BIOCHEMICAL AND MOLECULAR IDENTIFICATION OF *ENTROCOCCUS* ISOLATED FROM TRADITIONAL FERMENTED MILK AND ASSESSMENT OF THEIR ANTIMICROBIAL ACTIVITY AGAINST SOME PATHOGENS

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ABSTRACT

The purpose of the present work was to characterize promising starter culture strains of *Enterococcus faecium* and *Enterococcus durans* isolated from traditional fermented milk. The antimicrobial activity of *E. faecium* and *E. durans* against *Staphylococcus aureus* (MRSA), *Pasteurella multocida* and *Pseudomonas fluorescens* was evaluated. A total of 20 isolates of lactic acid bacteria were characterized using morphological characters and all the isolates were Gram-positive and catalase-negative lactic acid bacteria. Eight isolates were identified as *Enterococcus* spp using API kit and confirmed by using *Enterococcus* genus–specific primers (*Sod* A). Based on 16SrRNAgene analysis, four isolates were identified as *E. faecium* and four isolates were identified as *E. durans*. The plasmid contents and profiles of the strains were showed, all tested strains carried a certain number of plasmids ranged from 2 to 8 with different molecular sizes. The effectiveness of the antimicrobial activity of *Enetrococcus* is strictly related to the species and strain of the target microorganism. Commercial kits for species identification of *E. durans* and *E. faecium* are in some cases incorrect to distinguish species of enterococci, so the molecular identification of *Enterococcus* strain play an important role in food industry as starter cultures to improve food quality and safety.

Keywords: *Enterococcus* spp; Antimicrobial activity; phenotypic characterization; 16SrRNA; Genus– specific primer; plasmids; Pathogenic bacteria.

1. INTRODUCTION

Enterococci are lactic acid bacteria (LAB) widely distributed in foods and environment. These microorganisms, as commensals in the human gastrointestinal tract, show a long history of use in foods and fermented products (Haghshenas *et al.*, 2014 and Nami *et al.*, 2014). They can be found in soil, surface water, foods of animal origin (milk and cheese), vegetables and plant materials because of their ability to survive heat treatments and adverse environmental conditions (Giard *et al.*, 2001). Many kinds of food are still naturally fermented without the use of starter cultures by autochthonous lactic acid bacteria which form the characteristic properties of the products. These

natural isolates of lactic acid bacteria from spontaneous fermentations could be used as specific starter cultures or as adjunct strains after phenotypic and genotypic characterization and they represented a possible source of potentially new antimicrobial metabolites (Wouters *et al.*, 2002 and Topisirovic *et al.*, 2006). *Enterococcus* strains have various helpful functions in the dairy industry as starters; enterococci fulfill a considerable function in developing flavor progress and quality of cheeses. LAB able to be used for healing of gut disorders in both humans and animals since these bacteria contributes to the intestinal health of the host by the development of gut microbial balance (Rehaiem *et al.*, 2014). Enterococci play an important role in the development of the sensory characteristics of fermented foods and cheeses (Sánchez *et al.*, 2007). Furthermore, some enterococcal strains have been successfully used as preservatives to inhibit the growth of food spoilage microorganisms. The production of antimicrobial compounds like bacteriocins, organic acids, hydrogen peroxide, and fatty acids is of the well-designed properties applied to characterize probiotics (Rehaiem *et al.*, 2014). Lactic acid bacteria (LAB) also, show antagonistic actions against spoilage and pathogenic organisms (Ouwehand and Vesterlund, 1998). Production of bacteriocins (enterocins) is the useful biotechnological trait of *Enterococcus* bacteria (Franz *et al.*, 2011). Lactic acid reduces the pH that can cause the disruption of cellular substrate transport systems through altering the cell membrane permeability or collapsing the electrochemical proton gradient (Ammor *et al.*, 2006). LAB also produce antimicrobial substances such as bacteriocins, which are generally defined as ribosomally synthesized peptides or proteins with bactericidal actions that often target bacterial species closely related to the producer strain (Klaenhammer, 1993). These compounds have attracted great interest because of their potential use as food preservatives, therapeutic agents against Gram positive bacteria and several viruses, and important in modifying gut microflora (Shearer *et al.*, 2014). The identification of the isolates at strain level is of great importance not only in epidemiological and phylogenetic studies, but also for ecological and industrial purpose (Kuhn *et al.*, 1995).

