Probiotic Potential of *Lactobacillus plantarum* Isolated from *Zabady*

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ABSTRACT

Twenty one isolates of lactic acid bacteria (LAB) were isolated from *Zabady*, an Egyptian yoghurt, and screened for their antibacterial activities against 8 pathogens (*Bacillus cereus* ATCC 49064, *Clostridium perfringens* ATCC 13124, *Escherichia coli*, *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 19116, *Listeria ivanovii* Li4 (pVS2), *Salmonella enterica* ATCC 25566, and *Yersinia enterocolitica* ATCC 23715) as indicator strains. One isolate was selected as a promising candidate.The selected isolate was identified as *Lactobacillus plantarum* by phenotypic and genotypic methods. Afterwards, the identified isolate was evaluated for a number of probiotic characteristics including tolerance to simulated gastric juice, bile salt resistance, cell surface hydrophobicity, resistance to low phenol concentration, autoggregation, coaggregation, adhesion to rabbit epithelial cells, and reduction of cholesterol. This isolate showed; sensitivity to simulated gastric juice adjusted at pH 2.0 - 3.5, tolerance to 0.3% bile concentration and antimicrobial activity against *Salmonella enterica* ATCC 25566 ((25600 Activity unit /ml), was *Bacillus cereus* ATCC 49064 (25600 Activity unit /ml), *Yersinia enterocolitica* ATCC 23715 (400 Activity unit /ml) and *L. monocytogenes* ATCC 19116 (200 Activity unit /ml) that is probably due to production of active peptides. Furthermore, the results demonstrate that this isolate was highly tolerated to different tested phenol concentrations, moderate cell surface hydrophobicity, weak autoaggregating and reasonable adhesion level to rabbit epithelial cell.

Keywords: ***Antibacterial activity, probiotics, lactic acid bacteria, food pathogenic bacteria, cell surface hydrophobicity.***

INTRODUCTION

The use of probiotics evolved from a theory proposed by the Noble Prize-winning scientist Elie Metchnikoff, who suggested in 1907 (Metchnikoff, 1907) that the prolonged life span of Bulgarian peasants was a result of their consumption of fermented milk products (yoghurt containing lactobacilli), which eliminate putrefactive intestinal bacteria (Stanton *et al.*, 2001, Duggan *et al.*, 2002). The word 'probiotic' originated from Greek meaning 'for life'. Several definitions of probiotic have been suggested by different investigators. Probiotic has been defined as "a live microbial food supplement which beneficially affects the host animal by improving its microbial balance" (Fuller, 1989, Goldin & Gorbach, 1992). In 2001, FAO/WHO defined probiotics as live microorganisms, which when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2001).

A number of health benefits are claimed in favour of products containing probiotic organisms including: antibacterial activity against pathogens, causing improvement in lactose metabolism, antimutagenic properties, anticarcinogenic properties, reduction in serum cholesterol, anti-diarrhoeal properties, immune system stimulation, improvement in inflammatory bowel disease and suppression of *Helicobacter pylori* infection (Kurmann & Rasic, 1991, Shah, 2000 & 2004). Health benefits imparted by probiotic bacteria are strain specific, and not species- or genus specific. It is important to note that no strain will provide all proposed benefits and not all strains of the same species will be effective against defined health conditions. Most probiotics belong to the genera of *Bifidobacterium*, *Enterococcus* and *Lactobacillus* (Klein *et al.*, 1998). However, species belonging to the genera *Saccharomyces* and *Propionibacterium* are also considered as probiotics due to their health-promoting effects (Sanders & Huis in't Veld, 1999, Blandino *et al.*, 2003).

The basic requirements of the strains to be used as probiotics include the following criteria: (1) they

should be generally recognized as safe (GRAS), (2) they should be tolerant to acid and bile, (3) they should be able to adhere to the intestinal epithelium of the hosts, (4) they are able to demonstrate antagonistic activity against pathogenic bacteria, and (5) they should be able to keep their viability during processing and storage (Rial. 2000, Lonkar *et al.*, 2005, Lin *et al.*, 2006). The probiotic must also present some desirable characteristics, such as low cost; maintain its viability during the processing and storage, facility of application in the products, resistance to the physicochemical processing of the food. Therefore, in order to claim that a strain has a probiotic effect, *in vitro* tests are useful to gain knowledge of strains and the mechanism of the probiotic effect.

The objectives of the current study were to isolate, lactic acid bacteria (LAB) from *Zabady* (Egyptian yoghurt), and to screen them against 8 Gram- positive and negative bacteria in order to select a promising antagonistic isolate. One promising isolate was further identified and tested for tolerance to acidic pH and bile, and adhesion to intestinal mucous. The competitiveness of selected isolate against pathogenic bacteria, autoaggregation, coaggregation, adhesion to host epithelial cells and reduction of cholesterol were also determined.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The pathogenic indicator strains and other LAB used in this study were obtained from Microbiological Resources Center (Cairo MIRCEN) Faculty of Agriculture, Ain shams University, Cairo, Egypt. The indicator strains used were *Bacillus cereus* ATCC 49064, *Clostridium perfringens* ATCC 13124, *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 19116, *Salmonella enterica* ATCC 25566, *Yersinia enterocolitica* ATCC 23715. LAB strains used including *Lactococcus lactis* subsp. *lactis* ATCC 19435, *Lactococcus lactis* subsp. *lactis* JCM 7638 and *Streptococcus thermophilus* CCUG 30577. *Listeria ivanovii* Li4 (pVS2) was kindly supplied by Dr. Lars Axelsson (Matforsk, Norwegian Food Research Institute, Akershus, Norway). *Lactobacillus sakei* NCDO 2714, *L. sake* LTH 673 and CTC 494 were kindly provided by Prof. Ingolf Nes (Laboratory of Microbial Gene Technology, Agriculture University of Norway). *E. coli* was isolated and identified at our laboratory. Media used and incubation temperatures applied for all bacterial strains are summarized in Table (1). The cultures used in this study were maintained as frozen stocks at −80°C and propagat-

Table 1. Indicator strains used in the present study and their growth conditions (media and temperatures).

