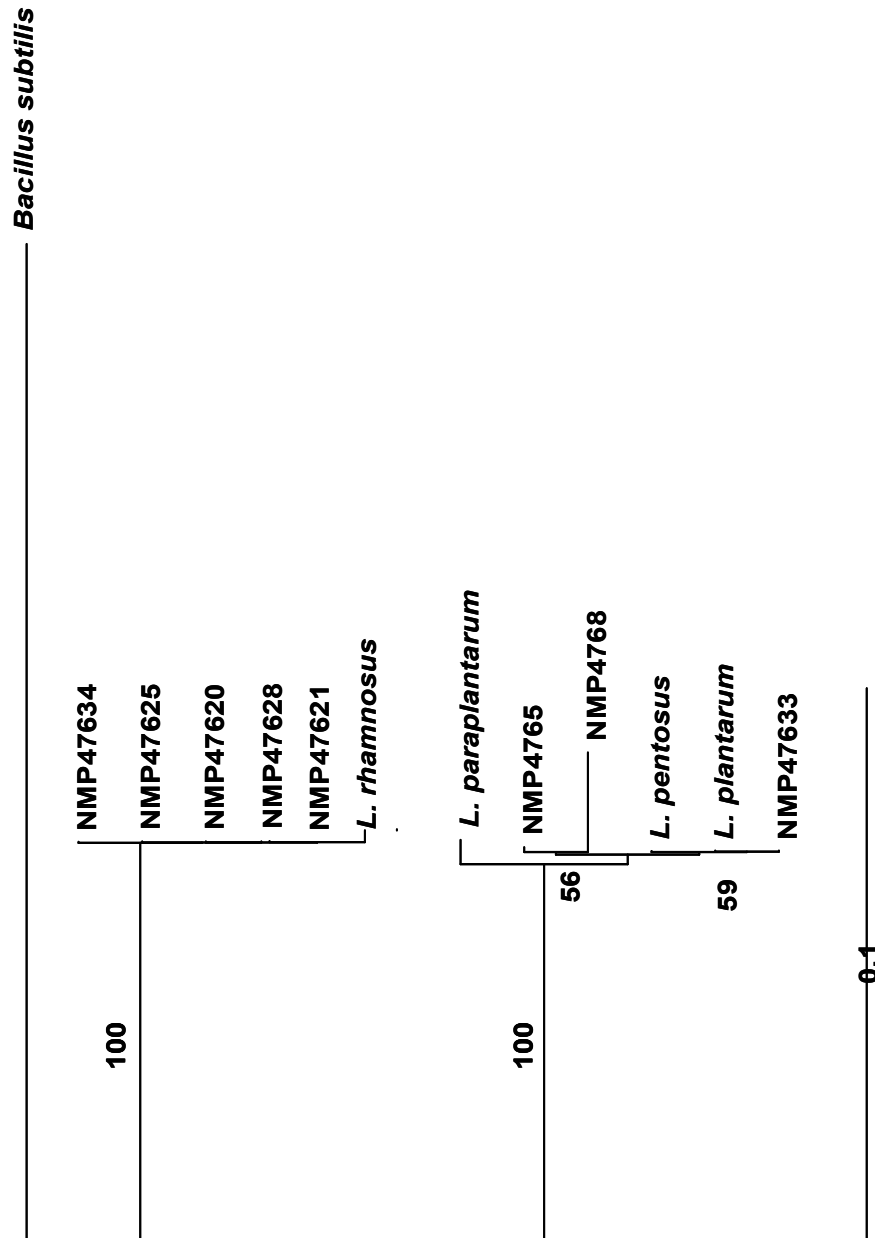
Fig. 1. Neighbor joining phylogenetic tree of partial 16S rRNA sequence of Lactobacillus isolates. The scale bar represents 10% nucleotide substitutions. Percentages of bootstrap values recovered from 100 trees are presented on the nodes.
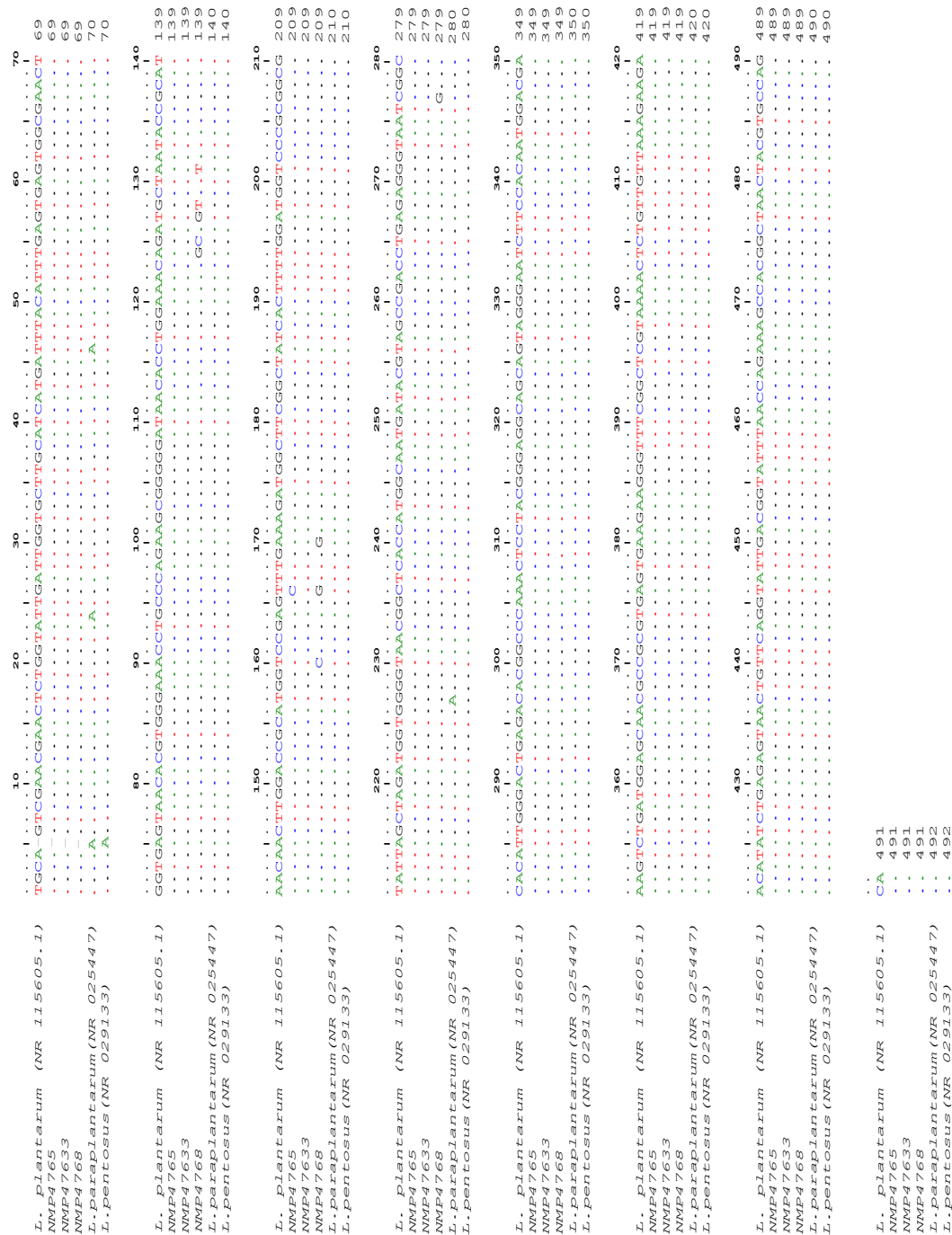
Fig. 2. Alignment of the first 350 nucleotide sequences of 16S rRNA gene of the *L. plantarum* isolates. The dots indicate conserved nucleotides. The alignment showed that the isolates are more related to *L.plantarum* than *L. pentosus* or *L. paraplantarum* in positions 5, 24, 46 and 226

