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PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM EGYPTIAN SALTED FOOD

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ABSTRACT: A total of 12 autochthonous lactic acid bacteria (LAB) were isolated from a traditional homemade salted foods (n=14) in Egypt. The salted fish (n=2), mixed pickles (n=3) and mish (n=9) at one to 48 months of fermentation were collected from Sharkia Governorate (Zagazig, Menya-Elkmh, Belbeas, Fakous and Kafr-Suqr districts), North Sinai Governorate (El-Areesh and Rafah cities), Sohag, Luxor and Aswan cities. Identification process to species and strain level was accomplished by biochemical, morphological characteristics and 16S rDNA gene analysis. The isolates were evaluated according to safety, antibacterial and antibiotic susceptibility criteria. The most frequently observed genus identified by 16S rDNA sequencing analysis was *Enterococcus faecium* (SHM7, ELM33 and ELM38). The most predominant genus identified by biochemical were *Lactobacillus rhamnosus* < *Lb. frumenti* < *Enterococcus faecium* < *Lb. plantarum*. The highest percentages of antibiotic resistance were to cefotaxime and cefaclor (91.67%), and ampicillin, kanamycin and levofloxacin (83.33%). All the selected isolates were sensitive to tetracycline and penicillin (25 and 50%, respectively).The results also revealed that 33.33% of the isolates were haemolytic, while 66.67% were non-haemolytic and only one isolate (SHM4) was induced β-haemolytic activity. All the selected isolates showed important levels of antibacterial activity against tested food-borne pathogens (*Salmonella enterica, Listeria monocytogenes* and *Escherichia coli)*. The results suggest the potential of the obtained isolates to improve the quality of these traditional fermented food products in Egypt.

Key words: Lactic acid bacteria, *Enterococcus*, salted foods, mish cheese, pickles, salted fish.

INTRODUCTION

Traditionally fermented foods have been consumed for thousands of years for their beneficial properties such as increased shelf-life and enhanced flavour and texture of the final product. There is a rich source of traditional fermented foods and enriched with beneficial bacteria, consumed by millions of people. Most of these traditional fermented foods are enriched with beneficial bacteria named probiotics. The probiotic potential of the lactic acid bacteria (LAB) from traditional fermented foods has been well documented (Abdulla *et al*., 2014). From literature studies, a number of probiotic LAB species such as *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus sakei, Lactococcus lactis, Leuconostoc* species, *Enterococcus faecium, Streptococcus thermophillus, Bifidobacteria, Lactobacillus casei, Weissella* species have been isolated and identified (Abdulla *et al*., 2014; Rao *et al*., 2015; Marwa *et al*., 2017; Domingos-Lopesa *et al*., 2017). Some of the LAB species such as *Lactobacillus casei* and *Lactobacillus plantarum* with potential properties were also identified from the fermented food products (Lee *et al*., 2011; Rao *et al*., 2015). Despite the wide range of LAB isolated from several fermented foods with diverse probiotic aspects, there are still

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many traditional fermented foods that have not been exploited for potential probiotic applications.

Probiotics are "live microorganisms that confer a health benefit to the host when administered in adequate amounts" (FAO/WHO, 2002). Studies in the area of probiotics have advanced significantly due to the growth of interest in the products with these microorganisms. The features associated with the probiotics include maintenance of the balance of intestinal microbiota (Arvola *et al*., 1999), control of diarrhea (Arvola *et al*., 1999), stimulation of the immune system (Isolauri *et al*., 2004), reducing hypersensitivity to allergenic substance and eczema in children (Kalliomäki *et al*., 2001; Kukkonen *et al*., 2007) and prevention of intestinal inflammations (Isolauri *et al*., 2000; Kalliomäki *et al*., 2001). Enterococci are a wide group of microorganisms that can be found in different niches, including the human gastrointestinal (GI) tract (Nueno-Palop and Narbad 2011) and foods products, especially fermented meats and dairy products (Gomes *et al*., 2008; Martín-Platero *et al*., 2009). Enterococcal species as starter cultures have been used in the dairy industry for decades and they have also been shown to act as probiotics (Eaton and Gasson, 2001). However, enterococcal species also contain pathogenic strains that can be responsible for serious infections in both humans and animals (Carlos *et al*., 2010) and safety assessment of enterococci with regards to antibiotic resistance and virulence traits is crucial in the selection of these strains for industrial purposes and more importantly as probiotics (Nueno-Palop and Narbad, 2011). However, in Egypt, there is a limited knowledge on the probiotic potential of microbial community associated with salted fermented food. In the present study, salted fermented food were obtained from Sharkia Governorate (Zagazig, Menya-Elkmh, Belbeas, Fakous and Kafr-Suqr districts), North Sinai Governorate (El-Areesh and Rafah cities), Sohag, Luxor and Aswan cities for screening, characterization, identification and *in vitro* evaluation of the probiotic potential of lactic acid bacteria from these foods from different regions of Egypt.