Characterization and identification of enterococci by using the traditional phenotypic differentiation can be a tedious process requiring numerous tests. Strains are classified based upon growth in various media, biochemical reactions in those media, motility, and pigmentation (Facklam *et al.*, 2002). The genus *Enterococcus* is not a phylogenetically and phenotypically coherent and homogeneous genus, automated systems, principally based on phenotypic characteristics, not always permit the correct identification of more rarely encountered *Enterococcus* species (Tsakris *et al.*, 1998). The molecular identification methods such as PCR and sequencing 16SrRNA overcome the disadvantages of classical identification methods (Naser *et al.*, 2005). The screening of natural isolates from traditional fermented

milk is generally an efficient tool for the characterization of useful and safety strains for industrial and scientific purposes. Therefore, the present study aims to (1) isolate *Enterococcus* from tradiontal fermented milk (2) identify the most promising isolates based on phenotypic and molecular characterization include, genus-specific primer and16SrRNA gene sequencing (3) assess the potential antimicrobial activity of *Enterococcus* isolates against *S. aureus, P. multocida* and *Ps. fluorescens*

2. MATERIALS AND METHODS

2.1. Bacterial isolates, culture conditions and maintenances

The *Enterococcus* isolates used in this study were isolated from different fermented milk samples collected from local market in Fayoum, Egypt. One gram sample of each fermented milk was aseptically transferred into a sterile flask, serial dilutions of the samples were made in sterile physiological saline and pour plated into de Man Rogosa Sharpe broth (MRS) agar and incubated at 37°C and sub culturing in MRS medium and incubated anaerobically at 37°C overnight. Ten µl of each active broth culture was inoculated into petri dish contain MRS agar medium and incubated anaerobically at 37°C for 24-48 h. Colonies showing morphological characteristics for lactic acid bacteria were selected and inoculated into MRS broth. After appropriate incubation period, the cultures were examined microscopically for purity. The pure culture was preserved in MRS broth supplemented with 20% glycerol (v/v) for long- term storage and maintenance at -20 °C until use.

2.2. Identification of *Enterococcus* isolates

The *Enterococcus* isolates were characterized based on Gram's stain reaction, cell morphology, motility according to Lalam *et al.* (2015). Putative Enterococci were identified to species level based on the API 20 strip (bioMérieux, Marcy l'Etoile, France) according to the instructions of the manufacturer, and by the database provided by Biomerieux (Kim *et al.*, 2006). All the isolates were overnight cultured in MRS broth, and then added individually to the wells of the API strips. The inoculated strips were incubated at 37°C and then monitored for changes in the color of the medium after 24 h. Discrimination between isolates was based on the principle of a pattern matching manual as described by the manufacturer.

2.3. Test microorganisms

Eighteen strains of various Gram-negative and Gram–positive bacteria were selected and used as test microorganisms (8 strains of *Staphylococcus aureus* (MRSA), 5 strains of *Pasteurella multocida* and 5 strains of *Pseudomonas fluorescens*. The strains of *S. aureus* and *Ps. fluorescens* were obtained from the Agricultural microbiology department, while the strains of *P. multocida* were obtained from Genetics department, Fayoum University, Egypt.

2.4. Antimicrobial activity assay of *Enterococcus* isolates

The antibacterial activity was assessed against *S. aureus* (MRSA), *P. maltucida* and *Ps. fluorescens* using the well agar diffusion method (Naghmouchi *et al.*, 2006). The cell free supernatants (CFS) used for antibacterial activity measurement were obtained by centrifuging (10000 rpm 20 min, 4°C), cultures of *Enterococcus* grown at 37°C for 18–24 h, on MRS broth. Wells were performed in solid agar and 100 µl of each cell-free supernatant (CFS) were poured into the wells. The Petri plates were left at room temperature for 1 h before incubation for 24 h at adequate temperature for each pathogenic strain. After this period of incubation the antibacterial activity was detected by observing the inhibition zones around the well containing the CFS. The clear zones were regarded as inhibitory zones and recorded in mm.

2.5. Genomic DNA extraction

Genomic DNA was extracted using phenol-chloroform method according to Hefzy *et al.* (2015).

2.6. Genus-specific PCR from *lactobacillus* isolates

The primers for *sod*A gene were designed on the basis of the available sequences at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and the internal fragment (458 bp length) of the *sod*A gene was amplified using primers F (5'-CAGGAGCAATCAAAGACGCA-3') and R (5'-TGGCAGTAGAAGTGATGGCA -3'). The PCR reaction was performed in a final volume of 25 µL containing 250 ng of DNA template, 1x Master Mix ((One PCR™, GeneDirex, Taiwan), PCR-grade H_2O, and 10 pmol of each primer. PCR protocol by Poyart *et al.* (2000) was slightly modified: the initial denaturation was 3 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 60 s, and elongation at 72 °C for 60 s. The final extension step was 7 min at 72 °C. A single DNA band corresponding with the expected length of 438 bp was observed in all cases on 1.5% agarose gel with ethidium bromide (Poyart *et al.*, 2000).

