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ORIGINAL RESEARCH

Characterisation of *Lactobacillus helveticus* strains isolated from home-made dairy products in Iran

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The aims of this study were the isolation and characterisation of a number of lactobacilli strains from traditional dairy products. Fifteen home-made samples were pour-plated onto MRS and predominant colonies were randomly picked up. Nine isolated lactobacilli were grouped using rep-PCR fingerprinting, and partial sequencing of 16S-rRNA of group's representatives confirmed them as Lactobacillus helveticus. *Detection of two CEP (*prtH *and* prtH2*) genes and examination of acidification and growth in milk revealed intradiversity among isolates. The findings indicate the possibility of isolating novel wild strains of* L. helveticus *from home-made products and emphasises on the necessity of both genetic and technological characterisation for deeper differentiation of strains.*

Keywords Isolation, Genetic identification, *L. helveticus*, Cell-envelope proteinases, Acidification.

INTRODUCTION

Spontaneous fermentations and backslopping, as low-cost alternatives to using starter cultures, still occur in the production of a wide range of traditional fermented food especially in developing countries (Ravyts *et al.* 2012). These products can be considered as a potential resource of novel micro-organisms, as wild native micro-organisms present in raw materials play the most important role in fermentation and flavour formation due to a high biosynthetic capacity (Leroy and De Vuyst 2004). In fermented dairy products, the dominant microbial community belongs to the lactic acid bacteria (LAB) group that has considerable effects on the typical aroma, taste and physical and chemical properties of fermented products (Ravyts *et al.* 2012).

The rapidly increasing usage of commercial starter cultures has resulted in the growth in consumption of commercial products instead of traditional ones in over the last two decades. This development has seriously hastened the disappearance of rich resources of LAB found in the traditional products. Therefore, isolation and identification of wild LAB strains found in uninvestigated niche products is a valuable research (Ortu *et al.* 2007). A comprehensive evaluation of the safety and biotechnological properties of these isolated LAB might result in new complementary or replacement strains to current dairy starters and probiotics currently used dairy starters and probiotics with newly isolated strains (Wang *et al.* 2010; Leroy and De Vuyst 2004).

Lactobacilli, because of their high diversity and key technological properties, have a special position among LAB. Isolation and appropriate identification of lactobacilli from niche resources has been the subject of numerous studies (Marroki *et al.* 2011; Sedláček *et al.* 2010; Ortu *et al.* 2007; Majhenic *et al.* 2007; Henri-Dubernet *et al.* 2004; Coeuret *et al.* 2003). Accordingly, this study was designed to investigate a number of home-made dairy products collected from the rural regions of Iran, to achieve a better perspective of on the diversity of the predominant lactobacilli in these kinds of products. In the second part, intraspecies diversity of isolated strains pursued.

MATERIALS AND METHODS

Dairy samples and isolation of lactobacilli

Twelve samples of yoghurts and three samples of butter were obtained randomly from individual households in the rural areas of different localities of the Chaharmahal-Bakhtiari province in Iran.

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Samples were collected aseptically in sterile bottles, kept in an ice-box container and transported to the laboratory for analyses. Ten grams of each sample was aseptically weighed and homogenised with 90 mL of sterile 2% (w/v) sodium citrate solution and serially diluted; dilutions 10^{-5} to 10^{-7} were pour-plated onto MRS agar and incubated at 30 and 42 °C for 48 h under aerobic conditions. In plates where growth was observed, 1–2 colonies were randomly picked as the predominant colonies. Isolates were purified through two subsequent subcultures on MRS and characterised by examining their cellular morphology, by undertaking Gram staining and by determining their catalase activity. The 24-h lactobacilli isolates in MRS broth were stored with 20% (v/v) glycerol in 1.5 mL eppendorf tubes at −20 °C.

