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Selection, Molecular Identification and Testing of Potentially Probiotic Bacteria Recovered from Popular Artisanal Egyptian Cheeses

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Abstract

The present study was aimed to select potential probiotic and functional strains among lactic acid bacteria (LAB) isolated from some artisanal Egyptian dairy products. For this, 75 samples comprising karish (fresh skimmed), mish (aged salted skimmed) and Domiati (brined ripened) cheese varieties were surveyed in this study. Approximately, 300 lactic acid bacterial (LAB) strains were isolated and initially screened for their antibacterial activity against the two common food-borne pathogens; *Staphylococcus aureus* and *Escherichia coli* O157:H7. Using cell free extract of isolated LAB strains, various antimicrobial inhibition patterns have been noted against the tested pathogens. Upon further proteolytic and neutralization treatments, only 16 strains were proved to have potent antimicrobial attribute. Based on 16S rRNA gene sequencing identification of those 16 strains, 8 different species were identified (*Lactobacillus plantarum*, *Lb. fermentum*, *Pediococcus acidilacticii* and *Enterococcus faecium*). Additionally, those 16 strains were tested for different probiotic, functional and safety criteria (acid and bile resistance, salt tolerance, milk acidification ability, heamolytic activity and antibiotic sensitivity). The present study showed that artisanal Egyptian artisanal chesses were proven to be sources of safe potentially probiotic LAB with interesting physiological properties, thus it could be further incorporated in manufacture of various dairy products as starter and non-starter cultures.

KEYWORDS
Dairy products, LAB, Probiotic, 16S rRNA gene sequencing, Antibiotic Resistance.

INTRODUCTION

Currently, an extensive trend has been arising towards healthy functional foods which combine both desirable aromatic characteristics and containing bacteria having health-promoting effects; the so called "Probiotics". In this sense, cheeses and fermented dairy products are largely occupying advanced rank in consumers' interest.

Among typical artisanal chesses in Egypt, Karish, Mish and Domiati cheese varieties are extensively manufactured in the rural areas and are desired by a wide variety of consumers. Domiati cheese is the most popular soft white pickled cheese in Egypt and constituting about 75% of the locally-manufactured and consumed type in this country (Zhang *et al*., 2003; Abou-Donia, 2008). This cheese is made of either cow or buffalo whole milk or a mixture of both (El-Baradei *et al*., 2007). The principle difference of Domiati cheese from other varieties of pickled cheese, such as Feta, Brinza, or Telema cheeses, is that the milk is salted at the first step of its processing in a percentage varying from 5 to 14% depending on the season and on the ripening temperature (Abou-Donia, 1986). Afterwards, this salted milk is enzymatically-curdled either in a fresh state or subjected to pasteurization, but without the addition of defined starter cultures (El-Baradei *et al*., 2007). The cheese can be consumed either as a "fresh Domiati cheese"; which is less salted and stored for a few weeks under refrigeration or, more often, as "stored Domiati cheese"; which is highly salted following pickling in salted whey or a brine solution for few months (El-Baradei *et al*., 2007).

On the other hand, Karish cheese is a type of acid-coagulated cheeses made from defatted or skim cow or buffalo's milk in a special earthenware pots on which the partly skimmed milk sours and clots (Baraheem *et al*., 2007; Abou-Donia, 2008; Alnakip, 2009; Korish and Abd Elhamid, 2012). Later, the curd is poured onto a special mat which is tied and hung with its contents to allow the drainage of the whey over a period of 2-3 days until the desired texture of the cheese is obtained. Finally, the cheese is salted and cut into suitable pieces to be left for a few hours in the mat till no more whey drains out. Like Domiati type, Karish cheese can be ready to be consumed as a "fresh Karish" within 1-2 weeks or can be pickled in brine earthenware pots; the so called "Mish cheese" to maintain a valid shelf-life for up to a year (Abou-Donia, 1986; Baraheem *et al*., 2007; Abou-Donia, 2008). This cheese is one of the most popular types of soft cheese consumed in Egypt, especially in the countryside and among athletics owing to its high protein content (30%), low fat and lowered price (Ahmed *et al*., 2005; Abou-Donia, 2008; Alnakip, 2009; Korish and Abd Elhamid, 2012). Actually, the quantity of Karish cheese produced in Egypt, is unknown, however, it is believed that about 50% of the total milk produced is utilized for its manufacturing (Baraheem *et al*., 2007).

As inferred from production process, incubation conditions of those products are relatively uncontrolled because the facil-

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ities are quite primitive. Therefore, a wide variety of natural microflora particularly; LAB, are existing and contributing to fermentation process (Fahmy and Youssef., 1978; Dufour and Collin, 1995; El-Soda *et al*., 2003; El-Baradei *et al*., 2007; El-Baradei *et al*., 2008; El-Baradei *et al*., 2008; Alnakip, 2009). LAB are a group of Gram-positive non-sporing, non-respiring cocci or rods that are widely distributed in nature and mainly characterized by producing lactic acid as a major end product during the fermentation of carbohydrates (Axelsson, 2004). LAB represent 20-30% of total bacterial count in raw milk, but several factors as production conditions, season, breeding and the animal origin can influence their abundance and diversity (Gaya *et al*., 1999; Drakoularakou *et al*., 2003; Verdier-Metz *et al*., 2009). However, only LAB belonging to beneficial and nonpathogenic genera have traditionally been used in the food industry. From a practical, dairy-technology point of view, the following genera are considered the principally used LAB: *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus*.

