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Full Length Research Paper

High-level acetaldehyde production by an indigenous *Lactobacillus* strain obtained from traditional dairy products of Iran

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Isolation and characterization of new *Lactobacilli* strains with high level acetaldehyde production, from traditional fermented milk, in different provinces of Iran, are reported. In this study, a total of 112 local dairy products such as yoghurt and doogh were collected from four provinces in the west of Iran, namely Kordestan, Kermanshah, Ilam and Lorestan. Ninety three isolates with extensive diverse phenotypic and biochemical characteristics were identified. These isolates were screened for productions of acetaldehyde during fermentation of skim milk. 67 isolate were positive for acetaldehyde production. Two strains (KR43 and IL121) with the highest acetaldehyde production were further characterized by amplification and sequencing of 16S rRNA genes and their phylogenetic trees were constructed. The results showed that the two strains were similar to *Lactobacillus fermentum*. 2-dimensional electrophoresis analysis was used to investigate the proteome of the cytosolic fraction extracted from stationary phase cells of the KR43 strain. Also some spots of KR43 strain were analyzed by MS/MS subunits of acetoin dehydrogenase involved in the production of acetaldehyde from acetoin, which has not been reported in *Lactobacilli* yet. To our knowledge, this is the first report in *Lactobacilli*.

Key words: Acetaldehyde production, acetoin dehydrogenase, *Lactobacillus*, Iran, proteomics, fermented foods, dairy products.

INTRODUCTION

Lactic acid bacteria (LAB) are important in the dairy industry; and are used as starter cultures for the production of fermented foods, such as yoghurt and cheese. During the fermentation process, LAB influence the sensory properties of the product including the flavour development (Ayad et al., 2002). In the dairy industry, the quality and reproducibility of fermented milks and processes are ensured by using appropriate starters. This has led some researchers to isolate and characterize artisanal strains in order to provide sensorial properties similar to those of traditional products. Therefore, new strains of lactic acid bacteria have been isolated from different dairy products, such as raw milk (Wouters et al., 2002) fermented milks (Cogan et al., 1997; Ayad et al., 1999; Abdelgadir, 2001) or several types of cheeses (Cogan et al., 1997). For the development of novel or improved dairy products, flavour characteristics are generally a primary target (Pastink et al., 2008). One of the basic parameters through which starter cultures are characterized is their ability to produce aroma compounds such as acetaldehyde (Yuguchi et al., 1989). Acetaldehyde is considered as the most prominent and typical aroma compound in yoghurt and other yoghurt-related products (Ott et al., 1999).

LAB have several metabolic pathways for acetaldehyde formation, including amino acids, nucleotide and pyruvate metabolism. Pyruvate is a key catabolic intermediate in many lactic acid bacteria and is converted into a variety of end products, such as lactic acid, acetic acid, acetaldehyde, ethanol, diacetyl, and acetoin (Hickey, 1983; Kandler, 1983). The characteristic buttery and

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nutlike flavor of dairy products is attributed to diacetyl and acetoin (Heath and Reineccius, 1986), which are formed by two metabolic pathways that both require pyruvate as a precursor (Kempler and McKay, 1981; Speckman and Collins, 1968). In homofermentative lactic acid bacteria, most of the pyruvate must be converted to lactate to regenerate NAD. Thus, little or no diacetyl and acetoin are produced unless in the presence of an additional source of pyruvate, such as citrate (Cogan et al., 1981; Harvey and Collins, 1962; Kaneko, 1990; Tsau and Montville, 1990). Harvey and Collins (1961, 1963) proposed that lactic acid bacteria produce diacetyl and acetoin to dispose the toxic amounts of pyruvate not required for biosynthesis (Harvey and Collins, 1963).

