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SCREENING OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA FROM DIFFERENT SOURCES BY *In vitro* TESTS

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ABSTRACT: A total of 87 isolates of lactic acid bacteria (LAB) were isolated anaerobically from different food sources (yoghurt; Domiati cheese; fresh and fermented milk; mixed pickles and green olives), as well as human breast milk and infant stools. Only ten isolates showed high tolerance to pH 3.0 for three hours and therefore they were chosen for other studies. The selected isolates were identified based on physiological, biochemical and MALDI- TOF mass spectrometry identification. The most frequently observed genus was *Lactobacillus* (8 isolates) and one isolate seemed to be *Enterococcus faecium* and another isolate showed that it is *Bifidobacterium bifidium*. All of the tested species with a score value between 2.000 to 2.484 (100%) were correctly identified by MALDI -TOF-MS to the genus and species levels. The majority of LAB species were tolerant to 0.3% bile salts for up to 4 hrs but *L. fermentum* was the most tolerant. Four species exhibited partial bile salt hydrolase activity. All of the species survived in 1 mg/ml pancreatin for 4 hrs. However, two of them showed 1/10 decrease in their numbers. Eight species were non haemolytic. Most of the tested species were resistant to penicillin (10 µg), ciprofloxacin (5 µg), gentamycin (10 µg) and streptomycin (10µg). However other species showed variable resistance against the ten tested antibiotics according to NCCLS. The cell free supernatant of *L. acidophilus* (IS9) showed the highest antimicrobial property against all the indicator pathogens tested specially *Staphylococcus aureus* and *Klebsiella pneumonia*e. Therefore, these 10 species were found, *in vitro*, to possess desirable properties in order to use as probiotic for human consumption.

Key words: Lactic acid bacteria, probiotic, antimicrobial and antibiotic resistance.

INTRODUCTION

Probiotics are live microorganisms defined according to **FAO/WHO (2002)** as: "Live microorganisms whose administration in adequate amount to the body is able to confer a health beneficial effect on the host". The term probiotic, literally meaning “for life”, was first addressed by **Lilly and Stillwell (1965)**. Nowadays, the term refers to viable, nonpathogenic microorganisms (bacteria or fungi) that, when ingested, are able to reach the intestines in sufficient numbers to confer health benefits to the host (**De Vrese and Schrezenmeir, 2008**). Commonly used bacterial probiotics include various species of *Lactobacillus, Bifidobacterium* and *Streptococcus*, as well as *Lactococcus lactis* and some *Enterococcus* species. Currently, the only probiotic yeast used is the nonpathogenic *Saccharomyces boulardii* (**Morrow *et al.*, 2012**). In order for a probiotic specie to exert its beneficial effect on the host, it has to be able to survive passage through the host’s digestive tract. Researchers have mainly focused on species sensitivity towards low pH, proteolytic enzymes and bile salts (**Conway *et al.*, 1987; Charteris *et al.*, 1998a; Du Toit *et al.*, 1998; Jacobsen *et al.*, 1999**). Another relevant property is the ability of probiotic bacteria to assimilate cholesterol (**Du Toit *et al.*, 1998**). This has been linked to the bile salt

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deconjugation activity of some species because of the enzyme bile salt hydrolase (BSH).

Most of the studies published about physiological properties of species intended to be used as probiotics are performed on species from human or animal internal cavities, considering that species of these origins would be better adapted and colonize the human/ animal gastrointestinal tract (**Johansson *et al.*, 1993; Parasad *et al.*, 1999; Ouwehand *et al.*, 2002; Ruiz- Moyano *et al.*, 2009; Zacarías *et al.*, 2011; Xanthopoulos *et al.*, 2012**). On the other hand, research on probiotic functions of lactic acid bacteria isolated from foods like dairy products has started to increase (**Maragkoudakis *et al.*, 2006; Bao *et al.*, 2010; Espeche *et al.*, 2012; Monteagudo-Mera *et al.,* 2012**), dry sausages (**Papamanoli *et al.*, 2003; De Vuyst *et al.*, 2008**), foods of plant origin (**Husmaini *et al.,* 2011**) and fruits, cereals, meat or fish (**Rivera-Espinoza and Gallardo-Navarro, 2010**). Traditional fermented foods are a plentiful source of microorganisms and some of them show probiotic characteristics, although the research of these matrices as raw material for probiotic microorganisms is still scarce compared with their dairy counter part (**Rivera-Espinoza and Gallardo- Navarro, 2010**).