Probiotic as a term is describing a group of mainly LAB bacteria when administered in adequate quantity, confer beneficial effects for the host (FAO/WHO, 2007). The guidelines accredited by FAO/WHO for evaluating probiotics in foods impose that proper in vitro investigations which should establish the potential health benefits of tested organisms prior to undertaking in vivo trials. Moreover, individual strains have to be tested for each property because characteristics ascribed to a probiotic are strain-specific (Verdenelli *et al*., 2009). Accordingly, to be considered as a probiotic, several characteristics must be possessed by the bacterial strain including, their ability to resist and survive gastrointestinal tract (GIT) conditions represented in gastric acidity (pH 2.5-3) and duodenal bile acids (Holzapfel *et al*., 1998), possessing pathogen antagonizing mechanisms possibly via production of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide, bacteriocins and bacteriocin-like substances (Saarela *et al*., 2000), and finally having adhesive ability to gut epithelium (Monteagudo-Mera *et al*., 2012). Concurrently, safety or non-pathogenicity of probiotic strain is a non-debatable concept, which principally includes non-harboring of antibiotic resistance (AR) patterns or virulence factors. Absence of AR feature is essential to ensure non-spread of AR genes from proposed probiotic bacteria to pathogenic and potentially-pathogenic bacteria (Monteagudo-Mera *et al*., 2011; Monteagudo-Mera *et al*., 2012). AR genes' spread may lead to serious concerns because of elevating AR levels within regular medication (Mathur and Singh, 2005; Pinto *et al*., 2006; Mattia and Merker, 2008) and possible reconstitution of the intestinal microflora of patients suffering from antibiotic-associated colitis (Salminen *et al*., 1998; Danielsen and Wind, 2003).

Most studies investigating probiotics were performed on strains inhabiting internal cavities of human or animal, considering the better adaptability to colonize the human/animal GIT (Monteagudo-Mera *et al*., 2012). Meanwhile, few studies investigated the behavior of dairy-originated strains under GIT conditions. However, the latter studies are more interesting since these strains are better adapted to the dairy products environment in which they are intended to be used. Also, some strains from plant and dairy origin like Saccharomyces cerevisiae subtype boulardii and Bifidobacterium animalis/lactis have internationally recognized as probiotics although they do not have a human or animal origin (Monteagudo-Mera *et al*., 2012). The objectives of the present work were to isolate and identify; on genomic base, the bacterial strains with both high probiotic potentiality and missing AR, among LAB which exist in traditional artisanal Egyptian fermented dairy products for the aim of their further incorporation in dairy industry.

MATERIALS AND METHODS

Study design

Artisanal Egyptian cheeses, which are manufactured in rural areas, were surveyed to isolate their native LAB. Following phenotypic characterization of selected isolates, testing for their anti-pathogenic potential was done using LAB cell free extract. In order to definitely select bactericinogenic strains, cell free extract was neutralized, treated with proteolytic enzymes and catalase deactivated. Strains that proved potent anti-pathogenic potential against food-borne pathogens, were subjected to precise discrimination based on 16S rRNA gene sequencing. Finally, these isolates were assessed for functional, technological and safety parameters.

Samples Collection and Preparation

Seventy-five samples of different traditional Egyptian artisanal Karish, Mish and Domiati cheeses (25 each), were collected from different households and localities at Elsharkia Governorate, Egypt. For cheeses varieties, approximately 50 gm was aseptically taken into a sterile wide mouth and air-tight sampling-jars, and directly transported unopened to laboratory. All samples were transported immediately to our laboratory in a 4°C vehicle-mounted refrigerator. Buffered peptone water (BD Difco) was used to prepare serial dilution with addition of 2% sodium citrate in case of Domiati cheese to ensure full fat saponification.

Isolation of LAB and Preliminary Identification

0.1 mL of appropriate dilution of each sample was spread plated on the surface of de Man, Rogosa, and Sharpe agar (MRS) (Difco Laboratories, Detroit, MI) adjusted to pH of 5.5 (De Man *et al*., 1960). MRS plates were incubated anaerobically (BBL Gas pak plus Anaerobic System) at 30°C for 72 h. Colonies with distinct morphological differences (color, shape, and size) were selected from each plate and further purified by re-streaking two successive times on fresh MRS plates. Nearly, three distinct colonies were selected for each sample. All Gram-positive and catalase-negative isolates were maintained as frozen 30% glycerol stock culture at -80°C until further use (Quintela-Baluja *et al*., 2013; Alnakip, 2014).

Food-borne Pathogenic Strains and Growth Conditions

Pathogenic *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) (previously isolated and molecularly identified based on 16s rRNA GS (Sánchez-Rubio *et al*., 2016; Kamal *et al*., 2018; Alnakip *et al*., 2019) were used as reference strains. *S. aureus* and *E. coli* were inoculated into Brain Heart Infusion broth (BD, Difco) and incubated at 37°C for 24 h prior to their incorporation.

Cell free supernatant (CFS) preparation and treatments

LAB isolates were anaerobically grown on MRS broth at 37 oC for 48 h. Incubated broths were then centrifuged (5000 rpm at 4°C for 10 minutes) and filtered using 0.45 µm pore membrane (Millipore). CFS kept refrigerated until further use. For exclusion of inhibitory effect of produced organic acids, CFS were initially neutralized using sterile 0.1N NaOH and named neutralized CFS (nCFS). To determine whether H_2O_2 and/ or bacteriocins have contributed to LAB's antimicrobial effect, CFS was divided into two portions; one was heated at 80°C for 10 min (hCFS) and the

second was treated with proteolytic enzymes (pCFS) (trypsin, proteinase K and alpha chemotrypsin, 1 mg/mL) (Nair, 2000 and Darwish, 2006).

Antibacterial Activity for LAB isolates

Agar well diffusion method was used to detect the antibacterial activity of our LAB isolates against food-borne bacteria (*S. aureus* and E.coli) as described by (Kamal *et al*., 2018). Muller Hinton agar (MHA) (BD Difco) at 45°C were inoculated with 500 µL of an overnight culture of the reference pathogenic strain and poured into a Petri dishes. After solidification, wells with a diameter of 5 mm were made in the agar layer and 50 µL of each of CFS, nCFS, hCFS and pCFS of each strain were placed in separate wells. MHA plates were incubated at 37°C for 24 h. The antibacterial activity was determined by appearance of clear inhibition zones of more than 4 mm diameter around wells. The diameter of inhibition zones was measured in millimeters.

