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Diversity of lactic acid bacteria isolated from raw milk in Elsharkia province, Egypt

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Abstract

A total of 50 raw cow's milk samples were collected from different areas of Elsharkia province, Egypt for characterizing lactic acid bacteria (LAB) load. Using 16S rRNA gene sequencing, a total of 41 LAB isolates have been identified corresponding to *Enterococcus* sp. (51.22 %) as the most predominant LAB genus, followed in order by *Aerococcus* (26.82 %), *Lactococcus* (7.32 %), *Lactobacillus* (7.32 %), *Leuconostic* (4.88 %) and *Pediococcus* (2.44 %) genera. All isolates were identified to species level with exception of one strain (*Lc. lactis* subsp. *cremoris*) that has been assigned to subspecies. The phylogenetic dendrogram created has allowed good discrimination between all isolated LAB species identified with this study. Results showed a wide diversity among isolated LAB from raw milk in Elsharkia province. The impact of LAB presence in raw cow's milk on dairy safety has been discussed.

Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria widely distributed in different foods. LAB were first isolated from milk and subsequently discovered that LAB are occurring naturally as indigenous microflora in raw milk. This bacterial group is united by a constellation of morphological, metabolic, and physiological characteristics; generally by being non-sporing, non-respiring cocci or rods, and ferment carbohydrates with production of lactic acid as the major end product[9]. Nearly 30% of raw milk bacterial counts is related to LAB, and production conditions, season and animal species usually affecting their numbers and diversity[45]. From a practical dairy technology point of view, the following genera are considered the principal LAB: *Aerococcus, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Streptococcus*.

LAB presence in raw milk may be attributed to various origins, which can explain their diversity among seasons, animal species, etc. They can directly come from milk, but also from the surrounding animals' environment. Indeed, *Leuconostoc* sp. come from vegetation and roots but can easily propagate and persist in various niches which later on contaminate raw milk[27]. The ubiquitous genera *Lactococcus* and *Lactobacillus* may come from plants, feces or udder skin. Meanwhile, enterococci mainly inhabit milk as a result of fecal pollution of either human or animal routes[29].

The discrimination of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the resulted lactic acid, ability to grow at high salt concentrations, and acid or alkaline tolerance[9]. However, these methods

turned unfit nowadays due to time consuming nature, huge amount of materials and labor in addition to low diagnostic specificity and sensitivity[3,6,40]. Chemotaxonomic markers such as fatty acids composition as well as constituents of the cell wall are also used in classification[9]. New tools for classification and identification of LAB are currently replacing and/or complementing the traditional phenotype-based methodologies such as PCR-based fingerprinting and protein fingerprinting techniques [3,6,40,41]. In this sense and on the basis of the 16S rRNA gene sequencing (GS), the bacterium can then be identified and assigned to species or even subspecies level against phylogenetically-related strains located in different databases (**e.g.** NCBI GenBank). The aim of this work is to characterize the predominant LAB isolated from raw cow milk samples collected from Elsharkia province, Egypt, based on 16S rRNA GS and to discuss the impacts of isolated species on dairy safety.

Materials and Methods

Collection of milk samples: Fifty raw cow's milk samples were collected from different individual households in Elsharkia province. About 50 mL of each milk sample was aseptically collected and transported to laboratory in a 4°C vehicle-mounted refrigerator to be analyzed microbiologically within few hours.

Isolation of LAB from raw milk samples: Serial dilutions were made for each sample using 0.85% sterile physiological saline and 0.1 mL of each dilution was spread plated in duplicates of de Man, Rogosa, and Sharpe agar (MRS) (Difco Labs, Detroit, MI) adjusted to PH of 5.5[14]. Plates were incubated anaerobically (BBL Gas pak plus Anaerobic Sys.) at 30°C for 48 h. Colonies with distinct morphological differences were selected from each plate and further purified by re-streaking two successive times on fresh MRS plates. All isolates were maintained as frozen cultures in MRS broth and 50% glycerol at -80 °C.