2.7. PCR and sequencing of 16S rRNA gene and phylogenetic analysis

The 16S r RNA gene was amplified by PCR using universal eubacterial primers F (5-AGAGTTTGATCGTGGCTCAG-3and R5- GGATACCTTGTTACGAATTC-3). PCR amplification was carried out according to Drago *et al.*, (2011), in 25 µl volumes containing 1 µl of bacterial DNA and 24µl of amplification mix, which contained the following components: 10 pmol each primer, 0.2 mM (each) dNTPs, 2.5 µl of 10 x PCR buffer, 2.0 µl of 25 mM $MgCl_2$ and 1.25 U of Taq DNA polymerase. The PCR amplification was performed in the thermal cycler 2720 (Applied Biosystems, USA). Samples were incubated for 5 min at 95°C to denature the target DNA and went through 30 cycles of 95°C for 30 s, 55°C for 50 s and 72°C for 2min. The samples were then incubated at 72°C for 10 min for a final extension and were maintained at 4°C until they were tested. The amplicons of 550 bp was expected were purified using Montage PCR

Clean up kit (Millipore), and sequenced using ABI 3730 xl automated DNA sequencer (Applied biosystem USA) through Lab Biotechnology Company, Egypt. Similarity search was conducted using the BLAST software by NCBI ((National Center Biotechnology Information, http//:www.ncbi.nml.nih.gov). Phylogenetic analysis of the 16S rRNA gene sequences was conducted with MEGA software version 4.0 (MEGA4; http://www.megasoftware.net). Tree was generated by neighbour joining using the maximum composite likelihood model (Tamura *et al.* 2007).

2.8. Total Plasmid DNA Isolation

The plasmid DNA isolated from selected isolates using the alkaline method according to Birnboim (1983) with modifications. Each culture was grown in MRS broth at 37 °C overnight and 10 ml of each culture sediment by centrifugation at 13000 rpm for 10 min. at 4 °C. The cells were washed with 500 µl 1X phosphate saline solution (PBS), pH 7.4 and resuspended in 200 µl of a solution containing 25% sucrose, 50 mM Tris pH 8.0, 0.1 M NaCl , 30 mg mL^{-1} lysozyme, 120 U mL^{-1} mutanolysin and 40 µg mL^{-1} RNase A. The samples were incubated for 15 min. at 37 °C. Cells debris and chromosomal DNA was removed from the extracts by addition of 400 µl of a solution containing 3% SDS and 0.2 N NaOH. Mix immediately by immersion and incubated 7 min at 21 °C. Added 300 µl of potassium acetate 3 M pH 4.8 and mix immediately. Without vortex, followed by centrifugation at 13000 rpm for 15 min at 4 °C. The plasmidic DNA in supernatant was precipitated and concentrated by addition of 650 µl isopropanol, centrifugation at 13000 rpm for 15 min at 4 °C and resuspension in 320 µl of ultrapure water. The DNA preparation was purified and deproteinated by adding 200 µl of 7.5M ammonium acetate contained 0.5 mg/ml of ethidium bromide and 350 µl a mixture of phenol - chloroform - isoamyl alcohol (25:24: 1) and centrifugation at 13000 rpm for 10 min at 21 °C. Plasmid DNA was precipitation from the aqueous phase with 1 ml of ethanol absolute for 12 hours at -20 °C and recovered by centrifugation at 12500 rpm for 45 min at -10 °C. The precipitated DNA was washed with 1 mL of 70% ethanol, sediment by centrifugation at 12500 rpm for 30 min at -10 °C, and suspended in 10 mM Tris buffer pH 8.0 and stored frozen at -20 °C until further analysis.

3. Results and discussion

3.1. Isolation, morphological and biochemical identification of *Enterococcus* spp.

A total of 20 isolates of lactic acid bacteria were isolated from traditional fermented milk. All isolates were Gram- positive, non-spore former, non-motile, facultative anaerobic cocci-shaped bacteria and identified as Lactic acid bacteria. Identification of the twenty lactic acid bacterial isolates to species level was carried out on the basis of their carbohydrate fermentation patterns obtained by API 20E system Kit. Based on API profile index 8 out of 20 (40%) isolates was *Enterococcus* spp. The *Enterococcus* isolates could be classified

into four species as the following: three isolates were identified as *E. fecium* (E9, E11 and E 13), Three isolates were identified as *E. durans* (E5, E25 and E48), one isolate (E1) *as E. asini* and one isolate (E2) as *E. avium* (Table 1). However, the characterization of some *Lactobacillus* to species level according to biochemical and morphological characters is not sufficient and may lead to an overlap and misidentification of the isolates (Schleifer et *al.*, 1995 and Klein *et al.*, 1998). The morphological and biochemical identification of *Lactobacillus* isolates was confirmed by molecular identification based on 16S rRNA gene.