Indicator microorganisms	Medium and growth temperature
Pathogenic bacteria	
Bacillus cereus ATCC 49064	Nutreint Broth, 37 °C
Clostridium perfringens ATCC 13124	Trypticase Soya Broth, 37 °C
Escherichia coli	LB[1] Broth, 37 °C
Listeria innocua ATCC 33090	BHI[2], 37 °C
Listeria monocytogenes ATCC 19116	BHI, 37 °C
Listeria ivanovii Li4 (pVS2)	BHI, 30 °C
Salmonella enterica ATCC 25566	Nutrient Broth, 37 °C
Yersinia enterocolitica ATCC 23715	Tryptose Broth, 37 °C
Lactic acid bacteria	
Lactococcus lactis subsp. *lactis* ATCC 19435	MRS[3], 37 °C
Lactococcus lactis subsp. *lactis* JCM 7638	MRS, 37 °C
Lactobacillus sakei LTH 673	MRS, 37 °C
Lactobacillus sakei CTC 494	MRS, 37 °C
lactobacillus sakei NCDO 2714	MRS, 37 °C
Streptococcus thermophilus CCUG 30577	MRS, 37 °C

[1] LB broth: Luria-Bertani medium. [2] BHI: Brain Heart Infusion broth medium (Oxoid).
[3] MRS: De Man, Rogosa and Sharpe broth medium (Difco).

ed twice (1% inoculum) before experimental use. Agar plates and soft agar were prepared by addition of 1.5 or 0.75% granulated agar (Difco laboratories Ltd., Chad Well Heath, Essex, England) to broth medium, respectively.

Isolation of lactic acid bacterial strains

Many samples of *Zabady* (Egyptian yoghurt) were obtained from various local supermarkets at Alexandria, Egypt. Samples were transported in ice box and stored at 7°C for up to 48 hr. Thirty gram sample was taken aseptically and decimal dilution serials in pepton physiologic salt solution (PPS; NaCl 8.5 g/L and peptone 1 g/l) were prepared. Then diluted samples were spread on de Man, Rogosa and Sharpe (MRS) agar medium. Plates were incubated at 37 or 42°C for 24 hr, white and creamy colonies were randomly selected and further purified on MRS agar. The pure cultures were characterized using Gram stain, cell morphology and catalase reaction according to standard procedures (Sharpe, 1979). Gram-positive and catalase-negative isolates were selected and stored at -80°C in MRS broth plus 28% glycerol (El-Soda *et al.*, 2003). The isolates were twice activated by subculturing in MRS broth before use.

Antibacterial activity of bacterial isolates

The antibacterial activities of isolated bacteria were evaluated against 8 pathogenic strains (Table 1) using the spot-on-lawn assay as described by Barefoot & Klenhammer (1983). The LAB isolates were cultivated in MRS for 16-18 hr with inoculum 1%, cells were removed from MRS medium by centrifugation (6,500 x*g* for 10 min, 4°C) to obtain cell free supernatant. Lawns of indicator strains were prepared by adding 0.125 ml ($2x10^7$) of 10× diluted overnight culture to 5 ml suitable soft agar (0.75%). The contents of culture tubes were gently mixed and poured over the surfaces of pre-poured MRS agar plates. Ten µl of each cell free supernatant were spotted on the surface of the soft agar plate. Clear zones around the spots indicate the antibacterial activity of isolated bacteria.

Quantification of antibacterial activity was carried out on some selected isolates as described by Parente *et al.* (1994) and Deraz *et al.* (2005). Serial two-fold dilutions were carried out in the medium used for growth of the indicator strain. Activity was quantified by taking the reciprocal of the highest dilution that exhibited a clear zone of inhibition and was expressed as activity units (AU) per milliliter of culture media. The titer (AU/ml) of the antibacterial substance was calculated according to the following equation: $(1000/d)\,D$, where D is the dilution factor and d is the amount of supernatant in µl.

To prove the proteinions nature of the selected cell free supernatant, the susceptibility of the neutralized (pH 6.5) and catalase treated supernatant to various proteases was performed by incubating the culture supernatant with 1 mg/ml trypsin (Universal Fine Chemical PVT, LTD, India), pepsin (P-7000, Sigma- Aldrich, St. Louis, MO, USA) and papain (Titan Biotech, LTD) for 1 hr at 37°C. After incubation, the treated supernatants were tested for their antibacterial activity against *Bacillus cereus* ATCC 49064 and *Salmonella enterica* ATCC 25566. Further screening against other LAB (Table 1) was also tested with the most promising isolate.

Identification of bacterial isolates:

Phenotypic characterization

The isolates were phenotyped as described in Bergey's Manual of Systematic Bacteriology (Kandler & Weiss, 1986, Logan & De Vos 2009). The following tests were applied: cell morphology; growth at 10, 25, 37 and 45°C; growth in MRS containing 2.5, 4.0, and 6.5% NaCl; and in MRS broth adjusted to pH 4.5 and 9.6.

Genotypic identification

Isolation of bacterial DNA.

The DNA was isolated and purified from the bacterial cells under study using a procedure reported by Birnboim & Doly (1979). The culture of promising isolate grown in MRS broth at 42°C for 24 hr was used. Cells were collected by centrifugation at 6400 rpm for 5 min using a microcentrifuge (Hettich, MIKRO 20, Germany). Pellet was washed with 1.0 ml distilled water, then resuspended in lysis buffer for 30 min at 65°C with occasional shaking every 5 min and centrifuged for 10 min at 1400 rpm. Briefly, 100 µl supernatant was mixed with 700 µl cold isopropanol and precipitated at room temperature for 10 min, then centrifuged for 15 min at 14000 rpm. The DNA was then washed twice with 70% ethanol and left in air to dry and resuspended in 50 µl sterile water. The DNA sample was mixed with the loading dye on 6:1 volume bases, after which the DNA sample was loaded into the gel and electrophoresis was carried

out at 90 volts for 45 min. The gel was visualized under UV-transilluminator (Syngene Bio Maging, Canada) and photographed.