Screening for stress tolerance of viable cells

In preliminary experiments, the 8 LAB isolates were screened for their stress tolerance compared to a control (Fig. 3). The pH stress response patterns are shown in Fig. 3a, 3b and 3c. At pH 2.5, the eight isolates showed considerable growth when sub-cultured after incubation for 3 h, whereas growth decreased greatly after 6 h (10-50%) for all isolates. Maximum growth (>200%) was recorded for isolates NMP47621, NMP47634 and the reference strain. At pH 3.5, the percentage of viable cells after 3 h ranged from approximately 100 to 300%. After 6 h, the same pattern was recorded for all isolates, the percentage of viable cells ranged from 130 to 180% and maximum growth was again observed for isolates NMP47621 and NMP47634. At pH 9, maximum growth (350-400%) was observed for the same isolates, NMP47621 and NMP47634, for both incubation times (3 and 6 h). These two isolates clearly survived acidic and alkaline conditions better than the other isolates, including the reference strain. McDonald et al. (1990) showed that "*L. plantarum* is able to maintain pH homeostasis down to an external pH of 3.0 and growth stops only when the internal pH reaches 4.6–4.8". Furthermore, Sawatari & Yokota (2007) revealed that "*L. plantarum* and *L. pentosus* strains can grow in alkaline pH up to 8.2–8.7, with some variability among strains". These authors also investigated the physiological responses of many *Lactobacillus* strains to alkaline stress and determined that "the pH max of many lactobacilli ranged between 6.7 and 8.9, indicating the alkali tolerance diversity in lactobacilli". Thus, LAB developed specific mechanisms to survive environmental stresses and changes (the stress-sensing system and defences) (van de Guchte et al., 2002). Indeed, it has been postulated that bacterial amino acid decarboxylase functions to control pH by consuming hydrogen ions throughout the carboxylation reaction (Cotter & Hill, 2003).
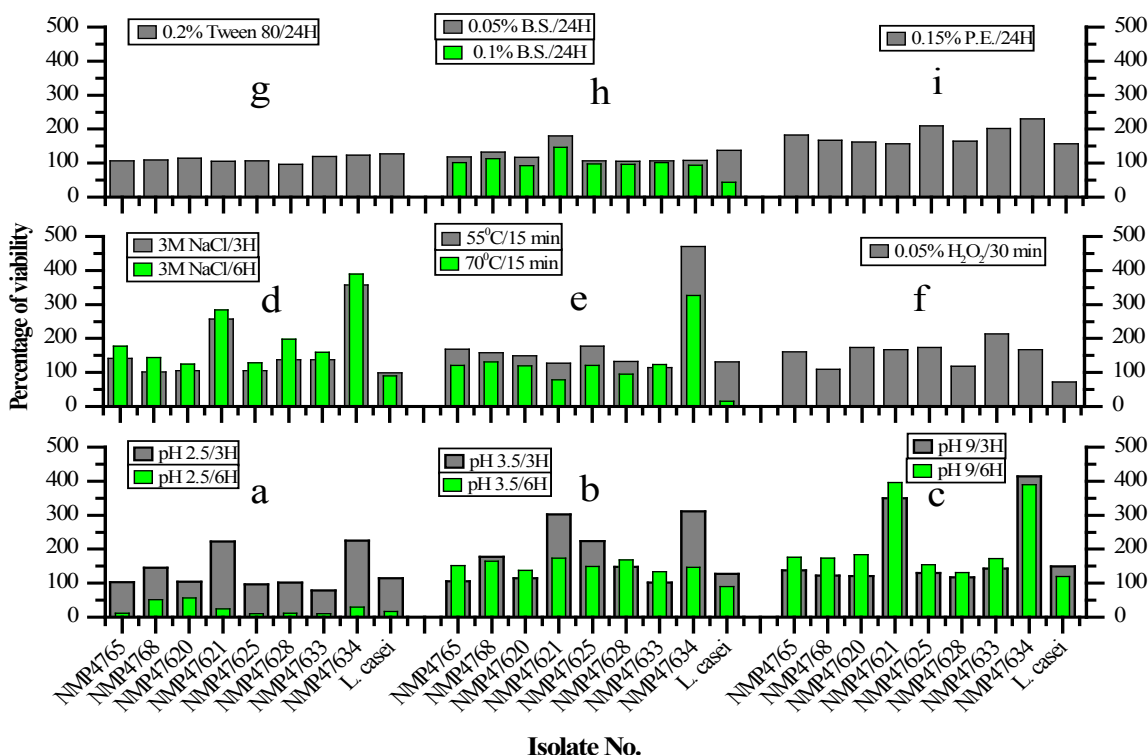

