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ISOLATION AND MOLECULAR IDENTIFICATION OF THE LACTIC ACID BACTERIA FROM RAW CAMEL MILK

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ABSTRACT

Morphological and biochemical characterisation of lactic acid bacteria (LAB) were conducted to identify 2 bacterial strains from camel milk. Two isolates of lactic acid bacteria were identified by PCR amplification and sequencing of a fragment of 16S rRNA. The sequences obtained from both isolates were compared to those of reference strains held in Gene Bank. A similarity of 95% was considered to provide identification of these isolates to be related to the genus *Enterococcus*. A confirmation of identification was obtained when a relationship between these isolates and other *Enterococcus* taxa was constructed. Neighbour-joining method provided somewhat accurate identification of the 2 *Enterococci* isolates obtained from camel milk.

Key words: Arabian , 16S rRNA, *Enterococcus*, lactic acid bacteria, PCR

The World Health Organisation has defined probiotics as live microorganisms that when administered in adequate amounts confer a health benefit to the host (Corsetti and Valmorri, 2011). *Lactobacillus*, *Bifidobacterium* (Yateem *et al,* 2008) and *Enterococcus* (Ogier and Serror, 2008) strains are the most commonly studied probiotic bacteria.

Lactobacilli are gram-positive organisms that belong to the general category of lactic acid bacteria are found in a variety of habitats, especially in milk and dairy products (Dana *et al,* 2010). Their habitats include the gastrointestinal tracts of animals and are used in the manufacture of fermented foods (Kandler and Weiss, 1986). Some *Lactobacillus* strains are extensively used in the food and pharmaceutical industries due to their healthful properties (Isolauri *et al,* 1991; Walter *et al,* 2001; Kwon *et al,* 2004). The phenotypic identification of *Lactobacillus* isolates requires determination of bacterial properties beyond the common fermentation tests like cell wall analysis and electrophoretic mobility of lactate dehydrogenase (Kandler and Weiss, 1986). Sequences of 16S ribosomal DNA (rDNA) for *Lactobacillus* provide an accurate basis for phylogenetic analysis and identification (Amann *et al,* 1995; Gurtler and Stanisich, 1996; Vandamme *et al,* 1996). The sequence obtained from an isolate can be compared to those of *Lactobacillus* species held in data banks. Although the whole gene sequence is more accurate for its identification (Stackebrandt and Goebel, 1994) which means that about 1.5 kb of DNA would have to be sequenced, the partial sequence for the first half of this gene is also important in its identification.

This study was undertaken to isolate and identify the lactic acid bacteria from raw camel's milk obtained from Taif governorate in Saudi Arabia. The identification tests were applied using the phenotypic and genotypic methods. Our goal is the selection of potential probiotic strains from camel's milk.

Materials and Methods

Isolation of lactic acid bacteria

Raw unpasteurised milk samples of camel were collected from the local area of Taif during lactation period under aseptic conditions in a sterile screw cap tubes, processed within three hours and used for further studies.

One ml from each sample was inoculated in 10 ml sterilised reconstituted skim milk (RSM) (12.5% w/v). These samples were incubated at 30°C (mesophilic LAB), 42°C (thermophilic LAB), and 37°C (both LAB) for 24 h to facilitate their enrichment.

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One ml of the milk culture was transferred into 10 ml broth culture media; Ml7 for incubation at 30°C, 37°C and 42°C (Terzaghi and Sandine, 1975), MRS for incubation at 30°C, 37°C and 42°C (De Man *et al,* 1960), then incubation for another 24 h.

The inoculated broth cultures were streaked in petri dishes contained different media included Ml7 agar at 30°C, 37°C and 42°C, MRS agar at 30°C, 37°C and 42°C, incubated for 24- 48 h (Biolife, Italy) to facilitate isolation. Representative colonies were randomly picked according to their shape and color in proper isolation broth media and then incubated for 24h.

Purified isolates were streaked into fresh agar plates, followed by microscopic examination. The strains displaying the general characteristics of lactic acid bacteria were chosen from each plate for further studies. The purified strains were stored in duplicates at (-20°C) in sterile reconstituted skimmed milk (12.5% w/v) supplemented with 15% glycerol.