MATERIALS AND METHODS

Microbiological Analysis and Isolation of LAB

The mish samples (the liquid medium used for the ripening of Karish starter cheese), Molooha fish and mixed pickles were obtained from the household of different regions of Egypt (Zagazig, Menya-Eikmh, Belbas, Fakous and Kafr-Suqr districts, Sharkia govemorate, El-Areesh and Rafah cities of North Sinai, Sohag, Luxor and Aswan cities, Egypt (Table1) were collected in a sterile cold plastic containers and stored under 4ºC until analysis for isolation of LAB. The conventional serial dilution and pour plate technique were used for enumerating and isolating bacteria from the samples. Total bacterial count (TBC) were performed according to Marth (1978). In addition, LAB were isolated and counted (cfu/g) on MRS-agar (DE-Man,Rogosa and Shap, 1960 and Oxoid, CM 361) and M17-agar (Biakar, Beauvais, France) plates as described by Ozgun and Vural (2011). The plates were incubated at 37ºC for 48 hr. The well grown, discrete colonies were enumerated for total colony forming unit and sub-cultured isolates were stored at -20ºC in 20% glycerol for further studies.

Biochemical Characterization of LAB

Biochemical and morphological characteristics were studied due to the standard protocol of Cappuccino and Sherman (2004). Initially, the selected isolates were tested for Gram staining, catalase test, arginine hydrolysis, bile salt hydrolase, different carbohydrates fermentation tests (glucose, L-arabinose, D-fructose, maltose, lactose, mannitol, raffinose, D-xylose and sorbitol, all from Hi-media). All the isolates were tested for their survival ability at different temperatures (15, 37 and 45ºC) and salinity (4 and 6.5% NaCl concentrations).

The identification processes were constructed with the online software ABIS online (http:// www.tgw1916.net/bacteria_logare_desktop.html). ABIS is a laboratory tool for bacterial identification and is open for public use. ABIS encyclopedia for phenotypical and cultural characteristics, ecology and pathogenicity data was consulted before making the final decision.

DNA Extraction and PCR Amplification

The selected isolates (SHM4, SHM6, ELM33, LUM35 and LUM38) were grown in 5 ml MRS broth in a rotary shaker at 37ºC overnight. The DNA was extracted as per the protocol described by Chennappa *et al*. (2014). The DNA was amplified using primer 27 F (AGAGTTTGA TCMTGGCTCAG) and 519 R (GGATTACCG CGGCCGCTG). PCR amplification reactions were carried out in a 25µl reaction mixture. 1µl of the DNA was amplified with 2.5µl of 109 PCR buffer, 2.5µl of 25 Mm $MgCl_2$, 2.0µl of 2 mM dNTPs, 1.0µl of 20 pmol primer 27 F, 1.0µLof 20 pmol primer 519 R, 0.125µl of LA Taq and water up to 14.875µl. PCR conditions were as follows: initial denaturation at 94ºC for 4 min, followed by 30 cycles of denaturation at 94ºC for1 min, annealing for 30sec., at 53ºC, extension at 74ºC for 2 min, followed by a final extension at 74ºC for 13 min. Amplified product was confirmed by agarose gel (1%). electrophoresis and documented using Syngene G-box gel documentation system.

Identification and Nucleotide Sequence Accession Number

The sequences were used for identification in NCBI BLAST database. All the sequences of *Enterococcus* strains were deposited in NCBI-Gen Bank. Accession numbers were: *Enterococcus faecium* (SHM4) (Gen Bank: KP137385.1) (SHM6), *Enterococcus faecium* (LUM38) (Gen Bank: KP137385.1) and Enterococcus *faecium* (LUM35) (Gen Bank: CP018071.1). The phylogenetic tree was constructed with online software MEGA 5.1 using Maximum Likelihood NJ method by (WWW.codencode aligner.com).

In Vitro Tests for Probiotic Potential of LAB Strains

Acidic pH tolerance test

The pH tolerance of the LAB isolates were tested according to the procedure described by Sahadeva *et al*. (2011). Phosphate buffer saline (PBS) solution was prepared for acid tolerance studies by adjusting the pH at 3, 4 and 5 using 1 ml Hcl. 1 ml of the tested LAB sample of 10^3 cells $ml^{-1}$was inoculated into the three test tubes containing 9 ml of PBS solution with pH 3, 4, and 5, respectively, mixed thoroughly and incubated for 48 hr., at 37ºC. After incubation, the growth density (OD_{600}), individually, was done for all tested isolates and acid tolerance was determined by comparing the OD_{600} in all the MRS broth tubes with control ones.