The main pathway of acetaldehyde biosynthesis in Lactic acid bacteria is through the activity of serine hydroxymethyltransferase (SHMT), possessing threonine aldolase activity, which converts threonine to acetaldehyde and glycine (Chaves et al., 2002). Over expression of this enzyme (SHMT), which is encoded by the *glyA* gene, resulted in the overproduction of acetaldehyde and disruption of the *glyA* gene inhibit the acetaldehyde production. This provides the ultimate evidence for the previously described acetaldehyde formation process by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp*. bulgaricus* through the breakdown of threonine to glycine (Chaves et al., 2002).

Both aerobic bacteria, such as *Bacillus subtilis* (Huang and Steinbuchel, 1999), *Ralstonia eutropha* H16 (Priefert et al., 1991) and *Pseudomonas putida* (Huang and Steinbuchel, 1994) as well as anaerobes, such as *Clostridium magnum* (Kruger et al., 1994) and *Pelobacter carbinolicus* DSM2380 (Oppermann and Steinbuchel, 1994) were shown to grow on acetoin and cleavage it to acetaldehyde and Acetyl CoA by the acetoin dehydrogenase complex. The formation of acetoin dehydrogenase complex is induced during growth on acetoin, which have been demonstrated in several related species, including *Pelobacter venetianus, Pelobacter acetylenicus, Pelobacter propionicus, Acetobacterium carbinolicum,* and *Clostridium magnum* (Oppermann and Steinbuchel, 1994).

To our knowledge there is no report about this pathway in *Lactobacillus* isolated in Iran. Following the previous report on the isolation and characterization of indigenous *Lactobacilli*, in traditional dairy products (Ghobadi Dana, 2010), in this research therefore the main objective is to isolate and characterise indigenous *Lactobacilli*, with high acetaldehyde production and analysis of its pathway using proteomics approach.

MATERIALS AND METHODS

Sampling

Four distinct and conserved geographical and sociological regions, in the Ilam, Lorestan, Kordestan and Kermanshah provinces of Iran were considered for this study. 112 different samples of homemade traditional yoghurt and doogh made from the milk of cows, sheep and goats were collected from the four mentioned regions. Yoghurt is a product obtained through milk fermentation with a specific yoghurt starter culture consisting of a mixture of two species of LAB, *L. delbrueckii* subsp*. bulgaricus* and *S. thermophilus*. Traditionally, doogh is referred to as a product obtained from dilution of full fat yoghurt after vigorous agitation. Doogh and yoghurt which were prepared by traditional methods and developed by nomads in their portable houses were sampled in June 2007. Each sample (3 ml) was collected in 50 ml sterile test tubes containing 15 ml MRS broth (Merck, Darmstadt, Germany) and transferred to the laboratory within 4 h under cold conditions.

Isolation of strains

Lactobacillus strains were isolated according to the procedure of Fitzsimons (Fitzsimons et al., 1999). The samples were cultivated in broth (MRS, and skim milk, Merck, Darmstadt, Germany). The inoculated MRS broth were incubated for 24 to 48 h at 37°C and skim milk for 4 h at 42°C. After this, the samples were sub-cultured (MRS and ROGOSA agar, Merck, Darmstadt, Germany) and incubated for 48-72 h at 37°C. The bacterial colonies from each sample with different morphologies were selected and examined microscopically. The Gram-positive, rod shaped bacteria were isolated and stored at -80°C in MRS broth supplemented with 15%(v/v) glycerol for further use.

Acetaldehyde production assay

The isolates were individually grown in skim milk to determine their acetaldehyde production abilities. The skim milk was sterilized at 110°C for 12 min, cooled up to 45°C and inoculated with 2% (v/v) (6×10^7 cells ml^{-1}) of the pure cultures (counts of cells were checked by preparing decimal dilution and culturing in MRS Agar and incubating for 48 h at 37°C before inoculation). They were incubated thermostatically for 20 h at 42°C until reached a pH of 4.3-4.4. The acetaldehyde UV-method Kit (R-Biopharm, Darmstadt, Germany) was utilized for quantization of acetaldehyde in the coagulated skim milk. Acetaldehyde production assay was based on the procedure provided by the manufacturer. In this procedure, acetaldehyde is oxidized to produce acetic acid and $NADH^+$ in the presence of aldehyde dehydrogenase (Al-DH) and nicotinamide-adenine dinucleotide (NAD).