Identification and characterisation of isolates

Sixteen strains isolated from camel milk were purified and phenotypically characterised for further examination. From these strains, 5 isolates were examined and only 2 were identified as lactic acid bacteria and these 2 strains were used for their molecular characterisation.

Phenotypic characterisation

Morphological characterisation and Physiological examination

The morphological shapes of the isolates were then determined by using oil lens of microscope. Cells were Gram stained according to Harigon and McCane (1976). Isolates which have homogenous cell morphology were classified into cocci or rod shaped. Gram-negative and samples showed heterogeneous cell morphology were eliminated.

1- Catalase test: LAB is known to be catalase-negative. To detect catalase activity; a drop of broth culture containing tested isolate, was transferred onto a clean dry slide, flooded with a drop of 3% hydrogen peroxide and observed for the production of effervescence that indicate catalase-positivity. Gram-positive and catalase-negative purified cultures were selected; 5 strains of camel milk and stored in duplicates in eppendorf tubes as frozen stock cultures at -20°C in (12.5% w/v) reconstituted milk powder containing 15% glycerol.

2- Production of carbon dioxide: The production of carbon dioxide was carried out as follows, 1.0 mL purified strain was inoculated in 2 mL MRS agar (for rods) and Ml7 agar (for cocci) at 40 - 45°C after that 2 mL water agar (1.5% agar) was added as an agar plug, incubated at 37°C and observed for gas production after 24-48 h. A control was carried out using yeast strain *Saccharomyces lactis* N.C.Y.C. 571 known for the production of carbon dioxide.

3- Growth at 45°C: The ability of strains to grow at 45°C was carried out; each tested strain was inoculated (1%) in broth medium MRS (for rods) and M17 (for cocci) and incubated at 45°C for 48 h. The growth was observed in the media and un-inoculated tube was incubated under the same conditions as control.

4- Growth at 10°C: The ability of strains to grow at 10°C was carried out; each tested strain was inoculated in broth medium MRS (for rods) and Ml7 (for cocci) at 1% and incubated in collar incubator at 10°C for 10 day. The growth was checked in comparison with un-inoculated tube which was incubated under the same conditions as control.

5- Growth in presence of 6.5% salt: This test was limited to *lactococci or enterococci*; salt tolerance was assessed after 3 days of incubation at proper temperature at concentration of 65 gL 1NaCl in Ml 7 broth.

6- Growth in pH 9.6: This test was limited to *lactococci* only; to distinguish between *Enterococcus* and *Lactococcus*, where *Enterococcus* only can be assessed after 48h of incubation in Ml7 broth (pH 9.6) at proper temperature.

Biochemical characterisation (carbohydrate fermentation)

Five strains from camel milk were selected, pre-identified and they were completely identified to species level on the basis of their sugar fermentation as described by MacFaddin (1976).

The fermentation of carbohydrates was determined in phenol red broth 0.04 g/µ1 (Difco, USA) as the pH indicator, and supplemented with 10 g/µ 1 (w/v) of the following carbohydrates: D-glucose ; lactose; saccharose; maltose; galactose. Each tube was topped up with 2 drops of sterile liquid paraffin after incubation (Samelis *et al,* 1994). Tests for phenotypic characterisation were conducted twice for each strain and incubated at proper temperature. When the sugar fermentation was taken place, the colour changed from red to yellow and turbidity was increased. Glucose fermentation included to positive control, and samples without sugar were used as negative control.

Well-isolated colonies with typical characteristics namely pure white, small (2 - 3 mm diameter) with entire margins, were picked from each plate and transferred to MRS broth. Identification of the lactic acid bacteria was performed according to their morphological, cultural, physiological and biochemical characteristics (Kandler and Weiss, 1986; Sharpe *et al,* 1979).

DNA extraction and PCR

DNA was extracted from samples with EZ-10 spin-column kits (Bio Basic Inc., Canada) according to the manufacturer's instruction. DNA was extracted from pellet of saturated bacterial suspension culture (about 106-107). DNA was spectrophotometrically quantified at 260/280nm and 100ng DNA was used for polymerase chain reaction (PCR).