Several mechanisms by which probiotics mediate their health benefits on the host have been suggested, and can be divided into three categories; certain probiotics have antimicrobial activity and can exclude or inhibit pathogens; probiotic bacteria can enhance the intestinal epithelial barrier; and probiotic bacteria are believed to modulate the host immune response (**Marco *et al.,* 2006; Lebeer *et al.,* 2008**). Probiotics can produce a wide range of antimicrobial metabolites, *i.e.* organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins. These antimicrobial activities can contribute in the microbiological safety by controlling the growth of other microorganisms, and inhibition of pathogenic bacteria (**Hobbs, 2000; Ouweh and Vest, 2004**). The reported health benefits of probiotics include: boosting of the immune system, inhibition of the growth of pathogenic microorganisms, prevention of diarrhea from various causes, prevention of cancer, reduction of the risk of inflammatory bowel movements, improvement of digestion of proteins and fats, synthesis of vitamins, and detoxification and protection from toxins **Sonomoto and Yokota (2011)**.

Probiotics could maintain gut microbiota during or after antibiotic treatment through receptor competition, competition for nutrients, epithelial inhibition, and mucosal adherence of pathogens (**Clemente *et al.,* 2012**). Many of probiotics, which are lactic acid bacteria and anaerobic bifidobacteria, have been reported to be useful in the treatment of disturbed gut microbiota and diarrheal diseases. Thus, probiotic-based approaches that restore gut homeostasis are viewed as promising therapies for bacteria caused disease.

The aim of this study was to isolate and identify novel probiotic species originating from different sources and to evaluate their probiotic potential and safety in order to be used as health-promoting, functional foods.

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria from Different Sources

Lactic acid bacteria (LAB) were isolated from yoghurt, Domiati cheese, fresh and fermented milk, mixed pickles and green olives. Also human samples represented in human breast milk and infant stools were collected from two healthy mother volunteers and their infants, respectively. The samples were collected using sterile bottles and stored in an ice box until delivering to the laboratory of Agric. Microbiology Department, Fac.Agric, Zagazig University for analysis. One gram/milliliter of each sample was diluted in 0.9% sterile saline solution to a final volume of 10ml and 0.1ml of each dilution was plated onto selective MRS-medium (DeMan, Rogosa-Sharpe, Oxoid, CM 361). MRS medium was supplemented with 0.05% cysteine hydrochloride to improve the specificity of this medium for isolation of *Lactobacillus acidophilus* **(Diba *et al.*, 2013)** and bifidobacteria (**Parasad *et al.*, 1999)**. The pH of the media was adjusted to 6.5 and 5.2, respectively using a digital electrode pH meter. Plates were incubated at 37±2°c anaerobically in jars with AnaeroGen sacks (Oxoid,UK) for 48 hrs. After incubation different colonies were

randomly collected from each sample, the selected colonies were purified by streak plate technique. The purified bacterial isolates were stored in MRS broth at 4°C for further studies. As probiotic bacteria should pass through a highly acidic stomach in order to reach the intestine and accordingly creating proper conditions for residence **(Maragkoudakis *et al.*, 2006; Argyri *et al.*, 2013).** Therefore, the first step in screening the probiotic isolates is selecting those which showed acid resistance.

Preliminary Identification of Lactic Acid Bacteria

Morphological and biochemical characters were used to identify the most acid resistant bacterial isolates according to **Logan and De vos (2009)**, the following tests were performed: cell morphology, Gram reaction, catalase test, ammonia production , growth at 10°C for 5 days and 45°C for 48 hr., in MRS broth, salt tolerance (4%, 6.5% NaCl in MRS). Sugar fermentation tests were applied using D-trehalose, lactose, raffinose, sucrose, cellobiose, galactose. Gram-positive, catalase negative rods that grew at 45°C or 10°C were considerd lactobacilli. Gram-positive, catalase negative cocci that grew in 6.5% at 45°C and 10°C were considered enterococci.

MALDI-TOF-MS Profile Acquisition

Identification of LAB had been confirmed by using matrix-assisted laser desorption/ Ionization time of flight mass spectrometry (MALDI-TOF-MS) in peptide and protein analyses.

One large colony of each of selected bacterial isolate (enough to fill about one half of a 10-µl inoculating loop) was suspended in 70% ethanol in a 1.5 ml microcenrtifuge tube and loaded three times onto ground steel MALDI target according to the manufacturer′s instruction (Bruker Daltonics, Breman, Germany). Matching between experimental MALDI-TOF-MS profiles obtained from bacterial isolates and the reference MALDI-TOF-MS profiles is expressed by a BioTyper according to a Log (score) and an associated-color code (green, yellow and red). Briefly, a BioTyper log (score) exceeding 2.3 (green color) indicates a highly probable identification at the species level. A Log (score) between 2.0 and 2.3 means highly probable identification at the genus level (green color) and probable identification at the species level. A Log (score) between 1.7 and 2.0 (yellow color) implies only probable genus identification, while score value under 1.7 (red color) means no significant similarity between the unknown profile and any of those of the database. Micro Flex mass spectrometeries were performed at Academic Park, Faculty of Medicine, Alexandria, University, Egypt, according to **Biswass and Rolain (2013) and Nacef *et al.* (2016).**

Characterization of Isolates Considered to Be Potential Probiotics

Major selection criteria (resistance to low pH, tolerance against bile, bile salt hydrolysis, haemolytic activity, pancreatin resistance, antibiotic resistance and antimicrobial activity) were used for the determination of probiotic properties of the selected isolates of lactic acid bacteria.