Antibiotic susceptibility test

Antibiotic susceptibility of the LAB isolates was tested against eight selected antibiotics using Hi-media antibiotic discs of: ampicillin -10 µg, kanamycin- 10 µg, penicillin-10 µg, levofloxacin -5 µg, cefaclor -5 µg, ofloxacin-5 µg, cefotaxime-30 µg and tetracycline -30 µg by disc diffusion method (CLSI, 2011). Test LAB of 24 hr., culture were inoculated onto MRS agar plates using sterile cotton swabs, then antibiotic discs were placed on the inoculated plates. After incubation at 37ºC for 24 hr., inhibition zones around the discs were measured and susceptibility was compared to the reference chart of zone size interpretative chart for antibiotics as per CLSI (2011).

Antibacterial activity test

Antibacterial activity is an important test for the assessment of probiotics. Neutralized cell-free supernatant (CFS) of LAB isolates was used to test the production of antibacterial compounds by well diffusion method (Nel *et al*., 2001). Three different pathogenic bacteria were used as the indicator, including *Salmonella enterica*, *Escherichia coli* and *Listeria monocytogenes*. (From Agricultural Microbiology Department, Zagazig University) A18-h-old cultures of indicator were swabbed on the Muller Hinton agar (Hi Media) plates. CFS (50 µl) was added to the wells and allowed to diffuse and incubated at 37ºC for 24 hr. The diameter (mm) of the inhibition zone was measured to determine the antibacterial activity.

Haemolytic activity test

The LAB isolates were tested for the haemolytic activity using the procedure described by Argyri *et al*. (2013) onto Tryptose Blood Agar plates enriched with sheep blood 5% (*W/V*). The plates were incubated for 48 hr., at 37ºC then examined for signs of β-haemolysis

(clear zones around colonies), α-haemolysis (green zones around colonies) or γ-haemolysis (no zones around colonies) (Asteri *et al*., 2009).

Bile tolerance

Potential probiotic LAB not only should be able to survive passage through the human stomach (high acid environment) but also capable to survive passage in the intestine, where bile juice secreted by gall bladder was added. Therefore, they must resist gastric juices and bile salts during their transition *via* the digestive canal as requested by FAO / WHO (2001). The tolerance of lactobacilli to bile salts (BS) was evaluated in MRS brouth supplemented with bile salts using a modified method described by Dora and Glenn (2002). Test lactobacilli isolates cultures were grown for 6hr., in MRS broth at 37°C. An aliquot of 1ml of the 6hr., old culture was inoculated into 100ml MRS broth with 0.2 or 0.4% (*W/V*) bile salts (Sigma, USA). Bacterial growth was monitored by determination of optical density at 650nm after 6 and 24 hr., incubation period at 37°C. The percent difference between the variation of optical density (DO) of culture without bile salts (ΔDO_0% BS) and the variation of optical density of culture containing 0.2 or 0.4% bile salts (ΔDO0.2 or 0.4%BS) would give an index of isolates surviving that can be expressed as follows:

$$\text{Surviving}\,(\%) = \frac{\Delta DO0\% - \Delta DO0.2 \text{ or } 0.4\%\ Bs}{\Delta DO0\%\ BS} \times 100$$

Tolerance to NaCl as a Technological Property

Domiati cheese is brined ripened soft cheese with 6.0% salt when fresh. Therefore, salt tolerant probiotic strains are needed to be incorporated in production of probiotic Domiati cheese (Hamad, 2015). 12 screened LAB isolates in the present study were tested for their capability to tolerate under such stress in the presence of different salt concentrations. Growth in the presence of 2.5%, 5%, 7.5% and 10% NaCl in MRS-broth was determined according to the method described by Holt *et al*. (1994) in which 9.0 ml of MRS-broth containing 2.5% NaCl in the first set, 5%NaCl in the second set, 7.5% NaCl in the third set and 10%NaCl in the fourth set were inoculated with one ml of 24 hr., old broth culture of each LAB isolate (10^5 cfu/ml) and incubated at 37°C were examined for growth (turbidity) after 48 hr. The results of bacterial growth were expressed as (-): fair growth, (+): moderate growth, (++): good growth, (+++): fast growth.

RESULTS AND DISCUSSION

Microbiological Analysis

The current study was conducted to determine the potential health benefits of lactic acid bacteria (LAB) isolated from the traditional fermented food: mish (n=9), salted fish (n=2) and mixed pickles (n=3). These traditional food were collected from different regions in Egypt.The microbial characteristics of the 14 samples are presented in Tables 1 and 2. The results indicated that total bacterial (TBC) counts are similar to that found in the LAB (81.58% - 99.21%) counts, thus the LAB are the (TBC) dominant group in all samples. The percentage of LAB from TBC in the samples ranged between 82 to 99%.The highest percentage (99 and 95%) of LAB was found in mixed pickles and mish from Sharkia, respectively while the lowest percentage (81.58%) was found in pickles. The lowest number of TBC and LAB counts (4.51 and 4.99 log cfu/g) in mish resulted in the period of ripened this samples reached (4 years) of Sharkia. The TBC and LAB levels in the samples ranged from 4.99 to 7.41 log cfu/g and 4.51 to 6.60 log cfu/g, respectively. The lowest numbers of TBC and LAB were recorded in the mish at 3 -4 years of ripening.