GoTaq® Green Master Mix (Promega, USA) was used in 25 µl total PCR volume for each sample (0.2mM dNTPs, 1.5mM $MgCl^2$ and 0.25U of DNA Taq-polymerase). Ten PM of each primer was used. Sequence of forward primer was 5'- AGA GTT TGA TCC TGG CTC AG -3', while that of the reverse one was 5'- CTA CGG CAA GGC GAC GCT GAC G -3' according to Versalovic *et al,* 1994. The reaction mixture was put into a 0.2 ml thin-walled PCR tube and amplification was performed in PXE 0.5 thermal cycler (Thermo Electron Corporation Co.) with the following profile: 94°C for 5min followed by 30 cycles of 94°C for 1min, 53°C for 1min and 72°C for 1min. A final strand elongation at 72°C was done for an additional 7min.

The resultant solutions were electrophoresced on a 1.5% agarose gel in TAE (40 mM Tris, 40 mM acetic acid and 1 mM ethylenediamine-tetra acetic acid) and the gels were stained with ethidium bromide. 100 bp DNA Lad-der (Biolabs) was used as a marker for the molecular weight size. The PCR products were then purified from gel with the use of Biospin gel extraction kit (BioFlux) according to the kit manual.

Sequencing reactions were performed in a MJ Re-search PTC-225 Peltier Thermal Cycler using a ABI PRISM. BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq-DNA polymerase (FS enzyme) (Applied Biosystems) following the protocols supplied by the manufacturer were used. A single-pass sequencing was performed on each template using the last mentioned PCR- primers. The fluorescent-labeled fragments were purified from the un-incorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

After reading the targeted gene, the nucleotide sequences have been treated with different software programs (DNASIS, MacClade and PAUP) that enabled to detect genetic relatedness between different strains. The sequenced genes were tested by BLAST program to check their relatedness to the published genes for other *Enterococci* found in the Genbank database. The16S rRNA fragments from closely related bacterial strains were taken with their accession numbers (AJ420801, AF028836, KC699215, AJ508749, AF326472, EF197994, X55133 and JX948102) for the necessary alignments and tree construction.

The obtained DNA sequences were aligned separately and manually using MacClade v.4. The unalignable and gap-containing sites were deleted so that 482 bp were left for the analyses. The tree analyses were done by Maximum-Parsimony (MP) and Neighbour-Joining (NJ) methods with PAUP* 4.0b10 (Swofford, 2002) by heuristic searches with the TBR branch swapping and 10 random taxon additions, respectively. The bootstrapping replicates were set to be 1000 with simple additions for the Neighbour-Joining method.

Results and Discussion

The initial isolation and identification were based on the morphological appearance and catalase test. After conducting some preliminary tests, a total of 5 isolates were picked for further identification. All the 5 isolates were found to be gram positive and catalase negative (Table 1, Fig 1), non- spore forming rods or cocci. These isolates were identified for further biochemical and physiological characterisation (Table 1). Based on their positive gram reactions, non motility, absence of catalase activity and spore formation and their rod or coccal shape, all the 5 isolates were considered LAB. They grew at 15°C, 30°C, 37°C and 45 °C but not at 10°C. Almost 60% of the 5 isolates were able to grow at 6.5% NaCl but none of them were able to grow at 15% NaCl. All isolates were unable to produce carbon dioxide. They all showed positive growth at pH 7.0 and pH 4.4 but few colonies recorded a positive growth at pH 9.6. The isolates were able to ferment sugars. The obtained isolates were cocci with spherical or ovoid morphology and appeared mostly as pairs or forming chains and therefore referred to *Enterococcus* as shown in (Figs 1 and 2). Similar studies were conducted for the camel milk and the authors were able to isolate and identify lactic acid bacterial strains (Ahmed and Kanwal, 2004; Sharma *et al,* 2013).

Two *Enterococci* bacterial strains have been identified in the camel milk based on the partial sequence of 16S rRNA gene. A total of 490 bp from this gene have been amplified and sequenced in this study. Two different media have been used for culturing the 2 strains and that these media (17M and MSR) have been described in materials and methods. The total identity in the 16S rRNA gene between the 2 strains was 95% and the total gaps in the aligned sequences were 6 (Fig 3). We searched the Blast program inside the NCBI package for identifying the present strains. The present strains could be identified as *Enterococcus* where the total identity between either of them and the taxa of this genus was 95%. We collected the same sequences for some of the taxa acquired 95% identity with our strains and used one of the taxa acquired lower than 95% identity as an outgroup. These sequences were manipulated and aligned individually and together by DNASIS and MacClade v.4 programs. The output file was analysed by PAUP program for construing the genetic relationship and the obtained tree was described (Fig 4).