Identification of LAB isolates and Phylogenetic Analysis Based on 16S rRNA GS: All procedures were done as previously described by[3,6,10,41]. Total genomic DNA was extracted from overnight cultures. The bacterial cells were lysed by the addition of 180 μL of lysis solution (Sigma-Aldrich) after incubation for 2 h at 37 ºC. Total genomic DNA was extracted and purified using the DNeasy Tissue Mini Kit (Qiagen). A fragment of the 16S rRNA gene was amplified by PCR using the universal primer pair: p8FPL(5′-AGTTTGATCCTGGCTCAG-3′) and p806R (5′-GGACTAC-CAGGGTATCTAAT-3′). All of the PCR assays were performed using a "My Cycler" Thermal Cycler (BioRad Labs, USA). Direct sequencing was performed using the "BigDye Terminator v3.1" Cycle Sequencing Kit (ABs, Perkin-Elmer, Foster city, CA) and the same primers used for PCR were also used for the sequencing. The sequencing reactions were analyzed in ABI3130 automatic GS sys. (ABs, USA). Entire 16S rRNA gene sequences were analyzed using Chromas software and aligned with Clustal-X software[44]. 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/)[8]. Consensus sequences were imported into MEGA 6.0 software, with which a sequence alignment and phylogenetic trees were conducted based on the NJ method and Kimura-2 parameter model.

Rooting-out the phylogenetic dendrogram: 16S rRNA gene sequences of six strains of LAB from previous studies[3,5,41] plus two *Lc. lactis* subsp. *lactis* strains from NCBI GenBank (gi_387286036, gi_387286035) were used to root-out the phylogenetic dendrogram (Table 1).

Table 1.Out-group strains used in the phylogenetic dendrogram.

Strain code	Species	GenBank Accession no.
CECT4039	*E. faecalis*	KC510231
CECT410	*E. faecium*	KC510233
USC13	*S. dysgalactiae subsp. dysgalactiae*	KC510218
USC17	*S. dysgalactiae subsp. dysgalactiae*	KC510219
USC30	*Lc. lactis*	KP001333.1
USC31	*Lc. lactis*	KP001334.1

Results and Discussion

During last decade, genotypic identification has been emerged as an alternative or a complement to established phenotypic methods within dairy diagnostics providing more accuracy, less labor and time saving. Among the phylogenetic marker genes used to discriminate among different species, 16S rRNA is well-established as a universal gold standard for the identification and phylogenetic classification of prokaryotic species, genera, and families[36]. Thus, 16S rRNA GS has been applied extensively within food safety diagnostic labs proving powerful identification and discrimination potentials[3,6,10,41].

The partial 16S rRNA GS (800 bp) of all the strains isolated in our study were compared with related bacteria in GenBank and sequence similarities were determined using the BLAST tool. The resulted 16SrRNA gene sequences of different isolates have been submitted to be deposited in the GenBank. Based on 16S rRNA GS, forty one strains of LAB identified in our work were corresponding to Enterococcus (51.22 %) as the most predominant LAB genus, followed in order by *Aerococcus* (26.82 %), *Lactococcus* (7.32 %), *Lactobacillus* (7.32 %), *Leuconostic* (4.88 %) and *Pediococcus* (2.44 %) genera (Table 2).

Table 2: Isolated LAB strains in this study.

Isolated LAB	No.	%
E. durans	1	2.44
E. faecium	8	19.51
E. hirae	2	4.88
E. faecalis	6	14.63
E. casselifavus	2	4.88
E. saccharolyticus	2	4.88
Pediococcuspentosaceus	1	2.44
Lc. garviae	2	4.88
Lc. lactis subsp. cremoris	1	2.44
Lb. plantarum	1	2.44
Lb. casei	1	2.44
Lb. fermentum	1	2.44
Leuc. mesentroides	2	4.88
A. viridians	11	26.82
Total	**41**	**100**