Polymerase chain reaction

Universal primers identifying LAB designed using the invariant region in the 16S rDNA sequences for LAB (Wang *et al.* 1996), was obtained from Sigma Company (South San Francisco, USA). The reaction mixture (25 µl) contained: 2 µl of DNA extract was added to 23 µl of PCR mixture containing 12.8 µl of nuclease-free water, 2 µl of each primer (5/ CATCCAGTGCAAACCTAAGAG 3/ and 5/ GATCCGCTTGCCTTCGCA 3/) as forward and reverse primers, respectively, 1µl of nucleotide (dNTP) mix, 2.5 µl of PCR buffer, 2.5 µl $MgCl_2$ (25 Mm) and 0.2 µl of Taq polymerase (Strata gene, California, USA).

The PCR amplification was carried out in the thermocycler PCR (Eppendorf, Hamburg, Germany) according to the following program: initial denaturation 94°C/3 min, amplification for 35 cycles [94°C/30 sec (denaturing), 60°C/1 min (annealing), 68 °C/2 min (extension)], then final extension 68°C/7 min. The products were analysed by gel electrophoresis in 20.0 g/L agarose with consecutive staining in ethidium bromide and visualization on a UV transilluminator (Syngene Bio Maging, Canada). The DNA marker 155–970 bp (Top-Bio s.r.o., The Czech Republic) was used as the molecular weight standard.

DNA sequencing

The DNA sequencing reactions were performed in the Central Lab, City for Scientific Research, Alexandria using an automated DNA sequencer (3130 genetic analyzer, Japan). Database searches were performed using the latest release of non-redundant DNA sequence database present at the National Center for Biotechnology Information (NCBI) website located at: http://www.ncbi.nlm.nih.gov/BLAST (Altschul *et al.*, 1997).

Growth pattern and antibacterial production

The growth pattern and antibacterial activity of *L. plantarum* were studied as described by Ivanova *et al.* (1998). An overnight culture of *L. plantarum* was inoculated (1% v/v) into 250 ml MRS broth and incubated at 42°C for 24 hr. Samples were taken at 2 hr. intervals along a period of 24 hr. The growth was followed by measuring absorbance (A) at 620 nm using apectrophotomer (Unico, UV-2102 PC, CO., LTD, USA). Antibacterial activity (AU/ml) was measured in the neutralized (using 1N NaOH) supernatant against *Salmonella enteric* ATCC 25566, one of the most sensitive indicator strain. The experiment was performed in triplicates and repeated twice.

In vitro determination of probiotic potential of *Lactobacillus plantarum*

Acid tolerance:

The tested isolate was propagated twice in MRS broth (1% v/v) for 16-18 hr. at 37°C before experimental use. The cells from 100 ml MRS culture were harvested by centrifugation (4300 ×*g*, 10 min), and washed three times in sterilized phosphate-buffered saline, pH 7.0. The washed cell pellets were then suspended in (1/10)× cultivation volume in the same buffer, hence obtaining a 10-fold increase in cell density. To 1 ml of the washed cell suspension, 5 ml of simulated gastric juice and 1.5 ml NaCl (0.5 w/v) were added. Simulated gastric juice was prepared fresh by suspending pepsin (3 g/L) in sterile saline (0.5% w/v) and adjusting the pH to 2.0, 2.5, 3.0 and 3.5 with concentrated HCl (Charteris *et al.*, 1998).

The materials were vortexed for 10 sec and incubated at 37°C for 3 hr. Aliquots of 0.1 ml were then removed at constant intervals (0, 1, 2 and 3 hr.) for determination of total viable count. Dilutions (in MRS) were made and cells were spreaded on MRS agar. Plates were incubated at 37°C for 72 hr before enumeration (Deraz *et al.*, 2007).

Bile tolerance

Bile containing MRS broth was prepared by adding 0.1, 0.3, and 0.5% (w/v) of ox bile (Sigma-Aldrich, St. Louis, MO, USA). The cells from 100 ml 16-18 hr MRS tested culture were collected by centrifugation (3400×g, 10 min), washed twice in saline (8.5 g NaCl/L) and resuspended in 10 ml MRS broth. This suspension was inoculated (1%) into MRS broth lacking or containing bile. After 0, 1, 2 and 3 hr of incubation at 37°C, viable counts on MRS agar plates were determined (Matijasic & Rogelj, 2000). Experiments of acid and bile tolerance were repeated three times in duplicate analysis.

Cell surface hydrophobicity

The ability of *L. plantarum* to adhere to hydrocarbons was determined according to Thapa *et al.* (2004) with few modifications. This method was

based on adhesion of cells to xylene droplets. *L. plantarum* was grown in 10 ml MRS broth, centrifuged at 6,000×g for 5 min and the cell pellet was washed twice with 10 ml of sterile Ringer solution (6% NaCl, 0.0075% KCl, 0.01% $CaCl_2$ and 0.01% $NaHCO_3$) and resuspended in 10 ml of the same buffer. The absorbance at 600 nm was measured. Cell suspension was mixed with equal volume of xylene and mixed by vortexing for 2 min. The two phases were allowed to separate for 30 min and absorbance at 600 nm of the lower phase was recorded. The percentage of cell surface hydrophobicity was calculated as:

$$\text{Hydrophobicity (\%)} = \frac{OD_{600}\text{ (initial)} - OD_{600}\text{ (with xylene)} \times 100}{OD_{600}\text{ (initial)}}$$

Spectrophotometric autoaggregation assay

Autoaggregation assays were performed according to Ekmekci *et al.* (2009) with certain modifications. Bacteria were grown under aerobic and anaerobic conditions for 16 hr. at 37°C in MRS medium. The activated cells were harvested by centrifugation at 10,000 rpm for 15 min, washed twice in sterilized phosphate- buffered saline (PBS) containing (g/L): NaCl, 8; KH_2PO_4, 0.34; and K_2HPO_4, 1.21 (pH 6.0) and resuspended in the same buffer to give a final absorbance (A_1) of 0.60 ± 0.02 at 600 nm as measured by a spectrophotometer (Hitachi, T1800, Tokyo, Japan). For aerobic and anaerobic autoaggregation experiments, cell suspensions (2 ml) were mixed by vortexing for 10 sec and autoaggregation was determined after 4 hr of incubation at 37°C. The absorbance (A_2) was measured at 600 nm after 4 hr. The percent autoaggregation was expressed as follows:

$$\text{Autoaggregation \%} = [(A_1 - A_2) / (A_1) \times 100]$$

Where A_1 represents the cell absorbance at zero time and A_2 the cell absorbance after 4 hr.

