Molecular identification and characterization of lactobacilli isolated from Egyptian inf nts as potential probiotic candidates

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ABSTRACT

The objective of this study was to identify and characterize the lactobacilli from the infant's feces as a potential probiotic strains. Feces samples were collected from breast fed Egyptian infants. On the basis of morphological and biochemical properties 33 isolates were selected for further characterization. Using primers specific for 16S rRNA the isolates were determined as Lactobacillus sp. Sequencing of 16S rRNA genes was used for determining the isolates at species and subspecies levels. The isolates Lactobacillus fermentumNM059, Lactobacillus brevisNM012 and Lactobacillus helveticusNM075 exhibited antagonistic action towards Gram-positive and Gram-negative bacteria and they also showed high values of hydrophobicity. Besides, they were tolerant to low pH, high concentration of bile salt and pancreatic enzyme. These findings imply that Lactobacillus fermentumNM059, Lactobacillus brevisNM012 and Lactobacillus helveticusNM075 might be considered as potential probiotic strains.

Keywords: *Lactobacillus, probiotics, identification, 16S rRNA sequencing analysis.*

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INTRODUCTION

Probiotics are defined as 'live microorganisms which when administered in adequate numbers confer a health benefit on the host' (FAO/WHO 2001). They function to activate the mucosal immune system and prevent pathogen colonization. They strengthen the mucosal barrier, interfering with pathogen colonization, and in some instances, secreting antibacterial substances (Chen and Walker, 2005). They are effective in the prevention and treatment of a large number of human gastrointestinal tract diseases. These disorders include intestinal infections, colonic transit disorder, colonic adenomas and other forms of intestinal cancer (Picard *et al.*, 2005). To date, probiotics have been mainly selected from the genera *Lactobacillus* and *Bifidobacterium*, because of their long history of safe use in dairy industry, and their natural presence in the human intestinal tract (Saxelin, 1997). The main criteria for selecting probiotic strains are their acid and bile tolerance, survival through the gastrointestinal tract, ability to adhere to intestinal surfaces, temporary colonization and antagonism against pathogens. Although the

pH of the stomach may increase up to 6.0 or higher after food intake (Johnson, 1977), it generally ranges from 2.5 to 3.5 (Holzapfel *et al.*, 1998). Fasting pH in the stomach may even be as low as 1.5 (Waterman and Small, 1998), which implies that survival in extreme acidic conditions is one of the first major physiological challenges faced by probiotic cultures upon oral administration. Following stomach passage, the small intestine is a second major barrier in the GI tract. Although the pH of the small intestine (7.0 to 8.5) (Thomson *et al.*, 2003) is more favorable toward bacterial survival, the presence of pancreatin and bile salts may have adverse effects.

Strains isolated from the human intestinal tract are generally recommended for probiotic use in humans (Huis In't Veld and Shortt, 1996; Lee *et al.*, 1999; Shortt, 1999). Strains of human origin are most suitable because some health-promoting benefits may be species specific and microorganisms may perform optimally in the species from which they were isolated (Stanton *et al.*, 2003). Although there is a reasonable number of well characterized probiotic strains available for commercial use around the world (Ezendam and van Loveren, 2006; Reid, *et al.*, 2003; Santosa, *et al.*, 2006), the isolation and characterization of new probiotic strains is still desirable. The aim of this work was to isolate and identify new probiotic strains from Egyptian infants for food and pharmaceutical industry.

MATERIALS AND METHODS

Sampling and bacterial isolation

Stool samples were collected from 15 healthy full-term Egyptian newborn breast-fed babies (1-24 month). One gram of stool was first put with a sterile stick in a sterile measuring tube together with 2 ml 0.9% sterile saline solution. The stool was pressed and mixed in this solution and the volume was completed to 10 ml with the 0.9% sterile saline solution. Anaerobic cultures were made with 0.1 ml of this dilution (Mitsuoka, 1992; Sepp *et al.*, 1997). For the isolation of *lactobacillus* strains, MRS agar plates were used as a selective medium for lactobacilli and incubated anaerobically in jars with Anaero-Gen (Oxoid) at 37°C for 48 hr. After incubation, 10 developing colonies were randomly picked up from each sample and subcultured onto MRS plates for further analysis.