Genomic DNA extraction, RAPD-PCR and partial 16S rRNA gene sequencing

1.5 mL of each isolate grown overnight in MRS broth was used for the extraction of DNA using Wizard genomic DNA extraction kits (Milan, Promega, Italy). Genomic DNA of strains was used as a template for repetitive element sequence-based (rep)-PCR fingerprinting using the primer $(GTG)_5$ with sequence 5′-GTG GTGGTGGTGGTG-3′ according to a previous report (Versalovic *et al.* 1994). Briefly, amplification reactions were performed in 20 μL mixture of 1.25× buffer, 1.5 mmol/L $MgCl_2$, 4.25 μmol/L of primer, 1 mmol/L of each dNTP, 1 U of Taq polymerase (GoTaq G2 Flexi DNA polymerase, Promega) and 1 μL DNA. PCR amplification was performed in a 2720 thermal cycler (Applied Biosystems, Darmstadt, Germany). One cycle of 95 °C for 7 min was followed by 40 cycles of 95 °C for 1 min, 40 °C for 1 min and 65 °C for 8 min. Final extension was performed at 65 °C for 16 min.

PCR products were subjected to gel electrophoresis (1 h at 90 V) in 1.5% (w/v) agarose gel. The images of gels were captured using FireReading (UVITEC Cambrige, England) and analysed using GelCompare II software (Applied Maths, Sint-Martens-Latem, Belgium). A dendogram was elicited from the matrix of similarities by the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm.

The 16S rRNA gene was PCR amplified using the primer set E8F and E1541R designed for amplification positions 8 to 1541 in the *Escherichia coli* numbering system (Baker *et al.* 2003). The 20 μL reaction mixture contained 5 μL 5× buffer with 2.5 mmol/L $MgCl_2$, 1 unit Taq DNA polymerase, 0.3 mmol/L of dNTP, 1.25 μmol/L of each primer and 1 μL of DNA. The PCR amplifying procedures were as follows: 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 20 s at 57 °C, 2 min at 72 °C and then 10 min at 72 °C.

Partial sequencing was performed by the genomic analysis centre TGAC (Koln, Germany). The obtained sequences were aligned and compared to the sequences deposited in the EzTaxon website (http://eztaxon-e.ezbiocloud.net/).

Species-specific PCR

To obtain species-specific identification of isolates, a pair of primers Hel I (GAAGTGATGGAGAGTAGAGATA) and Hel II (CTCTTCTCGGTCGCCTTG) was used to amplify internal transcribed spacers (ITS) according to the method and amplification conditions previously described (Tilsala-Timisjärvi and Alatossava 1997).

Detection of *prtH* and *prtH2* genes

A set of specific primers, PrtH-for-1/PrtH-rev-1 (5′-GGT ACTTCAATGGCTTCTCC-3′ and 5′-GATGCGCCATCAA TCTTCTT-3′, respectively) and PrtH2-for-3/PrtH2-rev-2 (5′-GTTGGTGCCGCAACTAAATC-3′ and 5′-TAGCATTTT GGTCAAAGACA-3′, respectively), was used to target the conserved region surrounding the active site of PrtH and PrtH2 proteinases, respectively, encoded by the *L. helveticus* CNRZ32 proteinase genes *prtH* and *prtH2* as was previously described (Genay *et al.* 2009). Briefly, amplification reactions were performed in 20 μL mixture of 1× buffer, 1.5 mmol/L $MgCl_2$, 2.5 μmol/L of each primer, 0.2 mmol/L of each dNTP, 1.25 U of Taq polymerase and 1 μL DNA. One cycle of 95 °C for 4 min was followed by 30 cycles of three steps of 30 s (95 °C, 58 °C and 72 °C). Final extension was performed at 72 °C for 10 min.

Growth and acidification of milk

All isolated strains and two commercial *L. helveticus* starter cultures, LH-BO2 and LH-32 (Chr-Hansen, Hørsholm, Denmark), were individually propagated in 10 mL of sterilised (121 °C for 15 min) skim milk powder reconstituted at 10 % (RSM) (Pegah Dairy Co., Esfahan, Iran). Cultures grown overnight were inoculated in 40 mL of RSM to evaluate growth and acid production during fermentation at 40 °C. Before that, the inoculum was standardised for each strain, to get an initial cell density in the order of 6.5 log cfu/mL.