Coaggregation assay

Coaggregation of *L. plantarum* with other LAB (*Enterococcus faecium*) and some pathogenic bacteria (*Bacillus cereus* ATCC 49064, *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 19116 and *Salmonella enterica* ATCC 25566) was determined as described by Basson *et al.* (2008). LAB and pathogenic bacteria were grown in MRS and Brain Heart Infusion (BHI) broth, respectively at 37°C for 24 hr. Cells were harvested (7000 *xg*, 10 min, 20°C), washed twice with sterilized PBS, suspended in sterile saline and then diluted to A_{660} nm=0.3. Cell suspensions (500 µl of each) were combined, incubated at 37°C for 60 min, then centrifuged (300 *xg*, 2 min, 20°C) and the absorbance (at 600 nm) of the supernatant was measured and coaggregation was calculated as follows:

$$\text{Coaggregation} = (A_{Tot} - A_S / A_{Tot})\ 100$$

Where A_{Tot} refers to absorbance immediately after the strains were paired. A_S refers to the absorbance of the supernatant after 60 min of incubation and centrifugation at 300 *xg* for 2 min at 20°C.

Experiments of hydrophobicity, autoaggregation and coaggregation were conducted in triplicate on two separate occasions.

Tolerance to phenol

Phenol tolerance experiments were performed as described by Aswathy *et al.* (2008) with some modifications. An overnight culture of *L. plantarum* was inoculated (1%) into MRS broth with or without various phenol concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 % v/v). Bacterial cells in the culture broth were measured by reading the absorbance (A) at 600 nm after 0, 2, 4, 6, 8, 10, 12, and 24 hr of incubation at 42°C. The experiments were repeated two times in duplicate.

Adhesion to rabbit epithelial cells

Epithelial cells were obtained from the small intestines of rabbit as described by Alwan *et al.*, (1998). Briefly, 7 cm sections of freshly collected duodenum from a healthy rabbit was slit open, washed with cold 0.1 M PBS (4°C), and incubated at 20 °C in PBS buffer containing 10 mM EDTA for 20 min. The section was then rinsed twice with PBS to remove the EDTA and placed in 5 ml of cold PBS. Epithelial cells were dislodged by rubbing the intestine with a sterile syringe plunger and the epithelial-rich supernatant (which was identified from other cell fractions, such as leukocytes, on the basis of cell size and morphology) was removed with a sterile Pasteur pipette, pelleted by centrifugation at 100 *xg* for 10 min, washed twice with PBS and centrifuged (100 *xg* for 5 min) then resuspended in 2 ml PBS. A cell count was performed with hymocytometer after staining with crystal violet blue solution and viewed under light microscope.

Adhesion of LAB to intestinal epithelial cells was determined by microscopic examination. Approximately 1 ml of *L. plantarum* syspension in

PBS containing 1×10^9 cells was mixed with an equal volume of epithelial cells (1×10^6 cells/ml) and incubated for 30 min at 37°C. Epithelial cells were then pelleted by centrifugation at 100 *xg* for 10 min and washed twice with PBS and centrifuged (100 *xg* for 5 min), to remove any unattached bacteria, and resuspended in 5 ml PBS. Then, 0.5 ml of this suspension was centrifuged at 150 *xg* for 5 min in a cytocentrifuge (Hettich, MikRo 20; Bavaria, Germany) to prepare microscope slides. The slides were air-dried at room temperature and stained with crystal violet solution. The number of LAB attaching to a single epithelial cell was quantitated at a magnification of x100 under oil immersion using a light microscope(LABomed, laboAmerica, Inc, USA). Experiment was repeated three times. For each experiment, 50 epithelial cells were viewed and the mean number of attached bacteria was calculated. As a control, *E. coli* was processed in the same manner.

Cholesterol assimilation

The capacity of *L. plantarum* to reduce cholesterol was carried out according to the method of Mishra & Prasad (2005). The study was conducted using MRS broth supplemented with 0.2% sodium thioglycolates and 0.3% oxgall as selectivity agent for bile tolerance. The medium was sterilized at 121°C for 15 min. The buffalo serum (cholesterol sources), was obtained from the slaughterhouse of farm belonging to Department of Animal Production, Alexandria University, Egypt. The buffalo serum was sterilized by syring filter in 0.22 µm. The initial total cholesterol content of buffalo serum was 110 ± 2.2 dl. The serum was added at a rate of 10% into sterile medium and mixed thoroughly, then 10 ml of medium were distributed in test tubes. Active test culture was then inoculated into each tube (1%). The uninoculated tube was kept as a control. The tubes were incubated at 37°C for 24 hr. The cells were removed by centrifuging at 10.000 rpm for 15 min and the spent broth was collected in clean and dry tubes. From the collected spent broth 0.5 ml was placed in a test tube and 3 ml of 95% ethanol was added followed by 2 ml of 50% KOH. After mixing thoroughly, the contents were heated at 60°C for 15 min in water bath and subsequently cooled to 4°C. To each tube, 5 ml of n-hexane were added and mixed followed by a further 3 ml of distilled water which were also mixed. The tubes were allowed to stand for 5 min at room temperature to separate the phase. After phase separation, 2.5 ml of upper hexane layer was placed in a clean dry test tube. The hexane was evaporated at 60°C (Mishra & Prasad 2005). To each tube, 1.5 ml working solution of $FeCl_3$ was added. After thorough mixing, the solution was allowed to stand for 10 min. Then, 1 ml of concentrated sulfuric acid was added and the solution was mixed and placed in the dark. Absorbance at 560 nm was measured 45 min later, (Rude & Morris, 1973). Absorbance was compared to a standard curve prepared by using suitable concentrations (1 mg cholesterol / ml ethanol).

Stock solution of $FeCl_3$ was prepared by dissolving 840 mg of $FeCl_3$. $6H_2O$ in 10 ml of glacial acetic acid. A work solution was prepared by making a 1: 100 dilution of stock $FeCl_3$ with glacial acetic acid. The work solution of $FeCl_3$ was prepared 1 day prior to use and was stable for several months (Rude & Morris, 1973). Each experiment was repeated three times in duplicate.