Isolation and Biochemical Characterization of LAB

A total of 14 LAB isolates were isolated from the mish, salted fish and mixed pickles samples analyzed in the present study (Table 3). The isolates of Gram positive and catalase negative were considered as presumptive LAB (Chennappa *et al*., 2014). All the isolates were Gram positive, rod or cocci shape, catalase negative, negative for gas production from glucose, and arginine hydrolysis negative. Biochemical characterization revealed that the isolates were homo-fermentative, producing only acid, but no gas from glucose and negative for arginine hydrolysis. The isolates were able to ferment glucose, maltose, mannitol, maltose and

Table 1. Production regions, food materials, and fermentation periods of products

Sample number	Governorate	City	Local name of food materials	Isolate code	Fermentation period
1	Sharkia	Menya-Eilkmh	Mish	SHM	12 months
2	Sharkia	Zagazig-1	Mish	SHM	24 months
3	Sharkia	Belbeas	Mish	SHM	48 months
4	Sharkia	Fakous	Mish	SHM	24 months
5	Sharkia	Kafr-Suqr	Mish	SHM	48 months
6	North Sinai	Al-Areesh	Mish	ARM	24 months
7	North Sinai	Rafah	Mish	RAM	24 months
8	Aswan	Aswan	Mish	ASM	36 months
9	Luxor	Luxor	Mish cheese	LUM	48 months
10	Sharkia	Zagazig-1	Mixed pickles	SHP	3 months
11	Sharkia	Zagazig-2	Mixed pickles	SHP	4 months
12	Sharkia	Zagazig-3	Mixed pickles	SHP	6 months
13	Luxor	Luxor	Salted fish	LUF	1-2 months
14	Sohag	Sohag	Salted fish	SOF	1-2 months

Table 2. Some bacteriological (log cfu/g) properties of the tested foods

Sample number	Sample code	PCA	LAB		Total of LAB	LAB/TBC (%)	No. of colonies	Number of isolates
		TBC	MRS	M17				
1	**SHM**	6.69	6.00	5.90	6.25	93.30	18	Un-cultivable
2	**SHM**	6.63	6.11	5.95	6.26	95.37	22*	SHM 4
3	**SHM**	6.81	6.23	6.07	6.46	89.55	29*	SHM6,7 and 8
4	**SHM**	4.99	4.51	4.43	4.77	95.35	60	Un-cultivable
5	**SHM**	6.30	5.62	5.47	5.85	92.51	72	Un-cultivable
6	**ELM**	6.65	6.04	5.84	6.25	93.59	18*	ELM33
7	**RAM**	6.78	6.14	6.07	6.20	91.26	26	Un-cultivable
8	**ASM**	7.33	5.59	6.44	6.82	93.20	67	Un-cultivable
9	**LUM**	7.41	6.60	6.51	6.86	92.34	73*	LUM35,36,37 and 38
10	**SHP**	6.20	5.90	5.81	6.16	99.21	146	Un-cultivable
11	**SHP**	6.32	5.95	5.85	6.21	98.15	163	Un-cultivable
12	**SHP**	6.38	5.96	5.89	6.23	81.58	171*	SHP26
13	**LUF**	5.99	5.11	4.95	5.34	89.80	22	Un-cultivable
14	**SOF**	6.35	5.69	5.43	5.88	92.35	67*	SOF43 and 45
		Total number of lactic acid bacteria isolates					954	12

*The isolates were taken for the next experiments.
PCA, Plate count agar, LAB, lactic acid bacteria, MRS: used to isolate Lactobacilli;
M17: used to isolate Lactoccoci, TBC: total bacterial counts.

Table 3. Morphological, Physiological and biochemical characterizations of the selected cultivable LAB isolates

Test	Isolates											
	SHM 4	SHM 6	SHM 7	SHM 8	SHP 26	ELM 33	LUM 35	LUM 36	LUM 37	LUM 38	SOF 43	SOF 45
Morphology	Cocci	Cocci	Cocci	Rod	Cocci	Cocci	Cocci	Rod	Rod	Cocci	Cocci	Rod
Spore formation	-	-	-	-	-	-	-	-	-	-	-	-
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-
Growth at different temperature												
10˚C	+	+	+	+	+	+	+	-	-	+	+	-
37˚C	+	+	+	+	+	+	+	+	+	+	+	+
45˚C	+	+	+	+	+	+	+	+	+	+	+	+
Growth at different nacl concentrations												
3%	+	+	+	+	+	+	+	-	-	+	+	-
5%	+	+	+	+	+	+	+	+	+	+	+	+
7%	+	+	+	+	+	+	+	+	+	+	+	+
Growth at different pH levels												
4.5	-	-	-	-	-	-	-	+	+	-	-	+
5.5	+	+	+	+	+	+	+	-	-	+	+	-
7.5	+	+	+	+	+	+	+	-	-	+	+	-
Bile salt (0.3% oxgal) hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate fermentation												
Glucose	+	+	+	+	+	+	+			+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-