The pH of every RSM sample was measured immediately after inoculation and also after 2, 4, 6, 8 and 24 h of incubation (Jenway, Felsted, UK). The viable colony-forming units per millilitre were determined at 0, 8 and 24 h of fermentation. After 24 h of fermentation, the samples were refrigerated (4 °C) and cell numbers (cfu/mL) were enumerated after 7 days.

Statistical analysis

The pH data were converted to ΔpH (the difference of the initial value (pH_o) and the value reached in every measurement (pH_i)) and modelled according to the Gompertz equation 9 (modified by Zwietering), through the nonlinear regression procedure of the statistical package Statistica 7.0 (StatSoft Inc, Tulsa, OK, USA) for windows (Servili *et al.* 2011).

Analysis of variance (ANOVA) of data was carried out using SAS version 8.2 (SAS Institute, Cary, NC, USA), and the mean comparisons were conducted using Fisher's least significant differences (LSD) at 0.05 level of probability.

RESULTS

Enumeration and isolation of lactobacilli

In this study, the distribution of mesophilic and thermophilic LAB in samples was investigated on MRS agar at two incubation temperatures. In Table 1, the distributions of LAB in source samples, and the isolates that tentatively identified as lactobacilli from every sample, have been listed. The LAB number in samples ranged between 5 and 8 log cfu/g depending on the sample analysed and the incubation temperature. Generally, higher LAB counts were obtained under incubation at 42 °C, reflecting the thermophilic nature of LAB in all samples. The highest number of LAB was noted from sample FMK. In total 21 isolates were randomly selected from plates on the basis of apparent differences between colonies. Preliminary analysis by microscopic investigation, catalase reaction and Gram staining verified 9 isolates as lactobacilli, which were chosen for the following examinations.

Genetic identification

For investigation of genetic diversity among strains, all nine isolates were subjected to PCR amplification of repetitive bacterial DNA elements (rep-PCR) technique. This technique has been successfully employed for differentiation among closely related lactobacilli, and $(GTG)_5$-PCR fingerprinting was presented as a useful method for high-resolution typing and intraspecies differentiation (Gevers *et al.* 2001). In Figure 1, the dendrogram and $(GTG)_5$-PCR banding patterns of all isolated strains are shown. According to the results, a high similarity, ca. 93%, was documented between 9 lactobacilli isolates. Two strains, VY22 and SY14 were chosen for 16S rRNA sequencing, and interestingly, both strains were identified as *L. helveticus* with 100% identity (under accession number ACLM01000202). On the basis of high similarity found in $(GTG)_5$-PCR fingerprinting, there was the possibility that all strains were *L. helveticus.* This fact was verified using species-specific identification, and the results are presented in Figure 2.

Thereafter, to evaluate strains regarding technological properties, the presence of two cell-envelope proteinases (CEP) coding genes, and behaviour of growth and acidification in RSM were investigated. Strain DY2 was eliminated in the subsequent examination, as it is considered to be the same as DY1. Both strains were isolated from the same source (DY) and showed high similarity (>99%) according to the $(GTG)_5$-fingerprinting.

Table 1 Source samples used for the isolation of lactobacilli strains in this study

Identification name	*Sample type*	*Colony number (cfu/mL)*		*Isolates confirmed as lactobacilli on the base of phenotypic identification*[a]
		30 °C	*42 °C*	
FMK	Yoghurt	1×10^7	3×10^8	FMK1
FM1	Yoghurt	7×10^5	2×10^7	FM11
FM2	Yoghurt	5×10^6	4×10^6	–
BY2	Yoghurt	nc	7×10^5	BY24
BM1	Yoghurt	4×10^6	1×10^7	–
AM	Yoghurt	2×10^6	2×10^6	–
HY	Yoghurt	nc	7×10^7	HY21
BY1	Yoghurt	3×10^6	5×10^6	–
SY	Yoghurt	6×10^5	2×10^6	SY14
PY	Yoghurt	4×10^7	1×10^6	–
DY	Yoghurt	3×10^6	4×10^7	DY1, DY2
VY	Yoghurt	nc	9×10^5	VY22
AK	Butter	7×10^6	2×10^7	–
FK1	Butter	nc	4×10^5	FK11
SK	Butter	1×10^6	3×10^6	–

nc: no colony observed in plates comprising −5 to −7 dilutions.
[a]Phenotypic analyses were Gram staining, catalase reaction and microscopic investigation.