RESULTS AND DISCUSSION

Isolation and screening of LAB

A total of 21 isolates were isolated from *Zabady* and characterized as Gram positive, catalase negative, oxidase negative and non spore-forming bacteria. These isolates were screened for antibacterial activity against 8 pathogens (Table 1) using spot on lawn method. Six isolates showed antibacterial activities against 2 or more of the tested pathogens. Quantitative determinations of antibacterial activity of the selected isolates were carried out (Table 2). One isolate (isolate No.4) was selected as a promising isolate. This isolate has strong antibacterial activity against *Bacillus cereus* ATCC 49064 and *Salmonella enterica* ATCC 25566 and moderate and weak antibacterial activity against *Yersinia enterocolitica* ATCC 23715 and *Listeria monocytogenes* ATCC 19116, respectively. Furthermore, isolate No.4 had no antibacterial activity against the 6 indicator LAB presented in Table (1). According to its potential antibacterial activity, isolate No. 4 was selected, identified using phenotypic and genotypic methods and subjected to *in vitro* characterizations to evaluate its potential probiotic capacity.

Identification of selected isolate (isolate No. 4)

Morphological, physiological characteristics were used to identify the selected isolate to the ge-

Table 2: Antibacterial activities of cell-free extract of selected bacterial isolates against various indicator strains

Isolate No.	Cell Morphology and Growth Temperature	Antibacterial activity against indicator strain expressed in AU/ml[1]			
		B. cereus ATCC 49064	*L monocytogenes* ATCC 19116	*S. enterica* ATCC 25566	*Y. enterocolitica* ATCC 23715
1	Gram positive cocci, 30 °C	800	-	25600	200
2	Gram positive cocci, 30 °C	200	-	25600	400
3	Gram positive rods, 37 °C	200	-	200	-
4	Gram positive rods, 42 °C	25600	200	25600	400
5	Gram positive cocci, 42 °C	800	-	25600	200
6	Gram positive rods, 42 °C	3200	-	200	200

[1]Activity unit/ml calculated according to the following equation: 1000/d x D (D: dilution factor, d: amount of supernatant used).

nus level. The isolate was characterized as Gram positive, catalase-negative, oxidase negative and non-spore forming bacterium. Other characteristics, include growth at different temperatures, growth in the presence of different (%) NaCl and growth at different pH were similar to those described in Bergey's Manual of Determinative of Bacteriology (Logan & De Vos 2009) for genus *Lactobacillus*.

The PCR amplification of 16S rDNA of the selected isolate of LAB resulted in the synthesis of characteristic single band of about 600 bp (Fig. 1) using the primer 5/ CATCCAGTGCAAACCTAAGAG 3/ and 5/ GATCCGCTTGCCTTCGCA 3/. The sequencing data of purified 16S rDNA amplicons of isolate was employed for bacterial identification. The sequences of the chosen isolate were aligned with the 16S rDNA sequences from the GenBank database to identify the studied microorganism. 16S rDNA sequencing data of the selected isolate clearly showed 96% homology to *Lactobacillus plantarum*.

Production of antibacterial substance during growth of *L. plantarum*

Production of antibacterial substances by isolated *L. plantarum* during its growth in MRS broth at uncontrolled pH for 24 hr at the optimum growth temperature of 42°C was studied (Fig. 2). The antibacterial activity was determined against *Salmonella enterica* ATCC 25566. Activity could not be detected during the lag phase. It was observed after 8 hr. of incubation during the exponential growth phase. Titers of antibacterial activity reached the maximum (25600 Au/ml) at the end of exponential phase (16 hr and remained constant during the stationary phase (Fig. 2). These results are similar with bacteriocin produced by *Lactobacillus plantarum* (Todorov *et al.*, 2010).

To determine the proteinaceous nature of the antibacterial substance, the effect of some proteolytic enzymes (trypsin, pepsin and papain) on the antibacterial activity of neutralized and catalase treated cell-free supernatant of isolated *L. plantarum* grown in MRS was tested. Supernatant samples were individually incubated for 6 hr at 37°C with each enzyme then tested against *Salmonella en-*

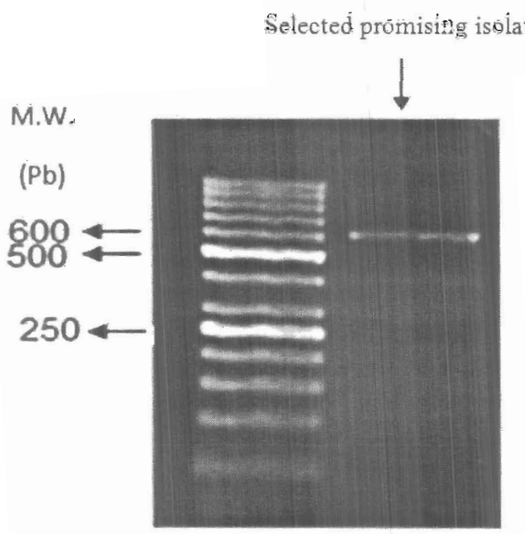

Fig. 1: 16S rDNA amplification products of selected promising isolate and DNA ladder gene RulerTM

