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Full Length Research Paper

High folate production by naturally occurring *Lactobacillus* sp. with probiotics potential isolated from dairy products in Ilam and Lorestan provinces of Iran

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Rapidly proliferating cells require large amounts of folate to support efficient DNA replication, repair and methylation indicating the importance of folate in human metabolism. Milk products are good sources of such vitamins which are produced by probiotics. In order to find suitable strains capable of high folate production, isolation and identification of *Lactobacilli* in traditional fermented milk from two different provinces located in the west of Iran were carried out. *Lactobacillus* bacteria were isolated according to the ISO 7889 standard procedure. The isolated bacteria were characterized phenotypically and were screened for their ability to produce folate during fermentation of skim milk. Folate production by the selected strains was between 2.8 to 66.6 µg/l. Two strains with the highest folate production were then selected. The 16SrRNA genes from these two strains were amplified and sequenced and a phylogenetic tree constructed. The sequencing results in combination with phenotypic and biochemical properties showed that both strains were similar to *Lactobacillus crustorum*. Therefore, two new strains with an ability of high folate production were isolated and identified. These could be used as probiotics in the dairy industry. Hence, exploiting natural food-grade microorganisms for the production of nutritive dairy products is possible.

Key words: Folate, *Lactobacillus*, probiotic, traditional dairy products, Iran.

INTRODUCTION

Gram-positive *Lactobacilli* belong to the general category of lactic acid bacteria that are found in a variety of habitats, especially in milk and dairy products. Some *Lactobacillus* strains are extensively used in the food and pharmaceutical industries due to their healthful properties (Isolauri et al., 1991; Kwon et al., 2004; Walter et al., 2001; Walter et al., 2000).

Numerous investigations regarding traditional dairy products have shown that they have a unique microflora which depends on the process conditions as well as on the ecological localities where they have been produced (Dewan and Tamang 2007; Mathara et al., 2008, 2004; Zamfir et al., 2006). Historically, Iranian people have been producing various kinds of fermented dairy products such as yoghurt and doogh. Traditionally, doogh is referred to as a product obtained from dilution of full fat yoghurt after vigorous agitation in special leather bags. It is now known as the 'Iranian National Drink'. Doogh, and various kinds of traditional yoghurt, made from cow, yak, goat or camel milk, have been an important part of the Iranian diet since ancient times. Analysis of traditional fermented dairy products for identification of microorganisms found naturally in these products is important for finding new strains with novel properties which may be used as a component of a starter or as a probiotic. Probiotics are commonly defined as viable microorganisms (bacteria or yeasts) that exhibit a beneficial effect on the health of the host (FAO/WHO, 2002).

Folate is produced by various kinds of microorganisms

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and green plants. Dairy products are the main sources of folate for humans. Among these, the fermented milk products, especially yoghurt, can contain even larger amounts of folate. Folate is an essential component of the human diet. It is involved, as a cofactor, in many metabolic reactions, including biosynthesis of the building blocks of DNA and RNA. The daily recommended intake for an adult varies from 200 µg in Europe to 400 µg in the United States. Since folate is known to prevent neural tube defects in newborns, a double dose is recommended for pregnant women (Van Der Put et al., 2001; Wald et al., 1991). There are a few published reports, which indicate that high-folate diets protect against cardiovascular disease (Boushey et al., 1995) and even some forms of cancer (Ames, 2001). In addition, several other reports have indicated that folate deficiency is common in various population groups, including women of childbearing age (Kiely et al., 2001; Konings et al., 2001).

It has been reported that lactic acid bacteria, such as *Streptococcus* sp., as an important feature, can produce folate during milk fermentation, thus acting as a probiotic (Lin and Young, 2000). However during the milk fermentation process, majority of bacteria such as *Lactobacillus* sp., with the exception of *Lactobacillus acidophilus* (Lin and Young, 2000; Rao et al., 1984) and *Lactobacillus plantarum* (Sybesma et al., 2003) could only utilize folate (Crittenden et al., 2003). Therefore isolation and use of naturally occurring *Lactobacilli* from traditional fermented milk which can produce folate is highly significant.