(+): positive ; (-): negative

L-arabinose. The isolates were not able to ferment sorbitol and raffinose. The optimum growth was shown at 37ºC, 3% NaCl and pH 5.5 Table 3. *Lactobacillus* strains isolated from other fermented foods, such as Sorghum-Based Traditional Fermented (Rao *et al*., 2015) and traditional Pico cheese (Domingos-Lopesa *et al*., 2017) showed similar results. The artisanal traditional fermented foods may harbour a rich source of diverse lactic acid bacteria (LAB) with interesting functional properties (Domingos-Lopesa *et al*., 2017).

Identification by 16S rRNA Sequencing and Phylogenetic Analysis

PCR amplification was carried out for three isolates using 27 F and 519 R primers, and the amplicon size was approximately 550 bp. Sequence analysis of the amplicons confirmed that the three strains (SHM7, LUM35 and LUM38) showed highest similarity (100 %) to *Enterococcus faecium*. The sequences were deposited in NCBI-Gen Bank and obtained Accession Numbers Table 4 and Fig. 1. The phylogenetic studies were carried out using BLAST algorithm and revealed Dominant LAB species identified from the study as: *Ent. faecium* (SHM7, LUM35 and LUM38) by (WWW.codencode aligner.com). The 16S rRNA sequence profiling is one of the cost-effective tools, and enables the researchers to identify the complex microbial communities up to species level at exceptional depth and resolution. Phylogenetic analysis was studied using the neighbor joining tree method to analyze the homology of the strains *Ent. faecium* (SHM7, LUM35 and LUM38).

In vitro Tests for Probiotic Potential of LAB Isolates

Acidic pH tolerance

Tolerance to acidic pH is one of the pre-requisite for the validation of probiotics. Table 5 show the tolerance of LAB to acidic pH. In this study, most of the isolates (75%) showed tolerance to acidic pH (pH 3) while the rest of isolates (25%) showed sensitive growth to acidic pH. The resistance of the *Enterococcus* strains to acidic pH environment represent the selection of acid tolerant strains. Similar results were reported by Turchi *et al*. (2013) who examined preliminary evaluation of probiotic potential of *Lb. plantarum* strains isolated from Italian food products where the LAB strains showed tolerance to acidic pH (pH 2).

Tolerance to NaCl as a technological property

Table 5 revealed high growth of all isolates in presence of 2.5% NaCl but some isolates were able to grow in presence of 5.0, 7.5 and 10.0% NaCl with some differences as the growth was decreased when concentration of NaCl was increased from 2.5 to 10.0%. On the contrary, at 10.0% NaCl, only 2 strains (SHM4 and SHM8) showed no growth. The present results are in accordance with the findings of (Ge *et al*., 2011; Reale *et al*., 2015). In dairy industry, such as Domiati cheese manufacture, LAB will exposed to osmotic stress due to high amounts of added salts. According to Ge *et al*. (2011), the osmotic stress may cause pronounced inhibition for bacterial growth and delayed ripening of cheese. For this best knowledge there is scarce studies related to the fate of *Enterococcus faecium* as probiotics in Domiati cheese manufacture. The present study highlighted information on intra-strain variability in osmotolerance in *Enterococcus faecium*. Therefore, it is concluded that osmotolerance is a crucial factor for the selection of strains for technological application in case of Domiati cheese manufacture that contain high salt concentration above 6%. Five screened LAB strains with high osmotolerance to high concentration of NaCl are worth of future investigation for their performance in production of probiotic Domiati cheese.

Antibiotic susceptibility test

Sensitivity to antibiotics is one of the criteria for the evaluation of probiotics.The antibiotic resistance profile was carried out with eight important antibiotics: ampicillin-10 µg, kanamycin- 10 µg, penicillin-10 µg, levofloxacin -5 µg, cefaclor -5 µg, ofloxacin-5 µg, cefotaxime- 30 µg and tetracycline -30 µg. Table 6 show the results obtained by comparing the reference chart of performance standards for antimicrobial disc susceptibility tests due to CLSI (2011). All the isolates were sensitive to amoxicillin, cefaclor, ofloxacin, cefotaxime and ampicillin. Isolates SHM7 and SHM6

Table 4 .Genotyping identification results of the isolated lactic acid bacteria as probiotic

Bacterial code number	Description	Accession number	Maximum Identity (%)
SHM7	*Enterococcus faecium* strain AT15 16S ribosomal RNA gene, partial sequence	KP137385.1	98
LUM35	*Enterococcus faecium* strain AT15 16S ribosomal RNA gene, partial sequence	KP137385.1	98
LUM38	*Enterococcus faecium* strain VRE001, complete genome	CP018071.1	98