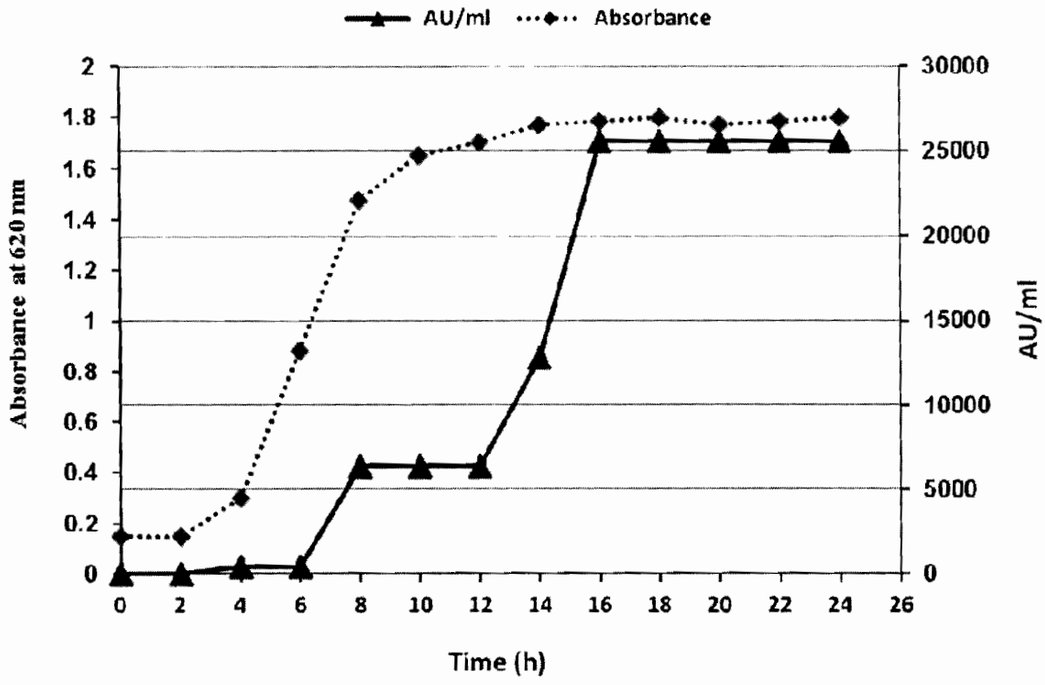

Fig. 2: Changes of absorbance at 620 nm (◆·····◆) and antibacterial activity against *Salmonella enteric* ATCC 25566 (▲——▲) during growth of isolated *L. plantarunm* in MRS at 42°C under uncontrolled pH.

terica ATCC 25566. The antibacterial activity was completely destroyed by papain while pepsin and trypsin had partial effect. These data clearly showed that the antibacterial substance is of proteinaceous nature, containing cleavage-sites suitable for the aformentioned proteases (Ivanova *et al.* 1998).

In vitro methods for testing selected *Lactobacillus plantarum* as potential probiotic

Gastric juice and bile tolerance

Potential probiotic strains should be able to survive within the gastrointestinal tract. Because tolerance to gastric juice and bile salts is seen as a prerequisite for strain survival through the gastrointestinal tract, therefore isolated *L. plantarum* was tested for gastric juice and bile tolerance.

The effects of gastric juice with pH 2.0, 2.5, 3.0 and 3.5 on the viability of the isolated *L. plantarum* are presented in Table (3). No viable cells of *L. plantarum* were detected after 1 hr incubation with gastric juice at pH 2.0. Remarkable tolerances were observed at pH 2.5 after 1 hr. and at pH 3.0 and 3.5 after 3 hr of exposure to gastric juice. The gastric juice tolerance of LAB is dependent upon the pH profile of H^+ ATPase and the composition of the cytoplasmic membrane, which is largely influenced by the type of bacteria, type of growth media, and the incubation conditions (Havenaar *et al.*, 1992, Hood & Zottola1988). Although the pH in the stomach can be as low as pH 1.5 to 3.0, probiotic strains can be buffered by food or other carrier molecules and in fact are not directly exposed to such a low pH in the stomach (Conway *et al.*, 1987, Prasad *et al.*, 1998).

The ability of isolated *L. plantarum* to survive for 3 hr was examined in the presence of bile salts in concentration ranged from 0.1 to 0.5% (Table 3). The tested isolate showed full tolerance to 0.1% bile up to 3h. The exposure to 0.3% bile salts for 1hr. did not affect the viability of the tested isolate and the highest reduction of bacterial survival occurred after 1 hr. of exposure to 0.5% bile. The average intestinal bile concentration is around 0.3% and may range up to an extreme of 2.0% during the first hour of digestion (Gotcheva *et al.*, 2002). Bile resistance of some strains varies a lot among the LAB species and between strains themselves. The effect of bile on the survival of *Lactobacilli* has

Table 3. Ability of isolated *Lactobacillus plantarum* strain to survive in the presence of various concentrations of bile salts (%) and pH

Treatments	Mean viable counts (log_{10}CFU/ml) at various exposure time (h)[1]			
	0	1 h	2 h	3 h
Acid Tolerance				
Control (pH6.5)	7.92 ± 0.45	7.64 ± 0.55	7.81 ± 1.05	7.34 ± 0.55
pH 2.0	7.79 ± 0.55	NG+	NG+	NG+
pH 2.5	7.14 ± 0.27	7.08 ± 0.28	NG+	NG+
pH 3.0	7.96 ± 0.65	7.77 ± 0.52	7.70 ± 0.36	7.08 ± 0.07
pH 3.5	7.49 ± 0.62	7.00 ± 0.1	7.07 ± 0.5	-7.39 ± 0.26
Bile (%)				
0.0	8.08 ± 0.02	8.09 ± 0.02	8.09 ± 0.01	8.10 ± 0.01
0.1	8.07 ± 0.09	8.08 ± 0.01	8.08 ± 0.09	8.09 ± 0.03
0.3	8.07 ± 0.03	8.07 ± 0.02	NG+	NG+
0.5	8.08 ± 0.02	NG+	NG+	NG+

[1]Each value in the table represents the mean value of three experiments each was carried out in induplicate ± standard deviation (SD). NG+ : No growth (at 10^{-4} dilution).

been investigated by several authors and is thought to be linked to the ability of strains to de-conjugate bile acids (Tannock *et al.*, 1989). Bile resistance of some strains is related to specific enzyme activity, bile salt hydrolase (BSH) which helps to hydrolyze conjugated bile, thus reduces its toxic effect (Du Toit *et al.*, 1998). Some studies have shown that the presence of bile salts in the bacterial culture medium is much more detrimental than the effects of low pH (Khalil *et al.*, 2007).