Identification procedures

Provisional identification of lactobacilli

Provisional identification was based on the ability of the isolates to grow in the MRS broth and catalase reaction (Holt *et al.*, 1994). Gram positive and catalase-negative rods (33 in total) were initially examined by morphology and simple physiological tests as follows: colony morphology and pigmentation; growth at 15 and 45°C in MRS broth; salt tolerance: 4, 6.5 and 8% NaCl in MRS (the tests were performed three times); production of carbon dioxide from glucose by sub-culturing the isolates in tubes with MRS broth containing Durham's bells.

Molecular identification of lactobacilli

Isolates were genetically identified using genus-specific primers specific for the 16S rRNA of lactobacilli. Chromosomal DNA from pure cultures was extracted using AxyGEN BIOSCIENCES DNA extraction kit according to manufacturer's instructions. All polymerase chain reaction amplifications were performed with the Taq DNA polymerase kit (Fermentas). Reaction mixtures consisted of 20 mm Tris–HCl (pH 8.4), 50 mm KCl, 3 mm $MgCl_2$, 50 mm each of the four deoxynucleoside triphosphates (dNTP), 1 U Taq polymerase, 5 pmol each primer and 1 µl of template DNA in a final volume of 50 µl .

Samples were amplified in a GeneAmp polymerase chain reaction System 2700 (Applied Biosystems) programmed as follows: initial denaturation of DNA for 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C; and extension of incomplete products for 7 min at 72°C. Polymerase chain reaction products were quantified by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. Polymerase chain reaction products obtained from the selected isolates were purified using QIAquick polymerase chain reaction purification KIT (Qiagen) and sequenced commercially by Sigma Company. The sequence was checked in the National Center for Biotechnology Information (NCBI) database using the standard nucleotide–nucleotide homology search blast (http://www.ncbi.nlm.nih.gov/BLAST) and the NEB cutter V2.0 database (http://tools.neb.com/ NEBcutter2). NEB cutter V2.0 is an on-line DNA sequence tool used to find large, non-overlapping, open-reading frames and works for all restriction enzymes. It provides a website, which allows users to check nucleotide sequences for restriction enzyme sites. The sequences were submitted and *Alu*I enzyme was chosen for digestion. Finally, gel photograph using 2% agarose was viewed.

Table (1): Strains used in this study.

Strains	Source
Staphylococcus aureus	ATCC29213
Escherichia coli	ATCC25922
Bacillus subtilis	ATCC6633
Pseudomonas aeruginosa	ATCC27953
Enterobacter cloacae	ATCC13047
Candida albicans	Clinical isolate
*Lactobacillus brevis*NM012	Current work
*Lactobacillus brevis*NM031	Current work
*Lactobacillus brevis*NM035	Current work
Lb. fermentum NM024	Current work
Lb. fermentum NM059	Current work
Lb. fermentum NM045	Current work
Lb. helveticus NM029	Current work
Lb. helveticus NM072	Current work

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table (1). *Lactobacillus* strains were grown on MRS (Difco) at 37°C. The indicator strains were cultivated in the following media: *Staphylococcus aureus* on Brain heart agar; *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* on LB agar; *Enterobacter cloacae* on nutrient agar and *Candida albicans* on 2% glucose, 0.3% yeast extract and 1% peptone (GYEP) agar.

Acid tolerance

Cultures were grown in MRS broth at 37°C overnight, and subcultured in 10 ml of fresh MRS broth adjusted to different pH values (2.5 and 3) with hydrochloric acid (3.0 M). The initial bacterial concentration was 10^6 cfu ml^{-1} and was checked by viable count determination on MRS; the samples were incubated for 3 or 6 hr at 37°C. Cells were serially diluted 10-fold in phosphate buffer (0.1 M, pH 6.2) in order to neutralize the medium acidity. The residual viable count was determined by plate count technique on MRS

agar after 24–48 hr of incubation. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial bacterial load.

Antimicrobial activity assay

For detection of antimicrobial activity, an agar well diffusion assay was used (Tagg and McGiven 1971). Corresponding soft agar (0.75%, w/v) containing approximately 10^5 cells ml^{-1} of indicator strains; *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Candida albicans* were overlaid individually onto appropriate plates for each strain. Wells were made in the lawn of the soft agar. Aliquots (100 µl) of fresh overnight lactobacilli cultures (16 hr) were poured into the wells. Plates were incubated for 48 hr at 37°C. After the 48 hr incubation, inhibition zones were measured.