3.2. Antimicrobial activity assay of *Enterococcus* isolates

Antimicrobial activity is a very important criterion for selection of starter and probiotic culture as natural antagonists of potentially harmful bacteria. Therefore, 20 *Lactobacillus* isolates from the traditional fermented milks were screened for their activity against three human pathogens. The cell-free filtrates obtained after cultivation of the selected *Enterococcus* in MRS were tested for their antimicrobial activities against various gram-positive (*S. aureus* MRSA) *and* gram negative bacteria (*P. maltucida* and *Ps. fluorescens*). In this study, the enterocin produced by *Enterococcus* spp, showed different activities against the three pathogenic bacteria .The data in Table (2) showed all selected *Enterococcus* isolates have relative inhibitive activity against the growth of *S. aureus* (MRSA), *P. maltucida* and *Ps. fluorescens.* On the other hand, some strains of pathogenic bacteria were showed resistance to some selected *Enterococcus* isolates (Fig. 1).The use of bacteriocinogenic cultures in biopreservation of foods may be considered as an additional tool to enhance the microbiological safety and reduce the risk of the development of spoilage microorganisms (Nascimento *et al.*, 2010). Ennahar and Deschamps, (2000) observed that enterocin A produced by *E. faecium* EFM01 inhibited 13 out of a total of 14 *L. monocytogenes* strains, while none of 7 *S. aureus* strains investigated were inhibited by this bacteriocin. On the other hand, Ammor *et al.* (2006) reported antimicrobial activity of enterocins produced by *E. faecium* against *S. aureus* strains. The increased production of lactic acid through fermentation reduces pH of the media, which is known to inhibit the growth of most food-borne pathogens. The antimicrobial effect is also due to the undissociated form of the acid and its capacity to reduce the intracellular pH, leading to inhibition of vital cell functions. (Kivans *et al.*, 2011). The activity against Gram-positive pathogens is mostly due to the bactericidal effect of protease sensitive bacteriocins (De Vuyst and Leroy, 2007) while the antagonistic effects towards Gram-negative pathogens could be related to the production of organic acids and hydrogen peroxide (Makras and De Vuyst, 2006).

Table (1): API 20 E system carbohydrate profile for the selected *Enterococcus* isolates.

No.	Substrate	*Enterococcus* Isolate							
		E1	*E9*	*E5*	*E48*	*E2*	*E25*	*E13*	*E11*
1	Oxidase	–	–	–	–	–	–	–	–
2	Catalase	–	–	–	–	–	–	–	–
3	pyruvate	+	+	+	+	+	+	+	+
4	hippurate	+	+	+	+	–	+	–	+
5	esculin +	+	+	+	+	+	+	+	+
6	Pyrrolidonyl 2 naphthylamide	+	+	+	+	–	+	+	+
7	6-bromo-2-naphthyl α D-galactopyranoside	–	–	–	–	–	–	+	–
8	naftol AS-BI β-D-glucuronat	–	–	–	–	–	+	–	–
9	2-naphthyil-β-D-galactopyranoside	–	+	+	-	–	+	+	+
10	2-naftilfosfat	–	+	–	-	+	+	+	+
11	L-leucina-2-naphthylamide	–	+	+	+	+	+	+	+
12	arginine	–	+	+	+	–	–	+	+
13	ribose	–	+	+	+	+	+	+	+
14	L- arabinose	–	+	–	–	+	+	+	–
15	mannitol	–	+	–	–	+	+	+	+
16	sorbitol	–	–	–	–	+	+	+	+
17	lactose	+	+	+	+	+	+	+	+
18	trehalose	+	+	+	+	+	+	+	+
19	inulin	–	–	–	–	+	+	–	–
20	raffinose	–	–	–	–	–	–	+	–
21	starch	+	+	+	+	+	+	–	+
22	glycogen	–	+	–	–	–	–	–	–
Preliminary identification		*E. asini*	*E. faecium*	*E. durans*	*E. durans*	*E. avium*	*E. durans*	*E. faecium*	*E. faecium*

Notes: Carbohydrate fermentation profiles were applied according to API 20 strips (BioMérieux, Lyon /France). The Score of the result tests: - negative growth; + growth.

Table (2): Inhibitory spectrum of enterocin from *Enterococcus* isolates as determined with the agar well diffusion assay.