Fig. 3. Stress tolerance response of the most representative Lactobacillus isolates at some stress conditions

The eight isolates exhibited different percentages of growth in MRS supplemented with sodium chloride concentrations as high as 3 M (Fig. 3d). Most isolates showed remarkable resistance to salt concentrations as high as 3 M for 6 h compared to the reference strain. Isolate NMP47634 was the most tolerant, demonstrating an increase in cell viability, which produced a maximum amount of biomass (350-400%). LAB accumulates compatible solutes of organic origin under hyperosmotic conditions (van de Guchte et al., 2002). The intracellular accumulation of compatible solutes prevents the loss of water caused by high external osmolarity (Piuri et al., 2003). These findings were in contrast with those of Kilstrup et al. (1997), who found that "the growth of *Lactococcus lactis* was inhibited at high salt concentrations, about 2.5% NaCl, that is normally found in some cheese types, which decreases the growth to 25 to 50% compared to control". However, Mahin et al. (2010) found that "*Lactococcus lactis* NZ9000 was reported to show improved tolerance to stresses induced by 5 M NaCl for 1 h".

Furthermore, the results illustrated that exposure to temperatures as high as 55 and even 70°C for 15 min did not negatively affect growth and in fact activated it (Fig. 3e), with viability ranging from 114 to 471% at 55°C and from 79 to 327% at 70°C. Isolate NMP47634 was the most heat-tolerant. The lowest viability at 70°C for 15 min was recorded for the reference strain (16%). Castaldo et al. (2006) evaluated the heat resistance of *L. plantarum* LM3 cells during stationary phase and revealed that "bacterial survival after exposure to 50 and 55°C for 1 h was decreased to 42 and 0.07%, respectively". This is considerably lower than what we have observed for *Lactobacillus* strains. In general, LAB heat resistance is a complex process involving proteins with different roles in cell physiology (De Angelis & Gobbetti, 2004).

In contrast, bacterial isolates were sensitive to oxidative stress and survived 30 min of exposure to low concentrations of H_2O_2 (0.05%) (Fig. 3f) but lost activity completely when exposed to 0.1% H_2O_2 for the same duration. Maximum growth (214%) at 0.05% H_2O_2 was observed for isolate NMP47633. These results agreed with the results of Machielsen et al. (2010), who found that exposure to 0.1% hydrogen peroxide caused a notable reduction in the viability of most tested *Lactobacillus* isolates. These authors also

indicated that all strains tolerant to heat stress were also tolerant to oxidative stress.

LAB are facultative anaerobes, meaning that oxygen is not needed for growth. Moreover, oxygen does not negatively affect the growth and survival of most bacteria. In general, LAB withstands oxygen but prefers nearly anaerobic conditions. In the presence of oxygen and during fermentation, H_2O_2 is liberated. Numerous species of LAB contain peroxidase to prevent the deleterious effects imposed by H_2O_2 (van de Guchte et al., 2002).

Bacterial isolates, including the reference strain, also showed tolerance to surfactants, such as Tween 80 (Fig. 3g) and pancreatic enzymes (Fig. 3h), but were slightly sensitive to bile salts (Fig. 3i). Maximum growth (124%) at 0.2% Tween 80 was observed for isolate NMP47634. Bacterial isolates displayed a lower tolerance to bile salts compared to their tolerance to pancreatin. The growth of the eight bacterial isolates ranged from 100 to 180% at 0.05% bile and between 90 and 140% at 0.1% bile for 24 h. Isolates NMP4768 and NMP47621 were the most tolerant. Mathara et al. (2008) indicated that the bile tolerance of some *L. plantarum* isolated from Maasai fermented products demonstrated greater variability. Moreover, Burns et al. (2011) found that "*L. delbrueckii* subsp. *lactis* 200 exhibited lower growth in the presence of 0.30% or 0.50% (w/v) of bile salts, compared to the control".