The main objective of this survey is the isolation, characterization and determination of *Lactobacilli* from traditional dairy products, yoghurt and doogh for their ability to produce folate.

MATERIALS AND METHODS

Sampling and isolation of *Lactobacillus* from traditional fermented milk

In this study, in order to find new strains with higher yields of folate, two distinct and conserved geographical and sociological regions of Iran, namely; Ilam and Lorestan with 6000 years of tribal residence and history were chosen. Dairy products in these two regions are not produced on an industrial scale but are all homemade instead. Therefore, these selected regions were considered suitable for collecting samples.

Doogh and yoghurt prepared by the traditional methods developed by nomads in their portable houses were sampled in June 2007. Three milliliters of samples were collected in 50 ml sterile tubes containing MRS broth and skim milk (Bergey et al., 2005). The samples were transferred to the laboratory within 4 h at 4 ± 2°C. Then innoculated skim milks were incubated at 37°C for 24 - 48 h and innoculated Man, Rogosa and Sharpe (MRS) medium incubated at 42°C for 4 h. After incubation time, these were subcultured on solid media (MRS and Rogosa agar, Merck, Darmstadt, Germany) and incubated at 37°C for 48 - 72 h. The bacterial colonies with typical morphologies were selected and examined microscopically. The Gram-positive rod shaped bacteria were then selected and stored as frozen stocks at - 80°C in MRS broth supplemented with 10% v/v glycerol for further analysis.

Phenotypic characterization

The isolated bacteria were primarily identified on the basis of the catalase reaction, esculin and gelatin liquefaction, nitrate reduction, indole production and motility test according to the procedure described previously (Collee, 1989). The ability of isolated bacteria to grow at two different temperatures (15 and 45°C) was checked. Furthermore, the coagulation of skim milk and acidification, coagulation and reduction of litmus milk and fermentation of 19 different carbohydrates were examined (Bergey et al., 2005).

Folate production assay

Four milliliters of sterile skim milk (Merck, Darmstadt, Germany) was inoculated with 40 µl of an overnight culture of isolated *Lactobacilli*. Vita fast folate kit (R-Biopharm, Darmstadt, Germany) was used to determine the folate level in coagulated skim milk. Total folate production was assayed based on the procedure provided by the kit manufacturer. The microbiological assay was performed in a 96-well microplate. The *Lactobacillus rhamnosus* strain was used as a control for the production of folate. The growth of *L. rhamnosus* is dependent on the supply of folate in the culture medium. Incubation of the culture was carried out at 37°C for 44 - 48 h in the dark. The intensity of metabolism or growth of *L. rhamnosus* in relation to the extracted folate was measured as turbidity and compared to a standard curve. The measurement was carried out using the microplate ELISA reader at 540 nm. Four ATCC strains (ATCC 9595 *L. rhamnosus*, ATCC 393 *Lactobacillus casei*, ATCC 314 *L. acidophilus* and ATCC 7830 *Lactobacillus lactis*) were quantified based on microbiological methods. For the strains with high folate production (more than 10 µg/l), the test was repeated three times and the results expressed with standard deviations (SD).

DNA extraction, primer design and polymerase chain reaction (PCR)

DNA extraction from the isolated *Lactobacilli* was carried out according to standard procedure (ISO-21571 2005). Based on the published data (Dubernet et al., 2002; Kwon et al., 2005), primer pairs, [(R16-1: 5'CTTGTACACACCGCCCGTCA) and (LbLMA1-rev: 5'CTCAAAACTAAACAAAGTTTC)] were used for verifying the putative *Lactobacillus* strains at genus level, for amplification of the desired fragment (250 bp); a 2.5 mM concentration of $MgCl_2$ was used. The following thermocycling programs was applied; initial denaturation at 95°C for 5 min followed by 30 cycles denaturation at 95°C for 40 s, annealing at 53°C for 40 s and extension at 72°C for 90 s, with a final extension at 72°C for 5 min.