Hydrophobicity

The ability of the organisms to adhere to hydrocarbons, as a measure of their hydrophobicity, was determined according to Vinderola and Reinheimer (2003). Cultures were harvested in the stationary phase by centrifugation at 12000 x g for five min at 5°C, washed twice in 50 mM K_2HPO_4 (pH 6.5) buffer and finally resuspended in the same buffer. The cell suspension was adjusted to an A_{560nm} value of approximately 1.0 with the buffer and 3 ml of the bacterial suspensions were put in contact with 0.6 ml of n-hexadecane and vortexed for 120 s. The two phases were allowed to separate at 37°C. The aqueous phase was carefully removed and the A_{560}nm was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula: $H\%=[(A_0-A)/A_0]x100$, where A_0 and A are the absorbance before and after extraction with n-hexadecane, respectively.

Bile tolerance

The method of Gilliland *et al.* (1984) was used to determine bile tolerance. The MRS broth was inoculated with 10^6 cfu ml^{-1} cultures. Test cultures were supplemented with Oxgall bile (0.3% w/v, Sigma). Samples were incubated for 24 hr at 37°C. Survival of control and test cultures (with Oxgall) was determined after 3 and 6 hr of incubation at 37°C by plating onto MRS agar plates and reincubation for 48 hr at 37°C.

Pancreatic enzyme tolerance

Tolerance for pancreatic fluid was tested according to the method of Rönkä *et al.* (2003). The strains were grown in MRS broth at 37°C for 24 hr. Aliquots of 30 µl incubated broth were inoculated in the wells of microtiter plates with 270 µl of the test medium (150 mM $NaHCO_3$ and 1.9 mg/ml pancreatin (Sigma); pH 8). Cultures were shaken for 3 hr and 6 hr at 37°C. Survival of strains was examined by plating onto MRS agar after 0, 3, and 6 hr.

RESULTS AND DISCUSSION

Conventional identification

Eighty isolates were obtained from the feces of 15 Egyptian infants as described in the Methods. Thirty three isolates out of 80 were shown to be *Lactobacillus* by their positive Gram reactions, absence of catalase activity and rods of cell morphology. Then, a few key conventional phenotypic tests were used to identify them tentatively. Moreover, all of them were identified mainly in sugar-fermentation (data not shown).

pH tolerance

Tolerance to low pH, bile and pancreatic fluid *in vitro* are expected to predict the survival of a strain in the conditions present in the gut (Rönkä *et al.*, 2003; Saarela *et al.*, 2000). Transit time can be from <1 hr to 3–4

hr depending on the individual and diet. Therefore, some authors have proposed that strains intended for probiotic purposes should be screened for tolerance to pH 2.5 in an HCl-acidified culture medium for 3-4 hr (Pennachia *et al.*, 2004; Klingberg and Budde, 2006). Initially, the Thirty three pre-selected lactobacilli isolates were tested for their ability to grow under these conditions. The exposure to pH 2.5 was found to be a highly discriminating factor where only eight out of the Thirty three strains were resisting adequately after 3 hr of exposure (Table 2). These eight lactobacilli isolates were also examined for their ability to survive in alkaline medium up to pH 9. These results suggested them as promising probiotic strains and were subjected for molecular identification.

16S rRNA identification

The eight isolates, selected as acid-resistant strains, were further identified by 16S rRNA gene sequence analysis. Genomic DNA was extracted as described previously.

Table (2): pH tolerance, survival % with respect to a control, following 3 and 6 hr of incubation in MRS broth at pH 2.5, 3.0 and 9.0.

Strains	pH 2.5		pH 3.0		pH 9.0	
	3hr (%)	6hr (%)	3hr (%)	6hr (%)	3hr (%)	6hr (%)
NM012	79.20	19.26	85.85	38.18	91.21	90.54
NM024	61.70	9.93	81.91	17.38	95.25	94.99
NM029	67.54	11.66	89.25	20.10	99.42	99.32
NM031	72.84	16.73	87.73	18.83	92.42	92.40
NM035	67.67	10.90	90.98	22.56	97.15	96.45
NM045	79.26	19.26	90.74	31.76	99.43	99.12
NM059	61.70	21.70	93.83	31.70	92.16	90.89
NM072	72.48	16.73	70.15	23.88	97.91	95.45

