Characterization of Lactic Acid Bacteria Isolated from Dairy Products in Egypt as a Probiotic.

Rasha H. Bassyouni.[1], Walla S. Abdel-all [2], Mostafa G. Fadl[2] Saed Abdel-all [2] and Zeinat kamel[3]*

[1]Medical Microbiology and Immunology Department, Faculty of Medicine, Fayoum University
[2]Microbiology Department, National Organization of Drug Control & Research
[3]Botany Department, Faculty of Science, Cairo University
zeinatk@yahoo.com

Abstract: *Lactobacilli* belong to lactic acid bacteria (LAB), generally recognized as safe primary fermentation end product from sugars is lactic acid and that is why foods are conserved. Lactic acid bacteria have been used for improving health host. Therefore, they are an important part of intestinal flora in human and animals as probiotic. This research aimed to isolate lactic acid bacteria with significant probiotic character from different dairy products. In this study, homo- fermentative LAB were isolated from different dairy products in Egypt. Isolates were identified by morphological, biochemical and physiological methods. Probiotic properties of isolates were investigated. The isolated bacteria were studied for antagonistic effects on clinically isolated *E.coli, Salmonella* spp. *Micrococcus* spp., *Staphylococcus* spp. A collection of fifty four isolates were obtained. Eight isolates from different dairy products were observed as potential probiotic safe for human use; where they found to be tolerant to low pH and bile salt and effective against isolated *E.coli, Salmonella* spp. *Micrococcus* spp. All isolates were screened for enzymatic activity using API ZYM Kits and antibiotic sensitivity. Biochemical and physiological results indicated that they were found to be related to the genus *Lactobacillus* and suggested to belong to *L. casei* (4 isolates), *L. Acidophillus* (3 isolates) and *L. lactis*(1 isolates) and that were effective on the isolated *E.coli ,Salmonella* spp. *Staphylococus* spp. and they have enzymatic activity. β-galactosidase was produced, which is beneficial for lactose intolerance. *Lactobacillus* spp. produced enzymes including leucinearylamidase, crystinearylamidase,acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and N-acetyl-β-glucosamidase so we concluded that human milk, yogurt and raw milk are considered a good source of potential probiotic strains also the isolated bacteria had no haemolytic activity so it consider as a great potential probiotic and safe for human use.
[Rasha H. Bassyouni, Walla S. Abdel-all b, Mostafa G. Fadl, Saed Abdel-all and Zeinat kamel **Characterization of Lactic Acid Bacteria Isolated from Dairy Products in Egypt as a Probiotic.]** *Life Sci J* 2012;9(4):2924-2933]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 428-2933]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 428.

Keywords: *Lactobacilli*, Probiotic .Bile salt, Acid tolerance, Enzymatic activity, Antimicrobial activity, Hemolytic activity.

1. Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive rods and cocci occurring naturally in a variety of niches, including the gastrointestinal tract, plants and fermented foods such as dairy products, meat and alcoholic beverages (**Hammes and Hertel, 2006; Mohania *et al.*, 2008**).Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on host" (the Food and Agriculture Organization/World Health Organization (FAO/WHO). Most probiotics commercially available today belong to the genera *Lactobacillus* and *Bifidobacterium.* LAB are the most important group of microorganism used in food fermentations, they contribute to the fast and texture of fermented products and inhibit food spoilage and pathogenic bacteria by producing antimicrobial substances (lactic acid, hydrogen peroxide ,bacteriocin) **(Phillip *et al.*, 2012).** Several mechanisms by which probiotics mediate their health benefits on the host have been suggested, and can be divided into three categories; (i) certain probiotics have antimicrobial activity and can exclude or inhibit pathogens; (ii) probiotic bacteria can enhance the intestinal epithelial barrier; and (iii) probiotic bacteria are believed to modulate the host immune response **(Ezendam and Loveren, 2006; Marco *et al.*, 2006; Lebeer *et al.*, 2008; Lebeer *et al.*, 2010**). The mechanisms of health promoting effects of probiotic bacteria have proven difficult to elucidate in detail, and traditionally most attention has been given to their anti-pathogenic properties (**Lebeer *et al.*, 2008)**. To perform their effect in the intestine, probiotic bacteria should be capable of surviving passage through the GIT(gastro intestinal tract). Thus, it is essential for the bacteria to have protection systems to withstand the low pH in the stomach, digestive enzymes and bile of the small intestine. Approximately pH 2.5 l of gastric juice (**Cotter and Hill, 2003**) and pH1 l of bile (**Begley *et al.*, 2005**) are secreted into the human digestive tract every day. Tolerance to gastric acid and bile has thus become important selection criterion for probiotic strains.(**Jensen *et al.*, 2012**).

Lactobacilli are ubiquitous and widespread commensal bacteria in the human and animal micro

flora. They are widely used by humans: as adjuvants against gastrointestinal disorders, as dietary supplements, and as biological food processors based on their fermentative properties (**Beasley *et al.,* 2004**). *Lactobacilli* are gram-positive, non-spore-forming rods. It is possible for this resistance to be transmitted to the human population through the food chain. Although many strains are not pathogenic, they could constitute a reservoir of genes conferring resistance to antibiotics which might be transferred to pathogenic strains **(Rattanachaikunsopon *et al.,* 2010)** The study of health-beneficial effects that probiotic bacteria can exert on humans and animals is at its beginning. Pending scientific questions include the identification of molecular markers of the health-promoting activity of specific strains, which may be used to select novel probiotic strains and to gain understanding of the mechanisms underlying their effects. LAB can be isolated from different sources **(Phillip *et al.,* 2012**) including African grape and wine sample also Selective enumeration of *lactobacillus* spp. Was isolated from cheese **(Karimi *et al.,* 2012**.), Similarly **Vitali *et al.* (2012**) Isolated novel probiotic bacteria from raw fruits and vegetables *lactobacillus* can be isolated from plant as reported by **Hurtado *et al.* (2012**), also isolated Lactic acid bacteria from fermented table olives (**Abriouel, 2012**). The aim of this study was to isolates safe and potential probiotic *lactobacillus* spp.From different dairy products**.**