Primer pairs for amplification of the complete 16S rRNA gene sequence were 16R08F (5'AGAGTTTGA TCCTGGCTCA) and 16R09R (5' TACCTTGTTACGACTTCACC), expecting a product size of approximately 1500 bp (Brosius et al., 1981). In this case, the $MgCl_2$ concentration was 2 mM. 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 40 s, extension at 72°C for 90 s and a final extension at 72°C for 5 min.

Sequence analysis of the 16S rRNA gene

The amplified fragments, were purified from the agarose gel (QIAGEN, Hilden, Germany), and was cloned into the pTZ57R/T T/A cloning vector (Fermentas, St.Leon-Rot, Germany). The recombinant vector was introduced to the recipient cell (*Escherichia coli* DH5α) by the procedure described by the manufacturer. The suspected *E. coli* DH5α colonies on amplicilin containing solid

Table 1. Location, source and number of samples.

Province	Yogurt			Doogh			Total
	Cow	Sheep	Goat	Cow	Sheep	Goat	
Ilam	4	6	1	9	9	3	
Lorestan	16	7	1	1			
Total	20	13	2	10	9	3	57

media were verified by amplification of the desired fragment with PCR using the 16R08F and 16R09R 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 MA 2007). The bootstrapped neighbor-joining phylogenetic tree was constructed by the Mega4 software. 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).

RESULTS AND DISCUSSION

Bacterial screening and identification

The household yoghurt and doogh made from cow's, sheep's and goat's milk were collected from the two mentioned regions. The number and description of samples are summarized in Table 1.

Fifty *Lactobacillus* strains were isolated from the 57 different samples of homemade traditional yoghurt and doogh (33 strains from Ilam and 17 strains from Lorestan). 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 fifty *Lactobacillus* strains were able to acidify, coagulate and reduce litmus milk. These strains were further characterized based on fermentation of 19 carbohydrates, the results of which are summarized in Table 2.

Most of the isolated strains were able to ferment glucose (94%), lactose (94%), fructose (86%), galactose (68%), sucrose (82%) and mannose (84%) but were not able to ferment sorbitol (96%), melezitose (90%), rhamnose (90%), cellobiose (76%), gluconate (76%), melibiose (76%), trehalose (76%) and xylose (72%). Some of them could ferment amygdalin (46%), arabinose (44%), maltose (32%), salicin (32%) and 28% of the strains could degrade esculin.

According to sugar fermentation, two strains (No.LO90, PTTC 1752 and No.IL125, PTCC1754) were similar to *Lactobacillus crustorum* except for degradation of esculin. *L. crustorum* could degrade esculin but these strains cannot. One strain (No.IL117) was similar to *Lactobacillus delbrueckii* subsp. *bulgaricus.* One strain (No.IL127) was similar to *L. plantarum* except fermentation of rhamnose. *L. plantarum* cannot ferment rhamnose but this strain can. The results of biochemical analyses are slightly different (5.2%-15.7%) from those in Bergey's table (Bergeys et al., 2005). This means that new strains or strains with new specifications may have been isolated in this study.

Conventional biochemical and physiological tests clearly have some limitations in discriminating between large numbers of isolates showing similar physiological characteristics (Mathara et al., 2008; Zamfir et al., 2006). Moreover, research results have not always contributed accurate and detailed information about microbial diversity in fermented milk, because they have relied only on culture methods based on phenotypic traits (Watanabe et al., 2008). Therefore, it is recommended that the genotypic technique be combined with phenotypic tests for confirmation purposes (FAO/WHO, 2002).

Dairy properties and folate production

The acidification and coagulation of skim milk at 43°C by isolated strains varied from 3.5 - 7 h when the inoculums was approaching 10^7cfu/ ml folate production by the 50 isolated *Lactobacilli* and the four ATCC strains were quantified based on microbiological methods (Table 3). The total folate content of strains ranged from 2.8 to 66.6 µg/l of fermented milk in single strain cultures. Folate production by ATCC strains in single strain culture conditions was very low (2.0µg/l). Other isolated *Lactobacilli* produced folate at different levels under single strain culture conditions which may reflect the varying abilities of different strains. A previous study has suggested that fermentation of milk affects the folate content which indicates that lactic acid bacteria differ in their abilities to produce or consume folate (Amann et al., 1995).