Tolerence to low pH

Tolerence to low pH is often indicative to stomach pH and was tested as described by **Conway *et al.* (1987)**. *Lactobacillus* cultures were grown anaerobically in MRS agar medium (Difco) at 37 ±2°C overnight and transferred to fresh MRS broth for a further 16 - 18 hr., (to stationary phase). Cultures were centrifuged at 10000 × g/ l0 min/4°C, washed once with sterile phosphate-buffered saline {(PBS) NaCL, 0.8%; 0.1 M KH_2PO_4, pH 7.2)} and resuspended to one-tenth of the culture volume. These suspensions were used for the *in vitro* survival studies, by the addition of 0.1 ml of each suspension into 2-ml of sterile PBS at pH 1, 2, and 3, and was maintained at 37 ±2°C. The growth of the species was determined by absorbance at OD 620 nm after 0, 1.0, 2.0 and 3.0 hr., reflecting the time spent by food in the stomach (**Bassyouni *et al.*, 2012).** Resistance percentage of species to stomach acid was determined by comparing optical denisty at zero time with optical denisty after 3 hr.

Tolerance to bile salts

The procedure of **Klaenhammer and Kleeman (1981)** was used to determine the tolerance of various species to bile (ox-bile) at final concentrations of 0, 0.3, 1.0% *W/V* on solid MRS growth media, considering the fluctuation of bile concentrations at different times.

Resistance was assessed in triplicates in terms of viable colony counts after incubation at 37±2°C for zero and 4 hr., reflecting the time spent by food in the small intestine.

Bile salt hydrolysis test

Fresh bacterial cultures were streaked in triplicates on MRS agar containing 0.5% (*W/V*) taurodeoxycholic acid (TDCA, Sigma). The hydrolysis effect was indicated by partial hydrolysis, also plates were examined for white precipitates as a sign of bile hydrolysis after 48 hr., of anaerobic incubation at 37±2°C (**Argyri *et al.*, 2013)**.

Haemolytic activity test

Fresh bacterial cultures were streaked on blood agar media containing 7% (*W/V*) human blood, and incubated at 37±2°C for 48 hr. Blood agar plates were examined for signs of β-haemolysis (clear zones around colonies), α- haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies) **(Hawaz, 2014).**

Tolerance to pancreatin

Lactic acid bacteria species overnight (18 hr.) cultures were harvested by centifugation (10,000 × g at 4°C for 5 min), washed twice with PBS (pH 7.2) then resuspended in PBS buffer solution (pH 8), containing pancreatin (Sigma-Aldrich) at 1mg/ml concentration. Tolerance was assessed in terms of viable colony counts and enumerated after incubation at 37±2°C for zero time and after 4 hr., reflecting the time spent by food in the small intestine **(Maragkoudakis *et al.*, 2006).**

Antibiotic susceptibility test

The antibiotic susceptibility test of the selected probiotic bacteria was determined towards 10 different antibiotics namely, penicillin 10µg, ampicillin 10µg, azithromycin 15 µg, erythromycin 15µg, ciprofloxacin 5 µg, ofloxacin 5 µg, choloramphenicol 30 µg, tetracyclin 30 µg, gentamycin 10 µg, and streptomycin 10 µg by using diffusion method. One milliliter of each actively growing cultures with an inoculum of approximately 10^5 cfu/ml of each species was mixed throughly with 10 ml of MRS agar and poured into Petri plates. After solidification, the antibiotic discs were placed on the solidified agar surface, and the plates were left for 15 min for diffusion of antibiotics, then incubated anaerobically at 37±2°C for 24 hr. Antibiotic susceptibility was determined according to methods described by **NCCLS (2002).** Diameters of the inhibition zones were measured by calipers in millimeters in which diameters is referred to as sensitive (S), intermediate (I) and resistant (R) **(Vlkova *et al.*, 2006).**

Antimicrobial activity test

Antimicrobial activity of the selected probiotic bacteria against 6 pathogens (*Staphylococcus aureus, E. coli, Klebsiella pneumoniae, Salmonella enteritidis, Pseudomonas aeruginosa* and *Candida albicans*) was checked by using well diffusion assay according to **Cardirici and Citak (2005) and Lailitha (2007)**. Species were tested for antimicrobial activity which were kindly provided by the Dept. of Microbiology, Faculty of Medicine, Zagazig Univ.