Phenol resistance

Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols (Suskovic *et al.*, 1997). These compounds can exert a bacteriostatic effect against some *Lactobacillus* strains. Thus, testing for the resistance to phenol may generate further information on the potential for survival of lactobacilli in gastrointestinal conditions (Xanthopoulos *et al.*, 2000). The examined strain showed different degrees of sensitivity towards different concentration of phenol (Fig. 3). *L. plantarum* was almost unaffected by 0.1% phenol concentration after 24 hr. Whereas, a slight gradual decrease in absorbance was reported with increasing phenol concentration to 0.3 %. The highest decline in growth was obtained at 0.5% phenol. *L. plantarunm* was highly tolerated to different tested phenol concentrations. Vizoso Pinto *et al.*, (2006) examined the tolerance of *L. plantarum* toward phenol. They found that four strains out of six tolerated phenol at 0.4% for 24hr as their numbers did not decrease from an initial inoculum of approximately log 7.6 to 8.0.

Cell surface hydrophobicity

The ability to adhere can give information about the possibility of probiotics to colonize and may modulate the host immune system. Several mechanisms were reported about the adhesion of microorganisms to intestinal epithelial cells (Savage, 1992; Naidu *et al.*, 1999). Cell hydrophobicity is one of the factors that may contribute to adhesion of bacterial cells to host tissues (Ram & Chander, 2003). The *in vitro* determination of microbial adhesion to xylene droplets was carried out (Table 4). This method was reported to be qualitatively valid to estimate the ability of a strain to adhere to epithelial cells (Kiely & Olson, 2000). A hydrophobicity of 50% was determined for isolated *L. plantarunm*. Hydrophobicities of some known cultures like *L. plantarum* ATCC 8014 , *L. pentosus* ATCC 804, *L. Casei* NCIMB 3254, *L. delbrueckii* NCIM 2025 were found to be 5.5 , 6.5, 6.2 and 3.7%, respectively (Aswathy *et al.*, 2008). All these hydrophobicities are much less than that of isolated *L. plantarum*. Bacterial cells with high hydrophobic properties usually form strong interactions with mucosal cells. Hydrophobicity may assist in adhesion, but is not a prerequisite for colonization (Todorov *et al.*, 2008).

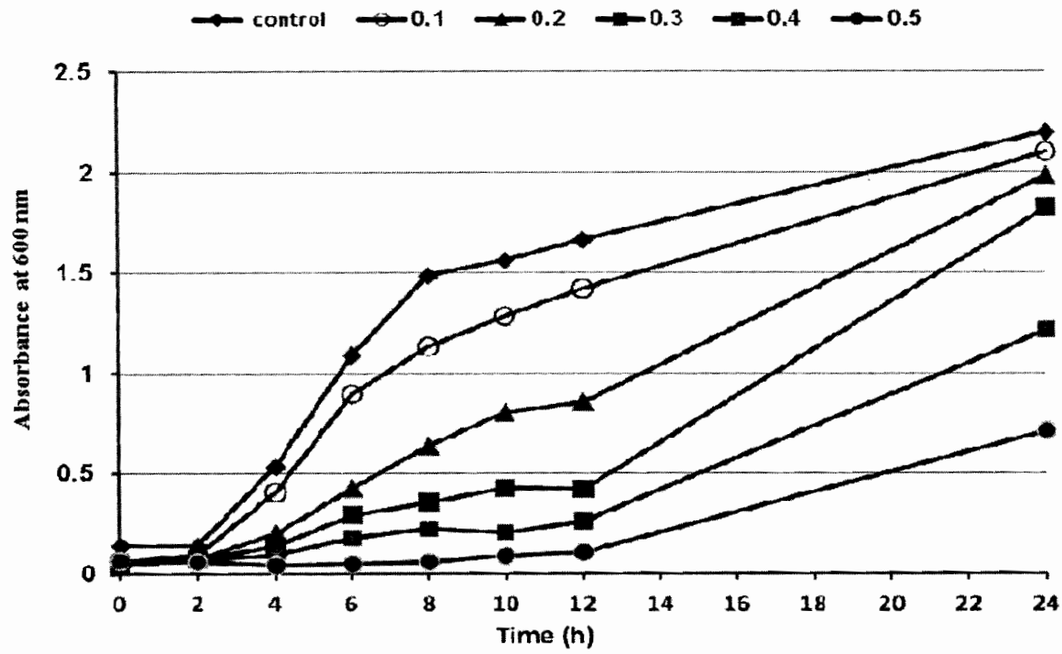

Fig. 3: Tolerance of *Lactobacillus plantarunm* to various increment phenol concentrations (0.1 to 0.5 %, v/v)

Autoaggregation, coaggregation and adhesiveness of *L. plantarunm*

Aggregation between cells of the same microorganism (auto-aggregation), or between cells of different species and strains (co-aggregation) as well as their ability to displace pathogens, are important properties of probiotic microorganisms and may have greater advantage over non-co-aggregating organisms which are easily removed from gastrointestinal tract (GIT) environment (Ocaña & Nader-Macias, 2002).

The autoaggregation rate of selected *L. plantarum* was measured after a period of 4 hr. *L. plantarum* exhibited a weak autoaggregating phenotype either under aerobic (12.8%) or anaerobic conditions (15.8%), (Table 4). The observed weak autoaggregation could be related to cell surface component, because it may be lost after washing and suspending of the cells in PBS. On contrary to our results, high autoaggregation was reported for *L. plantarum* H17 and T1 under aerobic (29 and 70%) and anaerobic conditions (44 and 76%), respectively (Ekmekci *et al.*, 2009).

Coaggregation of *L. plantaunm* with another potential probiotic strains (*Enterococcus faecium*) and some pathogenic bacteria were also examined, (Table 4). Results are expressed as the percentage reduction after 1 hr in the absorbance of mixed suspension compared with that of the individual suspension. The *L. plantarunm* demonstrated remarkable coaggregation with *Salmonella enterica* ATCC 25566 (13.1%), *Enterococcus faecium* (11.4%) and *Listeria innocua* ATCC 33090 (10.99%), (Table 4). The interaction of probiotic organisms with the natural gut flora is a key to the potential success of

Table 4. *In vitro* characterization of *Lactobacillus plantarum* as potential probiotic

Treatments	Percentage (%) Mean ± SD
Hydrophobicity	50.07 ± 0.62
Autoaggregation (aerobic)	12.8 ±3.29
Autoaggregation (anerobic)	15.8 ± 2.02
Coaggregation	
Bacillus cereus ATCC 49064	9.35± 1.01
Enterococcus faecium	11.4 ± 0.62
Listeria innocua ATCC 33090	10.99± 0.32
Listeria monocytogenes ATCC 19116	8.74 ±0.33
Salmonella enterica ATCC 25566	13.1± 0.40
Adhesion to epithelial cell of rabbit*	7.5 * ± 1.6
Cholesterol reduction (%)	32.7 ± 2.64

*Number of bacteria adhered to a single epithelial cell. *E.coli* showed adherence of 23.8 cells per epithelial cell.

the organism in terms of colonization and long-term persistence. Co-aggregation of probiotic bacterial strains has been suggested to enable them to form a physical-chemical barrier that prevents colonization by pathogenic bacteria (Collado *et al.*, 2007a).