2. Material and Methods:

1. Isolation of Lactic Acid Bacteria from dairy products

The isolation material was from different sources of dairy product obtained from market. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory. Once delivered to the laboratory, they were taken to the procedure for isolation. Pour plate technique was used to isolate the organisms. Samples were used directly and also diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile peptone water. 1 ml aliquot of the samples and dilutions were plated into selective medium MRS (Man, Rogosa and Sharpe) agar (Oxoid LTD, Basingstoke, England) according to **Dave and Shah (1996).** The plates were incubated at 37 °C for 3 days under aerobic conditions. After incubation, individual colonies were selected and transferred into sterile broth media. The selected colonies were purified by streak plate technique. The isolates were examined according to their colony morphology, catalase reaction and gram reaction survival at different temperature and tolerance to NaCL concentrations also methyl red and vogues proskaure. Gram positive and catalase negative bacilli colonies were taken as lactic acid bacteria stored in glycerol culture and kept for further investigation at -20°C.

2. Identification

2.1 Carbohydrate fermentations of isolates

The tested carbohydrates were D (+) cellobiose (Sigma, Detroit, MI, USA), D (+) galactose (Sigma), lactose (Sigma), fructose (Sigma), maltose l-hydrate (Sigma), D mannitol (Sigma), D (+) melezitose (Sigma), melibiose (Sigma), D (-) raffinose (Difco), rhamnose (Sigma), ribose (Sigma), sorbitol (Sigma), D (+) trehalose (Sigma), and D (+) xylose (Merck, Darmstadt, Germany);glucose (Sigma), and sterile water were used as positive and negative controls. During the test, stored in glycerol culture and kept for further investigation at -20°C.

2.2 Arginine hydrolysis test

For arginine hydrolysis test, base MRS broth(Oxoid LTD, Basingstoke, England) without glucose and meat extract containing 0.3% arginine and 0.2% sodium citrate instead of ammonium citrate was used. Arginine MRS medium and Nessler's reagent were used in order to see ammonia production from arginine. MRS containing 0.3% L-arginine hydrochloride was transferred into tubes as 5 ml and inoculated with 1% overnight cultures. Tubes were incubated at 37 °C for 24hrs. After incubation, 100 µl of cultures transferred onto a white back ground. The same amount of Nessler's reagent was pipetted on the cultures. The change in the color was observed. Bright orange color indicated a positive reaction while yellow indicated the negative reaction. A negative control, which did not contain arginine, was also used.

2.3. Growth at different temperature and growth at different NaCl concentrations was carried out according to method described by **Briugs (1953).** Gram stain, urea test, Methyl Red Test and Vogues Prosquer test were determined as described by **Prescott and Harley (2002).**

3. Probiotic Properties of Isolates

Major selection criteria (resistance to low pH, tolerance against bile salt and the antimicrobial activity) were choosen for the determination of probiotic properties of isolates.

3 1. Resistance to Low pH

Resistance to pH 3 is often used *in vitro* assays to determine the resistance to stomach pH. Because the foods are staying during 3 hrs, this time limit was taken into account for this purpose. Active cultures (incubated for 16-18 hrs) were used. Cells were harvested by centrifugation for 10 min at 5000 rpm and 4°C. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2). Then cell pellets were suspended in PBS (pH 3) and incubated at 37 °C. Viable microorganisms were enumerated at the 3 hours with pour plate techniques. Appropriate dilutions were done and

plates were incubated at 37°C under aerobic conditions for 48 h. Also growth was monitored by absorbance at OD620.

3.2. Tolerance against Bile

Because the mean intestinal bile concentration is believed to be 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 hrs **(Kumar and Murugalatha, 2012)**, the experiment was applied at this concentration of bile for 4 hrs. MRS medium containing 0.3% bile was inoculated with active cultures and incubated for 16-18 hrs. During the incubation for 4 hrs, viable colonies were enumerated for every hour with pour plate technique and also growth was monitored by absorbance at OD620.

3.3. Evaluation of Antagonistic Activity

Antimicrobial effects of presumptive strains of *Lactobacillus spp*. were determined by the agar diffusion method. The tested bacteria were incubated in nutrient broth at appropriate temperature for 24 hours. Approximately 10^5- 10^7 cfu/ml of the bacteria to be tested for sensitivity (indicator bacteria) were inoculated (1%) into 20 ml of nutrient agar and poured into the Petri dishes. To detect antibacterial activity of *Lactobacillus* spp., MRS containing only 0.2% glucose was used. 10 ml of broth was inoculated with each strain of *Lactobacillus* spp. and were incubated at 35 ˚C for 48 hours. After incubation, a cell-free solution was obtained by centrifuging (6000 × g for 15 min) the culture. Some supernatants were neutralized by 1 N NaOH to pH 6.5, supernatants of the strains of *Lactobacillus* spp. were checked for antibacterial activity against pathogenic bacteria in inoculated nutrient agar. Then 100 ml of cell free supernatants was filled in 8-mm diameter sealed wells cut in the nutrient agar. Once solidified, the dishes were stored for two hours in a refrigerator. The inoculated plates were incubated for 24 hours at 37 ˚C, and the diameter of the inhibition zone was measured by calipers in millimeters **(Lleo, 1998; Mir-hoseini, 2004).**