16S rRNA gene was amplified using primers FD1 (5′-AGA GTT TGA TCC TGG CTC AG) and RD1 (5′-AAG GAG GTG ATC CAG CC), resulting in PCR products of 1.5 kb. The PCR amplification, purification and sequencing were performed as described previously. The 1.5 kb obtained sequences were aligned and clustered with sequences from the NCBI database. 16S rRNA gene sequence analysis indicated that the eight isolates clearly belong to the *L. helveticus* (two strains), *L. brevis* (three strains), *and L. fermentum* (three strains) showing 16S rRNA gene sequence similarities of 99%. The analogical electrophoresis identified the results, which have been sequenced. Hence, strains with the same species have almost the same enzyme products and if the sequences are known, they can be digested with any enzyme that found in the database (Wang *et al.*, 2008). Indeed, by extensive application of this database, the homology of the strains of the same species and differences among the different species can be compared. In this study, the digestion by *Alu*I enzyme as shown in (Fig. 1) using the NEB cutter verified the results. The Figure shows that strains NM059, NM048 and NM024 are *L. fermentum*; strains NM035, NM031 and NM012 are *L. brevis*; while strains NM029 and NM072 are *L. helveticus*.

Antimicrobial activity

The eight isolates identified as *Lactobacillus* sp. were tested for antimicrobial activities and were investigated using an agar well diffusion assay against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Candida albicans* indicator

strains. The eight strains exhibited antagonistic action aganist the indicator strains (Table 3). The inhibition zones were classified as (-) no visible inhibition, (+) 1 to 7 mm inhibition, (++) 8 to 15 mm inhibition, and (+++) more than 15 mm inhibition. It is well known that the presence of lactobacilli is important for the maintenance of the intestinal ecosystem (Jacobsen *et al.*, 1999; Servin, 2004), as they can have inhibited pathogens.

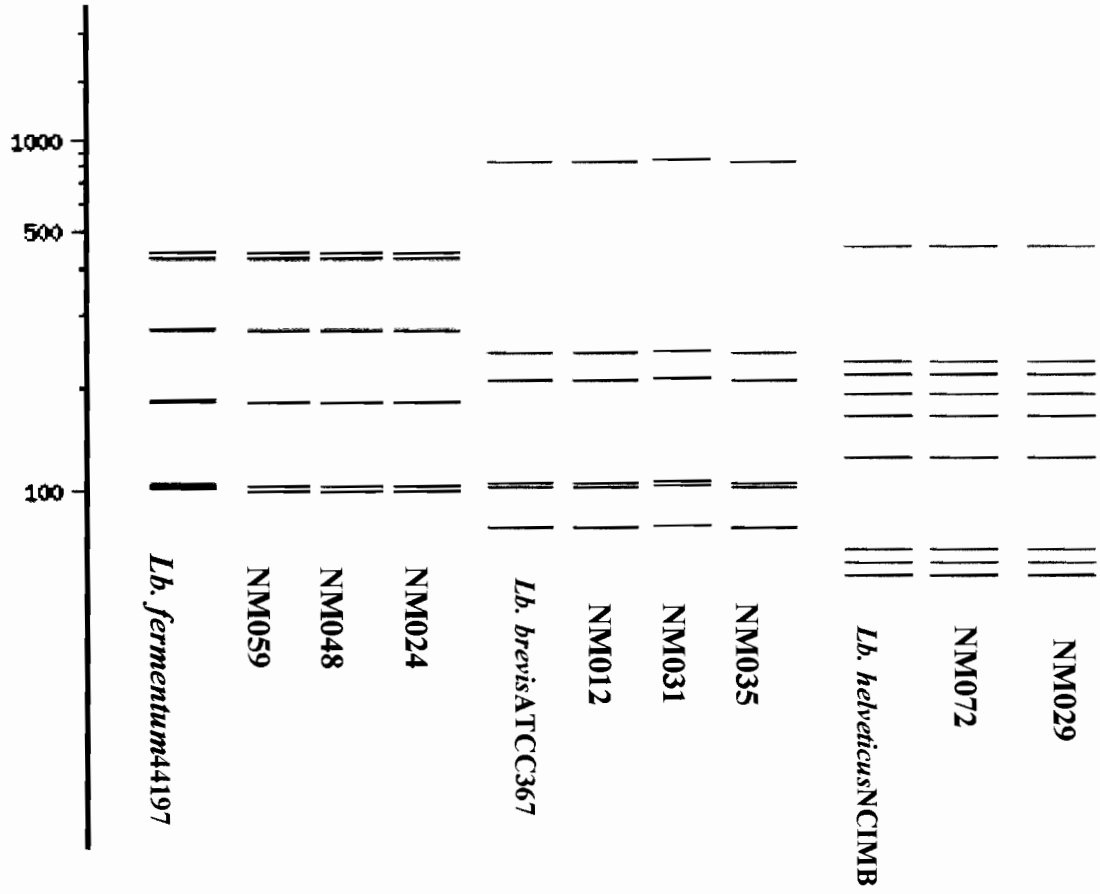

Fig. (1): The analogical electrophoresis of the selected eight isolates by "AluI" using NEBcutter2.0 compared to known lactobacillus strains from the data base (44197, ATCC367, NCIMB).