No.	Pathogens strains	Inhibition Zone Diameter (mm)							
		E1	*E9*	*E5*	*E48*	*E2*	*E25*	*E13*	*E11*
1	*Staph. aureus* strain 1	33	32	37	33	32	17	30	0
2	*Staph. aureus* strain 2	0	39	35	41	31	16	39	0
3	*Staph. aureus* strain 3	24	0	42	39	41	18	0	37
4	*Staph. aureus* strain 4	0	40	0	37	40	14	40	24
5	*Staph. aureus* strain 5	33	0	34	0	43	18	0	38
6	*Staph. aureus* strain 6	0	18	0	38	0	16	18	0
7	*Staph. aureus* strain 7	0	0	20	0	53	20	0	0
8	*Staph. aureus* strain 8	28	0	0	22	0	0	0	30
9	*Past. multocida* strain 1	18	31	0	0	24	0	31	17
10	*Past. multocida* strain 2	22	20	34	0	0	0	20	25
11	*Past. multocida* strain 3	26	33	22	37	0	22	33	13
12	*Past. multocida* strain 4	26	0	36	24	41	32	0	17
13	*Past. multocida* strain 5	0	31	0	40	24	30	51	17
14	*Ps. fluorescens* strain 1	28	0	36	0	34	0	0	18
15	*Ps. fluorescens* strain 2	0	31	0	41	0	28	31	17
16	*Ps. fluorescens* strain 3	0	25	34	0	37	17	25	17
17	*Ps. fluorescens* strain 4	39	0	28	37	0	0	0	13
18	*Ps. fluorescens* strain 5	0	43	0	30	41	11	43	14

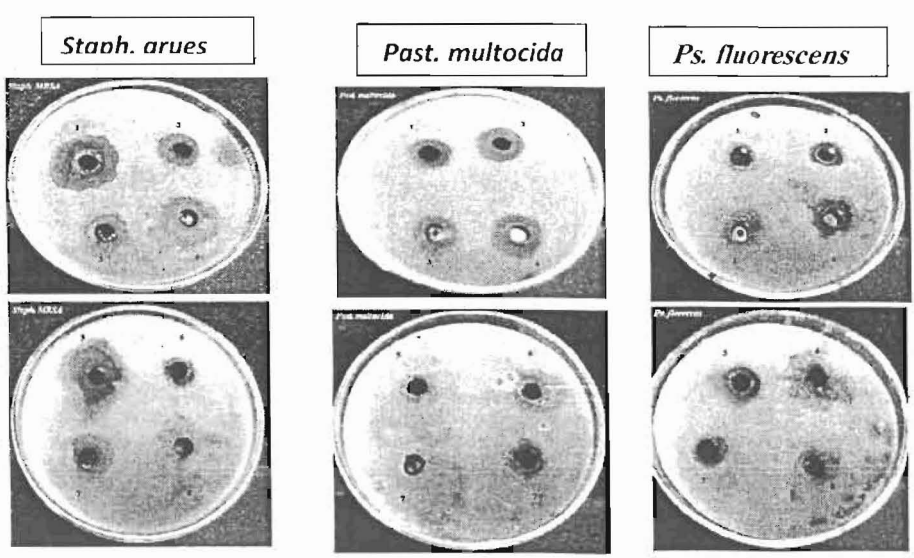

Fig. (1): Antibacterial activity of cell - free supernatants of *Enterococcus* isolates (CFS) against three indicator bacteria using agar well diffusion assay

3.3. Genus specific PCR from *Enterococcus* isolates

Enterococcus isolates were identified by using *Enterococcus* genus-specific primers *Sod* A according to Poyart *et al.* (2000). After agarose gel electrophoresis, amplified PCR fragments with size about 450 bp from genomic DNAs of *Enterococcus* isolates were observed (Fig. 2). Poyart *et al.* (1998) reported that identified the manganese-dependent superoxide dismutase gene *sod*A as an ideal gene for identification of enterococci. The superoxide dismutase gene has been used to distinguish genera and species of mycobacteria, streptococci, staphylococci, and enterococci (Scarpellini *et al.*, 2002). Genus and species-specific primers have been previously applied successfully by many researchers (Marino *et al.*, 2003 and Jackson *et al.*, 2004). By using the degenerate *sod*A gene encoding a manganese-dependent enzyme (Mn-Sod) in isolates identified as enterococci, a single amplification product having the expected size of 438 pb was observed with all selected strains (Ulrich and Müller, 1998).