Identification of LAB isolates and Phylogenetic Analysis Based on 16S rRNA GS

Total genomic DNA was extracted from overnight cultures as previously described by (Alnakip *et al*., 2020). The bacterial cells were lysed by the addition of 180 µL of lysis solution (Sigma-Aldrich, Saint Louis, MO) after incubation for 24 h at 37°C. The extraction and purification were carried out using the DNeasy Tissue Mini Kit (Qiagen, Valencia, CA, USA). A fragment of the 16S rRNA gene was amplified by PCR as described by using the universal primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) (Cheneby *et al*., 2000) and R1492 (5′-TACGGYTACCTTGTTACGACTT-3′) (Wang and Wang, 1996). All of the PCR assays were performed using a "My Cycler" Thermal Cycler (BioRad Labs, Hercules, USA), as previously described by (McCabe *et al*., 1999).

Sequencing reactions were prepared using PCR products and the ABI PRISM® BigDye™ Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Forster City, CA, USA) and the same primers utilized for PCR were also used for the sequencing. The sequencing reactions were analysed in an automatic sequencing system (ABI, 3130, Applied Biosystems, USA). Following generation of sequencing data, the entire 16S rRNA gene sequences were analysed using Chromas software version 2.33 (Griffith University, Queensland, Australia) and aligned with Clustal-X software (Thompson *et al*., 1997). Next, these sequences were identified by sequence homology alignment among published reference sequences using the web tool; NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) (Altschul *et al*., 1990). Consensus sequences were imported into MEGA 6.0 software, by which a sequence alignment and phylogenetic trees were conducted based on the neighbor-joining method and using Kimura-2 parameter model. The percentage of bootstrap confidence levels for internal branches, as defined by the MEGA program, was calculated from 1000 random re-samplings.

Testing of Probiotic properties of selected isolates

Acidic pH tolerance

After cultivating LAB isolate overnight in MRS broth, bacterial pellets were harvested by centrifugation at 5000 rpm for 10 min followed by washing once in phosphate-saline buffer (pH 7.2). Simulated gastric juice acidity was carried out by adjusting the pH of different sterile MRS broth tubes to 2.0, 2.5 and 3.0 with concentrated HCl. Re-suspension of bacterial cells was done in different-pH MRS broth tubes followed by incubation at 37°C for 3 h. For screening tolerance, aliquots of 1 mL of different-pH MRS broth were taken periodically each hour for the determination of total viable count.

Bile salts tolerance

The bile tolerance for LAB isolates was determined by inoculation of 1 mL from over-night incubated MRS into MRS broth containing bile (0.3% w/v). The mixture was incubated a 37°C for 4 h. without agitation. For the tolerance assay, aliquots of 1 mL were removed each hour for determination of total viable count.

Determination of antimicrobial production (lactic acid, hydrogen peroxide and Diacetyl production capabilities) (AOAC, 1990)

For these measurements, the isolates were grown on MRS broth for 72 hours and samples taken at 12 hours interval.

Lactic Acid: To 25 ml of broth culture of selected isolate, 3 drops of phenolphthalein were added as indicator. From the burette 0.1N NaOH was slowly added to the sample until pink Colour appears. Each ml of 0.1N NaoH is equivalent to 90.08 mg of lactic acid.

Hydrogen peroxide: Twenty five milliliter of dilute sulphuric acid were added to 25 ml of the broth culture of test isolate. Titration was carried out with 0.1N potassium permanganate. Each ml of 0.1N potassium permanganate is equivalent to 1.070 mg of H_20_2. A decolourization of the sample was regarded as end point.

Diacetyl: Twenty five milliliter of broth cultures were transferred into conical flasks and 7.5 ml of hydroxylamine solution were used for the residual titration. The flasks were titrated with 0.1N HCl to a greenish-yellow end point using bromophenol blue as indicator. The equivalence factor of HCl to diacetyl is 21.5 mg.

Testing of safety-related concerns of selected isolates

Antibiotic susceptibility testing and MIC determinations

Approximately 1 mL of overnightly grown MRS broth was well-mixed with 20 mL of semisolid MHA in Petri dishes and allowed to solidify. Different antibiotic discs corresponded to: Ampicillin (10 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Gentamycin (10 µg), Streptomycin (10 µg), Tetracycline (30 µg) and Vancomycin (30µg) were placed on the surface of MHA plates followed by incubation for 24 h at 37°C. AR was detected via appearance of inhibition zones around antibiotic discs, which were measured in millimeters.

Hemolytic activity

Blood hemolysis was evaluated on Columbia agar plates (Oxoid) supplemented with 5% sheep blood which were incubated at 37°C for 24 h (Lombardi *et al*., 2004).

Testing of technological properties of selected isolates

Milk Acidification ability

Acidification was determined by the change in pH (ΔpH) during time. Fifty mL of UHT skim milk was inoculated with 1% of LAB culture (10^6 cfu/ ml) and incubated at 37°C. The pH was measured using a pH-meter (Micro pH). The acidification value was calculated as the difference between the value immediately after inoculation and values after incubation (ΔpH = pH zero time – pH

at time). The LAB isolates were considered as fast, medium or slow acidifying when a ΔpH of 0.4 U was achieved after 3, 3-5 and > 5 h, respectively (Lombardi *et al*., 2002 and Ayad *et al*., 2004a).

Salt resistance

MRS broth tubes containing 4% sodium chloride were inoculated with a loopful of 18 h. active culture of a selected isolate. The tubes were incubated at 37°C and the growth was observed after 48 h.