In contrast, all eight isolates survived the stressful conditions caused by pancreatin, and their growth was greater than the growth observed with bile salts. These results agreed with the results of Temirova (2016), who reported that "some *Lactobacillus* strains showed high tolerance to pancreatic enzymes with a reduction in cell viability of only 0.07 - 0.19 log units". Sensitivity to bile salts is expected due to the toxic and deleterious effect of bile salts on bacterial cells. Bile salts induce intracellular acidification, meaning that many resistance mechanisms are activated in response to bile stress (Lebeer et al., 2008). Our results revealed that LAB were able to survive in stress environments without growing. Pre-adaptation of LAB to mild stress may lead to resistance to higher concentrations of the same stressors as well as to other stress-imposing conditions (O'Driscoll et al., 1996).

Qualitative determination of BSH activity
Cholesterol is the precursor of primary bile salts

that are formed in the liver and stored as conjugated bile salts in the gall bladder (Corzo & Gilliland, 1999b). Deconjugation of the conjugated bile salts by probiotic bacteria results in free bile acids that are less soluble and subsequently precipitate and are excreted from the body. Therefore, the deconjugation of bile salts reduces serum cholesterol levels, as new bile salts will be formed to replace those that have been depleted (Reynier et al., 1981). Some authors have suggested that deconjugation is a detoxification mechanism for the cell, and lactobacilli may express BSH as a protective mechanism against bile salt toxicity (Savage, 1992; De Smet et al., 1995 and Grill et al., 2000). In this study, the eight LAB isolates and the reference strain *L. casei* demonstrated a detectable ability to deconjugate bile salts, which was represented as an opaque halo around the colonies on MRS agar supplemented with 0.1% conjugated bile salts.

Quantitative determination of BSH activity

BSH enzyme expression is another method used by LAB to lower cholesterol. BSH-containing probiotic strains are preferred over BSH-negative strains because they help remove cholesterol. The BSHs described in several probiotic organisms are produced in the intracellular space; therefore,

overnight cultures were individually subjected to sonication and treated with 1% CTAB to chemically disrupt the bacterial cell walls and liberate intracellular BSH or were left untreated to use as active cells. The BSH activities of the eight isolates (Fig. 4) indicated that the active cells of the isolates exhibited the highest BSH enzyme activity, ranging from 90 to 142 U/mg. The active cells of three isolates had relatively high levels of activity (NMP4768, NMP47633 and NMP47634), while the other isolates (NMP4765, NMP47620, NMP47621, NMP47625 and NMP47628) exhibited moderate activities. The results also revealed that the sonicated cells presented the lowest BSH activities (ranging from 25 to 44%), while treatment with CTAB resulted in activity that ranged from 31 to 48%. The reference strain *L. casei* produced moderate amounts of the enzyme with the three different treatment methods compared to our isolates (approximately 30, 36 and 74 U/mg using sonicated, CTAB-treated or active cells, respectively). Tanaka et al. (1999) reported that BSH activity is sensitive to sonication and other cell disruption techniques, as well as to oxygen. This indicates that the use of active cells, with no need for enzyme extraction, is better for cholesterol reduction and will facilitate cholesterol reduction by LAB when used *in vivo*.
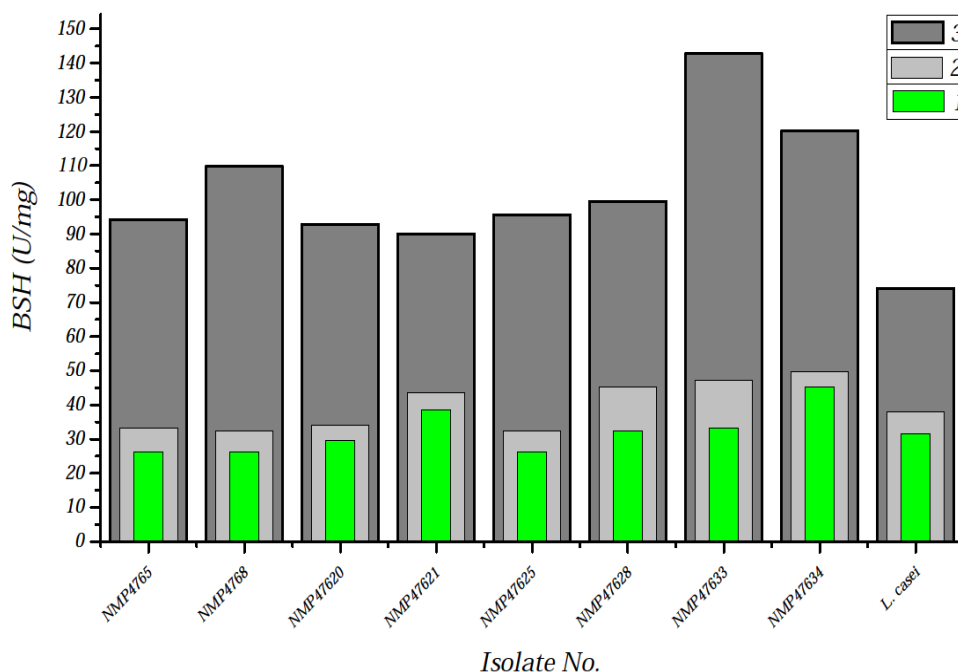

Fig. 4. Bile salt hydrolase activity of the most representative Lactobacillus isolates (1: sonicated cells, 2: CTAB-treated cells, 3: active cells).