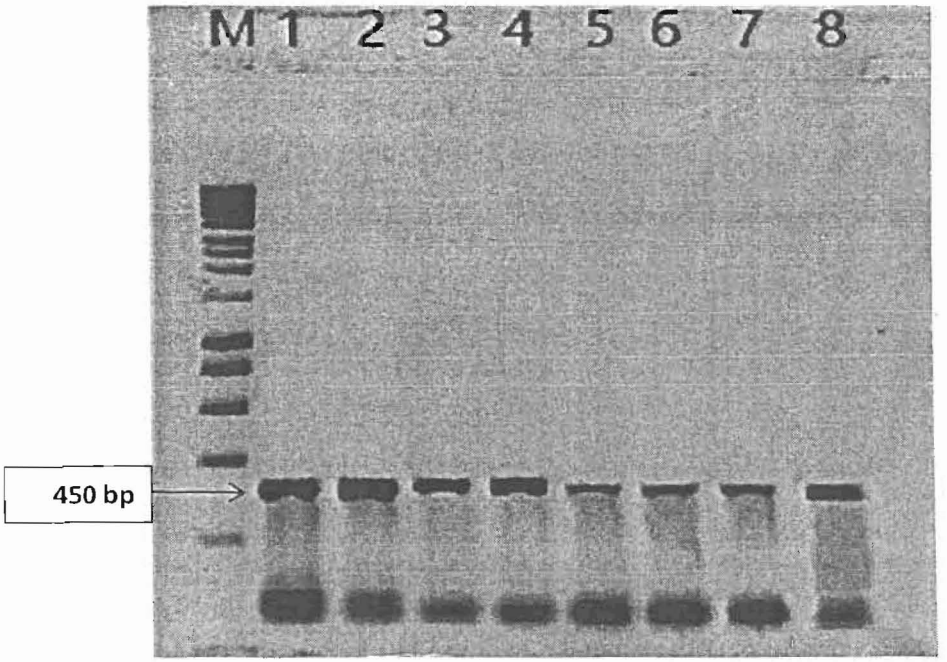

Fig. (2) Agarose gel analysis of PCR products from amplification of *Sod* A gene of the selected *Enterococcus* isolates. Lane M: molecular weight marker fragments were (250, 500,750, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 7000, 8000 and10000 bp), Lane 1 to lan8 represent the bacterial isolates (E1, E9, E5, E48, E2, E25, E13 and E11), respectively.

3.4. PCR and sequencing of 16S rRNA gene and phylogenetic analysis

The representative 16Sr RNA gene sequences of the eight selected *Enterococcus* isolates were chosen for phylogenetic and comparison with the sequences obtained from GenBank database (Fig.3). Sequencing of PCR products showed that *Enterococcus* isolates exhibited between 98% and 100% homology with different *Enterococcus* strains deposited in the GenBank. Eight *Enterococcus* isolates were identified as *E. faecium* (4 strains) and *E. durans* (4 strains). Results of the PCR assay correlated with those obtained using the API 20 E kit system for 6 out of 8 (75%) isolates identified as *E. faecium* (3 isolates) *and E. durans* (3 isolates). On the other hand, two isolates identified as *E. asini* and *E. avium* by the API system was found to be *E. faecium* and *E. durans* by sequencing 16S rRNA, respectively (Table 1 and Fig. 4). For this reason it is necessary to confirm the phenotypic characterization with molecular identification include genus-specific primer and sequencing of the 16S rRNA gene. Fortina *et al.* (2007) reported that the16S rRNA gene has been useful for the identification of *Enterococcus* genus and species. Jurkovic *et al.* (2006) found some discrepancies between results of enterococci identification from Bryndza cheese samples, obtained by commercial biochemical test and PCR method. Seven enterococci strains identified by commercial biochemical test were identified as *E. faecium* and by PCR method as *E. faecalis*. Three strains of *E. casseliflavus* were determined by PCR method as *E. faecium* (two strains) and *E. faecalis* (one strain). One major reason for the mismatch between phenotypic and genotypic data might be described to loosing or acquiring plasmids, which leads to metabolite inconsistencies, as some carbohydrate fermentation capacities are plasmid encoded (Ahrne *et al.*, 1989). The advantages of genotyping include the stability of genomic DNA, its composition being independent of cultural conditions or preparation methods, and amenability to automation and statistical data analysis. The comparative evaluation of phenotypic and genotypic results confirmed that the phenotypic test, in spite of giving information on the biochemical and metabolic traits of LAB, are not reliable enough for the identification of these microorganisms, although it is a useful tool for presumptive classification (Fitzsimons *et al.*, 1999).