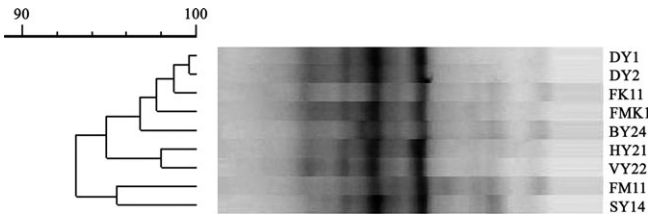

Figure 1 Dendrogram generated after cluster analysis of the digitised $(GTG)_5$-PCR fingerprints of the isolated lactobacilli strains.

Detection of the *prtH* and *prtH2* by PCR

To determine the presence or absence of the *prtH* and *prtH2* genes among isolated strains of *L. helveticus*, two pairs of primers (PrtH-for-1/PrtH-rev-1 and PrtH2-for-3/PrtH2-rev-2)

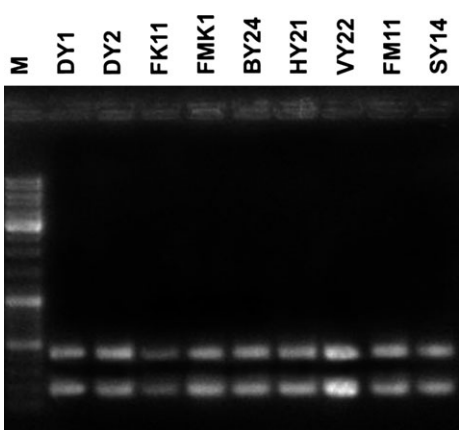

Figure 2 PCR amplicons from species-specific PCR assays for eight isolates. Electrophoresis was performed on a 1.5% (w/v) agarose gel in TBA 1× at 95 V for 40 min. The O'GeneRuler 100-bp DNA ladder was used as a molecular weight marker.

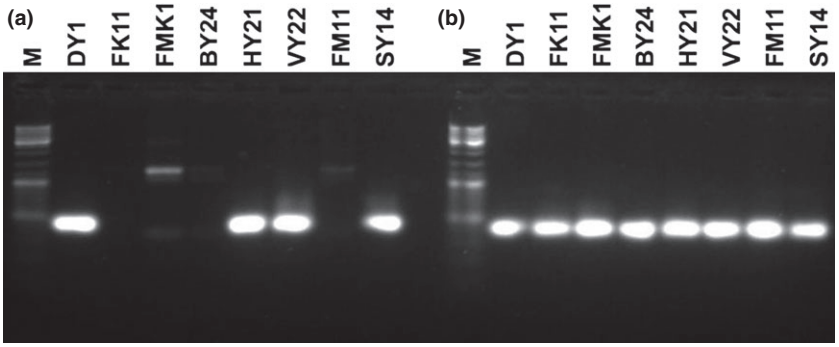

Figure 3 Results of amplification of the *prtH* (a) and *prtH2* (b) genes of eight isolates of *L. helveticus* . PCR products were obtained with the PrtH-for-1/PrtH-rev-1 (a) and PrtH2-for-3/PrtH2-rev-2 (b) primers and separated on a 1.5% (w/v) agarose gel in TBA 1× at 95 V for 40 min. The O'GeneRuler 100-bp DNA ladder was used as a molecular weight marker.

were used. PrtH and PrtH2 are two important CEPs in *L. helveticus* (Genay *et al.* 2009). The results are demonstrated in Figure 3. As illustrated in Figure 3 (b), all tested strains exhibited a band of ca. 400 bp corresponding to the *prtH2* gene, whereas only four strains (DY1, HY21, VY22 and SY14) of 8 exhibited a band of ca. 400 bp corresponding to the *prtH* gene (Figure 3a).