Study of β-Galactosidase Production: 20 mg/ml stock solution of X-Gal in dimethyl sulfoxide (DMSO) was Prepared. X-Gal stock solution was added to the molten agar at 45°C (5 ml./1liter). Active cultures (16-18 h) were spotted on modified MRS agar medium (lactose was used instead of glucose as a carbon source) and the plates were incubated at 37°C for 48 h anaerobically. Appearance of blue colonies indicated positive results.

Statistical analysis

All experiments were done in triplicates. Significant differences were considered at p value of < 0.05. Results were descriptively analyzed using SAS software (SAS, 2006). Results were reported as Mean value ± Standard error of mean.

RESULTS AND DISCUSSION

There is an increasing interest for the consumers towards traditional artisanal dairy products due their characteristic aromatic properties coupled by beneficial effects on the human health. One reason of being beneficial is explaining by their non-pathogenic/non-virulent LAB content which proven to have many important functions, so we try to study their probiotic potentiality.

Among typical artisanal cheeses existing in Egypt, Karish (skimmed milk cheese), mish (pickled ripened Karish cheese) and Domiati (pickled white soft cheese) cheese varieties are manufactured in the rural areas where no addition of starter occurs, and the fermentation is only dependent on subsistence of the wild microflora in the raw milk, particularly LAB (Alnakip, 2009; 2016). During the last decades, several studies declared the isolation of various LAB species from Domiati cheese, such as Lactococcus lactis subsp. lactis, Lactobacillus delbrueckii subsp. bulgaricus, L. casei (Fahmy and Youssef., 1978; Alnakip *et al*., 2016), L. farciminis, *L. alimentarius*, *Enterococcus faecalis*, *E. faecium* (Dufour and Collin, 1995), *Lactobacillus plantarum*, and *L. paracasei* (El-Soda *et al*., 2003). LAB play an essential role in the dairy industry due to the tremendous level of human consumption of various fermented products, mainly cheese and acidified/fermented milks and dairy products (Garabal, 2007).

LAB count in examined dairy products

The LAB were recovered on MRS agar with an anaerobic incubation. Data presented in this table 1 revealed that LAB were detected in all examined samples of Kariesh cheese samples (100%) while LAB could be detected in 80 and 60% out of the examined Domiatti and Mish cheese samples respectively. Many researchers isolated LAB strains from different Egyptian dairy products. Previous studies (El-Soda *et al*., 2003; Ayad *et al*., 2004b; 2004b) isolated LAB strains from Egyptian Kariesh and Domiatti cheeses. The recovery of LAB from Mish are rare. However, there were a few reports on isolation of LAB from Mish. Several LAB strains were isolated from Egyptian Mish cheese (El-Soda *et al*., 2003; Ayad *et al*., 2004a; 2004b).

The variation between Kariesh and Domiatti cheese samples in LAB count may be due to the difference in acidity, salt concentrations and the method of manufacture. Also, quality and heat treatment of milk used in the manufacture, ripening in brine solution, handling method, hygienic practices, transportation and conditions of storage and distribution play an important role in its microbial quality (El-Baradei *et al*., 2007). Manufacture of Kariesh cheese depends on natural fermentation of raw skim milk by LAB present normally in raw milk (Baraheem *et al*., 2007; Korish and Abd Elhamid, 2012). While, Domiatti cheese is made from raw or pasteurized either buffalo or cow milk or mixture of them (Dufour and Collin, 1995). It can be eaten either fresh or after pickling in a brine solution or salted whey for 2 to 4 months (El-Baradei *et al*., 2007).

Antibacterial activity of LAB isolates recovered from examined cheese samples against S. aureus and/or E. coli

Table 2 showed the numbers of LAB isolates having antibacterial activity against *S. aureus* and *E. coli*. A total of 180 LAB suspected isolates (75, 60 and 45 colonies from Kariesh, Domiatti and Mish cheese samples respectively) were isolated. Out of these isolates, 22 LAB isolates (8%), 13 (8.3%) and 10 (22.2%) isolates from Kariesh, Domiatti and Mish cheese samples respectively) showed antibacterial activity against *S. aureus*. While 20 LAB isolates (26.7%) and 8 (13.3%) isolates from Kariesh and Do-

Table 1. Statistical analytical results of LAB count/ml in examined artisanal Egyptian cheeses

Type of product	No. of samples	Positive Samples		LAB count/ml		
		No.	%	Min.	Max.	Mean ±S.E.M.
Kariesh cheese	25	25	100	8.2×10^5	5.8×10^7	$1.8\times10^7\pm0.33\times10^7$
Domiatti cheese	25	20	80	1.1×10^4	5.0×10^7	$4.7\times10^6\pm2.5\times10^6$
Mish cheese	25	15	60	2.4×10^4	1.6×10^5	$8.5\times10^4\pm1.3\times10^4$

Table 2. Incidence of LAB isolates showing antibacterial activity against *S. aureus* and/or *E. coli*.

Type of product	No. of LAB isolates	LAB isolates having antibacterial effect			
		S. aureus		*E. coli*	
		No.	%	No.	%
Kariesh cheese	75	6	8	20	26.7
Domiatti cheese	60	5	8.3	8	13.3
Mish cheese	45	10	22.2	0	0

miatti cheese samples respectively) showed antibacterial activity against *E. coli*. No LAB isolates from Mish cheese samples showed antibacterial activity against *E. coli*.

The antibacterial activities of all 180 isolates of LAB recovered from artisanal cheeses were carried out by preparing cell-free supernatants of overnight cultures of LAB and examining their antibacterial effect using *S. aureus* and *E. coli*. Table 3 show the diameters of the inhibition zones around wells previously filled with CFS from the examined LAB isolates. The antibacterial effect of LAB could be due to organic acids (lactic and acetic acids), hydrogen peroxide, acetaldehyde, diacetyl, reuterin, carbon dioxide, bacteriocins and bacteriocin-like substances (Rodrí guez *et al*., 2000; De Vuyst and Leroy, 2007).