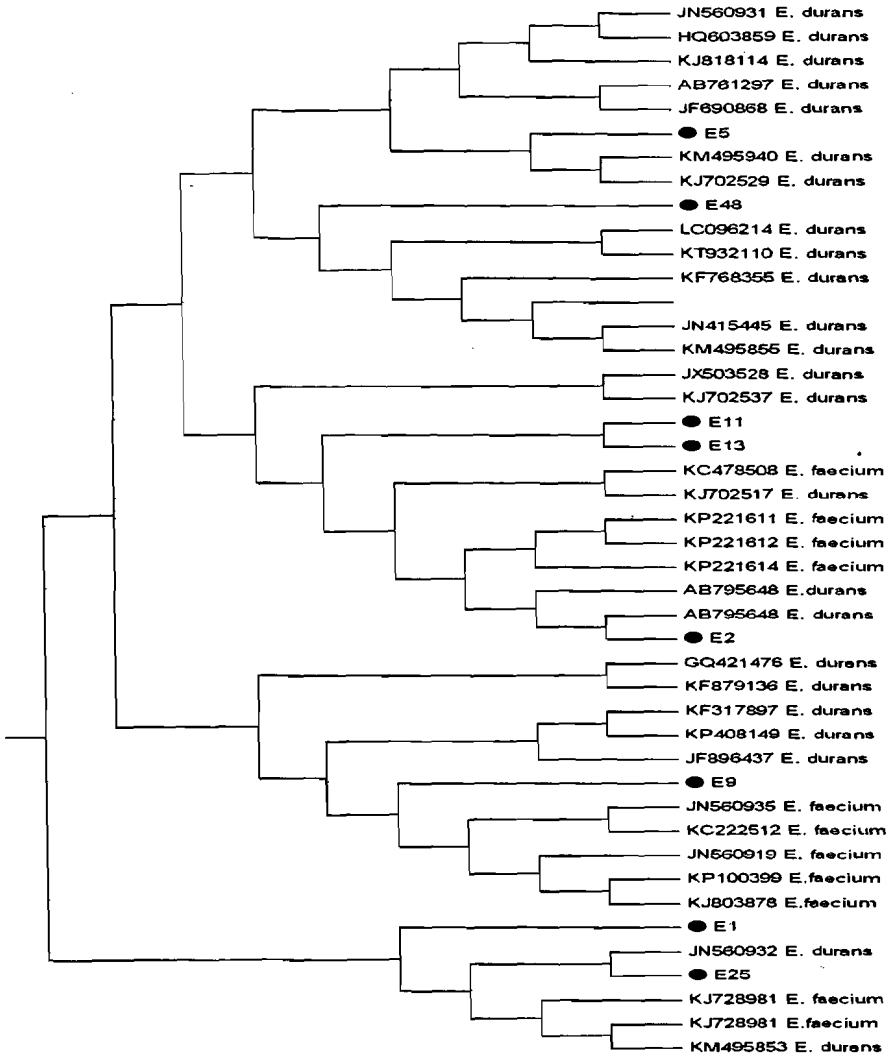

Fig. (3) Phylogenetic tree showing relationships among 16S rRNA gene sequences of *Enterococcus* strains and species representing different linages within the genus *Enterococcus.*

3.5. Plasmid DNA isolation

The plasmid contents and profiles of *E. faecium* and *E. durans* strains are shown in Figure (4). All tested *E. faecium and E. durans* strains carried a certain number of plasmids with different molecular sizes. The plasmid analyses showed that *Enterococcus* strains harbored 2–8 plasmids with different molecular sizes (Figure 4). E13 and E25 strains were observed with maximum number of plasmids and E1 strain had only two plasmids. Similar observations were reported by Coleri *et al.* (2004), and they determined that clinical *Enterococcus* isolates contained 1–11 plasmids, in molecular sizes ranged from 2.08 to 56.15 kb. Basci ,(2005) indicated that bacteriocin producer *E. faecalis*,

E. faecium and *E. durans* strains isolated from traditional white cheese samples from different regions in Turkey harboured 1 –10 plasmids with molecular sizes ranged between 2.1 and 61.1 kb. Ozmen Togay *et al.* (2010) showed that *E. faecalis* and *E. faecium* strains isolated from naturally fermented foods had 1–6 plasmids with different molecular sizes. Plasmid analysis is one of several useful methods for differentiating of isolates (Celebi *et al.*, 2007). On the other hand, plasmids are the most unstable genetic elements, which are influenced by environmental changes because of their possible gain or loss by horizontal or vertical transfer (Ustun *et al.*, 2009). For this reason, plasmid analysis should be used in combination with other molecular methods for differentiating of isolates.