4. Safety assessment

4.1 Test of isolates for antibiotic sensitivity

The isolates were tested for resistance to 15 antibiotics (Ampicillin/sulpactam, amoxicillin, claviolinicacid, Amoxicilin, Clarithromycin, Erothromycin, Naldixic acid, Trimethoprime/sulphamethoxazolin, Ciprofloxacin, tetracycline, Vancomycin, Rifampicin, Nitrofuruntoin, chloramphenicol, Tenadazole). This test was performed using the standard disc diffusion method **(National Committee for Clinical Laboratory Standards, 2000; SCAN, 2000; Herreros *et al.*, 2005; Phillip, 2012; Jensen, 2012).**

4.2. Analysis of Enzyme Activity of *lactobacillus* isolates.

The API ZYM kit (bio-Mérieux, France) was used to study enzyme activity production by isolates. Each identified isolate was grown overnight at 37oC on MRS broth. Sediment from centrifuged culture broth was used to prepare a suspension at 10^5 CFU/ml. After Inoculation, cultures were incubated for 4 h at 37oC. Placing a Surface-active agent (ZYM A reagent) in the cupules facilitated solubilization of the ZYM B reagent in the medium. Color was allowed to develop for at least 5 min, and values ranging from 0-5 were assigned corresponding to the colors developed. The approximate number of free n mole of hydrolyzed substrate was determined based on the color strengthen negative reaction; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, 40 nmol or higher

4.3 Test for hemolytic activity of *lactobacillus* spp.

Isolates were screened on blood agar plates containing 5% sheep blood and incubated at 37 °C for 48 hours. Hemolytic activity was detected as the presence of a clear zone around bacterial colonies **(Nour-Eddine, 2006)**

3. Results and Discussion:

1. Isolation and Identification of lactic acid bacteria

Fifty four isolates were isolated from different dairy product.

1.1. Morphological and Biochemical Identification

Isolates were characterized according to the method recommended by Bergey's manual of systematic Bacteriology **(Brinner *et al.*, 2001)**, 54 isolated bacteria were tested to select *lactobacillus* spp.which have the following characteristics aerobicity, gram-positive, catalase-negative and homofermentative bacilli that have yellowish, mocoid, rounded colonies. The species of *lactobacillus* identified by carbohydrates fermentation pattern, growth at10°C, 15°C, 30°C and 35°C ,growth at different NaCL concentration, arginine hydrolysis , urease test and carbohydrate fermentations as reported by **Cullimore (2008)**. (Table 1)

Table1: Stander identification of *genus of lactobacillus*

NO.	FAMILIES	MOT.	AERO.	G35°C	CAT.	H2S	A gluc
A	*LACTOBACILLUS*	-	FA	+	-	-	+
B	*ERYSIPELOTHRIX*	-	FA	+	-	+	+

Mot, motile; aero, aerobicity; fa, facultative anaerobic ;SA be ,strict aero, G35°C ,growth at 35°C; cat,catalase; H_2S, hydrogen sulphide produced ; Agluc, acid from glucose**.**

Table 2: Stander identification of *lactobacillus* spp.

	Ac	Ae	Al	Am	Ar	Ag
L. lactis	+/-	+	+	-	-	+/-
L. acidophillus	+	+	+	-	-	+
L. casei	+	+	+	+	+	+

(Ac, acid from cellobioseAe ,acid from esculin Al, acid from lactose Am, acid from mannitol; Ar ,Acid from raffinose, Ag,acid from galactose.)

According to the biochemical characteristics (Tables2,3), all isolates did not produced gas from glucose and did not produce ammonia from arginine .They tolerated 2%NaCl and 6.5%NaCl and 10%NaCl concentrations (Tables 3,4) and grew at 30°C, 35°C, 45 °C (Table 3). Isolates S8, S7, S3 gave positive results with the carbohydrates, glucose ,ramnose ,mannose, arabinose, raffinose, galactose, sucrose and lactose..Isolates S2, S4, S6, S5. gave positive test results with sugars ,glucose, ramnose, mannitol, fructose, maltose ,raffinose ,galactose, maltose, sucrose, fructose and lactose .and B.M .(breast milk) give positive result ramnose ,mannitol ,fructose ,inositol ,raffinose, mannose ,maltose ,lactose. Based on these biochemical. It seems that S8,S7,S3 is like to be *Lactobacillus acidophilus* (3 isolates) ,S2 ,S4 ,S6 may be identified as *Lactobacillus casei*(4 isolates) and B.M. is like to be *Lactobacillus lactis*. **(Roos *et al.*, 2005; Hammes and Hertel 2006).**The physiological and biochemical and morphological characters of identified Lactobacillus are reported in table 6.LAB are the most important group of microorganism used in food fermentation are predominant participant in many industrial products and plant and dairy fermentations various species of lactobacillus are the most commonly used probiotic microorganism **(Ranadheera *et al.*, 2012)** and played the dual role of starter and probiotic **.**Recently, significant attention has been paid to fermented dairy products containing probiotic bacteria Isolation of LAB from dairy product obtained in the present results was similar to many recent reports (**Wang *et al.*, 2010; Duskoval *et al.*, 2012**). In relation to the present result *L. acidophilus* was isolated from cheese (**karimi *et al.*, 2012). Liu *et al.* (2012**) found that *L.lactis* and *L.casei* were considered as the predominated species in fermented dairy product (Tarag). LAB play an important role in the production of a range of traditional fermented foods and have also previously been reported to be present in high numbers in many foods including fura **(Owusu *et al.*, 2012),** green table olive **(Abioue *et al.*, 2012**),cereal foods (**Oguntoyinbo *et al.*, 2012)** and curd and cucumber **(Patil *et al.*, 2010).**