Statistical Analyses

Each treatment was run in three replicates and the results were statistically analysed by CoStat version 6.311 Copyright(c) 1998-2005 CoHort Software, http://www.cohort.com

RESULTS AND DISCUSSION

Isolation and Preliminary Screening of Isolated LAB

A total of 87 isolates were recovered *i.e.,* 43 from dairy products, 15 from mixed pickles, 12 from human milk and 17 from infant stools as shown in Table 1, most of them were characterized as Gram positive, catalase negative and non spore forming bacteria. From the preliminary screening only 10 isolates out of 87 were selected based on their tolerance to high acidity according to **Bassyouni *et al.* (2012)**. These 10 isolates which survived in low pH (1-3) for one to three hours were then identified using phenotypic and genotypic methods and subjected to *in vitro* characterization to evaluate their potential probiotic capacity.

Identification of the Selected Isolates

Morphological and biochemical characteristics were used to identify the 10 selected isolates

Table 1. Number of LAB isolates and their sources

Product abbreviation	No. of isolated LAB	Total percentage
Yoghurt (Y)	15	17.24
Domiati cheese (DC)	6	6.98
Fresh raw milk (FRM)	7	8.04
Fermented milk (FM)	15	17.24
Mixed pickles (MP)	11	12.64
Green pickled olives (GPO)	4	4.677
Human milk (HM)	12	13.79
Infant stools (IS)	17	19.54
Total	87	100%

(Table 2) according to **Logan and De vos (2009)**. Gram positive and catalase negative isolates were considered as presumptive LAB. All the isolates were Gram positive, catalase negative and non-spore forming, eight isolates were negative for gas production from glucose and ammonia from arginine, contrarely to DC1 and DC5 were positive in both tests (gas from glucose and ammonia from arginine)which revealed that they are hetero-fermentative, while most of the isolates are homo-fermentative.

According to morphological and biochemical tests all of the isolates grew at 15°C except isolate IS9, IS10 and DC5, all isolates also grew at 45°C except isolates DC1 and MP7. Eight isolates tolerated 4% of NaCl concentration and also 6.5% but MP7 and DC5 did not. All the isolates fermented lactose and sucrose showed various fermentation levels to other carbohydrates. Based on these results, isolates HM1, IS3, FM4 and FM11 tend to be *Lactobacillus paracaesi*, IS1 *Enterococcus faecium*, DC1 *Lactobacillus brevis*, MP7 *Lactobacillus plantarum*, IS9.

Direct identification of the Tested Bacteria Using MALDI-TOF-MS

The above mentioned isolates were identified at Academic Park Fac. Medicine Alex. Univ., Egypt, using MALDI-TOF- MS. (matrix-assisted lazer desorption ionization- time of flight mass spectrometry). Bacterial identification based on (MALDI)-time of flight (TOF) mass spectrometry (MS) is becoming a method of choice for determining the genus, species and even subspecies of bacterial isolates **(Carbonnelle *et al.,* 2012; Dušková *et al.,* 2012).** Also, this technology is achievable for other microorganisms (*e.g.* yeasts, fungi,) from various sources **(Chalupová *et al.,* 2014)**. Using this advanced method, the identification was confirmed and the prospective species with their numbers as conserved in the International Cultural Center for Microorganisms. The score values for the bacterial isolates are shown in Table 3. All of the isolates showed a score value between 2.000 to 2.484 (100%) and were correctly identified to genus and species levels. All the tested bacterial species were type species that are included in the Bruker Database, and all spectrum scores were greater than 2.0. Thus, all of the tested LAB were correctly identified to genus and species levels with biotype software score values greater than 2.0, and all of them had high degree of precision. **(Bizzini *et al.,* 2010; Wang *et al.,* 2013)** reported that the 16S rRNA sequencing results agreed with MALDI-TOF- MS identification in most cases presumably owing to co-evolution of ribosomal proteins and ribosomal nucleic acids.

Survival Under Condition Simulating the Human GI Tract

As probiotic, LAB must be able to survive in the acidic conditions in the stomach and resist bile acids at the beginning of the small intestine **(Argyri *et al.,* 2013).**

Table 2. Some morphological and biochemical characteristics of the selected LAB isolates