Table 1. Morphological, physiological and biochemical characteristics of lactic acid bacteria isolated from camel milk.

Characteristics	Case	Isolate	
Cell morphology	strain	Cocci	Cocci
	Gram staining	+	+
Physiology	Spore formation	-	-
	Catalase activity	-	-
	Growth at 10°C	+	+
	Growth at 15°C	+	+
	Growth at 30°C	+	+
	Growth at 37°C	+	+
	Growth at 45°C	+	+
	production of CO_2	-	-
	Growth in 6.5% NaCl	+	+
	Growth at pH 7	+	+
	Growth at pH 4.5	+	+
	Growth at pH 9.6	+	+
Sugar fermentation	Galactose	+	+
	Sucrose	+	+
	D- Glucose	+	+
	Maltose	+	+
	Lactose	+	+

Unambiguous nucleotides of 499 bp and 484 bp from 16S rRNA gene were sequenced for M17 and MRS lactic acid bacterial strains. These data were deposited in NCBI GenBank database with their accession numbers (KJ489098, KJ489099). In order to estimate the base composition and frequencies for the obtained sequences, the data were aligned with their counterparts of other related bacterial taxa and the gab-containing sites were deleted so that 482 bp were left for analysis. The data showed base frequencies of A = 25.3%, C = 21.5%, G = 31.5% and T = 21.7%. Of the 482 nucleotides used for tree analysis, 431 were constant and 51 were variables. 47 of the variable sites were parsimony uninformative and 4 were informative under parsimony criterion. A single neighbour-joining tree (Fig. 4) was obtained from all data sets with reasonable statistical supports. The tree showed clustering of the present strains within *Enterococcus* bacteria with strong bootstrapping (97%). The strain

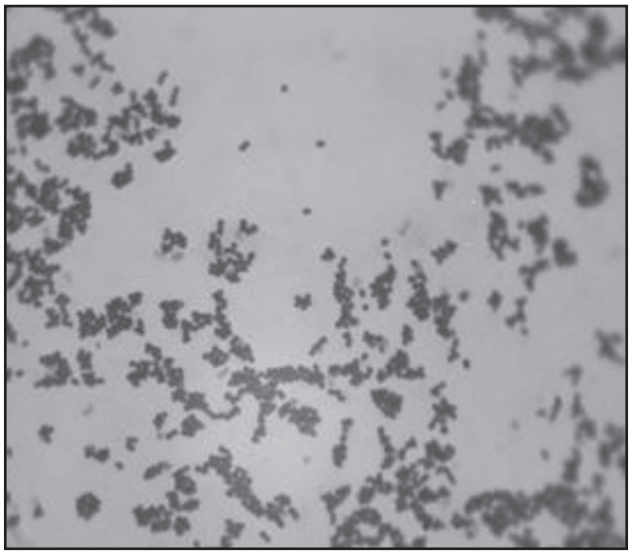

Fig 1. Growth profile for lactic acid bacteria on M17 agar plates.

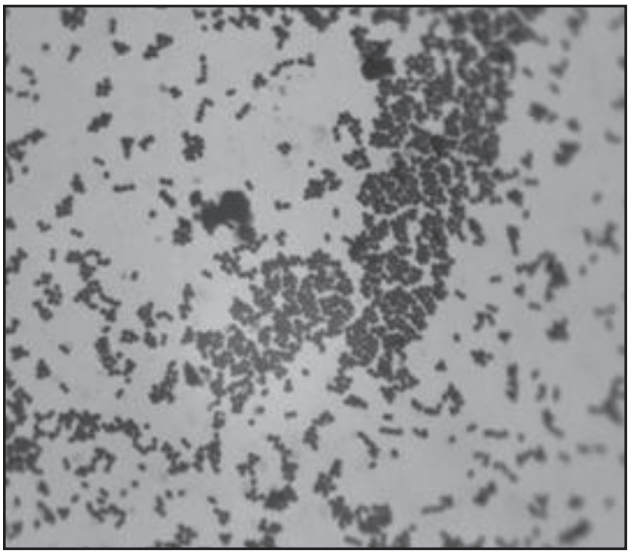

Fig 2. Growth profile for lactic acid bacteria on MRS agar plates.