Adherent probiotic strains are considered to colonize easily the intestine which is thought to be important for their positive effects (Ouwehand & Salminen, 2003). The adhesion of commercial probiotic strains showed a great variability depending on the strain, species and genus. The adhesiveness of *L. plantarum* to rabbit intestinal epithelium was also investigated. Microscopic examinations showed that this strain moderately adhered to epithelial cells with adhesion of 7.5 bacterial cells to one epithelial cell. While the tested *E. coli* showed adhesion of 23.8 cells / epithelial cell (Table 4). The adhesion level obtained for isolated *L. plantarunm* was comparable to the levels reported earlier for *L. plantarum* Lp-115 (Collado *et al.*, 2007b). Attachment of LAB to mucosal epithelial cells is important to prevent development of microbial infections in GIT. Blocking attachment of pathogenic microorganisms to the intestinal epithelium represents a potential strategy for disease prevention (Sherman *et al.*, 1983, Leung & Finlay, 1991, Peralta *et al.*, 1994).

Cholesterol assimilation

Elevated serum cholesterol in humans is generally a risk factor correlated with development of coronary heart disease. Modification of diets such as supplementation of diet with fermented dairy products or LAB-containing dairy products is a way that may be helpful in reducing serum cholesterol. The tested strain decreased cholesterol concentration from 110 ± 2.2 mg/dl to 74 ± 2.6 mg/dl in the 0.3% bile salt MRS broth with removal percentage of 32.7%. The LAB may alter serum cholesterol by two proposed mechanisms; directly binding of dietary cholesterol in the small intestine before cholesterol can be absorbed into the body (Gilliland *et al.*, 1985; Hosono & Tonooka, 1995) and deconjugation of bile acids to produce free bile acids (Walker & Gilliland, 1993). Previous studies reported the ability of some LAB such as *Lactobacilli* (Usman & Hosono 1999, 2000) to deconjugate bile salt.

CONCLUSION AND PERSPECTIVE SECTION

Twenty one isolates of LAB were isolated from *Zabady*. Among them, one isolate was selected as a promising probiotic candidate and identified as *Lactobacillus plantarum*. The selected *L. plantarum* showed sensitivity to simulated gastric juice adjusted at pH 2.0 - 3.5, tolerance to 0.3% bile concentration as well as antibacterial activity against *Salmonella enterica* ATCC 25566 (25600 AU/ml), *Bacillus cereus* ATCC 49064 (25600 AU / ml), *Yersinia enterocolitica* ATCC 23715 (400 AU /ml) and *L. monocytogenes* ATCC 19116 (200 AU/ ml). Furthermore, the strain demonstrated highly tolerance to different tested phenol concentrations, moderate cell surface hydrophobicity, weak auto-aggregating and reasonable adhesion level to the epithelial cells. However, additional *in vitro* and *in vivo* studies are needed, focused on determining the cholesterol reduction ability of this isolate.

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إمكانية استخدام *Lactobacillus plantarum* المعزوله من الزبادى كأحد الداعمات الحيوية

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تم عزل ٢١ عزلة تابعة لبكتيريا حامض اللاكتيك من الزبادى (ياغورت مصرى) وتم غربلتها بناءاً على نشاطها المضاد لثمانى سلالات بكتيرية ممرضة كالاتى *Bacillus cereus* ATCC 49064, *Clostridium perfringens* ATCC 13124, *Escherichia coli*, *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 19116, *Listeria ivanovii* Li4/pVS2, *Salmonella enteric* ATCC 25566 and *Yersinia enterocolitica* ATCC 23715. تم أختيار عزلة واحدة واعدة تم تصنيفها بأستخدام الطرق المظهرية والوراثية على أنها سلالة *Lactobacillus plantarum*. وتم تقييم هذه العزلة لاستخدمها كأحد الداعمات الحيوية وذلك عن طريق الصفات التالية : المقاومة للعصير المعدى وأملاح الصفراء ومدى ميل سطح خلاياها للامائية والمقاومه للتركيزات المنخفضة من الفينول وقدرتها على التجمع الذاتى و التجمع مع بكتريات أخرى وقدرتها على الالتصاق بجدران الخلايا الطلائية لأمعاء الارنب وكذلك قدرتها على خفض كوليسترول سيرم الدم.

أظهرت هذه العزلة حساسية للعصير المعدى عند رقم أس هيدروجينى من ٢ إلى ٣,٥ وكانت مقاومة لاملاح الصفراء عند تركيز ٠,٣ ٪ وذات نشاط مضاد لبكتيريا *Salmonella enterica* ATCC 25566 (قدرة ٢٥٦٠٠ وحده / مل) وضد *Bacillus cereus* ATCC 49064 (قدرة ٢٥٦٠٠ وحده / مل) وضد *Yersinia enterocolitica* ATCC 23715 (قدرة ٤٠٠ وحده / مل) وضد *Listeria monocytogenes* ATCC 19116 (قدرة ٢٠٠ وحده / مل) وقد يرجع هذا النشاط لوجود ببتيدات نشطة . وقد دلت النتائج على أن هذه العزلة مقاومه بدرجة كبيره لتركيزات الفينول المستخدمة فى الدراسة وكانت خلاياها لها ميل لامائى بدرجة متوسطه ولها قدره ضعيفه على التجمع الذاتى تلتصق بالخلايا الطلائية لأمعاء الأرنب بدرجة مقبولة.