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Isolation, identification and characterization of indigenous lactic acid bacteria for flavour improvement

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Abstract

The presence of lactic acid bacteria (LAB) in fermented foods improves the health safety of food. Flavour development in dairy fermentations rises from a series of biochemical processes, and to restore the unique characteristics of traditional dairy varieties is to evaluate new wild LAB strains being isolated from good quality raw-milk. In order to study LAB biodiversity and to evaluate their potential use, bovine milk samples were collected from different regions of Iran. A total of 26 LAB were isolated from the milk samples. The bacterial isolates were evaluated for autolysis, proteolysis, acidification and diacetyl production. Six LAB isolates were found to bear the best characteristics. These were applied to produce types of curds. The curds were tested for aroma and the best curd samples were identified by molecular techniques. Finally two new *Enterococcus faecium* subspecies were registered at GenBank data library of National Center for Biotechnology Information (NCBI). Rich biodiversity of natural environments can be considered as good sources of new LAB species or strains. The different combinations of strains LAB in cheese manufacturing can influence dairy products flavour, and acid production, autolysis, proteolysis and production of diacetyl can be considered for their use as starters or adjuncts.

Introduction

Taste and aroma are very important features of cheese and consumers make their choice primarily on the basis of flavour characteristics (Singh *et al*., 2003). Developing culture and enzyme technologies that result in consistent rapid development of cheese varieties with specific characteristic flavours, is the long term goal of this area of research (Steele *et al*., 2013). The use of bacterial strains for cheese ripening with enhanced flavour production is promising in order to respond to the demand for products with improved aroma characteristics (Marilley *et al*., 2004). Micro-organisms, particularly lactic acid bacteria (LAB) are important in dairy products. LAB are a group of Gram-positive, non-spore forming, cocci or rods, that excrete lactic acid as the main fermentation product into the medium if supplied with suitable carbohydrate (Kopermsub and Yunchalard, 2010). Traditionally, LAB have been defined by the formation of lactic acid as the main product from carbohydrate metabolism (Beresford *et al*., 2001). LAB are used in making starter culture for the production of fermented foods, such as yoghurt and cheese (Karna *et al*., 2007; All, 2011). Several studies focused on the characterisation of cheese-associated LAB (Giraffa *et al*., 2001; Aquilanti *et al*., 2006; Piraino *et al*., 2008) for their ability to improve flavour, texture (Ayad *et al*., 2001) and for safety of perishable raw materials such as milk, meat and vegetables. Comparing the odour properties of raw-milk cheeses with those of cheeses made from pasteurised milk indicated that milk indigenous microflora were determinant factors of the specific sensory properties of raw-milk cheeses (Callon *et al*., 2005). The organoleptic characteristics found in raw-milk cheeses is very much dependent on the microbiota and LAB occur naturally as a native microbiota in raw-milk which greatly contributes to its differences (Abriouel *et al*., 2008). Raw milk may contain a wide variety of LAB which may have interesting features for application in cheese, though they may not survive the cheese manufacturing process. Hence, isolation from milk, rather than ripened cheeses, may have the advantage of giving a higher choice of selection both in terms of species and strains. Development of aroma in cheese products results from the metabolic activities of bacteria, acidification, autolytic activity, proteolytic activity and diacetyl production are some of the most important technological characterisation in cheese making and flavour (Sarantinopoulos *et al*., 2001; Smit *et al*., 2005; Grattepanche *et al*., 2007; Coolbear *et al*., 2008). One strategy to restore the unique characteristics of traditional cheese varieties

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is to evaluate new wild LAB strains being isolated from good quality raw-milk for use as starters, which could increase biodiversity as well as improving the flavour (Peláez and Requena, 2005). Starter cultures used for the production of fermented dairy products play a key role in final product quality with respect to preservation, organoleptic quality and nutritional value. The impact on product quality is highly strain dependent and is caused by differences between strains with respect to the presence and activity of metabolic pathways (Van Hylckama Vlieg and Hugenholtz, 2007). The present study aims at isolation of LAB strains from raw bovine milk and evaluation of the potential of these isolates to improve flavour of curd or cheese.

Materials and Methods

Milk sampling

Six raw bovine milk samples were collected from highly regarded producers in Iran of which four were in Araak, one in Semnan and one in Isfahan cities. Freshly collected milk samples were immediately transported to the laboratory under hygienic conditions at 4ºC (All, 2011). In order to maintain initial raw milk microbial popula¬tion and to inhibit growth of psychrotrophic bacteria extended cold storage of raw milk was avoided (Samaržija *et al*., 2012). The samples were immediately subjected to microbiological analysis.