Kinetics of acidification and enumeration of lactobacilli

The initial pH of sterilised RSM was 6.62 ± 0.09, and the decrease in pH was determined based on the growth of *L. helveticus* isolates. Acidification curves (Figure 4) and kinetic parameters of acidification (Table 1) are shown. Although acidification rates differed depending on the strain, almost all strains were recognised as fast acidifying which could reduce pH of RSM to approximately 5.0 (strains FMK1, BY24 and reference culture LH-32) and below 4.6 (strains VY22, DY11, SY24, FM11 and FK11) during 8 h of fermentation. Strain HY21 showed a relatively slow acidifying rate and decreased pH to 5.54 ± 0.08 after 8 h, followed by the reference culture LH-BO2 (reaching 5.42 ± 0.07 in the same time period). According to the results, all strains, including reference cultures LH-32 and LH-BO2, lowered pH to a range between 3.17 ± 0.05 (strain DY11) and 3.45 ± 0.06 (strain HY21) after 24 h of fermentation. While the latency phase (λ) for all strains isolated in this study was between 2.06 ± 0.06 h (strain SY14) and 2.86 ± 0.03 h (strain BY24), this parameter for reference cultures LH-32 and LH-BO2 was 3.57 ± 0.04 h and 4.00 ± 0.08 h, respectively (Table 2).

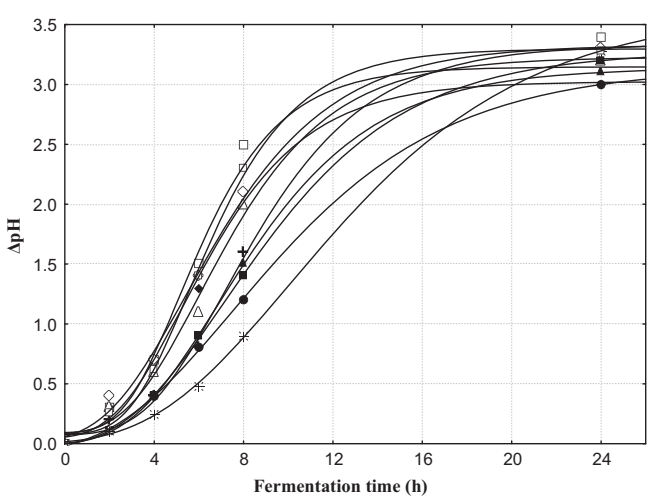

Figure 4 Acidification rate of 10% reconstituted skim milk fermented by FK11 (♦), HY21 (●), FMK1 (■), BY24 (▲), DY11 (□), SY14 (◊), FM11 (△), VY22 (○), LH-32 (✚) and LH-BO2 (✳) for 24 h incubated at 40 °C.

Figure 5 presents bacterial plate counts (log cfu/mL) of 10% RSM fermented by each isolate and by two reference cultures after 24 h and after 7 days of storage at 4 °C. After 24 h of incubation at 40 °C, the cell densities of all strains were more than 8.3 log cfu/mL with a maximum value of 9.17 log cfu/mL for DY11. The change in bacterial plate counts over storage at 4 °C for 7 days was strain dependent. While strains HY21 and FM11 did not show significant reductions ($P < 0.05$) (8.57 and 8.34 log cfu/mL after 24 h in comparison to 8.17 and 8.07 at 7 days of storage, respectively), strain DY11 recorded the greatest reduction of more than log 3.0 log cfu/mL to reach 5.7 log cfu/mL.

DISCUSSION

In the present study, the complete identification of nine lactobacilli strains, isolated from home-made dairy products, was achieved by $(GTG)_5$-PCR fingerprinting, 16S rRNA / sequencing of two representatives and species-specific PCR of all strains. The results demonstrated all isolates as *L. helveticus,* which it is a species with highly utilised technological abilities and which is one of the main starter cultures utilised for the production of cheese and functional fermented milks (Griffiths and Tellez 2013). Different strains of this species have been reported as being the most promising strains

Table 2 Acidification rate parameters of eight isolated *L. helveticus* strains and two commercial cultures of *L. helveticus* (LH-32 and LH-BO2) inoculated in 10% reconstituted skim milk after 24 h of fermentation at 40 °C