Folate is produced at different levels by different strains, as reported previously (Kariluoto et al., 2006; Lin and Young, 2000; Rao et al., 1984; Santos et al., 2008; Sybesma et al., 2003). Folate production by *Streptococcus thermophilus* (Rao et al., 1984) and *L. bulgaricus* (Lin and Young, 2000) have also been reported. Interestingly, some co-fermentation studies of *S. thermophilus* and *L. bulgaricus* have shown that folate produced by *S. thermophilus* is consumed by *L. bulgaricus* (Rao, 2000). Recently, it was shown that *Lactobacillus* species could produce folate at different levels; 41 µg/l by *Lactobacilli brevis* (Kariluoto et al., 2006), 19.8 ± 0.5 µg/l by *L. acidophilus* (Lin and Young, 2000), 21.10 µg/l of folate is produced by *Lactobacilli reuteri* (Santos et al.,

Table 2. Fermentation of carbohydrates by some lactobacilli strains isolated from traditional dairy products and ATCC strains.

Strains	Amigdalin	Arabinose	Cellobiose	Esculin	Fructose	Galactose	Glucose	Gluconate	Lactose	Maltose	Mannitol	Mannose	Melezitose	Melibiose	Raffinose	Rhamnose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose	Gowth 15°C	Gowth 42°C
ATCC9595	+	-	+	+	-	+	+	+	+	+	+	+	n	-	-	+	+	+	+	n	-	+	+
ATCC393	+	-	+	+	-	+	+	+	+	+	+	+	n	-	-	-	+	-	+	n	-	n	+
ATCC7830	+	-	+	-	+	-	+	-	+	+	-	+	n	-	-	-	+	-	+	n	-	n	+
ATCC314	+	-	+	+	+	+	+	-	+	+	-	+	n	+	+	-	+	-	+	n	n	n	+
LO74	n	n	-	-	+	+	+	-	+	-	-	+	n	n	-	+	n	-	n	+	-	-	+
LO75	-	-	-	-	+	+	+	-	+	-	-	+	-	n	-	-	n	-	+	n	n	-	+
LO76	-	-	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	n	+	n	n	-	+
LO77	n	n	-	-	+	-	+	-	+	-	-	+	-	-	+	-	-	n	+	-	n	-	+
LO80	+		-	-	+	+	+	-	+	-	-	-	-	-	-	-	-		+	-	n	+	+
LO81	n	n	-	-	+	-	+	-	+	-	-	+	-	-	+	-	n	+	+	+	+	-	+
LO83	+	+	-	-	+	-	+	-	+	-	-	+	-	-	+	-	n	n	+	-	+	-	+
LO84	+	-	-	-	-	-	+	-	+	-	-	+	-	+	+	-	-	-	+	-	-	-	+
LO86	+	-	-	-	+	-	+	-	+	-	-	+	-	-	+	-	+	-	+	-	+	-	+
LO90	-	-	-	-	+	-	+	-	+	-	-	+	-	-	+	-	n	n	+	+	+	-	+
LO92	+	+	-	-	+	-	+	-	+	-	-	+	-	-	+	-	-	n	+	-	-	-	+
LO93	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	-	-	+
LO94	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	n	+	-	-	-	+
LO95	n	+	-	-	+	-	+	-	+	-	-	+	-	-	+	-	n	n	+	-	+	-	+
LO96	+	+	-	-	+	-	+	-	+	-	-	+	-	-	+	-	n	n	+	+	+	-	+
LO97	n	n	-	-	+	-	+	-	+	-	-	+	-	-	+	-	n	n	+	+	+	-	+
LO98	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	n	+	-	-	-	+
IL99	+	+	-	+	+	+	+	-	-	+	+	+	-	+	+	-	-	-	+	+	n	-	+
IL100	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+	-	+	n	-	+	+
IL101	+	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	n	+	-	-	+	+
IL102	n	-	-	+	+	+	+	-	+	-	-	n	-	-	+	-	-	-	+	-	-	+	+
IL103	+	-	+	-	+	+	+	+	+	+	+	+	+	n	+	-	+	+	+	n	-	-	-
IL104	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	n	-	+	+
IL105	+	+	-	+	-	+	+	-	+	+	-	+	-	-	+	-	-	-	+	n	-	+	+

Table 2. Contd.