To determine whether acidity, bacteriocins and/ or H_2O_2 contributed to the inhibition effect by LAB, CFS were prepared from the previous LAB isolates showing antibacterial activities against *S. aureus* and/or *E. coli* and then neutralized to pH 6.5 to exclude the antibacterial effect of acidity. Neutralized supernatants were examined for antibacterial effect as the previous method.

The diameter of inhibition zones (Table 3) showed that only 16 strains displayed the highest inhibition against *S. aureus* and *E. coli* after neutralization compared with other LAB isolates. The antibacterial activities of selected 16 LAB isolates recovered from examined samples were carried out by preparing cell-free supernatants of overnight cultures of LAB and examining their antibacterial effect using *S. aureus* and *E. coli*. These isolates undergone further identification based on 16S rRNA gene sequencing. Table 4 show the diameters of the inhibition zones around wells previously filled with CFS from the examined LAB isolates.

Molecular Identification of selected strains based on 16S rRNA gene sequencing

The rapid and accurate identification of microbial species in dairy sector is a fundamental goal of diagnostic dairy microbiology. The traditional phenotypic identification methods became unsuitable nowadays with the technological revolution happened in last decades in microbial diagnostics, due to consumption of longer time, huge number of materials and labor and on the other hand, lowered diagnostic accuracy in comparison to the recent molecular approaches. In addition, when phenotypic methods are used to identify bacteria, interpretation of test results involves substantial subjective judgment (Stager and Davis, 1992; Caamaño-Antelo *et al*., 2015). As a consequence, During last decade, the identification, differentiation and taxonomical purposes for bacteria isolated from foods were carried out by

Table 3. Inhibition zones (mm) of LAB isolates (recovered from artisanal Egyptian cheese samples) having antibacterial activity against *S. aureus* and *E. coli*.

Type of product	Isolate No.	Diameter of inhibition zone (mm)		Isolate No.	Diameter of inhibition zone (mm)		Isolate No.	Diameter of inhibition zone (mm)	
		S. aureus	*E. coli*		*S. aureus*	*E. coli*		*S. aureus*	*E. coli*
Kariesh cheese	3b	-	8	8b	-	13	17c	9	7
	3c	-	7	10c	-	9	18b	-	9
	4a	-	9	13a	-	7	18c	-	13
	4b	-	8	13c	-	8	20a	-	9
	6b	-	10	14b	-	9	21c	8	-
	7b	9	7	14c	-	8	23b	7	-
	7c	8	10	16c	-	10	-	-	-
	8a	-	9	17a	8	10	-	-	-
Domiatti cheese	9b	14	-	19a	13	-	24a	15	-
	9c	15	-	19c	13	-	24b	-	11
	10a	-	13	20a	-	12	24c	-	11
	10b	-	12	20b	-	11	-	-	-
	10c	-	11	20c	-	13	-	-	-
Mish cheese	1a	8	-	11a	9	-	23b	8	-
	1b	9	-	11c	8	-	24b	8	-
	6b	7	-	15b	9	-	-	-	-
	7c	9	-	19c	9	-	-	-	-

(-): No inhibition zone

Table 4. Inhibition zones of 16 LAB isolates recovered from traditional dairy products against *S. aureus* and *E. coli* as indicator pathogenic bacteria.

Isolate	Diameter of inhibition zone (mm)		Isolate	Diameter of inhibition zone (mm)	
	S. aureus	*E. coli*		*S. aureus*	*E. coli*
Y1	12	11	K8	0	10
K1	9	7	K9	0	9
K2	0	9	D1	14	0
K3	0	10	D2	0	12
K4	0	8	D3	0	13
K5	0	10	D4	13	0
K6	8	10	D5	0	12
K7	9	7	D6	0	13

Table 5. Antibiotic sensitivity test for LAB isolates

Strain	Origin	Identification based on 16S rRNA GS	GenBank accession number	Testing of safety-related concerns								Effect of heating, addition of trypsin& proteinase K on the antibacterial activity of neutralized CFS			Testing of technological properties		
				Antibiotic resistance profile							Hemolysis	Heating (80°c/10min.)	Heating (100°c/30min.)	Treatment with trypsin & proteinase K	Acidifying ability	NaCL 4% tolerance	β- Galac-tosidase production
				AMP	CH	ER	GN	ST	TET	VAN							
FCVMZg1	Karish cheese	*Lactobacillus plantarum*	KX083537	R	S	R	R	S	R	S	γ-hemolysis	+	-	-	Fast	-	-
FCVMZg2	Karish cheese	*Lactobacillus fermentum*	KX083538	R	R	R	R	R	R	R	γ-hemolysis	-	-	-	Fast	+	++
FCVMZg3	Karish cheese	*Lactobacillus fermentum*	KX083539	S	R	R	R	R	R	R	γ-hemolysis	+	-	-	Fast	+	-
FCVMZg4	Mish cheese	*Lactobacillus fermentum*	KX083540	S	R	R	R	R	R	R	γ-hemolysis	+	-	-	Fast	+	++
FCVMZg5	Karish cheese	*Lactobacillus fermentum*	KX083541	S	S	S	S	S	S	S	γ-hemolysis	+	-	+	Fast	-	+
FCVMZg6	Karish cheese	*Lactobacillus fermentum*	KX083542	R	R	R	R	R	R	R	γ-hemolysis	+	-	-	Fast	+	++
FCVMZg7	Karish cheese	*Lactobacillus fermentum*	KX083543	S	S	S	S	S	S	S	γ-hemolysis	-	-	+	Fast	+	+
FCVMZg8	Domiatti cheese	*Enterococcus faecium*	KX083544	R	R	R	R	R	R	R	γ-hemolysis	-	-	+	Fast	+	++
FCVMZg9	Domiatti cheese	*Lactobacillus plantarum*	KX083545	S	R	R	R	R	R	R	γ-hemolysis	+	-	-	Fast	+	-
FCVMZg10	Domiatti cheese	*Pediococcus acidilactici*	KX083546	S	R	R	R	R	R	R	γ-hemolysis	+	-	-	Slow	-	++
FCVMZg11	Domiatti cheese	*Lactobacillus plantarum*	KX083547	R	S	R	S	R	S	S	γ-hemolysis	+	+	-	Medium	-	+
FCVMZg12	Karish cheese	*Lactobacillus fermentum*	KX083548	R	S	R	S	S	R	R	γ-hemolysis	-	-	-	Medium	-	-
FCVMZg13	Mish cheese	*Lactobacillus fermentum*	KX083549	R	R	R	S	R	R	R	γ-hemolysis	+	-	+	Medium	+	+
FCVMZg14	Karish cheese	*Lactobacillus fermentum*	KX083550	S	S	S	S	S	S	S	γ-hemolysis	+	-	-	Medium	+	++
FCVMZg15	Domiatti cheese	*Lactobacillus plantarum*	KX083551	R	R	R	S	R	R	R	γ-hemolysis	-	-	+	Medium	+	+
FCVMZg16	Domiatti cheese	*Lactobacillus plantarum*	KX083552	R	S	R	S	S	R	R	γ-hemolysis	+	-	-	Medium	+	++