Table 3: Biochemical characteristics OF *lactobacillus* isolates

Isolates No.	Acid from Glucose	methyl red	vogues prosquer	Urease test	Survival at 60 c for 90 min	Survival at 60 c for 60 min.	Growth at 45°C	Growth at 35°C	Growth at 30°C	Growth at 15°C	Growth at 10°C	2%NaCl	Gas from glucose	Motility	Catalase	Shape	aerobicity	Ammonia from Arginine
S1	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a	-
S2	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a.	-
S3	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a.	-
S4	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a.	-
S5	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a.	-
S6	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a.	-
S7	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a.	-
S8	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a.	-
B.M..	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	bacilli	f.a.	-

Table 4: Physiological characteristic. Of *lactobacillus* isolates

ISolate no.	15. % NaCl	10. % NaCl	6.5%NaCl	2%NaCl	Ammonia from Arginine	Gas from glucose	Catalase	Motility
S1	-	+	+	+	-	-	-	-
S2	-	+	+	+	-	-	-	-
S3	-	+	+	+	-	-	-	-
S4	-	+	+	+	-	-	-	-
S5	-	+	+	+	-	-	-	-
S6	-	+	+	+	-	-	-	-
S7	-	+	+	+	-	-	-	-
S8	-	+	+	+	-	-	-	-
B.M.	-	+	+	+	-	-	-	-

Table 5: carbohydrate fermentation of *lactobacillus* isolates(B.M., human milk)

SUGER	S1	S2	S3	S4	S5	S6	S7	S8	B.M.
RAMNOS	+	+	+	+	+	+	+	+	+
MANNITOL	+	+	_	+	+	+	-	-	+
FRUCTOS	+	+	_	_	+	+	-	-	+
INOSITOL	_	_	_	_	-	-	-	-	+
SALICIN	_	_	+	_	-	-	-	-	-
RAFINOSE	+	+	_	+	+	+	+	+	+
MANNOSE	-	-		-	-	-	+	+	+
MALTOSE	_	+	_	+	_	+	-	-	+
LACTOSE	+	+	+	+	+	+	+	+	+

Table 6: Morphological and biochemical properties of identified *Lactobacillus spp.*

Characteristics	*L. acidophilus*	*L. casei*	*L. lactis*
Cell shape	Bacilli	Bacilli	Bacilli
Catalase test	-	-	-
Motility	-	-	-
Aerobicity	f.a	f.a	f.a
Acid, from glucose	+	+	+
Gas from glucose	-	-	-
Ammonia from arginine	-	-	-
Growth at different temp. 35C 30C 15C 10C	+	+	+
Growth at NaCl 10,6.5,4,2.5	+	+	+
Growth at pH 3for 1 hour 2 hours 3 hours	+	+	+
Vogues prosquer	-	-	-
Methyl red	+	+	+
Urease test	-	-	-
RAMNOS	+	+	+
MANNITOL	-	+	+
FRUCTOS	-	+	+
INOSITOL	-	-	+
SALICIN	-	-	-
RAFINOSE	+	+	+
MANNOSE	+	-	+
MALTOSE	-	+	+
LACTOSE	+	+	+

2-Probiotic characterization

2.1. Survival of lactobacillus Spp. isolates in pH 3.0

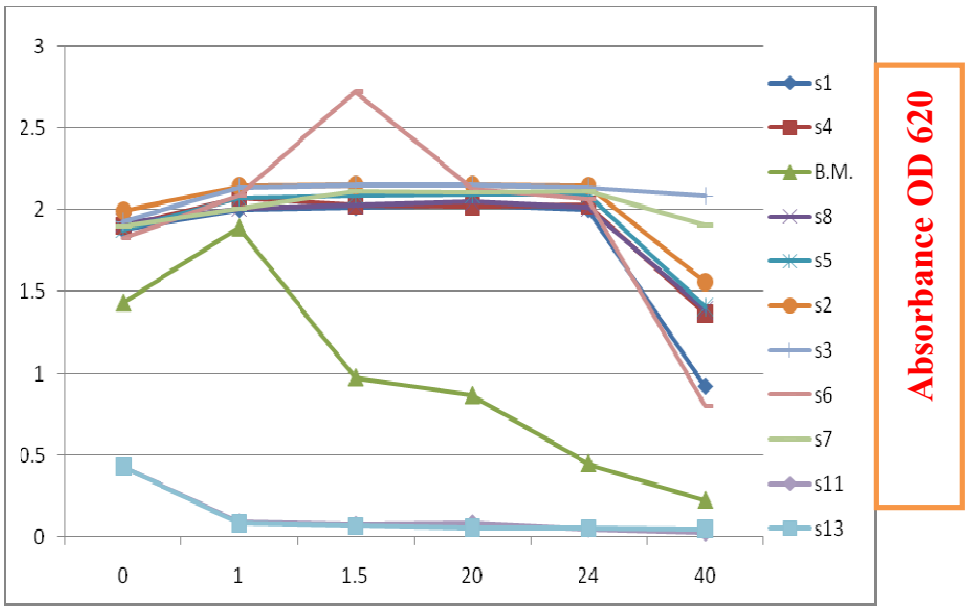

Figure 1: Survival of isolates in pH 3.0 – absorbance at OD_{620} values

Resistance to low pH is one of the major selection for probiotic strains **(Cakir, 2003).** LAB are indigenous habitants of human gastro intestinal tract and thought to among the dominant colonies of the small intestine **(Marco *et al.*, 2006).** To reach the small intestine ,they have to pass through stomach. Although in the stomach ,pH can be as low as in vitro assay pH 3.0 have been preferred . For selection of strain resistant to low pH 3.0 was used .The time that takes during digestion in stomach is 3 hours, so isolates were tested for resistance to pH 3.0 during 3.0 h. Figure 1 indicates that eight of eleven *lactobacillus* isolates were resistance to pH 3.0 during three hours.