Strains	Amigdalin	Arabinose	Cellobiose	Esculin	Fructose	Galactose	Glucose	Gluconate	Lactose	Maltose	Mannitol	Mannose	Melezitose	Melibiose	Raffinose	Rhamnose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose	Gowth 15°C	Gowth 42°C
IL106	n	n	-	-	-	+	+	-	+	-	-	+	-	-	+	-	-	n	+	-	-	+	+
IL108	+	+	+	+	+	-	n	+	+	+	+	-	+	-	+	-	+	n	+	-	-	-	+
IL109	-	-	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	n	-	+	-	-	+
IL110	+	-	+	-	+	+	+	-	+	+	-	+	-	-	-	-	+	-	-	+	-	-	+
IL111	-	+	+	+	+	+	n	+	+	-	+	+	-	-	-	-	+	n	+	+	-	+	+
IL112	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	n	+	-	-	-	-
IL113	-	-	-	-	+	+	+	-	+	+	-	+	n	-	-	-	+	-	-	n	-+	+	+
IL114	n	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	n	+	+	-	+	+
IL116	n	n	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	n	+	-	-	-	+
IL117	-	-	-	-	+	-	+	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
IL118	-	-	-	-	+	-	+	-	+	-	-	+	-	+	+	-	-	-	-	n	+	+	+
IL119	n	N	-	-	-	-	-	-	n	-	-	-	-	-	-	-	-	n	-	-	-	-	+
IL120	-	+	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	n	+	-	+	-	+
IL121	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-	+	-	-	n	+	+	+
IL122	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	n	-	-	+
IL123	+	+	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	n	+	-	-	-	+
IL124	-	+	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	n	+	-	-	+	+
IL125	-	-	-	-	-	+	+	-	+	+	-	+	n	-	+	-	+	-	-	n	-+	+	+
IL126	+	+	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	n	+	-	-	+	+
IL127	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	n	+	+	+	+	+
IL128	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	-	+	n	+	+	+	+	-
IL129	n	n	-	-	+	+	+	-	-	+	-	+	-	-	+	-	-	n	+	-	-	+	+
IL131	+	+	-	+	-	+	+	+	+	-	-	+	-	-	+	-	-	-	+	n	-	+	+
IL132	-	-	-	-	+	+	+	-	+	-	-	+	-	n	+	-	-	-	+	n	-	+	+
IL133	-	-	-	-	+	+	+	+	+	-	-	+	-	+	+	-	-	n	+	-	-	-	+
IL134	-	-	-	-	+	+	+	-	+	-	-	+	-	n	+	-	-	-	+	n	-	+	+

+: Positive reaction; -: negative reaction; n: not determined; IL:Ilam; LO: Lorestan; numbers: number of samples whose strains were isolated.

Table 3. Folate production by *Lactobacillus* strains.

Strains	Folate (µg/l)	Strains	Folate (µg/l)	Strains	Folate (µg/l)
ATCC9595	2.0 ± 0.5	*LO* 93	17.6 ± 2	IL115	17.2 ± 2
ATCC393	3.0 ± 0.5	*LO* 95	19.4 ± 2	IL117	16.2 ± 1
ATCC7830	2.0 ± 0.5	*LO* 96	6.1 ± 1	IL118	17.2 ± 3
ATCC314	4.2 ± 0.4	*LO* 97	9 ± 0.6	IL119	16.3 ± 2
LO74	29.3 ± 1.5	*LO* 98	7 ± 0.4	IL120	12 ± 1
LO 75	15.2 ± 1.8	*IL* 99	7 ± 1	IL122	13.3 ± 1.5
LO 76	16.7 ± 1.7	*IL* 100	15 ± 2	IL123	26.6 ± 2
LO 77	2.8 ± 0.7	*IL* 101	13.3 ± 2	IL124	6.5 ± 0.7
LO 79	12.6 ± 1.7	*IL* 102	9 ± 0.5	IL125	66.6 ± 1.7
LO 80	7.1 ± 0.5	*IL* 103	14 ± 2	IL126	41 ± 2.7
LO 81	6.2 ± 0.6	*IL* 104	9 ± 0.5	IL127	12.8 ± 1.3
LO 83	20.5± 2.6	*IL* 105	12 ± 2	IL128	19.2 ± 2
LO 84	9.1 ± 0.7	*IL* 108	4.4 ± 1	IL129	6.3 ± 1
LO 86	9.1± 1	*IL* 109	7 ± 0.5	IL130	6.7 ± 0.5
LO 87	47.6 ± 2.7	*IL* 110	46.6 ± 1.6	IL131	33 ± 2
LO 89	6.4 ± 1	*IL* 111	8.5 ± 1	IL132	13.5 ± 1
LO 90	55 ± 1	*IL* 112	41 ± 2.6	IL133	15.9 ± 2
LO 92	19.1 ± 1	*IL* 114	15.1 ± 1.6	IL134	18.3 ± 2.7