The amount of NADH is stoichiometric to the amount of acetaldehyde. NADH is determined by the means of its absorption at 340 nm. Based on this method four ATCC strains (ATCC 9595 *L. rhamnosus,* ATCC 393 *L. casei,* ATCC 314 *L. acidophillus* and ATCC 7830 *L. lactis)* were also quantified. The test was repeated twice and analysis of variance (ANOVA) of the results was expressed with standard deviation (SD) for the strains producing high levels of acetaldehyde (more than 10 mg/kg).

DNA extraction, primer design and DNA amplification

DNA extraction from the isolated *Lactobacilli* was carried out according to the standard procedure (ISO-21571, 2005). Based on the published data (Kwon et al., 2005) primer pairs R16-1 (5'CTTGTACACACCGCCCGTCA) and LbLMA1-rev (5'CTCAAAACTAAACAAAGTTTC) were used for verifying the *Lactobacillus* strains at the genus level. For amplification of the desired fragment (250 bp), a 2.5 mM concentration of $MgCl_2$ was used in the polymerase chain reaction (PCR) and the following thermocycling program was applied: Initial denaturation at 95˚C for 5 min followed by 30 cycles of denaturation at 95˚C for 40 s, annealing at 53˚C for 40 s and extension at 72˚C for 30 s and at 72˚C for 5 min as final extension.

Primer pairs, 16R08F (5'AGAGTTTGATCCTGGCTCA) and 16R09R (5' TACCTTGTTACGACTTCACC) were used for amplification of the complete *16S rRNA* gene (Brosius et al., 1981) with the concentration of 2 mM $MgCl_2$. For amplification of the desired fragments, the following thermocycling program was applied; initial denaturation at 95˚C for 5 min followed by 30 cycles of denaturation at 95˚C for 40 s, annealing at 57.6˚C for 90 s and extension at 72˚C for 90 s and at 72˚C for 5 min as final extension.

In order to detect *glyA* gene in the screened strains, PCR was carried out with chromosomal DNA by using *glyA*F1 *(*5'ATGAATTATGGAGAAAAAGATCCA-3´*),* and *glyAR1 (*5'TTAATAGGATGCTTATCTGTTAAT-3´) primers were designed based on the multiple alignments of the gene sequences encoded by *glyA* genes from different *Lactobacillus* (*L. gaseri* gi/116628683_288055-289290, *L. jansoni* gi/42518084_295759-296994, *L. acidophillus* gi/159162017_250398-251646, *L. reuteri* gi/148543243_509425-510660, *L. brevis* gi/116332681_c1260036-1258795, *L. salivarius* gi/90960990_c903870-902617, *L. plantarum* gi/28376974_c2145380-2144142, *L. casei* gi/116493574_1143033-1144265, *L. saki* gi/81427616_c1122490-1121122). For amplification of the desired fragment (1200 bp), a 1.5 mM $MgCl_2$ was used in the polymerase chain reaction (PCR) and the following thermocycling program was applied: Initial denaturation at 95˚C for 5 min followed by 35 cycles of denaturation at 95˚C for 40 s, annealing at 51˚C for 70 s and extension at 72˚C for 60 s and at 72˚C for 5 min as final extension.

The primer pair of *glyAF2 (*5'CGAATAAATATGCTGAAGGTTATCC-3´*),* *glyAR2 (*5'CCGGCAATATGAGCCATATCAT3´) from conserved regions of the glyA gene was also used for amplification of 600 bp fragment. A 2.5 mM $MgCl_2$ was used in the polymerase chain reaction (PCR) and the following thermocycling program was applied: initial denaturation at 95˚C for 5 min followed by 35 cycles of denaturation at 95˚C for 40 s, annealing at 46.4˚C for 40 s and extension at 72˚C for 60 s and at 72˚C for 5 min as final extension.