Table 2. Pairwise genetic distances among the different strains including the two lactic acid strains of the present study.

	E. durans	*E. hirae*	*E. faecium*	*E. ratti*	*E. sulfureus*	*E. thailandicus*	Lact-MRS	Lact- M17
E. alcedinis	0.023	0.023	0.023	0.029	0.025	0.021	0.023	0.07
E. durans	-	0.00	0.00	0.006	0.014	0.002	0.00	0.044
E. hirae		-	0.00	0.006	0.014	0.002	0.00	0.044
E. faecium			-	0.006	0.014	0.002	0.00	0.044
E. ratti				-	0.02	0.008	0.006	0.05
E. sulfureus					-	0.012	0.014	0.06
E. thailandicus						-	0.002	0.05
Lact-MRS							-	0.044

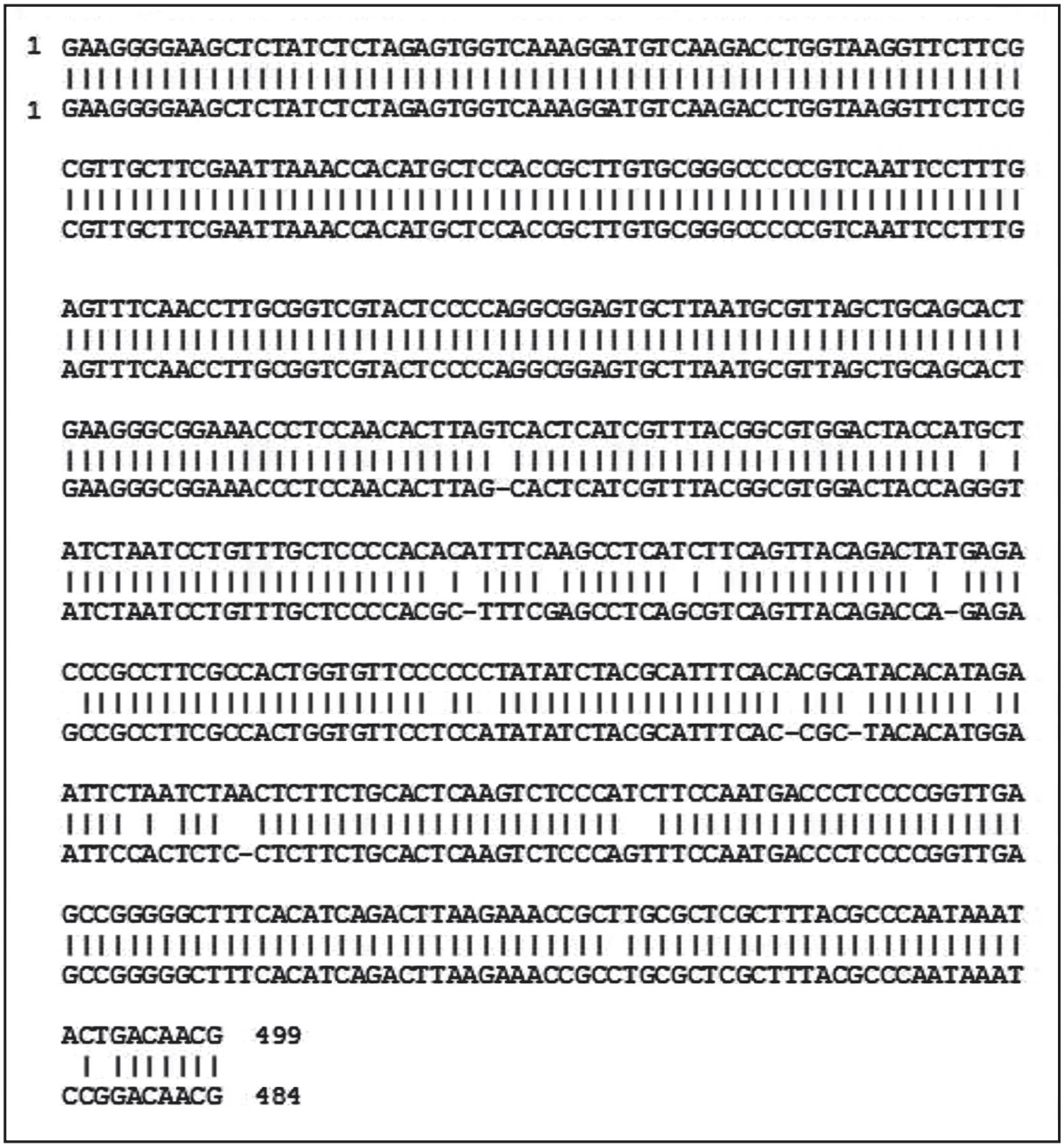

Fig 3. The aligned 16S rRNA gene fragment sequenced for the two lactic acid strains isolated in this study.