2008), 42 µg/l by *L. plantarum*, 29 µg/l by *Lactobacilli sanfransiscesis* (Kariluoto et al., 2006), and finally 22 µg/l by *Lactobacilli helveticus* (Sybesma et al., 2003). Therefore, to our knowledge, the highest reported folate production is 42µg/l; hence, the strains of this study with the ability to produce 66.6 ± 1.7 and 55.6 ± 1.6 µg/l of folate are superior and may thus be useful in the dairy industry. From the 50 strains analyzed for folate production, 4 strains had higher yields of folate. Two strains with the highest yields of folate were chosen for molecular analysis and sequencing of the complete 16S rRNA gene and subsequently was registered in the Persian type culture collection (LO90: PTCC 1752, IL125: PTCC1754). It should be noted that these two strains have to be evaluated for safety assessment, but since they are of food origin, the human study phase of the safety assessment, may not be difficult, since they can be certified as food grade probiotics.

Molecular analysis and construction of phylogenetic tree

Extraction of DNA from wild type strains was implemented. The fourteen selected strains with higher levels of folate production were genotypically characterized by using genus specific primers which amplified a 250 bp fragment in the conserved domain of the *Lactobacillus* ribosomal DNA and complete 16S rRNA gene sequences. Ribosomal RNA genes have generally been accepted as potential targets for identification and phylogenetic analysis of bacteria (Amann et al., 1995). PCR using 16S rDNA- or 23S rDNA-targeted primers has successfully detected and identified the corresponding *Lactobacillus* species (Kwon et al., 2004; Nour 1998; Ward and Timmins, 1999). Amplification of suspected fragments from 14 selected strains with genus specific primers, are presented in Figure 1. The 16S rRNA gene sequences of selected strains with the highest folate production have been deposited in the GenBank database, under the accession numbers FJ645923 and FJ645924. The nucleotide sequences of 16S rRNA were used in analysis of similarity using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). Sequence analysis showed that these two isolated *Lactobacilli* were similar to a recently isolated species, *L. crustorum* (Ilse Scheirlinck and Peter Vandamme 2007).

The phylogenic relationship for the two strains is presented in Figure 2. These two *Lactobacillus* strains numbered LO90 (PTCC 1752) and IL125 (PTCC 1754) are similar to each other and have the highest similarity with other *L. crustorum* strains. They show considerable