Criteria isolate	Presumptive name	Gram reaction	Endospore formation	Catalase test	NH_3 from arginine	Growth at Temp. (°C)		Growth at NaCl (%)		Gas from glucose	Acid from carbohydrates							
						15	45	4	6.5		Glucose	Lactose	Mannitol	Galactose	D- Cellobiose	Raffinose	D-Trehalose	Sucrose
HM 1	*L. paracasei*	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+
IS 1	*Enterococcus faecium*	+	-	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+
IS 3	*L. paracasei*	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+
DC 1	*L. brevis*	+	-	-	+	+	-	+	+	+	-	+	-	+	-	+	-	+
FM 4	*L. paracasei*	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+
FM 11	*L. paracasei*	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+
MP 7	*L. plantarum*	+	-	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+
IS 9	*L. acidophilus*	+	-	-	-	-	+	+	+	-	+	+	-	-	+	+	+	+
IS 10	*Bifidobacterium bifidium*	+	-	-	-	-	+	+	+	-	+	+	-	+	-	-	-	+
DC 5	*L. fermentum*	+	-	-	+	-	+	+	-	+	+	+	+	+	-	+	-	+

L.:Lactobacillus

Table 3. Rate classification results as determined by Bruker Daltonik MALDI Biotyper

Isolate code	Analyte name	Organism(best match)	Score value
IS 9	**(++) (A)**	*Lactobacillus acidophilus* DSM 20242 DSM	2.213
HM 1	**(++) (A)**	*Lactobacillus paracasei ssp paracasei* DSM 20312 DSM	2.175
DC 1	**(++) (A)**	*Lactobacillus brevis* DSM 2647 DSM	2.122
IS 3	**(++) (A)**	*Lactobacillus paracasei ssp paracasei* DSM 20006 DSM	2.046
IS 1	**(+++)(A)**	*Enterococcus faecium* 11037 CHB	2.484
FM 4	**(++) (A)**	*Lactobacillus paracasei ssp paracasei* DSM 20244 DSM	2.165
FM 11	**(++) (A)**	*Lactobacillus paracasei ssp paracasei* DSM 20207 DSM	2.145
MP 7	**(++) (A)**	*Lactobacillus plantarum* DSM 2601 DSM	2.116
IS 10	**(++) (A)**	*Bifidobacterium bifidium* DSM 27651 DSM	2.224
DC 5	**(++) (A)**	*Lactobacillus fermentum* DSM 12341 DSM	2.165

- Category A= species consistency (2.300-3.000). DSM: Deutsche Sammlung Von Mikroorganismen.

Tolerance to low pH

From Table 4, it can be shown that the 10 LAB were highly tolerant to pH 3 for three hours as 8 species remained viable when determined by absorbance at OD 620 nm. These results are in agreement with those obtained by **Du Toit *et al.* (1998), Jacobsen *et al.* (1999), Maragkoudakis *et al.* (2006), Xanthopoulos *et al.* (2012) and Argyri *et al.* (2013)** who reported that *Lactobacillus* species in food or animal and human origin were able to retain their viability when exposed to pH values of 2- 4.

Tolerance to bile salts and bile salts hydrolysis

Another key characteristic of probiotic bacteria is their tolerance and ability to grow in the presence of bile salts in order to survive in the digestive system. Although the bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% (*W/V*) and the staying time is suggested to be 4 hr., **(Parasad *et al.*, 1999)**. Results in Table 5 show that the majority of the tested species are resistant to bile salts even after 4 hr., of exposure, retaining their viability with negligible reduction in viable counts (≤ 1 log cycle). However, the numbers of nine of them showed decreases in their numbers ranging from log 0.9 to log 1.95. *L. fermentum* DC5 only showed negligible decrease. This is an important observation indicating that these species will not only survive *in vivo* in the low pH of the stomach but may be able to grow and colonize in the high bile environment in the intestine. These results are in harmony with those of **Jensen *et al.* (2012)**, who reported that *Lactobacillus* species tolerate gastric juice well with no reduction in viability.

Some species of LAB secrete bile salt hydrolase enzyme, which hydrolyses conjugated bile acids to release de-conjugated bile acids and amino acids **(Begley *et al.*, 2006; Sridevi *et al.*, 2009; Franz *et al.*, 2011).** When these salts are secreted from the gastrointestinal tract, the demand for cholesterol is increased, which in turn lowers cholesterol levels **(Driessen and de Boer, 1989; De Rodas *et al.*, 1996)**.

Tolerance to pancreatin

From Table 6 it can be shown that all species survived in the presence of 1mg/ml pancreatin for 4 hr., confirming their ability to survive in the passage through the GI tract well. However, their numbers dropped especially those of *L. acidophilus* IS9 and *L.paracasei* FM11. Specie *L. paracasei* HM1 showed the highest tolerance (the drop was from log 8.51 to 8.45).These results are in agreement with **Mansour *et al.* (2014)**, found that *Enterococcus faecium* NM1 13, NM2 13 and *Lactobacillus casei* NM5 12 showed high tolerance to low pH, bile salts and pancreatic enzymes.

Haemolytic activity

The results in Table 6 revealed that 8 species are non-hemolytic, while 2 of them (*L. paracasei* FM4 and *L. plantarum* MP7) induced α-haemolysis. Non haemolytic species are considered as a safe prerequisite for the selection of a probiotic species **(Hawaz, 2014)**.