Isolates S1, S2, S3, S4, S5, S6, S7, S8, B.M. were very stable in pH 3.0 which means that isolates are able to survive in this pH value the other *lactobacillus* isolates are able to tolerates pH 3.0 for one hour but they more sensitive to low Ph. In agreement with the present results **Martin *et al.* (2004)** isolated three *lactobacillus spp*. from human milk and were identified as *L. gasseri* and *L. fermented*. They survived Low pH and in gastrointestinal environment the strains especially *L. casei* result showed that these isolates can be used as potential probiotic strains. Our results were also similar to that reported by **Maragkoudakis *et al.* (2005)** who tested that 29 *Lactobacillus* strains of dairy origin for their probiotic potential. All of the examined strains were resistant to pH 3.0 during 3h most of them lost their viability in one hour in pH 1. Tolerance of probiotic *lactobacillus spp*. isolated in the present study are in accordance with previous results **(Jensen *et al.*, 2012; Hurtado *et al.*, 2012; Abioue *et al.*, 2012).**

2.2.Tolerance against Bile.

All *lactobacilli* isolates were tested for bile salt tolerance The strains, resistant to low pH became 8 isolates , were screened for their ability to tolerate the bile salt Strains were detected in 0.3% during 3hours (Table7).

Table 7: Tolerance of *lactobacillus* spp. isolates against 0.3% bile – cfu. values

	CFU/ml				
CFU/time	B.M.	S8	S1	S4	ATCC7830
0h	196	190	205	200	221
1h	189	187	170	185	183
2hrs	188	180	175	183	189
3 hrs	178	179	179	180	181

These results indicated that all isolates can tolerates the bile salt 0.3%concenteration. This in agreement with **Darilmaz, and and Beyatli (2012)** who reported that acid tolerance of isolated

Lactobacillus spp. varied at different pHs. Such difference was however lower between isolated Lactobacilli and *L. plantarum* .The isolated *Lactobacilli* were bile salt tolerant. Bile tolerance is essential for probiotic strains to colonize the small intestine (**Huang, 2004**) With the development of new delivery systems and the use of specific foods , Evidence clearly demonstrates that acid sensitive strains can be buffered through the Stomach. However, to exert a positive effect on the health and well-being of a host, probiotics need to colonize and survive in the small intestine **(Leverrier, 2005)** and it is the condition of this environment that may in fact ebe the essential selection criteria for future probiotics. Similarly **Abriouel *et al.* (2012)** reported that *Lactobacillus* strains isolated from fermented table olive tolerated 2% bile salt. **Jensen *et al.* (2012**) reported that *Lactobacillus spp*. tolerate gastric juice well with no reduction in viability and *L.pentoses* and *L.sakei* strains lost viability over 180 min. similarly **Vitali *et al.* (2012)**determined the probiotic potential of large number of lactic acid bacteria isolated from fruit and vegetables, the result indicated that 35 % of LAB. maintained high cell densities and survived gastric and intestinal conditions.

2.3. Antimicrobial Activity

The selected eight isolates were tested for their antimicrobial activity against clinical isolates .For this purpose, strains were tested against *Salmonella thyphimurium, Escherichia coli, Micrococcus spp., Staphylococcus spp*. Result shown in Table 8.

Table 8.Antimicrobial activity of lactobacillus isolates

	Diameter of inhibition zone (mm)			
No. of isolate	*E. coli*	*Salmonella thyphimurium*	*Micrococcus*	*Staphylococcus*
S1	14	13	13	14
S2	14	15	15	15
S3	13	12	12	14
S4	15	14	14	17
S5	18	10	20	17
S6	19	9	17	20
S7	20	20	17	22
S8	18	17	18	25
B.M.	21	18	22	25

Table 8 showed that all of the isolates have antibacterial effect on the tested microorganisms. The tests were applied two times and the averages of diameters of zones were given. From the result we found that B.M. has the most potent antimicrobial activity isolate followed by S5 then S6, S8 and the lowest antimicrobial activity was found in S1 ,S2, S3 LAB . This may be due to production of bacteriocins which are peptides with bactericidal activity usually against strains of closely related species (**Abriouel *et al.*, 2012).** Bacteriocins may enhance survival of LAB in complex ecological systems that focused on prevention of growth of harmful bacteria in the fermentation and preservation of dairy products. It is more interesting with respect to probiotics that individual strains may inhibit growth of or adhesion of pathogenic microorganisms by secreted products, and not merely an effect of acidic pH (**Atta, 2009**.) Also *Lactobacillus* isolates obtained from fermented millet drink are more effective than isolates from cow milk as regards their antagonism or inhibition (**Shehata, 2012**). An important property of probiotic strains is their Antagonistic activity against pathogenic bacteria. Propionic acid bacteria can produce Antimicrobial substances capable of inhibiting the growth of pathogenic and spoilage microorganisms. Propionic acid, acetic acid, and diacetyl in addition to the antimicrobial peptides are included among these compounds (**Havenaa, 1992).** similar result on antagonistic activity of LAB. was reported by several investigation and largely documented (**Abriouel *et al.*, 2012**). Also all LAB. Isolated from raw fruits and vegtables inhibited *E. coli* isolated from human sources .**Vitali *et al.* (2012**) isolated strains of different species (*90* isolates) from olives produced antimicrobial substances which were active against a number of potentially pathogenic gram negative and gram positive bacteria such as *S. aureus, E.facalis* , *Salmonella enterica* ,Also another study revealed that bacteriocin produced by *Lactobacillus* isolated from chicken showed inhibition against a number of food-borne pathogens, such as *L.monocytogenes*, *S.aureus* and *Salmonella* without inhibiting LAB (**Messaoudi., 2012**).