R: resistant, S: susceptible, AMP: Ampicillin, CH: Chloramphenicol, ER: Erythromycin, GN: Gentamycin, ST: Streptomycin, TET: tetracycline, VAN: Vancomycin

advanced molecular tools such as 16S rRNA gene sequencing (Alnakip, 2014; Alnakip *et al*., 2014; Caamaño-Antelo *et al*., 2015) and MALDI-TOF MS (Quintela-Baluja *et al*., 2013; Quintela-Baluja *et al*., 2014).

As previously illustrated in study design, the isolates, LAB strains with potent antipathogenic potential were identified by 16S rRNA gene sequencing. Using cell free extract of isolated LAB strains, various antimicrobial inhibition patterns have been noted against the tested pathogens. Upon further proteolytic and neutralization treatments, only 16 strains were proved to have potent antimicrobial attribute. Based on 16S rRNA gene sequencing identification of those 16 strains, 8 different species were identified (*Lactobacillus plantarum*, *Lb. fermentum*, *Pediococcus acidilacticii* and *Enterococcus faecium*) (Table 5). Later, the different functional, technological and safety parameters were assessed for those later strains. The results of !6S rRNA gene sequencing were deposited in NCBI GenBank and corresponding accession numbers of each strain are illustrated in Table 5.

PCR targeting 16S rRNA gene has been extensively the identification and phylogenetic analysis of representatives of LAB in dairy sector (El-Soda *et al*., 2003; Fernández-No *et al*., 2012; Quintela-Baluja *et al*., 2013; Alnakip *et al*., 2014). Notably, our work has proven effective discrimination, between different LAB species based on sequencing of first 800 bp of 16S rRNA gene with high reliability and accuracy that extended to species level as shown in Figure 1.

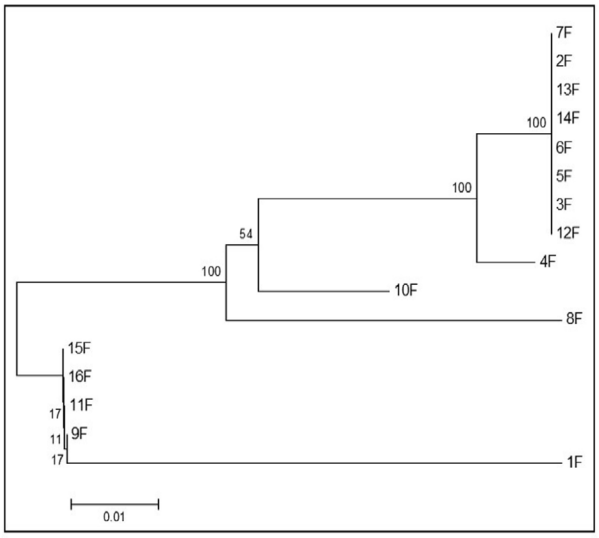

Figure 1. Phylogenetic tree of LAB isolates

Testing of Probiotic properties of selected isolates

The 16 strains were tested for different probiotic (acid and bile resistance), technological (salt tolerance, β-galactosidase production, milk acidification ability) and safety-related (heamolytic activity and antibiotic resistance) criteria. Potential probiotic bacteria should resist stressful conditions of the stomach to reach to the small intestine. Tolerance to low pH is an important criterion for any probiotic strains (Çakır and Çakmakçı, 2018).

Resistance to acidic conditions

The resistance to acidic condition of stomach is usually determined in vitro by detection of resistance to pH 3. The time needed during the digestion in the stomach is 3 hours so we used MRS broth pH-previously adjusted to 3.0 for selection of the resistant strains to low pH (Prasad *et al*., 1998). The results presented in Figure 2 showed that only 10 LAB strains could survive test period of 3h at pH 3.0 without decrease in survival percentage. *Lb. fermentum* stains (FCVMZg14, FCVMZg5 and FCVMZg7) and *Lb. plantarum* strains (FCVMZg9 and FCVMZg15) resist acidic conditions better than the remaining strains.