The results of Jiang et al. (2010) showed that some strains of *L. casei* and *L. salivarius* did not exhibit any BSH activity, whereas *L. helveticus*, *L. fermentum* and *L. gallinarum* strains only deconjugated taurine-conjugated bile salts. As such, their BSH activity was very low, ranging from 8.7 to 32.3 U/mg. Moreover, these authors mentioned that *L. plantarum* strains deconjugated both glycine-conjugated and taurine-conjugated bile salts with higher activities, ranging from 23.6 to 54.5 U/mg.

Antibiotic susceptibility test
The antibiotic susceptibilities of the eight

bacterial isolates to the five tested antibiotics are shown in Table 1. The results revealed that all bacterial strains, including the reference strain *L. casei*, were resistant to vancomycin and susceptible to amoxicillin, cloxacillin and penicillin, with the exception of isolate NMP4765, which was resistant to all the aforementioned antibiotics. For oxy-tetracycline and gentamycin, all strains were resistant or intermediately resistant with the exception of the reference strain *L. casei* and isolate NMP47625, which were susceptible to oxy-tetracycline.

TABLE 1. Antibiotic resistance of *Lactobacillus* isolates

Lactobacillus isolate No.	Antibiotic disc (30 mcg)				
	Vancomycin	Oxy-tetracycline	Gentamycine	Amoxycillin & Cloxacillin	Penicillin
NMP4765	R	R	R	R	IR
NMP4768	R	R	R	S	S
NMP47620	R	R	IR	S	S
NMP47621	R	IR	IR	S	S
NMP47625	R	S	IR	S	S
NMP47628	R	IR	R	S	S
NMP47633	R	R	R	S	S
NMP47634	R	R	R	S	S
L. casei	R	S	IR	S	S
R	Resistant (no inhibition zone)				
IR	Intermediate resistant (inhibition zone 1.5 cm or less)				
S	Susceptible (inhibition zone larger than 1.5 cm)				

The acquisition of antibiotic resistance in bacteria is common in species living in human and animal intestines, which are frequently treated with antibiotics (Teuber et al., 1999). Lactobacilli showed intrinsic resistance towards some frequently consumed antibiotics, such as vancomycin. This resistance to vancomycin was due to the presence of D-alanine and D-alanine ligase-related enzymes (Elisha & Courvalin, 1995). *Lactobacillus* resistance to vancomycin distinguishes Lactobacilli from other Gram-positive bacteria on vancomycin selective media (Simpson et al., 1988).

Cell surface hydrophobicity test
Bacterial adhesion to hydrocarbons has been widely used to measure cell surface hydrophobicity in

LAB. Hydrophobic characteristics of LAB cells are, to a certain extent, related to epithelial adhesion. The tested bacterial isolates, including the reference strain *L. casei*, were highly hydrophobic (>70%) (Table 2). Cell surface hydrophobicity ranges from 73 to 89%. None of the strains were included within the category of low cell surface hydrophobicity (0–35%). Isolates NMP4768, NMP47628 and NMP47633 were the most hydrophobic strains. The hydrophobic nature of the outer surface of lactobacilli is involved in bacterial attachment to host tissues, which contributes to the colonization of mucosal surfaces. This property confers a competitive advantage, which is important for bacterial maintenance in the human GIT (Naidu et al., 1999).

TABLE 2. *In vitro* **hydrophobicity of** *Lactobacillus* **isolates on n-hexadecane**

Lactobacillus isolate No.	H%	*Lactobacillus* isolate No.	H%	*Lactobacillus* isolate No.	H%
NMP4765	83	NMP47621	81	NMP47633	88
NMP4768	89	NMP47625	82	NMP47634	82
NMP47620	73	NMP47628	87	*L. casei*	81

The results of Duary et al. (2011) revealed that "*L. plantarum* Lp91 showed 35.73 percentage hydrophobicity", while the results of Blajman et al. (2015) revealed that "*L. salivarius* DSPV 003P showed a higher percentage hydrophobicity (78%)" for n-hexadecane.

Conclusion

In conclusion, the data revealed that the eight bacterial isolates investigated in this study are probiotic bacteria that possess cholesterol reduction properties with the ability to survive conditions in the human gastrointestinal tract. The isolates belonged to *L. plantarum* and *L. rhamnosus* and were active cholesterol reducers, as indicated by bile salt deconjugation, BSH activity, and high hydrophobic activity. Isolate NMP47634 was the most tolerant strain and holds potential as a probiotic.

References

Araki, Y.I., Lee, S., Sugihara, G., Furuichi, M., Yamashita, S. and Ohseto, F. (1996) New cationic surfactants derived from bile acids: synthesis and thermodynamic and biophysicochemical properties such as membrane perturbation and protein solubilizing abilities. *Colloids Surf., B: Biointerfaces,* **8,** 81-92.

Blajman, J., Gazianob, C., Zbrun, M.V., Sotoa, L., Astesana, D., Berisvil, A., Scharpen, A.R., Signorini, M. and Frizzo, L. (2015) *In vitro* and *in vivo* screening of native lactic acid bacteria toward their selection as a probiotic in broiler chickens. *Res. in Vet. Sci.* **101**,50-56.

Bove, P., Gallone, A., Russo, P., Capozzi, V., Albenzio, M., Spano, G. and Fiocco, D. (2012) Probiotic features of *Lactobacillus plantarum* mutant strains.

Appl. Microbiol. and Biotechnol. **96,** 431-441.