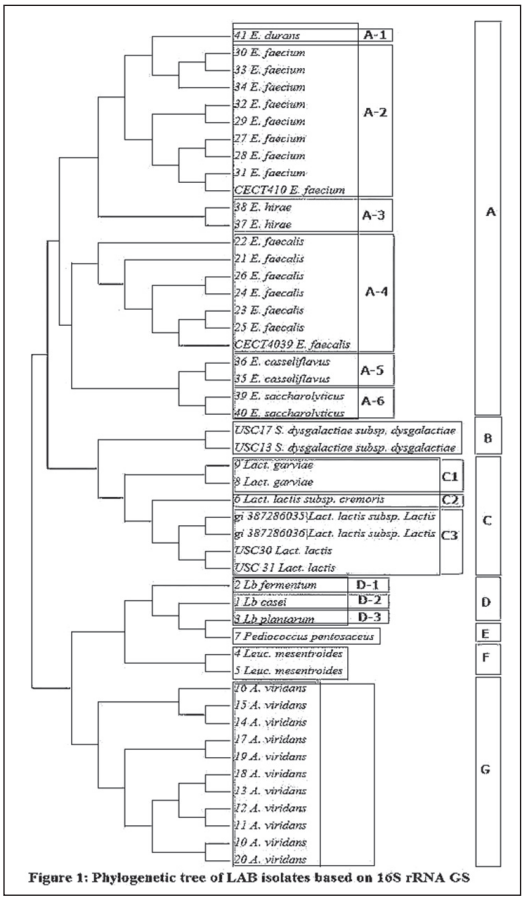

Figure 1: Phylogenetic tree of LAB isolates based on 16S rRNA GS

Out of all isolates and according to identification evolved from 16S rRNA GS, 40 isolates (97.56 %) have been identified to the species levels, and only for one *Lc. lactis* subsp. *cremoris* isolate (2.44 %), the identification has been extended to the subspecies level. Nearly similar to our results, a study by[24] showed that 94% of isolated LAB in cow's milk included lactococci, enterococci and streptococci, while the remaining 6% isolates were lactobacilli (mostly *Lb. casei, Lb. delbrueckii, Lb. paracasei* and *Lb. plantarum*), *Leuconostoc* and pediococci. Also, other study by[2], most LAB recovered from raw cow milk samples in Khartoum, Sudan were corresponded to Enterococcus, *Lactococcus* and *Lactobacillus* species. Among relevant isolates in another investigation[16], *Streptococcus,* Enterococcus and *Aerococcus* were corresponding to 52, 26 and 15 %, respectively and remaining % were lactococci. The phylogenetic dendrogram has been created using MEGA 6.0 software as shown in Figure 1. Remarkably, 16S rRNA GS has allowed for a very good discrimination among all isolated LAB species with high bootstrap values. The phylogenetic tree has been separated into 2 main branches. The first branch included *Enterococcus, Streptococcus* and *Lactococcus* sp., corresponded to A, B and C sub-branches respectively. Meanwhile, the second branch included *Lactobacillus, Pediococcus, Leuconostoc* and *Aerococcus* sp., which corresponded to D, E, F and G sub-branches, respectively.

Presence of LAB in raw milk could be good candidates in biopreservation of processed dairy products[15], especially fermented foods such as mature cheeses, cream and yoghurt. LAB have an essential role in the nutritious and organoleptic properties of fermented milk production[11]. As a result of lowered pH following sugar fermentation and acid production, the development of the desirable organoleptic properties occurs[12]. Additionally, antimicrobial potentials have been linked to some LAB[32,37]. In this sense, *Leuc. mesenteroides* sp. *mesenteroides* FR 52, isolated from a raw milk, produced a bacteriocin which was named Mesenterocin 52[32]. This bacteriocin inhibited other *Leuconostoc* strains and several strains of *Enterococcus* and *Listeria* spp. For this, most LAB are belonging to the qualified Presumption of Safety (QPS) and generally recognized as safe (GRAS) lists which insure their safety for use in food[42]. However, some LAB are excluded from these advantages as *Enterococcus* sp. because of their roles in causing certain human infections and contribution to spread of antibiotic resistance[33] and more importantly, their presence in milk could indicate unsanitary production and fecal pollution of either human or animal routes or both as they are ubiquitously found in the intestinal microflora of humans and animals[29]. Unfortunately, our results indicated the unsanitary production associated with raw milk as *Enterococcus* sp. constituted alone 51.22% out of all LAB isolates. Enterococci are among predominant isolated LAB from raw milk[1,7,16,24]. Also, enterococci are well-known to be minor mastitis pathogens causing subclinical mastitis (SCM) in dairy animals with no apparent signs, or clinical form with abnormal milk, swelling of the udder, and fever[4,16].