Resistance to antibiotics

The antibiotic resistance profile was carried out with ten antibiotics belongs to three different groups depending on their mode of action as following: ampicillin and pencillin 10 µg (cell wall synthesis inhibitors), ciprofloxacin and ofloxacin 5 µg (DNA synthesis inhibitors), gentamycin 10 µg, streptomycin 10 µg, tetracyclin 30 µg (Anti 30S ribosomal subunit) and azithromycin 15 µg, erythromycin 15 µg and chloramphenicol 30 µg (Anti 50S ribosomal subunit). All the tested LAB species were resistant to penicillin, ciprofloxacin, gentamycin and streptomycin. *Enterococcus faecium*, *Bifidobacterium bifidium, L. plantarum, L. acidophilus, L. fermentum and L. paracasei* IS3 were resistant to all antibiotics but intermediate to ampicillin and chloramphenicol. Other *Lactobacillus* species showed variable resistance to the tested antibiotics according to the National Committee for Clinical Laboratory Standards **(NCCLS, 2002)** as shown in Table 7**. Maragkoudakis *et al.* (2006)** examined 29 *Lactobacillus* species for their probiotic potential and found that the majority of species were resistant to vancomycin but sensitive to chloramphenicol and tetracycline. **Xanthopoulos *et al.* (2012)** evaluated antibiotic susceptibility of 8 isolates and found some variations of susceptibility between isolates.

Table 4. Tolerance of LAB species to low pH and their survival percentage

LAB specie code	pH	OD at 620 nm at different periods (hr.)				Survival (%)
		0	1	2	3	
	1	1.120	0.404	0.259	0.240	21
IS 1	2	1.070	0.803	0.701	0.700	64
	3	1.080	0.971	0.960	0.927	86
	1	0.764	0.712	0.736	0.573	75
IS 3	2	0.766	0.735	0.675	0.622	81
	3	0.767	0.751	0.770	0.631	82
	1	0.144	0.121	0.097	0.051	35
IS 9	2	0.145	0.139	0.112	0.100	68
	3	0.148	0.141	0.135	0.120	81
	1	0.152	0.079	0.077	0.073	48
IS 10	2	0.150	0.139	0.100	0.078	51
	3	0.150	0.140	0.132	0.130	86
	1	0.313	0.250	0.183	0.161	51
FM 4	2	0.311	0.267	0.244	0.243	78
	3	0.313	0.275	0.269	0.256	81
	1	0.227	0.186	0.177	0.173	67
FM 11	2	0.236	0.198	0.196	0.160	76
	3	0.228	0.211	0.203	0.198	86
	1	1.254	0.935	0.899	0.878	70
HM 1	2	1.236	0.988	0.913	0.900	72
	3	1.248	1.484	1.183	1.001	80
	1	0.499	0.280	0.255	0.229	45
MP 7	2	0.501	0.461	0.448	0.357	71
	3	0.500	0.482	0.472	0.410	82
	1	1.194	0.875	0.815	0.746	62
DC 1	2	1.197	1.059	0.865	0.806	67
	3	1.196	1.115	1.025	1.002	83
	1	0.264	0.205	0.200	0.187	70
DC 5	2	0.267	0.245	0.243	0.194	72
	3	0.268	0.256	0.248	0.223	83
LSD 0.05		0.00120	0.00332	0.00116	0.0033	

OD: optical density, (%) of survival is calculated by dividing the OD of 3 hr., to 0hr at pH3

Table 5. Tolerance of selected species to bile salts and bile salt hydrolysis (BSH)

LAB specie	Log at 0 hr.			Log at 4 hr.			Bile salt hydrolysis (BSH)
	Bile salt concentration			Bile salt concentration			
	0.0%	0.3%	1.0%	0.0%	0.3%	1.0%	
***L. paracasei*HM1**	8.99	8.90	6.80	7.92	6.08	5.61	1
***E. faecium* IS1**	8.34	7.76	6.09	7.68	6.30	5.73	1
***L. paracasei* IS3**	8.41	6.93	6.05	7.99	6.60	5.83	0
***L. brevis* DC1**	8.40	7.19	6.02	7.35	6.39	5.93	0
***L. paracasei* FM4**	8.04	6.59	5.76	7.83	5.88	5.57	0
***L. plantarum* MP7**	8.28	6.79	5.98	7.67	5.95	5.52	1
***L. acidophils* IS9**	8.64	7.27	4.92	7.37	5.26	4.70	0
***Bifid. bifidium* IS10**	8.40	7.86	5.68	8.21	6.48	5.43	1
***L. fermentm* DC5**	8.21	7.92	7.05	7.97	7.91	6.75	0
***L. paracase*i FM11**	8.82	6.99	5.13	7.10	5.35	4.68	0
LSD 0.05		0.00927			0.00910		