Strain	*ΔpH (pH units)*	V_{max} *(ΔpH* h^{-1}*)*	*λ (h)*
VY22	3.16 ± 0.03^{de}	0.44 ± 0.01^{a}	2.60 ± 0.03^{e}
DY11	3.45 ± 0.05^{a}	0.45 ± 0.02^{a}	2.81 ± 0.00^{cd}
SY14	3.36 ± 0.05^{abc}	0.35 ± 0.02^{b}	2.06 ± 0.06^{f}
FM11	3.23 ± 0.01^{cd}	0.37 ± 0.03^{ab}	2.69 ± 0.04^{de}
HY21	3.18 ± 0.05^{de}	0.25 ± 0.04^{d}	2.18 ± 0.03^{f}
FMK1	3.04 ± 0.04^{ef}	0.29 ± 0.03^{cd}	2.66 ± 0.03^{e}
FK11	2.95 ± 0.02^{f}	0.36 ± 0.01^{ab}	2.19 ± 0.02^{f}
BY24	3.16 ± 0.02^{de}	0.31 ± 0.02^{cd}	2.86 ± 0.03^{c}
LH-32	3.38 ± 0.07^{ab}	0.32 ± 0.03^{cd}	3.57 ± 0.04^{b}
LH-BO2	3.28 ± 0.01^{bcd}	0.26 ± 0.04^{d}	4.00 ± 0.06^{a}

ΔpH = the difference in pH (units) between the initial value (pH_0) and the value reached in the stationary phase of fermentation (pH_i).

V_{max} = maximum acidification rate (measured in ΔpH per hour).

λ = latency phase (h).

Data are expressed as the mean ± standard deviations (SD) ($n = 3$).

Values in the same column with different superscript letters differ significantly ($P < 0.05$).

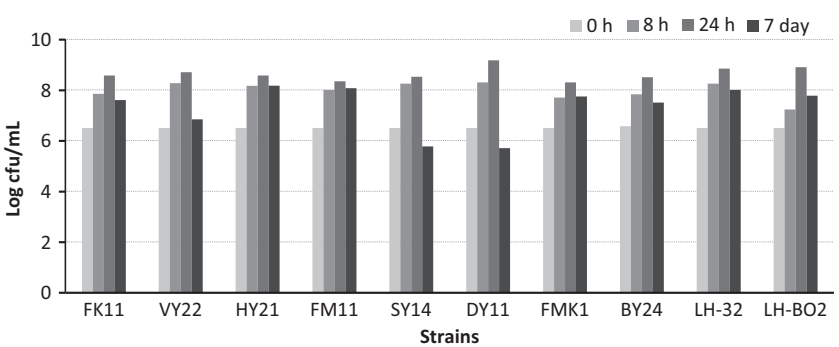

Figure 5 Cell numbers in skim milk fermented by eight isolated strains and two reference cultures of *L. helveticus* during 0, 8 and 24 h incubation at 40 °C and 7 days of storage at 4 °C.

among LAB for the production of bioactive peptides, which attributable to the strong proteolytic system of this species (Sadat-Mekmene *et al.* 2011). Regarding probiotic properties, it has been indicated that *L. helveticus* species can display functional characteristics similar to those of *L. acidophilus*, *L. rhamnosus*, and *Bifidobacterium animalis* subsp. *lactis* (Taverniti and Guglielmetti 2012). Recently, it has been shown that *L. helveticus* SBT2171, a starter strain in the production of Gouda-type cheese, can suppress the proliferation of immune cells, reduce the production of LPS-stimulated proinflammatory cytokines and have beneficial immunoregulatory properties (Yamashita *et al.* 2014).

According to literature, strains of *L. helveticus* have been isolated from different natural resources and represented various biotypes (Sedláček *et al.* 2010; Quiberoni *et al.* 1998). This species identified as the first, and the second predominant lactobacilli species was isolated from traditional fermented koumiss (Sedláček *et al.* 2010; Wu *et al.* 2009). A previous study in Iran indicated that this species constitutes 15.3% of all lactobacilli strains isolated from drinking yoghurt produced in Fars province of Iran (Azadina and Khan Nazer 2009).