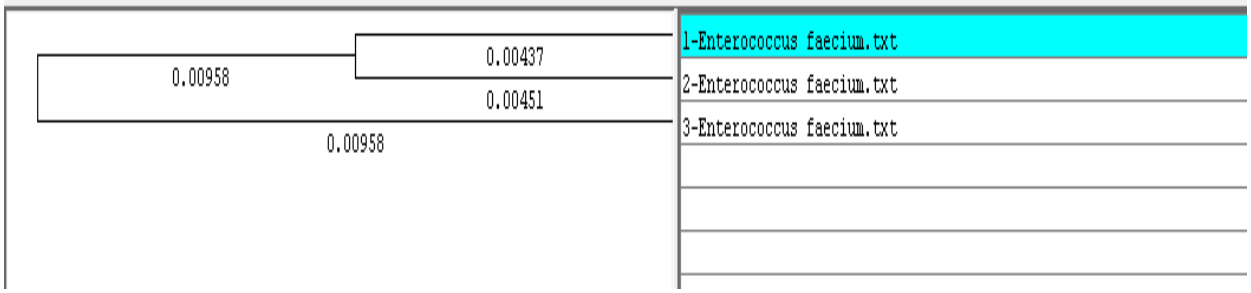

Fig. 1. Phylogenetic tree based on 16S rRNA of Enterococcus faecium strain

Table 5. Tolerance of LAB isolates for acidic pHand NaCl concentration

Isolates code	pH- level				NaCl concentration (%)				
	Control (5.7)	5	4	3	Control (0.5)	10	7.50	5	2.50
SHM-4	+++	+++	+++	+++	+++	-	+	++	+++
SHM-6	+++	+++	+++	+	+++	+	++	+++	+++
SHM-7*	+++	++	-	++	+++	+	++	+++	+++
SHM-8	+++	+	-	-	+++	-	-	+	++
ELM-33	+++	+++	++	+	+++	++	+++	+++	+++
LUM-35*	+++	+++	+++	+++	+++	++	+	++	+++
LUM-36	+++	+	+	-	+++	++	+++	+++	+++
LUM-37	+++	+++	+++	+++	+++	++	+++	+++	+++
LUM-38*	+++	+++	+++	+++	+++	++	+++	+++	+++
SHP-26	+++	+	-	-	+++	++	+++	+++	+++
SOF-43	+++	++	++	+	+++	++	+++	+++	+++
SOF-45	+++	+++	++	+	+++	++	+++	+++	+++

(-): fair growth, (+): moderate growth, (++): good growth, (+++): fast growth

*SHM7, LUM35and LUM38: were identified and belong to *Enterococcus faecium*

Table 6. The resistance patterns of the selected LAB isolates against 9 antibiotics

Isolates \ Antibiotic	Diameter of inhibition zone in mm									
	Ampicillin (10)	Kanamycin (10)	Penicillin (10)	Levofloxacin (5)	Cefaclor (30)	Ofloxacin (5)	Cefotaxime (30)	Tetracycline (30)	Avg. of isolates sensitivity	Resistant (%)
SHM-4	0(R)	0(R)	30(S)	0(R)	0(R)	0(R)	0(R)	30(S)	7.50	75
SHM-6	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0.00	100
SHM-7	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0.00	100
SHM-8	0(R)	0(R)	30(S)	0(R)	0(R)	0(R)	0(R)	35(S)	8.12	75
ELM-33	0(R)	0(R)	15(I)	0(R)	0(R)	0(R)	0(R)	20(I)	4.37	75
LUM-35	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	30(S)	3.75	87.5
LUM-36	12(I)	0(R)	0(R)	20(I)	22(S)	17(I)	25(S)	0(R)	24.0	37.5
LUM-37	0(R)	0(R)	15(I)	10(I)	0(R)	10(I)	0(R)	30(S)	8.12	50
LUM-38	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	25(S)	3.12	87.5
SHP-26	0(R)	13(I)	20(I)	0(R)	0(R)	0(R)	0(R)	30(S)	7.87	62.5
SOF-43	18(I)	0(R)	30(S)	0(R)	0(R)	0(R)	0(R)	23(S)	8.87	62.5
SOF-45	0(R)	28(S)	0(R)	0(R)	0(R)	10(I)	0(R)	27(S)	16.25	62.5
Avg. of antibiotic effect	2.50	3.41	11.66	2.50	1.83	2.25	2.08	20.83		
	83.33	83.33	50	83.33	91.67	75	91.67	25		