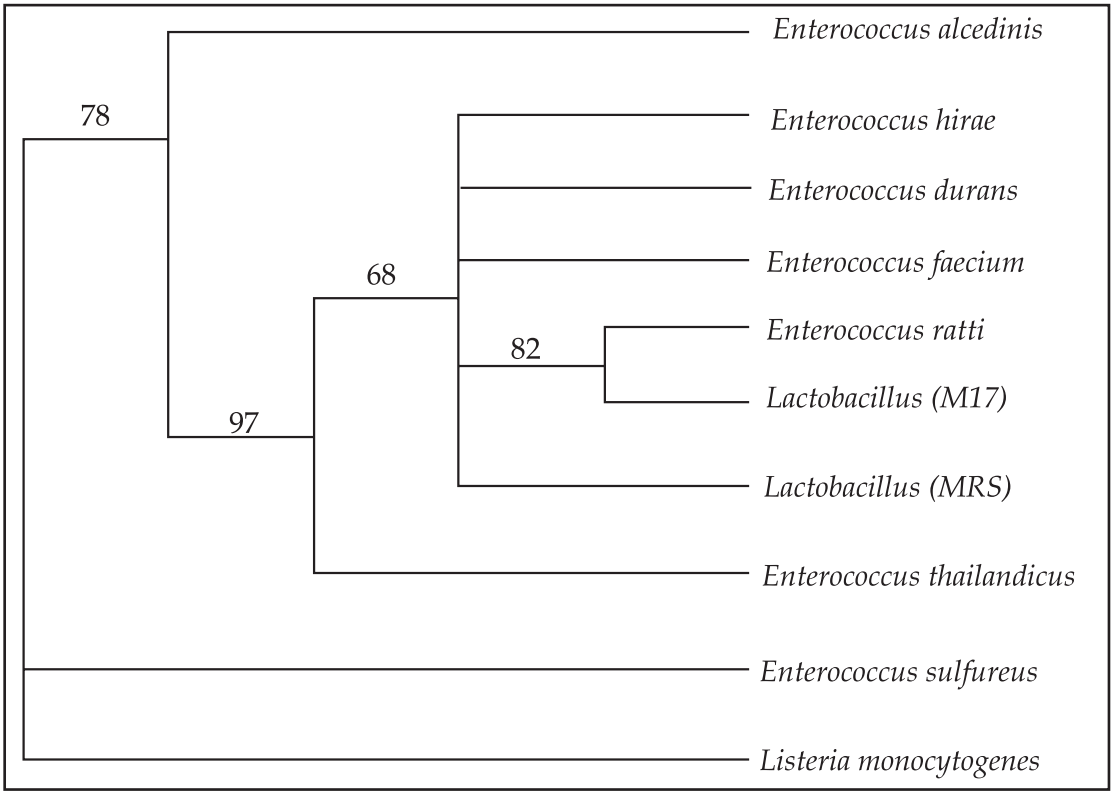

Fig 4. Neighbour-joining tree showing the genetic relationship of the two lactic acid bacterial strains isolated in this study. Values above nodes indicate the bootstrap probabilities.

which has been cultured in M17 showed sister relationship with *E. ratti* with reasonable statistical support (BP 82%). The genetic distance showed high value of 0.044 between the two bacterial strains of this study. Either of them showed smaller genetic distances with other *Enterococcus taxa* (Table 2). The tree topology supports that the identified lactic acid bacterial strains could be *Enterococcus* and one of them could be related to *E. ratti*. The total genomic DNA of needs to be subjected to sequence more data in order to identify the two strains more accurately. Several investigators used 16S rRNA gene for identification and characterisation of the lactic acid bacteria from the milk of many domestic animals (Cariolato *et al*, 2008; Nawaz *et al*, 2011; Hamed and Elattar, 2013).

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