In this study, intraspecific diversity among isolated *L. helveticus* was documented using CEP-encoding gene evaluation. CEPs located in the cell wall are the first bacterial enzymes hydrolysing milk caseins into peptides, which are later transported into the cell (Savijoki *et al.* 2006). Generally, *L. helveticus* proteolytic activity is considered the strongest among LAB with the highest diversity of proteinases among lactobacilli strains (Lozo *et al.* 2011; Genay *et al.* 2009). A comprehensive study of the proteolytic systems of several strains of *L.* helveticus strains showed that there is a high intraspecific diversity of genes encoding CEPs in the 51 analysed strains (Broadbent *et al.* 2011). Genome sequencing of CNRZ 32 revealed four proteinases genes: *prtH, prtH2, prtH3* and *prtH4* (Broadbent *et al.* 2008). Detection of proteinases genes in *L. helveticus* strains is a useful method in the investigation of their heterogeneity (Broadbent *et al.* 2011). Interestingly, according to the

results from this study, the *prtH2* gene is present in all eight strains, in comparison to *prtH* which exists only in four strains, which is completely in accordance with results of previous studies (Broadbent *et al.* 2011; Genay *et al.* 2009). This finding again confirms that the *prtH2* gene is ubiquitous, whereas the presence of the *prtH* gene is strain dependent. Broadbent *et al.* (2011) in a genetic screening of CEP enzymes among *L. helveticus* strains reported that only ca. 60% of tested strains contained more than one gene encoding CEP which is somehow similar to current results. However, it is important to note that the method used in this study can only detect paralogs of CEP genes found in CNRZ 32 (Broadbent *et al.* 2011), so the fragment detected in the isolate FMK1 (1300 bp, Figure 3) appears to encode a CPE enzyme similar to, but not identical with, PrtH. A similar case was also reported by Genay *et al.* (2009) for *L. helveticus* ROSELL 5089.

Generally, the rate and degree of acidification is one of the most important criteria for LAB with commercial applications (Ravyts *et al.* 2012) including *L. helveticus*. This species has been reported as the most acidifying and acid-tolerant LAB species, leading to a decrease in pH to pH 3.5, while *L. lactis* and *S. thermophilus* stop their acidification and growth at pH 4.3 (Nielsen *et al.* 2009). According to the classification presented in a previous report, it is possible to categorise all the strains isolated in this study as fast acidifying strains, as ΔpH_{24} was higher than 3.1 pH units (Fortina *et al.* 1998). However, Fortina *et al.* (1998), in a screening of 26 *L. helveticus* strains, reported that only four strains could be considered as fast acidifying.

Lactobacillus helveticus strains isolated in the current study showed good growth and adaptivity in skim milk and high stability in refrigerated conditions for most of them. While it was observed that the viable cell numbers increase continuously during 24-h fermentation, Leclerc *et al.* (2002) reported that the cell numbers of both *L. helveticus* strains studied (R211 and R389) increased over 9 h, followed by a slight decrease until the end of the fermentation (24 h). In the current study, the count of viable numbers of strains FK11, VY22, HY21, FM11, FMK1 and BY24 remained more than 10^6 cfu/mL after 7 days in the refrigerator. It is worth pointing out their number was comparable with two commercial cultures, LH-32 and LH-BO2, over the same duration.

Taken together, this research supports the findings of Quiberoni *et al.* (1998) and also presents greater clarity in the characterisation of the genetic and technological diversity in wild strains of *L. helveticus*.

CONCLUSION

This study represents an insight into the distribution and characterisation of prevailing lactobacilli isolated from home-made yoghurt and butter samples, which are produced in the rural localities of Iran. The results demonstrated that all nine isolated lactobacilli belong to *L. helveticus*, a strain with high technological properties. Although the results of fingerprinting indicated a high similarity between strains, the analysis of the presence of cell-envelope proteinases (CEP) genes, and acidification and growth behaviour in milk revealed interesting differences, which were comparable with commercial cultures. These findings suggest merit in future studies on the isolated strains such as evaluation of probiotic properties, induction of health-promoting effects and release of bioactive peptide profiles in fermented milk, as well as other technological abilities that are important in yoghurt and cheese production.

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