Bover-Cid, S. and Holzapfel, W.H. (1999) Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol.* **53**, 33-41.

Burns, P., Reinheimer, J. and Vinderola, G. (2011) Impact of bile salt adaptation of *Lactobacillus delbrueckii* subsp. lactis 200 on its interaction capacity with the gut. *Res. in Microbiol.* **162**, 782-790.

Castaldo, C., Siciliano, R.A., Muscariello, L., Marasco, R. and Sacco, M. (2006) CcpA affects expression of the groESL and dnaK operons in *Lactobacillus plantarum. Microb. Cell Fact,* **5**, 35.

CLSI (2006) Performance standards for antimicrobial disk susceptibility tests; Approved Standard e Ninth Edition. NCCLS: 940 West Valley Road Suite 1400, Wayne, PA, M2eA9.

Corzo, G. and Gilliland, S.E. (1999a) Measurement of bile salt hydrolase activity from *Lactobacillus acidophilus* based on disappearance of conjugated bile salts. *J. Dairy Sci.* **82**, 466-71.

Corzo, G. and Gilliland, S.E. (1999b) Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus. J. Dairy Sci.* **82**, 472-480.

Cotter, P.D. and Hill, C. (2003) Surviving the acid test: responses of Gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* **67**, 429-453.

De Angelis, M. and Gobbetti, M. (2004) Environmental stress responses in *Lactobacillus*: A review. *Proteom,* **4**, 106-122.

De Smet, I., van Hoorde, L., van de Woestyne, M., Cristiaens, H. and Verstraete, W. (1995) Significance of bile salt hydrolytic activities of lactobacilli. *J. Appl. Bacteriol.* **79**, 292-301.

Duary, R.K., Rajput, Y.S., Batish, V.K. and Grover, S. (2011) Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J. Med. Res.* **134**, 664-671.

Edwards, U., Rogall, T., Blocker, H., Emde, M. and Bottger, E. (1989) Isolation and complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* **17,** 7843–7853. Elisha BG and Courvalin P (1995) Analysis of genes encoding D-alanine: D-alanine ligase-related enzymes in *Leuconostoc mesenteroides* and *Lactobacillus* spp. *Gene,* **152**, 79-83.

FAO/WHO (2002) Guidelines for the evaluation of probiotic in food. http://www.who.int/foodsafety/ publications/fs_management/ probiotic2/en/.pp 1–11.

Grill, J.P., Cayuela, C., Antoine, J.M. and Schneider, F. (2000) Isolation and characterization of a *Lactobacillus amylovorus* mutant depleted in conjugated bile salt hydrolase activity: Relation between activity and bile salt resistance. *J. Appl. Microbiol.* **89**, 553-563.

Harrigan, W.F. and McCance, M.E. (1976) "*Laboratory Methods in Food and Dairy Microbiology*", Revised Edition. Academic Press Limited, London (UK).

Heuer, H. and Smalla, K. (1997) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. In: "*Modern Soil Microbiology*", van Elsas, J.D., Trevors, J.T. and Wellington, E.M.H. (Ed.), pp. 353-373. Marcel Dekker, New York.

Heuer, H., Krsek, M., Baker, P., Smalla, K. and Wellington, E.M.H. (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel electrophoretic separation in denaturing gradients. *Appl. Environ. Microbiol.* **63,** 3233-3241.

Ishikawa, J., Tsuchizaki, N., Yoshida, M., Ishiyama, D. and Hotta, K. (2000) Colony PCR for detection of specific DNA sequences in actinomycetes. *Actinomycetologia,* **14**(1), 1-15.

Isolauri, E., Sutus, Y., Kankaanpää, P., Arvilommi, H. and Salminen, S. (2001) Probiotics: Effects on immunity. *Am. J. Clin. Nutr.* **73**, 444-450.

Jiang, J., Hang, X., Zhang, M., Liu, X.., Li, D. and Yang, H. (2010) Diversity of bile salt hydrolase activities in different lactobacilli toward human bile salts. *Ann. Microbiol.* **60**, 81–88.

Joosten, H.M.L.J. and Northold, M.D. (1989) Detection, growth and amine-producing capacity of lactobacilli in cheese. *Appl. Environ. Microbiol.* **55**(9), 2356-2359.

Kilstrup, M., Jacobsen, S., Hammer, K. and Vogensen, F.K. (1997) Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis. Appl. Environ. Microbiol.* **63**, 1826-1837.

Kumar, A. and Kumar, D. (2015) Characterization of *Lactobacillus* isolated from dairy samples for probiotic properties. *Anaerobe* **33**,117–123.

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S.C.J. (2008) Genes and molecules of lactobacilli supporting probiotic action. *Microbiol. Mol. Biol. R.* **72**, 728-764.

Lee, Y.P. and Takahashi, T. (1966) An improved colorimetric determination of amino acids with the use of ninhydrin. *Anal. Biochem.* **14**, 71-77.

Liong, M.T. and Shah, N.P. (2005) Acid and bile tolerance and the cholesterol removal ability of lactobacilli strains. *J. Dairy Sci.* **88**, 55-66.

Lombardi, A., Gatti, M., Rizzotti, L., Torriani, S., Andrighetto, C. and Giraffa, G. (2004) Characterization of *Streptococcus macedonicus* strains isolated from artisanal Italian raw milk cheeses. *Int. Dairy J.* **14**, 967-976.

Lourens-Hattingh, A. and Viljoen, B.C. (2001) Yogurt as probiotic carrier food. *Int. Dairy J.* **11**, 1-17.