3. Safety assessment of probiotic *lactobacillus spp.*

3.1.Enzymatic Activities of lactobacillus isolates

All isolates were screened to enzymatic activity to detect any unfavorable enzyme like the carcinogenic enzyme, β-glucuronidase and presence of beneficial enzymes. Enzyme Production by isolates was an important criterion in its selection, because carcinogenic enzymes such as β-glucuronidase can be produced by microorganisms. When carcinogenic substances such as benzo(a) pyrene enter the human

body, their poisonous effects are counteracted because of conjugation with glucuronic acid in the liver. If this conjugated product is excreted with bile acid in the intestine, cleavage by β-glucuronidase can liberate these substances to become toxic once again. Result recorded in table 8 indicated that all isolates did not produce the carcinogenic enzyme, β-glucuronidase, whereas beneficial was produced, which is beneficial for lactose intolerance. These enzymes include leucine arylamidase, crystine arylamidase,acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and N-acetyl-β-glucosamidase. These results wee in agreement with **Chang-Won *et al.* (2008)**. Similarly, β-galactosidase was found in *lactobacillus* isolated from fermented oil as reported by **Abriouel *et al.* (2012)**.

Table 9: Enzyme activity of isolated *Lactobacillus* spp.

Enzyme	S1	S2	S3	S4	S5	S6	S7	B.M.	S8
Control	0	0	0	0	0	0	0	0	0
Alkaline phosphatase	0	0	0	0	0	0	0	0	0
Esterase	0	0	0	0	0	0	0	0	0
Esterase lipase	0	0	0	0	0	0	0	0	0
Lipase	0	0	0	0	0	0	0	0	0
Leucinearylamidase	2	2	2	2	2	2	2	3	2
Valinearylamidase	0	0	0	0	0	0	0	0	0
Cystinearylamidase	1	1	1	1	2	1	1	2	1
Trypsin	0	0	0	0	0	0	0	0	0
α-Chymotrypsin	0	0	0	0	0	0	0	0	0
Acid phosphatase	2	2	2	2	2	2	2	2	2
Naphthol-AS-BI-phosphohydrolase	5	5	5	5	5	5	5	5	5
α-Galactosidase	2	2	2	2	2	2	2	2	2
β-Galactosidase	5	5	5	5	5	5	5	5	5
β-Glucuronidase	0	0	0	0	0	0	0	0	0
α-Glucosidase	2	2	2	2	2	2	1	2	1
β-Glucosidase	2	2	2	2	2	2	2	2	2
N-Acetyl-β-glucosaminidase	3	3	3	3	3	3	3	3	3
α-Mannosidase	0	0	0	0	0	0	0	0	0
α-Fucosidase	0	0	0	0	0	0	0	0	0

Score 0 = 0 nmol, Score 1= 5 nmol, Score 2= 10 nmol, Score 3= 20 nmol, Score 4= 30 nmol, Score 5 ≥40 nmol.

3.2. Testing for resistance to antibiotics

All isolates were tested for antibiotics and show different degree of resistance which was also observed by **Herreros *et al.* (2005)**. Various reports indicate that LAB are normally resistant to the principal types of antibiotics, such as B-lactam, cephalosporin, aminoglycosides, quinolone, imidazole, nitrofurantoin and fluoroquinolines (**Halami *et al.*, 2000**).Transfer of resistance to antimicrobial substances is an essential mechanism in LAB if they are to adapt and survive in specific environments. Among the resistance mechanisms in use, enzymic inactivation of the antibiotics ,restricted import of antibiotics, active export of antibiotics or target modification may be high lighted (**Davies, 1997**). *Lactobacilli* are generally resistant to aminoglycosides (**Belletti *et al.*, 2009**).Vancomycin resistance is thought to be intrinsic, since nearly all the strains are constitutively resistant to low levels of the antibiotic (**Roland *et al.*,1992**.).

3.3. Hemolytic activity of *lactobacillus* spp.

All isolate were tested for hemolytic activity and gave negative result with this test confirming that LAB are safe for human use **Kumar and Murugalatha, 2012**).This result agree with **Sandra *et al.* (2012)** who reported that none of the fifteen putative probiotics was found to be B-hemolytic.

In conclusion the present study showed that human milk, yogurt and raw milk are sources of potential probiotic strains of LAB and the isolates meet several function features to be considered as suitable probiotic for application in food fermentation and the isolated bacteria are able to tolerate acidic medium and bile salt with favorable enzymatic activity and no hemolytic activity so we consider it great potential probiotic character and safe for human use.