Sequence analysis

The amplified DNA fragments were purified from the agarose gel (QIAGEN, Hilden, Germany), and cloned into the pTZ57R/T T/A cloning vector (Fermentas, St.Leon-Rot, Germany). The recombinant plasmid was introduced to the recipient cell (*E. coli* DH5□) by the procedure described by the manufacturer. The suspected *E. coli* DH5□ colonies on ampicillin-containing 0.2% solid medium were verified by amplification of the desired fragment with PCR using related primers. Authentic recombinant plasmids were purified by a plasmid purification kit (Fermentas, St. Leon-Rot, Germany) and sequenced by the di-deoxy chain termination method using M13 standard primers in both directions. Sequences were aligned using the CLUSTALW program (Larkin et al., 2007). The bootstrapped neighbor-joining phylogenetic tree was constructed by the Mega4 software (Tamura, 2007). The program CONSENCE was used for obtaining majority-rule consensus trees of all bootstrapped sequences and the bootstrap values were indicated as numbers on each internal branch (Bootstrap=1000, cut off=50).

Proteomics experiments

Protein extraction from the isolated lactobacilli was carried out according to the procedure described by Salasbury (1989).

SDS-PAGE analysis

Prior to gel electrophoresis, the protein concentration was determined using a Bradford protein assay, using BSA as the standard. SDS-PAGE was carried out according to the previously described procedure (Laemmli, 1970). Gel images were obtained by using a GS-800 calibrated densitometer (BioRad).

Immobilized pH gradient (IPG) and two dimentional electrophoresis (2-DE) analysis

L. fermentum KR 43 strain cytosolic proteins were first separated by IPG. Proteins (800 µg) were loaded on Immobiline Dry Strips (pH range 4–7, nonlinear, 17 cm long; BioRad) and operated at a constant temperature of 20℃. The strips, containing the samples, were first actively rehydrated at 50 V for 12 h. The IEF program was as follows: 300 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3500 V for 2 h, 5000 V for 12 h, and 8000 V for 2 h, to 70 000 Vh. Prior to running the second dimension, the strips were equilibrated for 20 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 20% v/v glycerol, 2% w/v SDS, 130 mM DTT. The strips were placed on 12.5% SDS-PAGE gels and covered with 0.5% agarose containing a trace of Bromophenol blue. The gels were electrophoresed at a constant voltage of 200 V until the marker dye had reached the base of the gel. The gels were stained with BioSafe colloidal Coommassie blue (BioRad) for 1 h and distained with three successive washes in deionized water. The images were obtained with a GS-800 calibrated densitometer (BioRad).

In-gel trypsin digestion of proteins

Spots were manually cut out from 2-DE gel, placed in 96 wells v-shape polypropylene plates and dried completely. The Ettan Spot Handling Workstation (GE Healthcare, UK) was used for automatic in gel digestion of samples. Each gel plug was soaked in 100 µL of washing solution (50% MeOH, 50 mM NH_4HCO_3) to re-swell and then washed two more times in the same solution. The gel plugs were further washed twice in 75% ACN, before being completely dried. Samples were then re-hydrated by the addition of freshly prepared trypsin solution (0.5 µg modified porcine trypsin in 25 µL 20 mM NH_4HCO_3), and incubated for 240 min at 37°C. Peptides were extracted from the gel plugs, by washing twice in 100 µl of 50% ACN, 0.1% TFA and transferred the solution to a fresh 96 well plate, where samples were dried. Tryptic peptides were resuspended in 3 µl of 50% ACN, 0.1%TFA. An aliquot of 0.3 µL of the resuspended tryptic peptides were spotted onto a steel Applied Biosystems 192 sample MALDI target plate, and mixed (while wet) with 0.3 µL of a 90% saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% TFA. The dried samples were analyzed using a MALDI-TOF/TOF MS (4700 Proteomics Analyzers, Applied Biosystems, UK), performing MS analysis and subsequent MS/MS analysis on up to 10 precursor peptides. Each sample was internally calibrated by reference to specific autolytic fragments of trypsin. The PMF and MS/MS information were automatically searched against the NCBI non-redundant database using the Mascot search engine (Matrix Science, UK). Mass tolerance settings of 1.2 Da for parent ion and 0.5 Da for fragment ions were applied. Search settings allowed one missed cleavage with trypsin and two modifications (carboxamidomethylation of cysteine and oxidation of methionine). Statistical confidence limits of 95% were applied for protein identification.