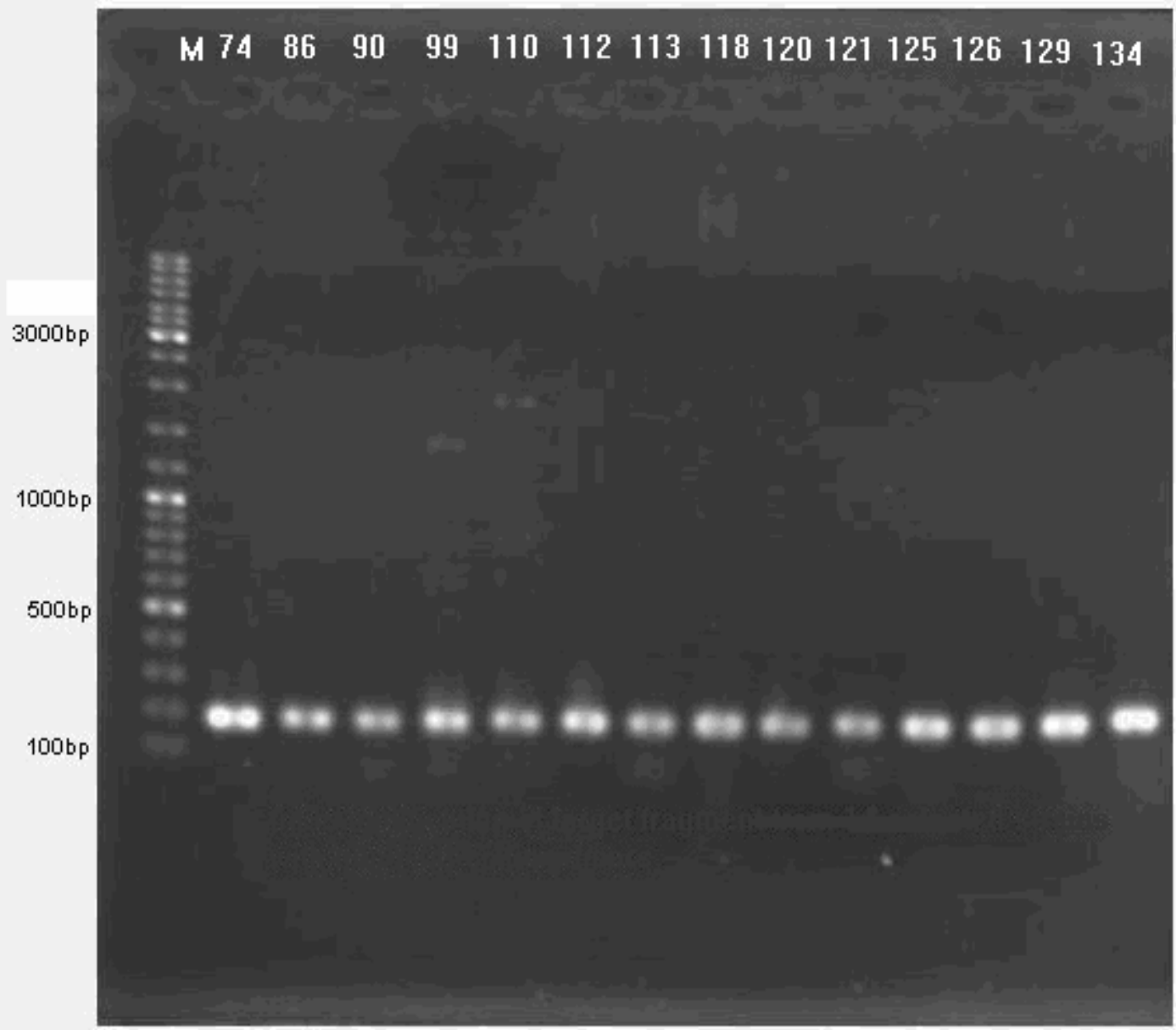

Figure 1. Amplification of suspected fragments from 14 selected strains with *Lactobacilli* genus specific primers. M: DNA molecular weight. Numbers of lanes are strain numbers.

similarity to *Lactobacillus farciminis*, and less similarity with *Lactobacillus kimchii* and *Lactobacillus paralimentarius*.

Screening and using high-folate-producing strains as part of the starter culture for making yoghurt, could result in production of high-folate-content dairy products (Wouters et al., 2002). Thus, it is expected that by using the two strains of this study for yoghurt or doogh production in combination with specific growth conditions, the current intake of folate from such products can be enhanced.

In addition, exploiting novel properties of these two strains as starters for development of yoghurt with characteristics similar to those of traditional products at an industrial scale should be further studied.

Conclusion

Traditional dairy products from two provinces of Iran as valuable sources for isolation of *Lactobacilli* with important specifications were evaluated. *Lactobacillus* strains with high-folate-production ability were identified. By using biochemical and preliminary molecular analysis, these two bacteria were found to be similar to *L. crustorum*.