Enumeration and isolation of microorganisms

Milk samples were homogenised and serially diluted in 0.1% peptone water (Grattepanche *et al*., 2007). As the LAB group includes both mesophilic and thermophilic species (Zannini *et al*., 2005), dilutions were plated and incubated as follows: (i) Under microaerophilic conditions on Man-Rogosa and Sharpe (MRS) agar (Merck KGaA) for 2 days at 30°C and 45°C for mesophilic and thermophilic LAB rods respectively. (ii) Under aerobic conditions on M17 agar plates (Medium for lactic Streptococci) for two days at 30°C for mesophilic LAB cocci; (iii) Under microaerophilic conditions on M17 agar for 2 days at 45°C for thermophilic LAB cocci; (iv) Aerobically on Slanetz-Bartley agar for 2 days at 37°C for enterococci (Kopermsub and Yunchalard, 2010). Total bacterial count (TBC) was obtained on plate count agar (PCA) added with 1g/L skimmed milk, aerobically incubated at 30°C for 24 h (Franciosi *et al*., 2009; Akpinar *et al*., 2011). Colonies showing different morphologies with the various types of culture media (Khunajakr *et al*., 2008) were analyzed by Gram's staining and catalase test. Colonies from each countable plate were isolated for each morphologically different colony type. The colonies with the Gram-positive and catalase-negative characteristics (Khunajakr *et al.*, 2008; Pang *et al*., 2011) and red colonies appearing on Slanetz-Bartley agar (Højberg *et al*., 2005) were purified by successive sub-culturing on M17, MRS and kanamycin aesculin agar (for enterococci) (KAA, HiMedia Laboratories Pvt. Ltd.). The purity was checked microscopically and stock cultures were maintained in glycerol at –70°C (Poznanski *et al*., 2004).

Acidification potential

LAB cultures were grown to OD_{600} nm of 0.5. Six mL from each culture was centrifuged at 8000×g for 5 min, washed with peptone water and inoculated in 10 mL 3% fat ultra-high temperature (UHT) milk. They were then incubated at their optimal growth temperatures on MRS agar (30 and 45°C), M17 agar (30 and 45°C) and KA agar (37 °C). The pH was measured at 2h intervals during the first 8h and thereafter at 24 h and 48 h post inoculation (Franciosi *et al*., 2009).

Autolytic activity

Six mL LAB cultures were centrifuged at OD600nm of 0.8^{-1} at 8000×g for 5 min, washed with potassium phosphate buffer (50 mmol/L, pH=6.5) and resuspended in the same buffer to attain OD nm=0.6–0.8 and were then incubated at 37°C for 48h. The degree of autolysis was expressed as the percentage decrease of the OD_{600}nm after 48 h (Mora *et al*., 2003).

Proteolytic activity

Proteolysis is probably the most important biochemical event during the ripening of most cheese varieties, with a major impact on flavour and texture. Techniques have been developed that permit quantitation of the principal ripening agents to proteolysis. For determination of proteolytic activity, LAB suspensions, prepared as autolytic activity, were spotted onto an agar medium (composition of 10% (w/v) skim milk powder and 2% (w/v) agar) and incubated at the optimal growth temperature for 4 days, and a clear zone around each colony was an indication to this phenomenon (Franciosi *et al*., 2009).

Diacetyl production

LAB were inoculated in UHT milk as described above. After incubation for 24 h at their optimal temperature, 1 mL of each cell suspension was added with 0.5 mL of a solution α-naphthol (1%, w/v) and

KOH (16%, w/v) and incubated at 30°C. Diacetyl generation is indicated by the formation of a red ring at the top of the tubes (Franciosi *et al*, 2009).

Odour intensity (sniffing) assay

The selected strains, singly or in combinations, were inoculated in full fat UHT milk. Pre-acidification or ripening of milk with starter culture brings about lower pH at renneting is lower than the natural pH of milk. The lower pH results in solubilization of colloidal calcium phosphate but little dissociation of casein (Lucey *et al*., 2003). The odour intensity test (Klein *et al*., 2001) was carried out after 3 days of incubation at the optimal growth temperatures. After 3 days of incubation at the optimal growth temperature, an odour analysis survey was conducted by involving 14 students randomly selected from Shahed University dormitory. The 14 assessor sensory panel was asked to score the intensity of odours using a three-level scale (unpleasant = –, moderate = +, pleasant = ++). The panelists were habitual consumers of traditional raw cow milk cheeses.