RESULTS AND DISCUSSION

Bacterial screening and identification

Ninety three *Lactobacillus* isolates (33 isolates from Ilam,

Table 1. Acetaldehyde production by *Lactobacillus* strains.

Strains	Acetaldehyde mg/kg	Strains	Acetaldehyde mg/kg	Strains	Acetaldehyde mg/kg	Strains	Acetaldehyde mg/kg
ATCC9595	2.4±0.5	Ko50	Nd	Lo94	7.7±0.5	IL129	8±0.5
ATCC393	3.3±0.5	Ko51	Nd	Lo95	Nd	IL131	9.8±0.5
ATCC7830	2.8±0.5	Ko54	7±1	Lo96	2.3±0.5	IL132	Nd
ATCC314	4.2±0.7	Ko55	Nd	Lo97	2±1	IL133	Nd
Kr5	nd	Ko56	7±1	Lo98	2.7±0.5	IL134	7±1
Kr7	nd	Ko57	6.3±1	IL99	5.2±0.5	IL136	2±1
Kr8	6.5±2	Ko59	6±0.5	IL100	Nd	IL137	4.7±0.5
Kr14	12±2	Ko60	8±0.5	IL102	Nd		
Kr15	6±2	Ko63	Nd	IL103	Nd		
Kr16	4±0.5	KO67	13±0.5	IL104	6.5±0.5		
Kr19	6.2±1	KO68	9.5±0.5	IL105	7.2±0.5		
Kr22	3.6±0.7	Ko69	6.3±1	IL108	4±0.5		
Kr24	10±1	Ko70	6±0.5	IL109	10.4±0.5		
Kr25	6.3±1	Ko72	8±0.5	IL110	6.4±0.5		
Kr27	2.7±2	Ko73	Nd	IL111	8.7±0.5		
Kr29	2±1	Lo74	Nd	IL112	17.1±1		
Kr30	7.9±0.5	Lo75	13±0.5	IL113	16±0.5		
Kr31	3±1	Lo77	5±0.5	IL115	12±1		
Kr33	18.6±1	Lo78	Nd	IL119	9.8±0.5		
Kr35	4±0.5	Lo80	12±0.5	IL120	13±0.5		
Kr36	8±1	Lo81	6.3±0.5	IL121	20±0.5		
Kr37	Nd	Lo82	8±1	IL122	4±0.5		
Kr38	10±1	Lo83	6±1	IL123	5.6±0.5		
Ko40	12±1	Lo84	8.4±0.5	IL124	4±1		
Ko41	Nd	Lo86	6.2±0.5	IL125	6±0.5		
Ko43	20±1	Lo87	14±1	IL126	8±0.5		
Ko48	Nd	Lo90	15±1	IL127	3±1		
Ko49	Nd	Lo92	Nd	IL128	5±1		

20 isolates from Lorestan, 23 isolates from Kermanshah and 17 isolates from Kordestan) were obtained from sample cultures. All of the isolates belong to the *Lactobacillus* species, which meant that they were non-spore forming, non-motile, Gram-positive and rod-shaped, demonstrating negative catalase reaction, negative indole production, negative nitrate reduction and negative gelatin liquefaction. All of these ninety three *Lactobacillus* strains were also able to acidify, coagulate and reduce litmus milk. The biochemical characterization results of 49 strains were reported previously (Ghobadi, 2010).

Acetaldehyde production

Out of 93 strains with ability of acetaldehyde production, two strains were able to produce higher yields (Table 1).