(S): sensitive; (I): intermediate; (R): resistant

showed resistance (100%) against all antibiotic tested while the other isolates showed variable resistant against the antibiotic tested ranged between 50 to 87.5%. The evaluation of antibiotic resistance profile was performed to promote the safety evaluation and development of potential probiotic LAB. The significance of the *Enterococcus faecium* strains (SHM7, LUM35 and LUM38) was the resistance to tetracycline. Furthermore, strain SHM7 and LUM35 were resistant to levofloxacin, kanamycin and penicillin. The previous report of Charteris *et al*. (1998) showed resistance of LAB to bacitracin at 10 U. Duskova and Karpiskova (2013) reported that fifteen strains of lactobacilli (17%) isolated from fermented foods were resistant to at least one antimicrobial agent, and one strain was multi resistant. The resistance profile was calculated based on zone size interpretative chart for antibiotics as per CLSI (2011). The natural resistance of the isolates for clinically important antibiotics may provide a way for the development of antibiotic/ probiotic combination therapies for conditions like diarrhoea, female urogenital tract infection and infective endocarditis (Charteris *et al*., 1998).

Antibacterial activity

Antibacterial activity of all the isolates was tested against *S. enterica*, *L. monocytogens* and *E. coli* are presented in Table 7. The isolates showed significant antibacterial activity against all the indicator bacteria. The zone of inhibition was measured and ranged from 5 to 26 mm. The isolates showed considerable antibacterial activity against Gram negative (*S. enterica* and *E. coli*) and Gram positive (*L.monocytogenes*) which are major food borne pathogens and of special concern with regard to food safety. *L. monocytogenes* and *S. enterica* were reported as the causal agents of several food borne diseases (Denny and McLauchlin, 2008; Scallan *et al*., 2011). The strains LUM38 and SOF45 showed higher antibacterial activity against all the tested food borne pathogens than all isolates tested, with inhibition zone 19.66 and 21.66 mm, respectively. The antibacterial activity differed among all the LAB strains, where certain LAB showed activity against specific indicator bacteria.

Table 7. Antibacterial activity (inhibition zone in mm) and Haemolytic pattern level of LAB isolates

Isolates	Antibacterial activity				Haemolytic pattern	
	S. enterica	*L. monocytogenes*	*E. coli*	Mean. inhibition activity	Haemolytic analysis zone (mm)	Type of Haemolytic analysis
SHM-4	15	11	8	11.33	22	β-haemolytic
SHM-6	10	0	5	5.00	12	α- haemolytic
SHM-7	16	0	17	11.0	0	Non- haemolytic
SHM-8	0	26	0	8.66	0	Non- haemolytic
ELM-33	12	13	12	12.33	0	Non- haemolytic
LUM-35	23	27	19	23.0	0	Non- haemolytic
LUM-36	5	0	7	4.00	0	Non- haemolytic
LUM-37	8	0	0	2.66	12	α- haemolytic
LUM-38	22	17	20	19.66	0	Non- haemolytic
SHP-26	7	5	7	6.33	5	α- haemolytic
SOF-43	12	15	13	13.33	0	Non- haemolytic
SOF-45	17	22	26	21.66	0	Non- haemolytic
Mean of haemolytic test						33.33%

The cell free supernatant was neutralized to deactivate the acids and to exclude the activity due to organic acids. Hence, the activity may be due to bioactive substances such as bacteriocin-like inhibitory substances, biosurfactants and other relevant molecules. *Enterococcus faecium* (LUM35, LUM38 and SOF45) strains showed the highest antimicrobial activity against food borne pathogens. Similar results were also reported with *Enterococcus faecium* (Araújo and Ferreira, 2013; Francois *et al*., 2013), also they observed the antimicrobial activity of *Enterococcus faecium* and *Lb. plantarum* against spoilage. In addition, Belicova *et al*. (2013) reported the inhibitory activity of LAB against *L. monocytogenes*.

Haemolytic activity

Table 7 shows the haemolytic activity assessed based on the presence or absence of clearing zone around the growth of the LAB on the blood agar plates. The results of the study revealed that 66.67% of the isolates are not inducing haemolysis, while 25% of the isolates induced α-haemolytic activity, 8% of the isolates induced β-haemolytic activity hence, the most strains are non-haemolytic. None of *En. faecium* isolated and characterized as probiotic bacteria by Muñoz-Atienza *et al*. (2013) exerted hemolytic activity.

Bile tolerance

In this study, LAB isolates were assayed for their ability to survive in different concentrations of bile salts as shown in Table 8. All 12 isolates resisted the different concentrations of bile used in the experiment with some fluctuation. However, increasing bile concentrations from 0.2 to 0.4% was accompanied by reduction in the ability to survive being clearly observed in the highest bile concentration. From Table 7 it could be shown that SHM7, LUM35 and LUM37 strain were promised to be selected for the purpose of this study based on their capability to tolerate the bile exposure under *in vitro* study especially at 0.4% bile salt concentration. It is obvious that from Table 7 that there is a variable distribution between LAB strains under study with their ability to tolerate different concentrations of bile salts. This may be explained as probiotic potentiality is strain