Lye, H.S., Rusul, G. and Liong, M.T. (2010) Removal of cholesterol by lactobacilli via incorporation of and conversion to coprostanol. *J. Dairy Sci.* **93**, 1383-1392.

Machielsen, R., van Alen-Boerrigter, I.J., Koole, L.A., Bongers, R.S., Kleerebezem, M. and Van Hylckama Vlieg, J.E.T. (2010) Indigenous and environmental modulation of frequencies of mutation in *Lactobacillus plantarum. Appl. Environ. Microbiol.* **76**, 1587-1595.

Mahin, A.A., Sugimoto, S., Higashi, C., Matsumoto, S. and Sonomoto, K. (2010) Improvement of multiple-stress tolerance and lactic acid production in *Lactococcus lactis* NZ9000 under conditions of thermal stress by heterologous expression of *Escherichia coli* dnaK. *Appl. Environ. Microbiol.* **76** 4277-4285.

Manson, J.E., Tosteson, H., Ridker, P.M., Sattereld, S., Herbert, P. and O'Conner, G.T. (1992) The primary prevention of myocardial infarction. N. *Engl. J. Med.* **326**, 1406–1416.

Marine´-Font, A., Vidal-Carou, M.C., Izquierdo-Pulido, M., Veciana-Nogue´s, M.T. and Herna´ndez-Jover, T. (1995) Les amines bioge`nes dans les aliments:

leur signification. Leur. analyse *Ann. Fals. Exp. Chim.* **88**, 119-140.

Mathara, J.M., Schillinger, U., Kutima, P.M., Mbugua, S.K., Guigas, C., Franz, C. and Holzapfel, W.H. (2008) Functional properties of *Lactobacillus plantarum* strains isolated from Maasai traditional fermented milk products in Kenya. *Curr. Microbiol.* **56**, 315- 321.

McDonald, L.C., Fleming, H.P. and Hassan, H.M. (1990) Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **56**, 2120-2124.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.

Naidu, A.S., Bidlack, W.R. and Clemens, R.A. (1999) Probiotic spectra of lactic acid bacteria (LB). *Crit. Rev. Food Sci. and Nut.* **38**, 13-126.

Noh, D.O., Kim, S.H. and Gilliland, S.E. (1997) Incorporation of cholesterol into the cellular membrane of *Lactobacillus acidophilus* ATCC 43121. *J. Dairy Sci.* **80**, 3107-3113.

Nomoto K (2005) Prevention of infections by probiotics. *J. Biosci. Bioeng.* **100**, 583-592.

O'Driscoll, B., Gahan, C.G.M. and Hill, C. (1996) Adaptive acid tolerance response in *Listeria monocytogenes*: Isolation of an acid-tolerant mutant which demonstrates increased virulence. *Appl. Environ. Microbiol.* **62**, 1693-1698.

Parente, E., Ciocia, F., Ricciardi, A., Zotta, T., Felis, G.E. and Torriani, S. (2010) Diversity of stress tolerance in *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*: A multivariate screening study. *Int. J. Food Microbiol.* **144**, 270-279.

Pereira, D.I.A. and Gibson, G.R. (2002a) Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. *Crit. Rev. Biochem. Mol. Biol.* **37**(4), 259-281.

Pereira, D.I.A. and Gibson, G.R. (2002b) Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. *Appl. Environ. Microbiol* **68**, 4689-4693.

Pigeon, R.M., Cuesta, E.P. and Gilliland, S.E. (2002) Binding of free bile acids by cells of yoghurt starter culture bacteria. *J. Dairy Sci.* **85,** 2705-2710.

Piuri, M., Sanchez-Rivas, C. and Ruzal, S.M. (2003) Adaptation to high salt in *Lactobacillus*: role of peptides and proteolytic enzymes. *J. Appl. Microbiol.* **95**, 372-379.

Preter, V.D., Vanhoutte, T., Huys, G., Swings, J., et al. (2007) Effects of *Lactobacillus casei Shirota*, *Bifidobacterium breve*, and oligofructose-enriched inulin on colonic nitrogen-protein metabolism in healthy humans. *Am. J. Physiol.* **129**, 358-368.

Recsei, P.A., Moore, W.M. and Snell, E.E. (1985) Pyruvoyl-dependent histidine decarboxylases from *Clostridium perfringens* and *Lactobacillus buchneri*. *J. Biol. Chem.* **258** (1), 439-444.

Reynier, M.O., Montet, J.C., Gerolami, A., Marteau, C., Crotte, C., Montet, A.M. and Mathieu, S. (1981) Comparative effects of cholic, chenodeoxycholic and ursodeoxycholic acids on micellar solubilization and intestinal absorption of cholesterol. *J. Lipid Res.* **22**, 467- 473 .

Roller, M., Femia, A.P., Caderni, G., Rechkemmer. G. and Watzl, B. (2004) Intestinal immunity of rats with colon cancer is modulated by oligofructose-enriched inulin combined with *Lactobacillus rhamnosus* and *Bifidobacterium lactis*. *Br. J. Nutr.* **92**, 931-938.

Ruiz, L., Ruas-Madiedo, P., Gueimonde, M., De Los Reyes-Gavilán, C.G., Margolles, A. and Sánchez, B. (2011) How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences. *Genes and Nutr.* **6** (3), 307-318.

Sanders, M.E. (2000) Considerations for use of probiotic bacteria to modulate human health. *J. Nutr.* **130**, 384-390.