Different bacteria use different pathways for acetaldehyde production, for instance *Lactobacillus lactis* produces acetaldehyde during the metabolism of lactose by pyruvate decarboxylation (Bongers et al., 2005) while *S. thermophilus* convert threonine into acetaldehyde and glycine by the activity of threonine aldolase (Chaves et al., 2002), whereas, the genome of *L. plantarum* has no homolog of threonine aldolase, it consumes threonine at significantly higher rates than required for biomass production, indicating the possible degradation of threonine into acetaldehyde (Teusink et al., 2006). The relatively high concentration of acetaldehyde found in yoghurt must be due to the lack of the main enzyme dehydrogenase for conversion of acetaldehyde into ethanol (Marshall, 1984). Analysis of variance (ANOVA) of acetaldehyde production showed significant differences between isolates. While some strains produced no detectable amounts of acetaldehyde, other strains produced a considerable amount (20 ± 1.7 mg/kg). Acetaldehyde production by the ATCC strains as a single strain cultures were very low. It has been reported that there is a large variation in flavour-forming

abilities among food fermenting LAB (Pastink et al., 2008). Flavour production is strain dependent and therefore, the composition of a starter culture can greatly influence flavour characteristics of the final product (Kieronczyk et al., 2003). This diversity does not only occur at the species level but also within the species, with a large strain-to-strain diversity (Wouters et al., 2002).

Variation in levels of flavour compounds has been reported by using different strains of LAB (Bottazzi et al., 1973). However, some authors, have not found acetaldehyde in yoghurt cultures (Imhof et al., 1995). It was also reported that *L. bulgaricus* produces more acetaldehyde than *S. thermophilus* (Beshkova et al., 1998; Chammas et al., 2006). There are reports of a comparatively lower level of acetaldehyde (0.18 to 9.7 mg/kg) (Yuksekdag et al., 2004). To our knowledge, the highest reported acetaldehyde production is 18.3 mg/ kg in kefir with *L. delbrueckii subsp. Bulgaricus* (Beshkova et al., 2003), while strains from this study produce 20±1.7 mg/kg acetaldehyde. The data obtained from this study (20 ± 1.7 mg/kg) is similar to those of Kneifel et al. for yoghurt-type products (Kneifel et al., 1992).

Molecular analysis and phylogenetic tree

Extraction of DNA from wild type strains were implemented and fourteen selected strains with higher level of acetaldehyde production were genotypically characterized by using genus specific primers which amplified a 250 bp fragment from the conserved domain of the *Lactobacilli* ribosomal DNA. Amplification of the suspected fragment from 14 selected strains with genus specific primers, confirmed the strains as *lactobacilli.* Two strains which produced 20 mg/Kg of acetaldehyde as single strain cultures were selected for sequencing and analysis of the complete *16S rRNA*. These two strains were from Kermanshah and Ilam. As a result, both strains were identified as *Lactobacillus fermentum* (99% identity) and the *16S rRNA gene* sequences were deposited in Gene Bank, under the accession numbers of FJ645922 and FJ645925. The strains were registered in the Persian Type Culture Collection (PTCC*1751* and *PTCC1753)*.

The phylogenic relationship for these two strains (KR43 and IL121) and in comparison with the genomes of other available *Lactobacillus* is presented in Figure 1. These strains which were from Kermanshah (KR43) and Ilam (IL121), were similar to each other and have a high similarity with other *L. fermentum* which were from Europe. *L. fermentum* is a closely related species based on a sequence analysis of the 16S rRNA gene (99% identity) and on phenotypic properties, including being an obligate heterofermentative organism. *L. fermentum* show considerable similarity to *Lactobacillus reuteri*. Previously *L. fermentum* and *Lactobacillus reuteri* were classified as a single species, *L. fermentum* but were subsequently separated based primarily on DNA hybridization and GC content (Kelin, 1998).

Acetaldehyde biosynthesis pathway analysis

In order to specify the acetaldehyde biosynthesis pathway, the presence of *glyA* gene in the KR43 and IL121 strains were investigated, using primers for amplification of *glyA* by PCR. Although, different PCR conditions were applied, we were not able to amplify the *glyA* gene in the strains. The 600bp DNA fragment was amplified by nested PCR, but its sequence revealed different gene instead of desired gene (*glyA*). Here after, proteome of the KR43 strain was analyzed for probable enzymes involved in the biosynthesis of acetaldehyde that is presented in Table 2.