References:

Abriouel H., Benomar N., Cobo A., Caballero N., Fuentes M.A., Pérez-Pulido R., Gálvez A. (2012). Characterization of lactic acid bacteria from

naturally-fermented Manzanilla Aloreña green table olives * Food Microbiology 32: 308-316

Atta H.M. (2009). Application of Biotechnology for Production, Purification and Characterization of Peptide Antibiotic Produced by Probiotic Lactobacillus plantarum, NRRL B-227 Global Journal of Biotechnology & Biochemistry. 4 ((2): 115-125.

Beasley S. (2004). Isolation, identification and exploitation of lactic acid bacteria from human and animal microbiota. PhD thesis, Helsinky: University of Helsinki

Begley M., Gahan C.G.M. and,Hill C. (2005). The interaction between bacteria and bile. FEMS Microbiology Reviews 29: 625–651.

Belletti N., Benedetta-bottari M.G., Tabaneli G and Gardini A.F. (2009). Antibiotic Resistance of *Lactobacilli* Isolated from Two Italian Hard Cheeses Journal of Food Protection,. 72(No. 10): 2162-2169.

Briugs M. (1953). The Classification of Lactobacilli by means of Physiological Tests J. gen. Microbial. 9: 234-2444.

Brinner D.J., Staley J.T., Kreig N.R. (2001). Classification of procaryotic organisms and the content of bacterial speciation In: BOONE dr,castenholz RW (eds, garrity GM (editor in chief)bergey's manual of sydtematic bacteriology 2nd edn,vol1,thearchaea and thgedeeply branching and phototrophic bacteria

Çakır İ. (2003). Determination ofsome probiotic properties on Lactobacilli and Bifidobacteria. *Ankara University Thesis of Ph.D*.

Chang-Won K., and Paik H-D. (2008). Screening of *Lactobacilli* Derived from Chicken Feces and Partial Characterization of *Lactobacillus acidophilus A12 as Animal Probiotic* J. Microbiol. Biotechnol. 18(2): 338-342.

Cotter P.D., Hill, C. (2003).Surviving the acid test: responses of Gram-positive bacteria to low pH. Microbiology and Molecular Biology Reviews 67, 429–453

Cullimore R. (2008). Practical atlas for bacterial identification .p. 91 chapter gram positive rods.

Darilmaz D.O. and Beyatli Y. (2012) Acid-bile, antibiotic resistance and inhibitory properties of propionibacteria isolated from Turkish traditional home-made cheeses Anaerobe 18 122-127

Dave R. I. and Shah N. P. (1996). Evaluation of media for selective enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus acidophilus and Bifidobacterium spp*. J. Dairy Sci. 79:1529–1536.

Davies J.E. (1997). Origins, acquisition and dissemination of antibiotic resistance determinants. In: Chadwick, D.J., Goode, J. (Eds.), Antibiotic Resisance. Origins, Evolution, Selection and Spread. Wiley, Chichester, pp. 15–27.

Dušková M., KateřinaKšicová O.Š., ZbyněkZdráhal , RenátaKarpíšková, (2012). Identification of *lactobacilli* isolated from food by genotypic methods and MALDI-TOF MS. International Journal of Food Microbiology 159: 107-114.

Ezendam, J., Loveren, H. (2006).Probiotics: immunomodulation and evaluation of safety and efficacy. Nutrition Reviews 64:1–14.

Hammes W.P. and Hertel, C. (2006). The genera *Lactobacillus* and *Carnobacterium*, In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), third ed. The Prokaryote, vol.4. Springer, New York, NY, pp. 320–403.

Halami, P.M., Chandrashekar, A., Nand, K., 2000. *Lactobacillus farciminis* MD, a newer strain with potential for bacteriocin and antibiotic assay. Lett. Appl. Microbiol. 30, 197–202.

Havenaar, R., Brink N.G., Huisin'tVed J.H.J. (1992). Selection of strains for probiotics use. In: Fuller R, editor. Probiotics: the scientific basis. London: Chapman and Hall;. p. 210-24.

Herreros, M.A., Sandoval H., Gonzáleza L., Castro J.M., Fresno J.M. and Tornadijo M.E. (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). Food Microbiology,.22: 455–459.

Huang, Y. and Adams M.C. (2004).*In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. Int J Food Microbiol; 91:253-60.

Hurtado, H.,C.R., Bordons A., Rozès N., (2012). Lactic acid bacteria from fermented table olives. Food Microbiology 31: 1-8.

Jensen H., Grimmer S. , Naterstad K., Axelsson L. (2012), In vitro testing of commercial and potential probiotic lactic acid bacteria International Journal of Food Microbiology (153): 216-222.

Karimi R., Mortazavian A.M., Amiri-Rigi A. (2012). Selective enumeration of probiotic microorganisms in cheese Food Microbiology 29: 1-9

Kumar M. (2012), Isolation of Lactobacillus plantarum from cow milk and screenin for the presence of sugar alcohol producing gene.Journal of Microbiology and Antimicrobials. 4 (1): 16-22.

Lebeer S., Vanderleyden J., De Keersmaecker, S.C. (2008). Genes and molecules of lactobacilli supporting probiotic action. Microbiology and Molecular Biology Reviews 72, 728–764.

Lebeer S., Vanderleyden J., De Keersmaecker S.C. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nature Reviews Microbiology 8, 171–184.

Leverrier P., Fremont Y., Rouault A., Boyaval P. and Jan G. (2005). In vitro tolerance to digestive stresses of propionibacteria: influence of food matrices. Food Microbiol; 22:11-8.

Leclerqu R., S.D.-M., Duval J. and Courvalin P., (1992). Vancomycin Resistance Gene vanC Is Specific to *Enterococcus gallinarum* Antimicrobial

agents and chemotherapy, 36(9): 2005–2008. .