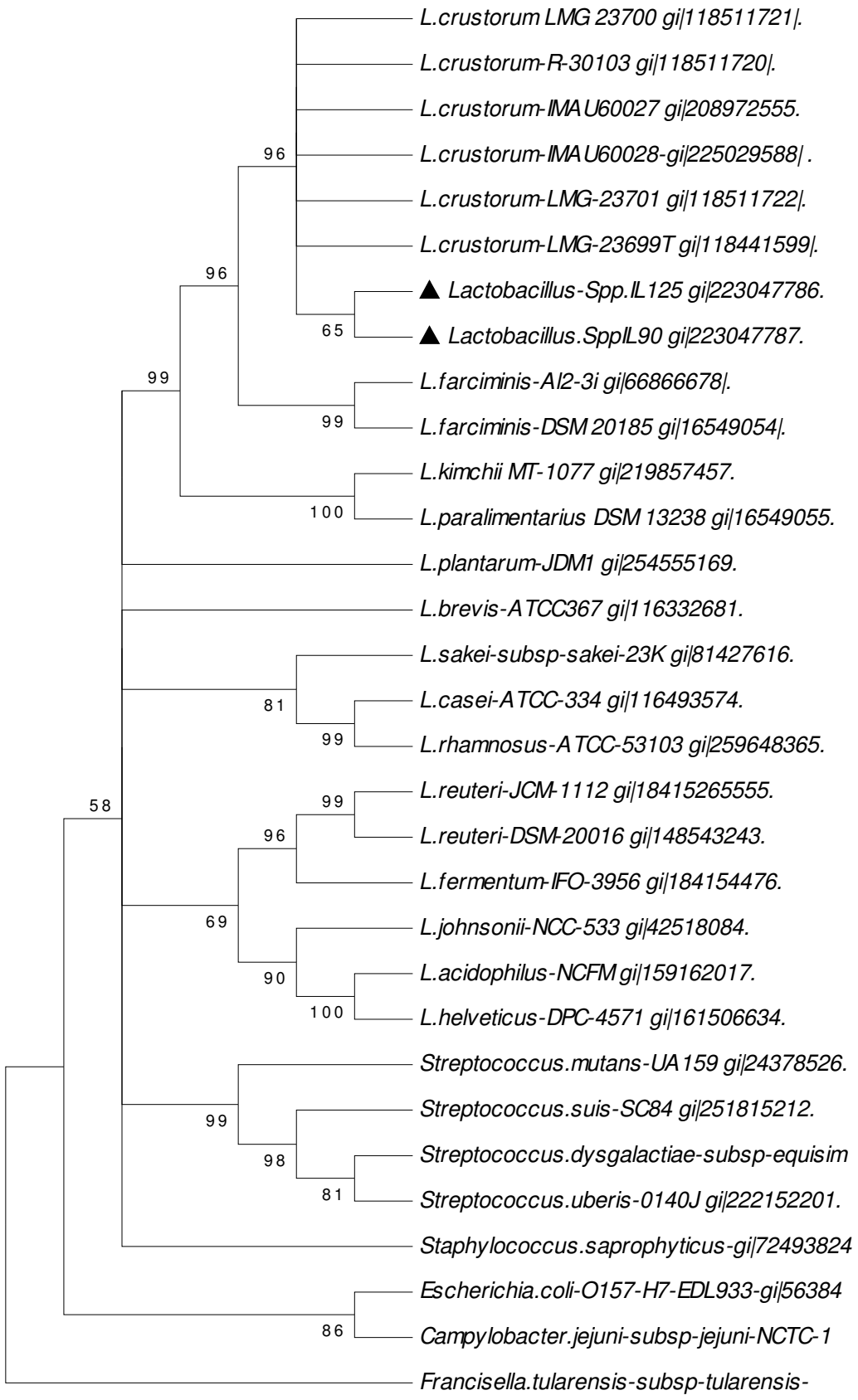

Figure 2. The phylogenetic relationship between two strains from this study (*Lactobacillus* sp.IL125, *Lactobacillus* sp.90) and other strains present in the NCBI database.

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