Resistance to bile conditions

The mean bile concentration in small intestine was suggested to be 0.3% (w/v) and the staying time of food in it was believed to be 4 h (Prasad *et al*., 1998). In turn, these conditions were adopted in this study to explore the ability of strains to resist intestinal

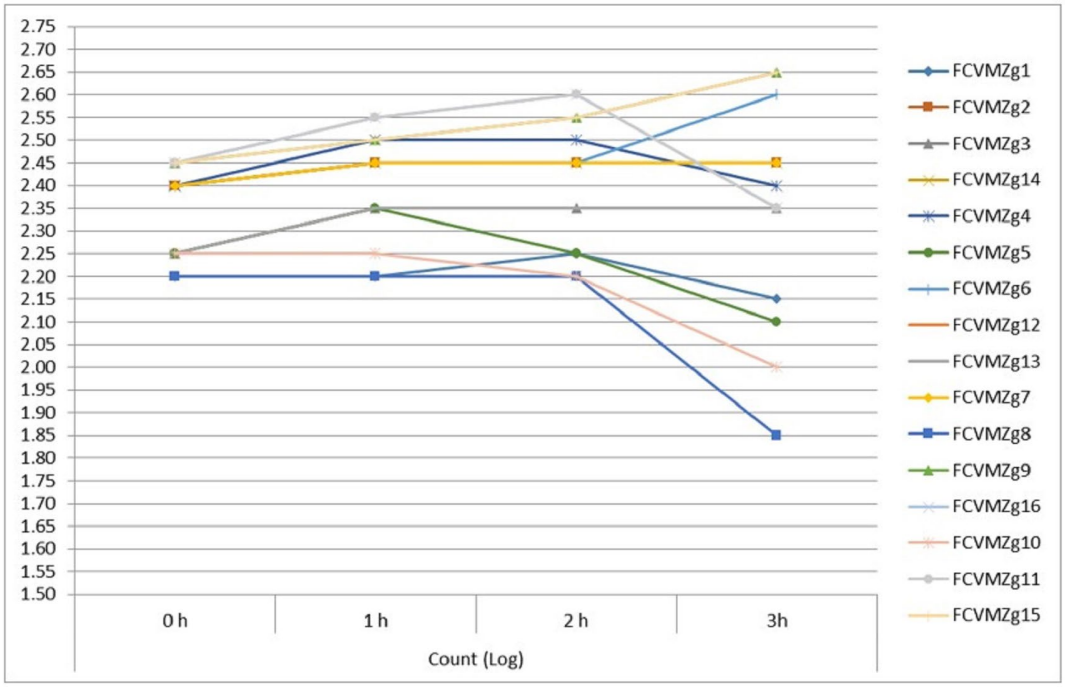

Figure 2. Acid tolerance of LAB strains

condition. The results reported in figure 3 showed that only 8 tested strains could resist these conditions. *Lb. plantarum* strains (FCVMZg9 and FCVMZg15) showed the highest tolerance. While other strains had showed variable survival percentage. It was established that the bile salt resistance differs a lot among the species of LAB and even between strains themselves. Bile resistance of some strains is due to presence of specific enzyme activity; bile salt hydrolase (BSH) that helps in hydrolyzing of conjugated bile, thus reducing its toxic effect (Prasad *et al*., 1998).

To conclude acid/bile tolerance tests, only six strains had both properties of acid and bile tolerance. These isolates were identified as *Lb. fermentum* (FCVMZg2), *Lb. fermentum* (FCVMZg14), *Lb. fermentum* (FCVMZg12), *Lb. fermentum* (FCVMZg7), *Lb. plantarum* (FCVMZg9) and *Lb. plantarum* (FCVMZg15). Therefore, these isolates could be good candidates as probiotics.

Testing of safety-related criteria of selected isolates

Antimicrobial resistance of selected 16 LAB isolates

The absence of resistance to antibiotics in starter cultures is very important for safety reasons because bacteria which resist antibiotics may transfer their resistance to other bacteria. When the previously mentioned 16 LAB strains were tested for antibiotic resistance, as shown in table 5, three *Lb. fermentum* strains (FCVMZg4, FCVMZg6 and FCVMZg10) were found to be sensitive to seven different antibiotics. Therefore, these isolates could be good candidates as probiotics. However, Many LAB are resistant to antibiotics but these resistance attributes are often intrinsic and non-transmissible (Salminen *et al*., 1998). In this regard, some studies showed that, some LAB may carry potentially transmissible plasmid encoded antibiotic resistance genes and any strains harboring antibiotic resistance plasmids are considered unsuitable for use as human or animal probiotics (Klupsaite *et al*., 2017). On the other hand, the importance of intrinsic antibiotic resistant strains which may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to administration of various antimicrobial agents had also been reported (Kullen and Klaenhammer, 1999 and Lavanya *et al*., 2011).

Blood hemolysis

Hemolysis on blood agar is one of the safety tests which was done to all selected isolates to ensure safe administration. Non-hemolytic activity was considered as a safety prerequisite for the selection of a probiotic strain. Also, absence of hemolytic activity should be a selection criterion for starter strain for dairy use (De Vuyst and Leroy, 2007). Hemolysis was evaluated using Columbia blood agar plates containing 5% (v/v) sheep blood and incubated at 37°C for 48 h. Characteristics of hemolysis on blood agar were shown as β-, α-, and γ-hemolysis. Our results presented in table 5 showed that all examined strains exhibit γ-hemolysis (no hemolysis). Thus, these isolates had not exhibited any pathogenicity and regarded as safe organisms due to their non-hemolytic activity.

Testing of technological properties of selected isolates

Acidifying ability

A rapid decrease in pH during the initial step of cheese preparation is of definitive importance in the manufacture of cheese because it is very essential for coagulation and prevention or reduction of the growth of adventitious microflora. The fast-acidifying strains are good candidates in the dairy fermentation process as primary starter organisms. Whereas, the poor acidifier strains can be used as adjunct cultures based on their other important properties, e.g., proteolytic, and autolytic activity. With respect to the acidifying activities of the identified 16 LAB strains in our study table 5, most LAB strains showed a fast or medium acidification activity.