Extraction of genome and 16SrRNA analysis

Bacterial isolates from pleasant odour samples were selected for molecular identification. The bacterial cells from pure cultures were grown overnight in LB (Luria-Bertani) broth. 1.5 mL from the bacterial suspensions were centrifuged at 5000 × g for 3 min and the pellets were re-suspended in HTE (HCl Tris EDTA) buffer. The genomic DNA was extracted according to the protocol of Sambrook and Russell (2001). The 16S rRNA gene sequence coding region was amplified by PCR performed in a PCR thermal cycler with the prokaryotic 16S ribosomal DNA universal primers i.e. F: AGAGTTTGATCCTGGCTC and R: ACGGCTACCTTGTTACGA. PCR reactions were performed in 30 µL volumes containing 0.5 µL DNA polymerase, 3 µL 10xPCR buffer, 18.5 µL D.D.W, 1 µL Primer Forward, 1 µL Primer Reverse, 2 µL $MgCl_2$, 2 µL dNTP mix, 2 µL DNA Template. PCR products were purified with Low Melting Point Agarose and kit (Bioneer). The PCR product sequences were determined with genetic sequencer. Sequence similarity searches were performed in the GenBank data library of NCBI (National Center for Biotechnology Information). The isolates were identified by comparing the sequences with known sequences in GenBank using a BLAST (Basic Local Alignment Search Tools) program in the National Center for Biotechnology Information (NCBI) nucleotide database *(http://www.ncbi.nlm.nih.gov/)*. The percent sequence identity was determined by BLASTP using the default setting without a filter. The molecular classification of these isolated strains was determined by 16S rDNA sequence analysis.

Results

Microbial cell counts on specific culture media are given in Table 1. A total of 26 isolates were screened on specific culture media (MRS agar, M17 agar and KA agar) based on the morphology of the colonies. The LAB strains isolated on specific culture media were morphologically different. Four isolates (Nos. 1-4) were selected from M17 medium at 30°C. Six isolates (No. 5-10) were selected from M17 medium at 45°C. A single isolate (No. 12) was selected from MRS medium at 30°C and two isolates (Nos. 11 and 13) were selected from MRS medium at 45°C. Thirteen isolates (Nos. 14-26) were selected from KA medium at 37°C.

Acidification findings showed that during the first 24 h the enterococci isolated on Kanamycin Aesculin agar (KA agar) were more effective than the other LAB in decreasing milk pH (Figure 1). Isolate No. 25 was the strongest acidifier LAB after 24 hours (Figure 1). The highest autolytic activity was seen in isolates Nos. 6, 8, 23, 25 (Figure 2). The isolate No. 8 *(Enterococcus faecium)* possessed the highest autolytic activity in raw milk. Isolate No. 26 did not grow well and was therefore excluded from this parameter.

The presence of a clear zone around an isolate was indication of proteolytic activity. Six isolates (1, 2, 3, 4, 18, 26) exhibited this feature of which, number 4 had the highest proteolytic activity. The flavour compound, diacetyl, generated as an end product of citrate metabolism by certain LAB was at 5 levels of high, medium, low, very low and none (Table 2). At the end of the technological characterization, finally 6 isolates (4, 8, 23, 24, 25, 26) were selected and the odour test was performed.

The strains abilities to impart odour to milk during fermentation was evaluated by odour intensity assay. General odour intensity was ranked by the 14 assessors and the rankings were summed for each strain. Isolate 4 was rejected and the isolates 8, 23, 24, 25 and 26 were selected as pleasant. The results from odour intensity test the combinations of isolates, summarised in Table 5, led to the selection of a mixture of 4+8+25 as giving the most pleasant cheese. These isolates were analyzed by molecular techniques.

The genome of isolates Nos. 4, 8 and 25 were extracted and 16S rRNA genes were amplified by PCR. A blast search at NCBI indicated that the

Table 1. Enumeration of microorganisms (log cfu.mL^{-1}) in raw milk samples using specific culture mediaa

Sample	M_{17} (45°C)	M_{17} (30°C)	MRS (45°C)	MRS (30°C)	Slanetz-Bartley agar	PCA
1	-	3.0	-	-	-	6.6
2	3.4	7.1	3.2	3.4	2.1	6.1
3	2.8	7.4	3.1	6.5	3.4	5.9
4	-	2.8	-	2.7	-	7.3
5	2.4	7.4	2.1	5.8	3.8	8.3
6	5.7	7.4	4.9	6.0	6.2	7.0

[a]Medium abbreviations: M_{17}, medium for lactic streptococci; MRS, Man–Rogosa and Sharpe; PCA, plate count agar.