Proteome analysis did not show serine hydroxymethyl transferase (SHMT) in the proteome profile. These results indicated that, in *L. fermentum KR43* strain SHMT is not the main enzyme for acetaldehyde formation and presence of a putative alternative pathway for acetaldehyde production could be considered. On the other hand, acetoin dehydrogenase enzyme (EC:1.1.1.5) with high score was identified in the proteome. It seemed that the pathway for production of acetaldehyde in this strain is different from hypothesized pathway.

Significantly the formation of the enzyme acetoin dehydrogenase had the same subunits in both *Pelobacter carbinolicus* and KR43. These subunits included acetoin dehydrogenase (E1 component, consist of acetoin dehydrogenase α and acetoin dehydrogenase β subunits), dihydrolipoamide acetyltransferase (E2) and dihydrolipoyl dehydrogenase (E3) that were identified with a high range score in protome of KR43.

It seems that the main structure of the enzyme acetoin dehydrogenase might have the same formula in each species; but in different genus, repetition of subunits may vary.

This protein which is induced by the presence of acetoin, has been purified from acetoin –grown cells, and catalyzed *in vitro* thiamine diphosphate –dependent oxidative-hydrolytic cleavage of acetoin, methylacetoin, and diacetyl in the presence of NAD (Oppermann, 1991). Purified acetoin dehydrogenase could catalyze the reaction coupled to reduction of 2,6-dichlorophenolidophenol (DIPCP).

The compound 2,6-dichlorophenolidophenol was also found in the proteome of KR43 which confirms the presence of enzyme acetoin dehydrogenase in KR43. This shows that, this bacterium has the capability to produce acetaldehyde through acetoin degradation's pathway. In addition, dihydrolipoyl lysine-residue acetyltransferase component of pyruvate dehydrogenase complex was found in the proteome of KR43.

It is proposed that the yoghurt starters do not have any alcohol dehydrogenase for the conversion of acetaldehyde to ethanol, resulted in the presence of high