Savage, D.C. (1992) Growth phase, cellular hydrophobicity and adhesion *in vitro* of lactobacilli colonizing the keratinizing gastric epithelium in the mouse. *Appl. Environ. Microbiol.* **58**, 1992–1995.

Sawatari, Y. and Yokota, A. (2007) Diversity and mechanisms of alkali tolerance in lactobacilli. *Appl. Environ. Microbiol.* **73** (12), 3909-3915.

Simpson, J.M., Kocherginskaya, S.A., Aminov, R.I., Skerlos, L.T., Bradley, T.M., Mackie, R.I. and White, B.A. (2002) Comparative microbial diversity in the gastrointestinal tracts of food animal species. *Integ. Comp. Biol.* **42**, 327-331.

Simpson, W.J., Hammond, J.R.M. and Miller, R.B. (1988) Avoparcin and vancomycin: Useful antibiotics for the isolation of brewery lactic acid bacteria. *J. Appl. Bacteriol.* **64**, 299–310.

Straub, B.W., Kicherer, M., Schilcher, S.M. and Hammes, W.P. (1995) The formation of biogenic amines by fermentation organisms. *Z. Lebensm. Unters. Forsch.* **201**, 79–82.

Tabas, I. (2002) Cholesterol in health and disease. *J. Clin. Invest.* **110**, 583–590.

Tanaka, H., Doesburg, K., Iwasaki, T. and Mierau, I. (1999) Screening of lactic acid bacteria for bile salt hydrolase hctivity. *J. Dairy Sci.* **82**, 2530-2535.

Tanasupawat, S., Okada, S. and Komagata, K. (1998) Lactic acid bacteria found in fermented fish in Thailand. *J. Gen. Appl. Microbiol.* **44**,193-200.

Taranto, M.P., de Ruiz Holgado, A.P. and de Valdez, G.F. (1995) Bile salt hydrolase activity in *Enterococcus faecium* strains. *Microbiologie-Aliments-Nutrition,* **13**, 375-379.

Temirova, G. (2016) Antibiotic resistance and probiotic properties of lactic acid bacteria isolated from camel milk and shubat. *MSc Thesis.* Institute of Public Health and Clinical Nutrition.

Teuber, M., Meile, L. and Schwarz, F. (1999) Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek,* **76**, 115-137.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D. and Maguin, E. (2002) Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek,* **82**, 187-216.

Vinderola, C.G. and Reinheimer, J.A. (2003) Lactic acid starter and probiotic bacteria: A comparative *'in vitro'* study of probiotic characteristics and biological barrier resistance. *Food Res. Int.* **36**, 895-904.

Ward, S., Lloyd, J.M. and Pandor, A. (2007) A systematic review and economic evaluation of statins for the prevention of coronary events. *Health Technol. Assess.* **11**(14), 1-160.

Wood, B.J.B. and Holzapfel, W.H. (1995) "*The Genera of Lactic Acid Bacteria*", vol. 2. Blackie Academic and Professional, Glasgow (UK).

خصائص المدعمات الحيوية و نشاط إنزيم **Bile Salt Hydrolase** لبكتيريا حمض اللاكتيك المعزولة محليا

أسماء نجم الدين، عزة محمد نور الدين، سحر طلبة*، إيناس حامد الشطورى*، غادة أحمد عواد، محمد خالد إبراهيم*و محمد عبد الفتاح فريد

قسم كيمياء المنتجات الطبيعية والميكروبية ـ المركز القومى للبحوث ـ و*قسم الميكروبيولوجى ـ كلية العلوم ـ جامعة عين شمس ـ القاهرة ـ مصر.

تم عزل ثماني عزلات من بكتيريا حمض اللبنيك من مصادر غذائية وغير غذائية و تم تعريفها جينيا بواسطة 16S rRNA. أظهر التعريف الجيني للعزلات أنها تنتمى لنوعين من البكتيريا، *Lactobacillus plantarum* و*Lactobacillus rhamonsus* ثم تمت مقارنة هذه السلالات مع سلالة معرفة وهى *Lactobacillus casei* لتقييم خصائصها كمدعمات حيوية، مثل نشاط تحلل الدم، والقدرة على تكوين الهستامين، ومقاومة الظروف القاسية ، ومقاومتها للمضادات الحيوية والقدرة على الإلتصاق بالوسط الحيوى. كما تم تقييم نشاط إنزيم BSH. أظهرت النتائج أن كل العزلات لم تظهر أي نشاط لتحلل الدم أو تكوين الهستامين. كما أظهرت قدرتها على تحمل الظروف الحمضية والقلوية (2.5 pH ، 3.5 و 9) لمدة 3 و 6 ساعات، وكذلك الاسموزية (3M كلوريد الصوديوم) والحرارة (55 و 70 درجة مئوية) ، ولكن كانت أكثر تأثرا بالتعرض للأكسدة وأملاح الصفراء. وأنتجت العزلات البكتيرية أيضا كميات عالية من إنزيم BSH بنسب تتراوح من 90 إلى 142 وحدة/ مج في الخلايا النشطة، بالمقارنة مع *Lactobacillus casei* (74 وحدة / مج)، والتي قد تكون مفيدة في خفض الكوليسترول. وأبدت جميع العزلات البكتيرية مقاومة للفانكومايسين وحساسية لأموكسيسيلين، كلوكساسيلين والبنسلين. وكانت لجميع العزلات قدرة عالية على الإلتصاق بالوسط الحيوى (< 70٪)، مما يؤكد قدرتها على الإلتصاق بسهولة بالأمعاء.