In one study[16], several enterococcal species have been isolated from subclinical intramammary infections (IMIs) in dairy cows corresponded to *E. faecalis, E. faecium, E. durans* and *E. hirae.* Also, other investigations showed isolation of enterococci as predominant LAB from raw milk, and the most isolated enterococcal species were *E. faecalis, E. facium*[1,7] and *E. durans*[24]. Like streptococcal IMIs, those caused by enterococci may represent poor responsiveness to antibiotic therapy[38]. Biofilm-formation by enterococci is thought to contribute to this resistance[35]. Enterococci can be responsible for variety of defects in processed dairy products such as cheese, causing excessive softening, splits and cracks, off flavors and abnormal colors[30]. In human, enterococci are incriminated as direct or indirect agents of diseases[18,25]. Some enterococci can cause food-poisoning especially *E. faecium*

if predominated in the food. Enterococcal food-intoxication caused is greatly attributed to production of biogenic amines[25]. In addition, *E. faecalis* has associated with a large number of gastroenteritis outbreaks and implicated in urinary tract and wound infections, intra-abdominal abscesses and endocarditis[18].. It is thought that enterococcal toxins behaving and producing symptoms similar to but less acute than those of staphylococcal enterotoxins[28].

Several *Lactococcus, Lactobacillus* and *Leuconostoc* species have potential technological applications within dairy industry[21,22,24]. In other studies, *Lb. plantarum, Lb. fermentum, Lb. acidophilus, Lb. paracasei* and *Lb. rhamnosus* were the frequent isolated lactobacilli[2,31]. In our study, isolated lactobacilli were only limited to 3 species in lowered incidences; *Lb. plantarum, Lb. fermentum* and *Lb. casei* (1 strain of each). Among isolated lacococci, *Lc. garvieae* constituted 4.88 % out of LAB. *Lc. garvieae* has been reported recently as a majority component of the autochthonous microbial populations of certain artisanal cheeses[21] and fermented milk products[17]. *Lc. garvieae* cause lactococcosis in fish due to owing several virulence factors, meanwhile, *Lc. garvieae* isolates of dairy origin have shown absence of virulence determinants[22], suggesting that *Lc. garvieae* dairy strains are unrelated to the pathogenic ones[23]. However, the isolation of *Lc. garvieae* from milk of bovines with SCM was reported[16,38]. Moreover, isolates of *Lc. garvieae* of dairy origin are incriminated to carry antibiotic resistance genes[46], which might contribute to antibiotic resistance in both animal and human. Also, *Leuc. mesentroides* has been identified recently to be as a sporadic infectious agent in immune-compromised humans[13], but further studies are needed to show if strains of dairy origin are possessing such virulence factors.

Aerococcus sp. exhibit many biochemical and physiological similarities with *Pediococcus, Enterococcus, Lactococcus and Leuconostoc species,* and are often confused with *Streptococcus* sp.[20]. *A. viridans* is a catalase-negative Gram-positive cocci resembling staphylococci by Gram stain, but have biochemical and growth characteristics of streptococci and enterococci[19]. The typing of *A. viridans* by some commercial biochemical systems may be not sufficient to achieve 100% identification accuracy[43], thus, the genotypic typing was recommended. In our study, accurate identification to the species level in 100% of *Aerococcus* sp. isolates based on 16S rRNA GS has been obtained, which agreed with recommendation by[43] for *A. viridians* identification. In current study, *A. viridians* has been isolated with incidence of 26.82%. In a study by[47], *A. viridans* was detected in 50% of 48 bulk tank milk samples from 48 dairy farms in USA. The contribution of *A. viridans* in causing clinical, subclinical or latent types of mastitis have been described[16,43], although the exact role of the m.o was not clear. It has reported in a study by[16] that *A. viridans* constituted 15% of Gram-positive, catalase-negative, aesculin-degrading cocci isolated from clinical and subclinical bovine mastitic cases, but in a different study[34], only two *A. viridans* isolates were detected among 100 isolates incriminated in causing mastitis. In Humans, many infections such as endocarditis, urinary tract infections, arthritis, or meningitis have been associated with *A. viridans*[26,39]. However and like *Lc. garvieae,* it must be demonstrated if dairy *A. viridans* isolates are possessing virulence factors of human-clinically associated strains.

Conclusion

A wide diversity in LAB isolated from raw cow milk samples collected from Elsharkia province, has been shown. Several beneficial LAB have been isolated and could be of potential application within future researches. On the other hand, the results showed presence of enterococci in high incidence which reflected bad sanitary production processes within individual households, which

reflect necessity to commitment to healthy specifications and showed that further attention of the health authorities towards individual households should be directed.

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