Table 8. Tolerance of LAB isolates to bile salt (OD_{600})

Isolates	Bile salt Resistance after 6 hr. (OD_{600})			Bile salt Resistance after 24 hr. (OD_{600})		
	Control	20%	40%	Control	20%	40%
SHM-4	0.565	0.558	0.555	1.204	1.145	1.135
SHM-6	0.730	0.710	0.660	1.236	1.158	1.128
SHM-7	0.900	0.801	0.792	1.205	1.234	1.244
SHM-8	0.804	0.763	0.720	1.269	1.230	1.229
ELM-33	0.058	0.055	0.042	1.190	1.089	1.064
LUM-35	0.068	0.020	0.018	2.130	2.100	2.083
LUM-36	0.094	0.080	0.075	1.266	1.220	1.140
LUM-37	0.171	0.155	0.166	1.720	1.678	1.377
LUM-38	0.660	0.664	0.555	1.160	1.208	1.241
SHP-26	0.002	0.002	0.002	1.008	1.20	1.048
SOF-43	0.050	0.035	0.017	1.252	1.218	1.127
SOF-45	0.022	0.011	0.009	1.195	1.166	1.080

specific. Similar reports were reported by other researchers and are in accordance with this explanation (Solieri *et al*., 2014; Zhang *et al*., 2014; Reale *et al*., 2015).

Conclusion

Artisanal mish, salted fish (molooha) and mixed pickles may be a rich source of diverse lactic acid bacteria (LAB) with interesting functional properties. In the present study, LAB from traditional mish, salted and mixed pickels were isolated, identified and characterized in order to get better knowledge of the indigenous bacterial population responsible for this mish, salted fish and mixed pickles unique flavor. The species isolated from mish, salted fish and mixed pickles were, in decreasing order of importance, *E. faecium*, *Lb. rhamnosus*, *Lb. plantarum* and *Lb. frumenti*. These LAB are essential for developing the diversity and typical features of cheese, salted fish and mixed pickles variety. A high variability of functional probiotic potential was found among isolates and could be the basis for the selection of specific strains to be used as adjunct cultures in the production of traditional foods. In addition, the selected specific strains needed to meet safety criteria by the absence of potential pathogenic factors. Indeed, a low percentage of strains produced β-haemolytic reaction (one strain). Remarkably, all enterococci were susceptible to tetracycline. According to the present study, these strains are promising candidates for application as adjunct cultures in food production.

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خصائص بكتيريا حمض اللاكتيك الحيوية المعزولة من الأغذية المصرية المملحة

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تهدف هذه الدراسة إلى عزل مجموعه من بكتيريا حمض اللاكتيك الحيوية(LAB)، حيث قد تم عزل (١٢ عزلة) من: المش (الذي يصنع بواسطة الأسر وعادة يتم تحضيره بحرفية) من المناطق المختلفة بجمهورية مصر العربية، تم تخمير المش في خلال عام إلي أربعة أعوام من التسوية وكذلك تم عزل الـ LAB من الأسماك المملحة والمخللات المختلطة وكانت فترة التسوية والأنضاج تتراوح ما بين شهر وستة أشهر وقد تم الحصول علي العينات من مدينة الزقازيق بمحافظة الشرقية ومن مدن العريش و رفح بمحافظة شمال سيناء، ومن سوهاج، والأقصر وأسوان، وقد تم تعريف هذه العزلات بواسطة الاختبارات المورفولوجية والكيموحيوية ودراسة جين 16S rDNA، وتم تقييم العزلات وفقا للسلامة، والتضاد للبكتيريا ومعايير الحساسية للمضادات الحيوية، وكان جنس *Enterococcus faecium* من أكثر الأجناس والتي تم تحديدها من خلال تحليل 16S rDNA، في حين أن جنس *Lactobacillus* من أكثر الأجناس التي تم تحديدها بواسطة الاختبارات الكيموحيوية، وكانت أكثر الأنواع البكتيرية السائدة *Lb. rhamnosus* يليها *Lb.frumenti* ثم *En. faecium* ثم.*Lb plantarum* وكانت أعلى نسبة من المقاومة للمضادات الحيوية في سيفوتاكسيم وسيفاكلور (٩١.٦٧%)، والأمبيسلين وليفوفلوكسين والكاناميسن (٨٣.٣٣%)، وكانت معظم العزلات حساسة للتتراسيكلين والبنسليين بنسبة ٢٥و٥٠ على التوالي، وكانت أكثر العزلات لا تحلل الدم كما تمت دراسة السلالات التي تم اختيارها لإمكانيات بروبيوتيك الوظيفية، أظهرت جميع السلالات مستويات هامة من النشاط البكتيري ضد مسببات الأمراض المنقولة عن طريق الأغذية، حيث تشير النتائج إلى إمكانية تحسين نوعية هذه المنتجات الغذائية التقليدية التخمرية في مصر بواسطة هذه العزلات.

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