Liu W., QiuhuaBao, Jirimutu, Qing M., Siriguleng, Chen X., Sun T., Meihua Li , Zhang J., Jie Yu, Bilige M. , Tiansong Sun T, and Zhang H. (2012). Isolation and identification of lactic acid bacteria from Tarag in Eastern Inner Mongolia of China by 16S rRNA sequences and DGGE analysis Microbiological Research 167: 110– 115.

Lleo M.M., Tafi M.C., Canepari P. (1998). Non culturable Enterococcus faecalis cells are metabolically active and capable of resuming active growth *Syst Appl Microbiol*. 21(3):333-9.

Maragkoudakis P.A., Zoumpopoulou G., Miaris C., Kalantzopoulos G., Pot, B., Tsakalidou, E. (2005). Probiotic potential of *Lactobacillus* strains isolated from dairy products. *International Dairy Journal* 16:189-199.

Marco M.L., Pavan S., Kleerebezem M. (2006). Towards understanding molecular modes of probiotic action. Current Opinion in Biotechnology 17: 204–210.

Martin R., Langa S., Reviriego C., Jimenez E., Marin L.M. Olivares M., Boza J., Jimenez J., Fernandez L., Xaus J. and Rodriguez J.M. (2004).Tthe commensal microflora of human milk: New perspectives for food bacteriotherapy and probiotics. *Trends in Food Science & Technology*15 :121-127.

Messaoudi S., Kergourlay G., Dalgalarrondo M., Choiset Y, Mir-hoseini M. (2012). Study of effect of nisin and producer bacteria of nisin on *Listeriamonocytogenes* and *Bacillus cereus*] [Isfahan: University of Isfahan]

Mir-hoseini M. (2004). Study of effect of nisin and producer bacteria of nisin on *Listeria monocytogenes* and *Bacillus cereus*. Isfahan: University of Isfahan.

Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain,S., Marotta, F., Singh, V., Parkash, O., Yadav, H. (2008) . Molecular approaches for identification and characterization of lactic acid bacteria. Journal of Digestive Diseases 190–198.

National Committee for Clinical Laboratory Standards (2000). Performance standards for antimicrobial disk susceptibility tests. Villanova, PA: NCCLS, Approved Standard: M2-A7. 7th ed.

Nour-Eddine K.M., .K. (2006). *In-vitro* pre-selection criteria for probiotic *LACTOBACILLUS PLANTARUM* strains of fermented olives origin.international Journal of Probiotics and Prebiotics. 1. 27-32.

Oguntoyinbo F.A and ,Narbad, A. (2012) Molecular characterization of lactic acid bacteria and in situ amylase expression during traditional fermentation of cereal foods.Food Microbiology 2012.31 : 254-262.

Owusu- J,K., , Fortune Akabanda , Dennis S. Nielsen , KwakuTano-Debrah, Richard L.K. Glover , Lene Jespersen (2012). Identification of lactic acid bacteria isolated during traditional fura processing in Ghana Food Microbiology 32 (2012) 72-78.

Patil, m.m., and Ajay pal, T.(2010).isolation and characterization of lactic acid bacteria from curd and cucumber ,Indian Journal, 9: 166-172.

Phillip, S.,Mtshali, B.D., Maret du Toit (2012) *Identification and characterization of Lactobacillus florum strains isolated from South African grape and wine samples* International Journal of Food Microbiology 153:106-113.

Prescott, L.M.andHarley ,J.P. (2002). Appendix h: Reagents,solutions ,stains ,and tests .in laboratory excercises in microbiology. 5th ed. New york :mcgraw hill.

Ranadheera C.C.S., Evans C.A C.A., Adams M .C .M.C. and Baines S. K. S.K. (2012). Probiotic viability and physico-chemical and sensory properties of plain and stirred fruit yogurts made from goat's milk.Food Chem 135(3):1411-8.

Rattanachaikunsopon, P., Phumkhachorn P (2010). Lactic acid bacteria: Their antimicrobial compounds and their uses in food production *Ann Biol Res*.; 1:218-28.

Roos, S., Engstrand L., Jonsson H. (2005). *Lactobacillus gastricus sp. nov., Lactobacillus antri sp. nov., Lactobacillus kalixensis sp. nov. and Lactobacillus ultunensis* sp. nov., isolated from human stomach mucosa. International Journal of Systematic and Evolutionary Microbiology 55:77-82.

Sandra, T. S., Janine Barlow, Adele Costabile , Glenn R. Gibson and Ian Rowland (2012) In vitro evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids J. Anaerobe 18 530-538.

SCAN (2000): Report of the Scientific Committee on Animal Nutrition on the Safety of Use of Bacillus Species in Animal Nutrition. European Commission Health & Consumer Protection Directorate-General. http://europa.eu.int/comm/food/fs/sc/scan/out41.pdf

Shehata A. (2012). Molecular Identification Of Probiotics Lactobacillus Strain isolates by: Amplified Ribosomal DNA Restriction Analysis (ARDRA). Science Journal of Microbiology, ID SJMB-175, 8 Pages, 2012. doi:10.7237/sjmb/175.

Vitali B., Minervini G., Rizzello C.G., Spisni E., Simone Maccaferria S., Brigidi P., Gobbettib M and Di Cagno R. (2012). Novel probiotic candidates for humans isolated from raw fruits and vegetables.Food Microbiology.31: 116-125.

Wang, J., Chen, X., Liu, W., Yang, M., Zhang, H., (2008).Identification of Lactobacillus from koumiss by conventional and molecular methods. European Food Research and Technology 227, 1555–1561.