Salt tolerance

Because addition of salt in cheese manufacture essential, thus probiotic strains should possess the ability to grow in the presence of 4-6.5% NaCl to ensure their application in dairy industry as primary starter cultures, or as adjunct cultures based on other technological properties. Salt tolerance of strains was measured as growth after 48 h, incubation periods in liquid MRS medium containing Nacl 4%. In our study, All LAB strains could grow well

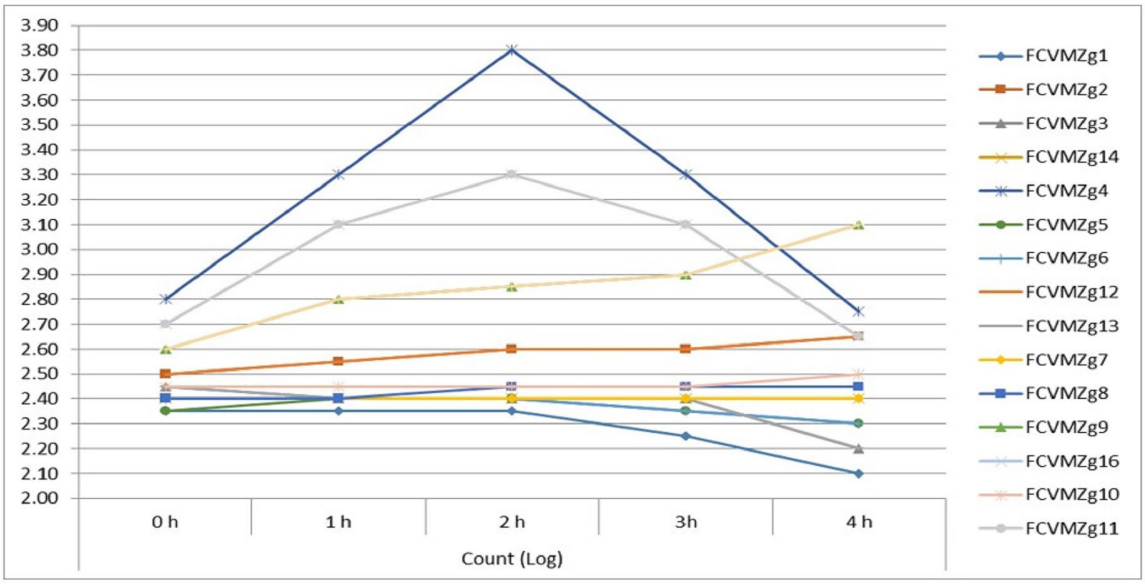

Figure 3. Bile tolerance of LAB strains

at Nacl 4% as shown in table 5. These results are similar to those reported by (Ayad *et al*., 2004a).

Galactosidase production

β-galactosidase had been widely used for industrial as well as medical applications. In dairy industries, β-galactosidase had been used to prevent crystallization of lactose, improve sweetness and increase the solubility, flavor and digestibility of the milk products (Gheytanchi *et al*., 2010). Enzymatic hydrolysis of lactose by β-galactosidase is one of the most popular technologies to produce lactose reduced milk and related dairy products for consumption by lactose intolerant people (Gheytanchi *et al*., 2010; Sumathy *et al*., 2012). In our study, the 10 LAB strains were screened for their β-galactosidase activity with X- gal and colonies with blue color were regarded as bacteria containing β-galactosidase enzyme as shown in Table 5.

Similar to our work, the identification, technological and enzymatic properties and some virulence traits of 11 LAB strains were previously reported (Monteagudo-Mera *et al*., 2011). It was demonstrated that these strains as starter or adjunct cultures in dairy products had advantages such as a good acidification ability at 30°C in ewe's and cow's milk as well as the presence of aminopeptidase activities. In addition, all LAB strains showed a high b-galactosidase activity, which have beneficial effects for human health. In the present work, only the strains that did not show any virulence factor in these works, were further tested for their tolerance to low pH, pancreatin and bile salts, their capacity to adhere to the human colon carcinoma cell line (Caco-2) as well as their ability to inhibit pathogens.

The increased trend towards incorporation of probiotics in industrial food production was accumulated after intensive investigations. The antagonistic mechanisms of probiotic LAB against pathogens have attracted worthy of attention since most LAB are belonging to the qualified presumption of safety (QPS) and generally recognized as safe (GRAS) lists (Pinto *et al*., 2006; Bernardeau *et al*., 2008; Rossetti *et al*., 2009). Additionally, several literatures showed that the use of LAB cultures and/or pasteurized milk for cheese manufacture results in loss of flavor and a reduction in the diversity of existing microflora. Sensorial differences between raw and pasteurized milk cheeses could be minimized by using LAB strains isolated from raw milk cheeses (Menéndez *et al*., 2004; Pavunc *et al*., 2012), which is considered among aims of our current study; to isolate and characterize LAB Strains with probiotic proprieties from traditional Artisanal Egyptian fermented dairy products. However, it is very important to assess the safety of LAB species intended for use as food additives (Pinto *et al*., 2006; Bernardeau *et al*., 2008; Rossetti *et al*., 2009; Verdenelli *et al*., 2009).

In 2012 at the Functional Food Center (FFC) 10th International Conference in Santa Barbara, CA, we announced a new proposed definition for "functional food": Natural or processed foods that contains known or unknown biologically-active compounds; which in defined amounts provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease (Martirosyan and Singh, 2015). Functional food science has gained momentum recently in response to the changing health status of developed countries. As healthcare costs and average life expectancy rise, the public has sought ways to become healthier and develop higher qualities of life (Martirosyan and Singh, 2015). This trend has gained momentum recently in response to the changing health status in several countries arising from pursuit of developing higher qualities of life in lower costs (Martirosyan and Singh, 2015).

CONCLUSION

The present study showed that artisanal Egyptian cheesses of rural origin have proven to be sources of safe potentially probiotic LAB with interesting physiological properties, thus it could be further incorporated in manufacture of various dairy products as starter and non-starter cultures.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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