Table (3): Antimicrobial activity and hydrophobicity % of the tested isolates. The inhibition zones were classified as (-) no visible inhibition, (+) 1 to 7 mm inhibition, (++) 8 to 15 mm inhibition, and (+++) more than 15 mm inhibition.

Strains	Inhibition of						Hydrophobicity %
	E. coli	*Staphylococcus aureus*	*Candida albicans*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Enterobacter cloacae*	
Lb. fermentum NM059							56.0 ± 0.9
Lb. fermentum NM024							42.7 ± 0.8
Lb. fermentum NM048			-				43.0 ± 0.9
Lb. brevis NM031			-				62.5 ± 2.0
Lb. brevis NM035			-				59.7 ± 1.3
Lb. brevis NM012							66.3 ± 3.2
Lb. helveticus NM029			-			-	42.8 ± 0.7
Lb. helviticus NM072			-				44.0 ± 0.9

All the eight isolates tested in this study exhibited some inhibitory activity against the pathogenic bacteria used, except *L. helveticus*NM029 has no activity against *Enterobacter cloacae*. Only three strains (*L. fermentum*NM059, *L. fermentum*NM024 and *L. brevis*NM012) exhibited antagonistic activity against *Candida albicans*.

Hydrophobicity

Several mechanisms are involved in the adhesion of microorganisms to intestinal epithelial cells (Savage, 1992). The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue (Rosenberg, *et al.*, 1980; Kiely and Olson, 2000). This property could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract (Naidu *et al.*, 1999). The determination of microbial adhesion to hexadecane as a way to estimate the ability of a given strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Kiely and Olson, 2000). In this study, the hydrophobicity values found ranged from 42.7 to 66.3% for the selected eight strains (Table 3) suggesting their good adhesion ability to epithelial cells. The highest values of hydrophobicity were found for the *L. brevis* strain NM012. It was demonstrated that the bacteria can activate the gut mucosal innate immunity by interacting with macrophages from the peritoneal capacity of mice (Vinderola *et al.*, 2006). Therefore, the high hydrophobicity of *L. brevis*NM012 strains could imply a potential good capacity to activate the gut immune response, but this must still be determined in an *in vivo* model.

Bile salt and pancreatic enzyme tolerance

The ability of probiotic bacteria to survive the passage through the stomach was reported to be variable and strain-dependent (Clark *et al.*, 1993; Charteris *et al.*, 1998, Zavaglia *et al.*, 1998; Chung *et al.*, 1999). Out of 200 strains of lactobacilli and bifidobacteria, Prasad *et al.*, (1998) did select only a few strains with satisfactory acid resistance. In the present study, the survival of the eight strains in MRS broth containing 0.3% bile salts and test medium containing 1.9 mg/ml pancreatin was tested. Results revealed that not all the tested strains survived after 3 hr, only strains *L. fermentum*NM059, *L. brevis*NM012, and *L. helveticus*NM072 showed good survival in the presence of bile salt and pancreatin after 3 h incubation (Fig. 2).

The effect of these strains in reducing serum cholesterol should also be investigated. One mechanisms of bile salt resistance could be the capacity of deconjugating bile salts which has been also related to the capacity to

remove cholesterol from the intestinal environment (Begley *et al.*, 2005).

CONCLUSION

The use of sequencing 16S rRNA gene plus a set of simple *in vitro* tests allowed to identify among 80 intestinal human isolates, three potential candidates for the formulation of new probiotic foods and for pharmaceutical uses. The methodologies used permitted us to determine that *L. fermentum*NM059, *L. brevis*NM012, and *L. helveticus*NM072 possess some proper characteristics required for a probiotic strain, but still, future and more complex studies are needed.