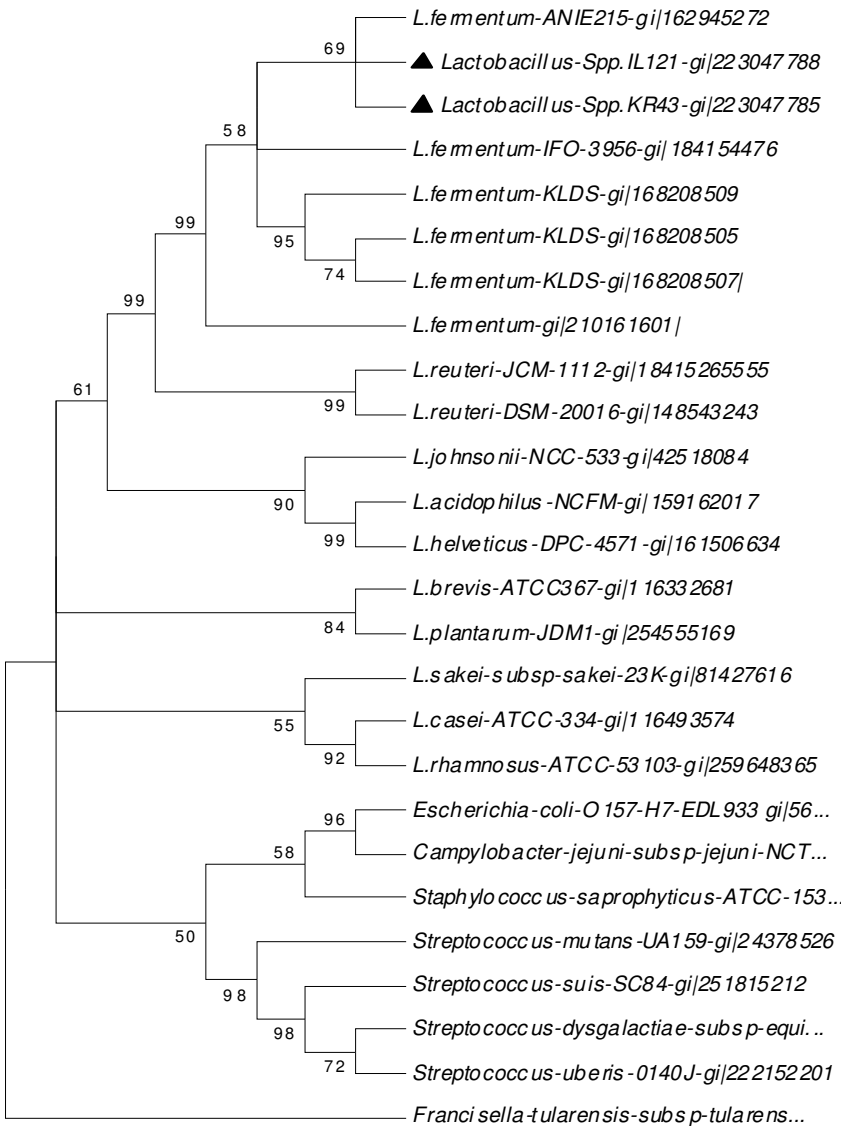

Figure 1. The phylogenetic relationship between two strains from this study (KR43, IL121) and other strains present in the NCBI database.

amount of acetaldehyde in yoghurt (Marshall, 1984). But surprisingly; alcohol dehydrogenase was detected within the proteome of KR43, and acetaldehyde still remained in a high amount, in spite of the production of ethanol.

Screening and using high acetaldehyde producing strains as part of the starter culture, for yoghurt, could result in the production of high acetaldehyde content dairy products.

Conclusions

Traditional dairy products from various provinces of Iran are valuable sources for isolation of lactobacilli with important biochemical properties. The two *Lactobacillus* strains with high acetaldehyde production ability, through new pathway were identified by biochemical and preliminary molecular analysis, and were found to be similar to *Lactobacillus fermentum*. From the result of this study the possibility of using some indigenous strains as the starters for the development of fermented milks with characteristics similar to traditional products, at an industrial scale, is very promising.

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Table 2. Some probable enzymes involved in the biosynthesis of acetaldehyde was analyzed in proteome of the KR43 strain.

Spot code	Protein name	Protein MW	Protein PI	Protein score
90	Acetoin dehydrogenase, E1 component, alpha subunit	35024.8	4.79	122
91	Acetoin:2,6-dichlorophenolindophenol oxidoreductase subunit beta	36987	4.66	313
197	Acetoin:2,6-dichlorophenolindophenol oxidoreductase subunit beta	36987	4.66	130
171	Dihydrolipoyl dehydrogenase	49886.8	4.84	240
50	Acetoin dehydrogenase	12568.5	4.94	114
158	Branched-chain alpha-keto acid dehydrogenase subunit	46336.3	4.57	396
142	Branched-chain alpha-keto acid dehydrogenase subunit E2	46642.6	4.8	189
196	Branched-chain alpha-keto acid dehydrogenase subunit E2	46336.3	4.57	105
175	Dihydrolipoamide dehydrogenase	49612.6	4.8	263
172	Dihydrolipoyl dehydrogenase	49886.8	4.84	345
143	Dihydrolipoyl lysine-residue acetyltransferase component of pyrovate dehydrogenase complex	46974.9	4.88	215
171	PYROVATE /2oxoglutarate dehydrogenase complex dihydrolipoamide dehydrogenase (E3) component	50080	5.07	81
183	Pyruvate dehydrogenase E1 component alpha subunit	41489	4.98	258
90	Pyruvate dehydrogenase E1 component subunit beta	35245.2	4.6	244
228	Pyruvate dehydrogenase E1 component,alpha subunit	41489	4.98	177

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