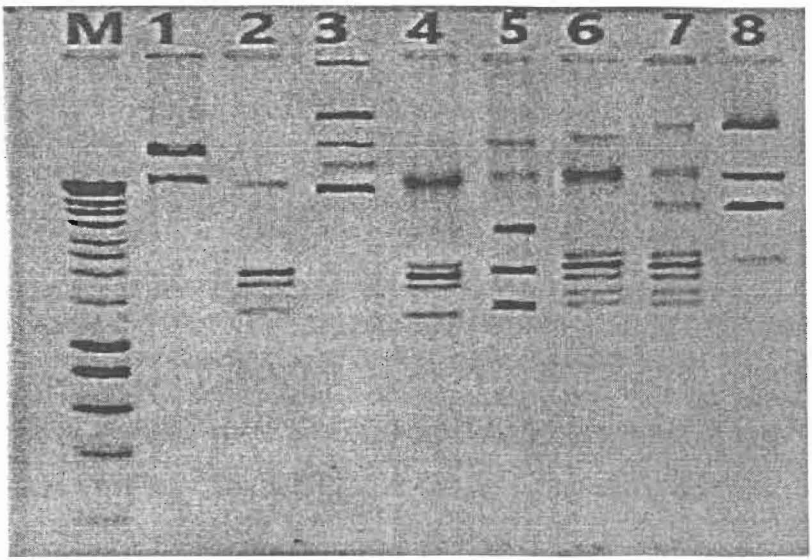

Fig. (4) Plasmid profiles of *Enterococcus* strains. laine M, molecular weight marker fragments were 250, 500,750,1000,1500, 2000, 2500, 3000, 4000, 5000, 6000,7000, 8000 and10000 bp ; Lane 1, *E. faecium* E1; lane 2, *E. faecium* E9; lane 3, *E. durans* E5; lane 4, *E. durans* E48; lane 5, *E. durans* E2; lane 6, *E. durans* E25; lane 7, *E. faecium* E13; lane 8, *E. faecium* E11.

CONCLUSION

The effectiveness of the antimicrobial activity of *Enterococcus* is strictly related to the species and strain of the target microorganism. Lactic acid bacterium shows interesting characteristics for use as an additional safety provision within the context of hurdle technologies to interact with other barriers for the control of foodborne Gram-positive pathogens in dairy products. They may play an important role in the food industry as starter cultures to improve food quality and safety. The combination of biochemical test and molecular characterization are necessary for identification of *Enterococcus* spp.

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التوصيف البيوكيميائي والجزيئي لبكتريا الانتيروكوكس والمعزولة من عينات ألبان متخمرة بالطرق التقليدية مع تقدير نشاطها المضاد لنمو بعض الانواع البكتيرية الممرضة

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الملخص العربي:

تضمنت هذه الدراسة عزل ٢٠ عزلة من بكتيريا حمض اللاكتيك من عينات ألبان متخمرة بالطرق التقليدية وتم تعريف العزلات البكتيرية بالاختبارات المورفولوجيه ووجد أن جميعها يتبع بكتيريا حامض اللاكتيك. وبناء على نتائج التعريف البيوكيميائي باستخدام API Kit تم تحديد ثمانية عزلات تنتمي الى جنس الانتيروكوكس (٣ عزلات انتيروكوكس فاسيوم، ٣ عزلات انتيروكوكس ديورانس، عزلة واحدة انتيروكوكس اسني وعزلة انتيروكوكس أوفيم) وتم تأكيد تلك النتائج باستخدام تفاعل البلمرة المتسلسل وباديء متخصص لجنس الانتيروكوس ونتج عن تفاعل البلمرة حزمة واحدة ذات وزن جزيئي ٤٥٠ زوج من القواعد لجميع العزلات. تم التصنيف الكامل للعزلات الثمانية وذلك بإستخدام تقنية الـ 16S rRNA حيث وجد أن العزلات الثمانية تقع تحت نوعين فقط هما (انتيروكوكس فاسيوم انتيروكوكس ديورانس). ولوحظ أن العزلتين (E1 و E2) والتي سبق تعريفهم باختبار API kit على أنهم (انتيروكوكس اسني وانتيروكوكس أوفيم) لا ينطبقان مع نتائج تعريف الـ 16S rRNA والذي بين انهم ينتميان الى الانواع انتيروكوكس فاسيوم و انتيروكوكس ديورانس بدرجة تشابه ٩٩%. تم عزل البلازميدات وأظهرت صورة الجيل إختلاف العزلات فى محتواها العددي من البلازميدات ما بين ٢ الى ٨ بلازميدات. كذلك تم دراسة النشاط الميكروبي للعزلات كمضادات لنمو ثلاثة أنواع بكتيرية ممرضة هي باستيوريلا مالتوسيدا، استافيلوكوكس أوريس وسيدوموناس فلورسنس ووجد أن لها نشاط ملحوظ يختلف حسب العزلة و نوع الميكروب الممرض.

ونستخلص من هذه الدراسة ضرورة تعريف العزلات البكتيرية وخاصة المستخدمه فى صناعة الالبان كباديء بالطرق الجزيئية بجانب الطرق المورفولوجيه والبيوكيميائية ، حيث أن الاعتمادعلى التعريف المورفولوجي والبيوكيميائي فقط قد يعطي نتائج غير دقيقة فى بعض الاحيان مما قد ينتج عن ذلك استخدام عزلات بكتيريه كبادي فى عمليات تصنيع الاغذيه قد تكون ضاره بصحة الانسان ومؤثرة على جودة المنتجات الغذائية.

الكلمات الدالة: بكتيريا الانتيروكوكس، النشاط الميكروبي، التوصيف البيوكيميائي، التوصيف الجزيئي، تفاعل البلمرة المتسلسل، البلازميدات، البكتيريا الممرضة.