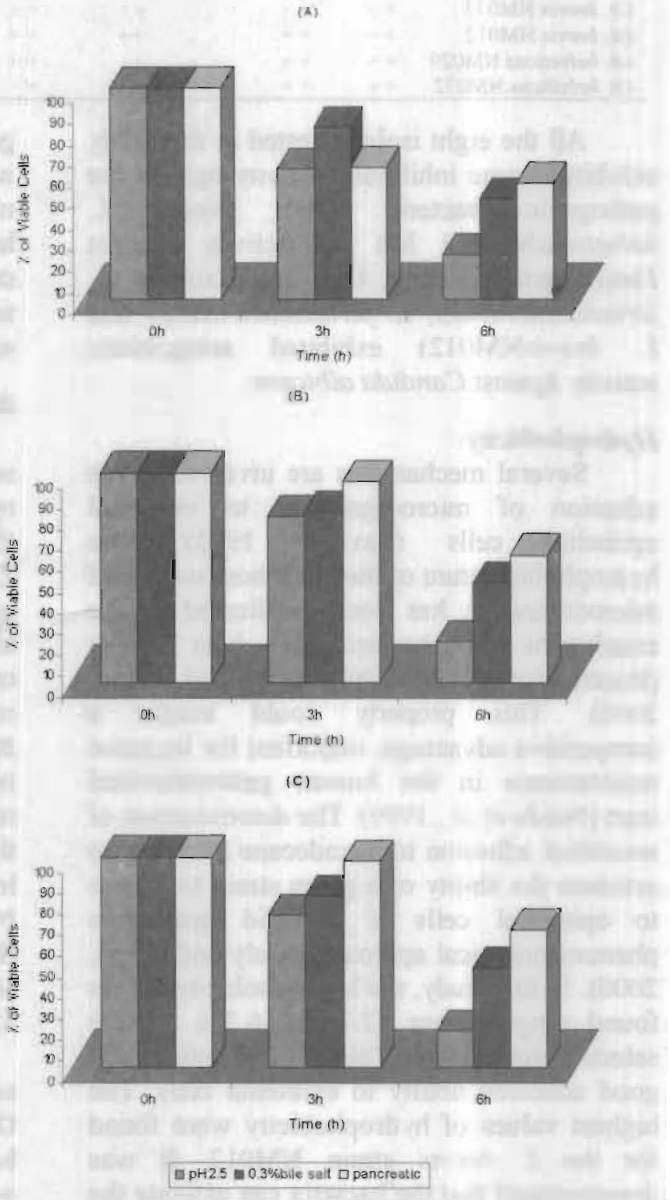

Fig. (2): The pH, bile and pancreatic tolerance. Survival following 3 and 6 hr of incubation in MRS broth at pH 2.5; MRS broth with 0.3% bile salts; Test medium containing 1.9 mg/ml pancreatin was observed for strains L. fermentumNM059 (A), L. brevis NM012 (B) and L. helveticus NM072 (C).

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الملخص العربي

تعريف و توصيف سلالات جديدة من جنس اللاكتوباسلاى معزولة من الاطفال المصريين كمدعمات حيوية

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*قسم كيمياء المنتجات الطبيعية والميكروبية- شعبة العلوم الصيدلية

** مجموعة الفاكسينات-مركز التميز العلمى للعلوم المتقدمة. المركز القومى للبحوث. شارع البحوث- الدقى- مصر

الهدف من هذة الدراسة هو عزل وتوصيف و تعريف سلالات جديدة من جنس اللاكتوباسلاى من الاطفال المصريين كمدعمات حيوية يمكن توظيفها فى صناعة الأغذية و الأدوية لاغراض علاجية و صحية . قد تم تجميع عينات البراز من الرضع الذين يتلقون الرضاعة الطبيعيةحيث تم عزل ٣٣ عزلة من جنس *Lactobacillus* وفقا للخصائص المورفولوجية والبيوكيميائية ومن ثم تم تقدير خواص ال probiotic للعزلات واستخدم جين ١٦S rRNA فى تعريف السلالات المنتقاة. نتيجة هذا البحث أسفرت عن ثلاث سلالات عرفت ب: *L. fermentum*NM05, *L. brevis*NM012, *L. helveticus*NM075. التى أظهرت خواصا جيدة كprobiotic، حيث أنه لهم المقدرة على تحمل الوسط الحمضى والقلوى (pH 2.5–9) وتحمل النمو فى الأملاح المرارية حتى 0.3% والأنزيمات البنكرياسية و اظهروا خواصا لل Hydrophobicity جيدة مما يؤهلهم للتعايش داخل الجهاز الهضمى. هذا بالأضافة لمقدرتهم على تثبيط نمو بعض انواع البكتيريا الممرضةالمستخدمة فى هذا البحث سواء الموجبة أو السالبة لجرام.