L: Lactobacillus, E: Enterococcus, Bifid: Bifid bacterium, 0: no hydrolysis, 1: partial hydrolysis

Table 6. Tolerance to pancreatin (1 mg/ml) and haemolytic activity

LAB specie	Log at zero time	Log after 4 hr.	Haemolytic activity
***L. paracasei* HM1**	8.51	8.45	γ
***E. faecium* IS1**	8.24	7.35	γ
***L. paracasei* IS3**	8.38	7.56	γ
***L. brevis* DC1**	8.37	8.13	γ
***L. paracasei* FM4**	7.97	7.74	α
***L. plantarum* MP7**	8.19	7.52	α
***L. acidophilus* IS9**	8.56	7.32	γ
***Bifid. bifidium* IS10**	8.35	7.52	γ
***L.fermentum* DC5**	8.05	7.39	γ
***L. paracasei* FM11**	8.76	7.67	γ
LSD 0.05	0.00983	0.00883	

L: Lactobacillus, E: Enterococcus, Bifid: Bifidobacterium γ: no zone α: greenish zone

Although probiotics with the resistance genes may increase the risk of potential transfer in gut, in this respect antibiotic-resistant probiotic may be advantageous in the case of antibiotic administration to human and animal and the establishment of the beneficial microorganisms in the gut for prolonged periods **(Kim and Austin, 2008**). The natural resistance of the isolates for clinically important antibiotics may provide a way for the development of antibiotic/ probiotic combination therapies for condition like diarrhea, female urogenital tract infection and infective endocarditis **(Charteris *et al.*, 1998b).**

Antimicrobial activity

The cell free supernatants of LAB species were tested for antimicrobial activity against 6 pathogens, *Staphylococcus aureus, E. coli, Klebsiella pneumonia*e, *Salmonella entritides*, *Pseudomonas aeruginosa*, *Candida albicans* by using well diffusion method.

From Table 8 it can be shown that the spectrum of inhibition was different among the tested species. *L. acidophilus* (IS9) showed the highest antimicrobial activity against all the tested pathogens. Also *E. faecium* (IS1), *L. plantarum* (MP7) *Bifidobacterium bifidium* (IS10) and *L. fermentum* (DC5) showed antimicrobial property against all the tested pathogens except *Candida albicans*, with highest inhibition zone against *E. coli* (18,17,19 and 22 mm, respectively).

Similar results were also reported by **Araujo and Ferreira (2013)** and **Francois *et al.* (2013)** on *E. faecium* and *L. plantarum* against spoilage and pathogenic bacteria. Also, **Abdel - Raouf *et al* (2017)** showed that LAB isolated from salted fish and mixed pickles had a good antimicrobial activity against *Salmonella entritides* and *E. coli* (Gram negative) which are major food borne pathogens. However **Yateem *et al.* (2008)** reported that LAB are capable of producing antimicrobial compounds such as formic and benzoic acids, hydrogen peroxide, diacetyl, acetoine and bacteriocins such as nicin.

The production levels and the proportions among those compounds depend on the specie, medium compounds and physical parameters **(Tannock, 2004)**. The inhibitory activities of LAB against Gram positive pathogens have been mostly shown to be due to the bactericidal effect of protease sensitive bacteriocins **(Jack and Tagg, 1995).** However, the antagonistic effects of LAB towards Gram negative pathogens could be related to the production of organic acids and hydrogen peroxide **(Ito *et al*., 2003).**

Table 7. Susceptibility of potentially LAB to antibiotics using the disc diffusion method (diameter of inhibition zone in mm)

LAB species	Cell wall synthesis inhibitors		DNA synthesis inhibitors		Protein synthesis inhibitors					
					Anti-30S ribosomal subunit			Anti-50S ribosomal subunit		
	Ampicillin 10 µg	Penicillin 10 µg	Ciprofloxacin 5 µg	Ofloxacin 5µg	Gentamycin 10 µg	Streptomycin 10µg	Tetracycline 30 µg	Azithromycin 15 µg	Erythromycin 15µg	Chloramphenicol 30µg
HM 1	23 (S)	20 (R)	10 (R)	12 (R)	8 (R)	0 (R)	16 (I)	18 (I)	20(I)	21 (S)
IS 1	14 (I)	14 (R)	0 (R)	8 (R)	0 (R)	0 (R)	12 (R)	12 (R)	12 (R)	15(I)
IS 3	14 (I)	15 (R)	0 (R)	7 (R)	0 (R)	0 (R)	14 (R)	12 (R)	13 (R)	14 (I)
DC 1	20 (S)	19 (R)	9 (R)	12 (R)	8 (R)	0 (R)	16 (I)	14 (R)	18 (I)	17 (I)
FM 4	21 (S)	20 (R)	8 (R)	13 (I)	0 (R)	0 (R)	17 (I)	16 (I)	17 (I)	20 (S)
FM 11	15 (S)	19 (R)	8 (R)	15 (I)	0 (R)	0 (R)	12 (R)	17 (I)	16 (I)	15 (I)
MP 7	14 (I)	15 (R)	0 (R)	8 (R)	0 (R)	0 (R)	11 (R)	11 (R)	13 (R)	12 (I)
IS 9	13 (I)	15 (R)	7 (R)	8 (R)	0 (R)	0 (R)	10 (R)	10 (R)	11 (R)	16 (I)
IS 10	14 (I)	14 (R)	0 (R)	8 (R)	0 (R)	0 (R)	13 (R)	13 (R)	12 (R)	15 (I)
DC 5	13 (I)	15 (R)	0 (R)	0 (R)	0 (R)	0 (R)	10 (R)	12 (R)	13 (R)	14 (I)

R: resistant I: intermediate S: sensitive

Table 8. Antimicrobial activities of cell – free extract of selected LAB against various indicator pathogens in terms of the diameter of inhibition zone (mm)

Pathogen / LAB species	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Salmonella entritidis*	*Pseudomonas aeruginosa*	*E. coli*	*Candida albicans*
***L. paracasei* HM1**	12	0	0	10	14	0
***E. faecium* IS1**	10	12	8	15	18	0
***L. paracasei* IS3**	10	0	0	12	8	0
***L. brevis* DC1**	16	0	10	14	10	0
***L. paracasei* FM4**	7	10	0	5	13	0
***L. pracasei* FM11**	8	0	0	11	11	0
***L. acidophilus* IS9**	20	21	18	19	18	14
***L. plantarum* MP7**	16	17	8	16	17	0
***Bifid. bifidium* IS10**	10	16	12	15	19	0
***L. fermentum* DC5**	22	14	18	17	22	0

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انتخاب بكتيريا حامض اللاكتيك كداعمات حيوية من بعض المصادر المختلفة بواسطة الاختبارات المعملية

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تم عزل ٨٧ عزلة من بكتيريا حمض اللاكتيك لا هوائيا من مصادر غذائية مختلفة و هي الزبادي والجبن الدمياطي واللبن الطازج والمتخمر والمخلل الخليط والزيتون الأخضر، كما تم العزل من لبن اثنتين من الأمهات المتبرعات ومن براز أطفالهن الرضع، أظهرت عشر عزلات فقط من الـ ٨٧ عزلة تحملا عاليا لدرجة الحموضة ٣,٠ لمدة ثلاث ساعات، وقد تم تعريف هذه العزلات البكتيرية العشرة باستخدام الصفات المورفولوجية والبيوكيميائية وتم التأكيد باستخدام طريقة MALDI-TOF-MS، أوضحت النتائج أن أكثر الأجناس تواجداً هو جنس *Lactobacillus* (٨ عزلات) وعزلة واحدة *Enterobacter faecium* وعزلة واحدة *Bifidobacterium bifidium*.تم تعريف كل العزلات المختبرة بكفاءة عالية بقيمة وصلت إلى ٢,٠٠ – ٢,٤٨٤ (١٠٠%) بواسطة جهاز MALDI-TOF-MS وذلك لمستوى الجنس والنوع. تحملت معظم السلالات أملاح الصفراء بتركيز ٠,٣% لمدة تصل إلى ٤ ساعات و كانت *L. fermentum* أكثرهم تحملا. أظهرت أربع سلالات نشاطا للتحلل الجزئي لأملاح الصفراء كما تحملت العشرة سلالات وجود البنكرياتين ١ مجم/مل لمدة ٤ ساعات غير أن اثنتين منها انخفضت أعدادهما للعشر، كانت ٨ سلالات منها غير محلله للدم، أظهرت معظم هذه الأنواع المختبرة مقاومة للبنسيلين (١٠ ميكروجرام) وسيبروفلوكساسين ٥ (ميكروجرام) وجنتاميسين (١٠ ميكروجرام) وستربتوميسين (١٠ ميكروجرام) بينما باقي السلالات أظهرت مقاومة متباينة للمضادات الحيوية المختبرة طبقا لمعايير اللجنة الدولية للمعايير المعملية السريرية، أوضح اختبار النشاط المضاد للميكروبات الممرضة أن بكتريا (IS9) *L. acidophilus* كان لها أعلى نشاط ضد كل الميكروبات الممرضة المختبرة خاصة ضد *Staphylococcus aureus* و *Klebsiella pneumoniae*، وعليه وجد أن أغلب السلالات المختبرة معمليا تمتلك صفات مرغوبة لاستخدامها كبروبيوتك.

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