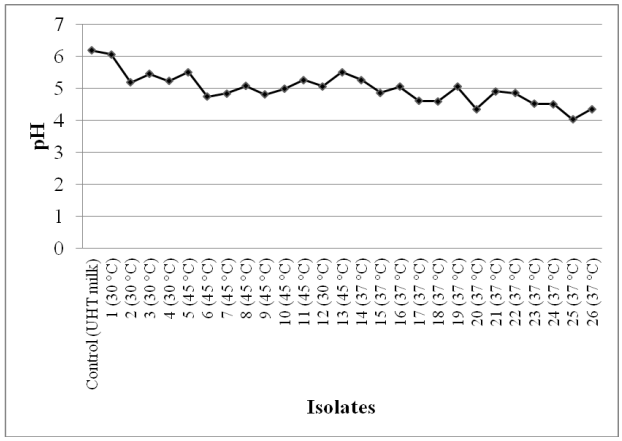

Figure 1. Changes in pH of raw milk after incubation for 24 hours at optimal growth temperatures

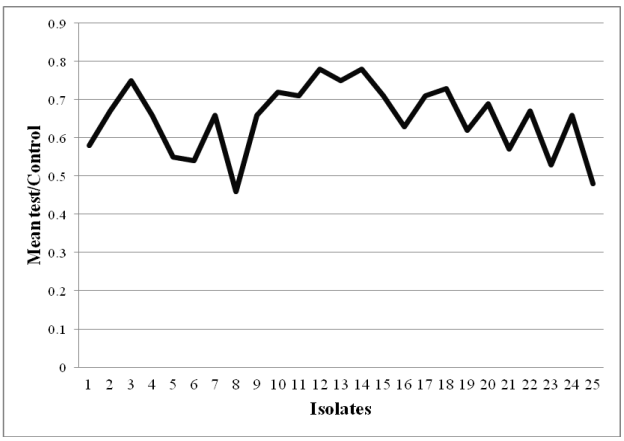

Figure 2. Autolytic activity of isolates after 48 h of incubation as a proportion of a control (The activity was calculated by dividing the initial OD of the bacterial suspension in potassium phosphate buffer by the OD taken after 48 hours. The degree of autolysis was expressed as the percentage decrease of the OD_{600nm} after 48 hours. The smaller the test/control, the greater is the autolytic activity)

strains were Enterococcus faecium. Strains 4 and 25 were registered as two new subspecies, *Enterococcus faecium* subsp. *Shahedfarahani* and *Enterococcus faecium* subsp. *Farahanirasooli*, accession numbers HM583338.1 and HM854854.1 respectively.

Discussion

The results of the routine microbiological assays on various culture media showed quantitative data on microbial diversity of raw cows' milk. The results (Table 1) show that LAB dominated the bacterial population in the milk samples. These findings are in agreement with the observations reported in different batches of Alberquilla cheese (Abriouel *et al*., 2008) and in traditional Greek cheeses (Asteri *et al*., 2009) showing that LAB composed the main microflora in all the tested samples. The plate counts showed dominance of LAB cocci over LAB rods (Table 1). More Lactococci than *Lactobacilli populations* has also been reported in Tetilla raw cows-milk cheeses (Menéndez *et al*., 2001). It was shown that the majority of the isolates belonged to the genera *Lactococcus* and *Leuconostoc* in Galicia raw cows' milk cheeses (Garabal *et al*., 2008). It was found that in enumeration of cheese microbial community the number of LAB grown on MRS agar were greater than those on M17 and that *Lactobacillus* was the most dominant genus. The enterococci were isolated on Kenner Fecal (KF) agar medium with *Enterococcus faecium* in larger number than the others (Abriouel *et al.*, 2008). The mesophilic lactococci were dominant over thermophilic cocci and the mesophilic rods were dominant over thermophilic rods (Wouters *et al*., 2002; Franciosi *et al*., 2009). Among the Lactobacilli, nearly all were characterized as mesophilic in raw cows' milk cheeses produced in Galicia (Garabal *et al.*, 2008). In the present study the isolates 4, 8 and 25 belong to mesophilic and thermophilic species indicating importance of both mesophilic (Albenzio *et al*., 2001) and thermophilic (Peláez and Requena, 2005) LAB in curd or cheese flavour and aroma.

Acidification of milk is an important step and predominant in curd formation from cheese manufacturing (Grattepanche *et al.*, 2007). Our results showed that during the first 24 h, milk pH decreased and the *Enterococcus faecium* (isolate No. 25) was the most effective species (Franciosi *et al.,*

Table 2. Diacetyl production by LAB isolated from raw cow milk

Level	High	Medium	Low	Very low	None
Isolate No.	25, 26	15, 16, 21, 23, 24	1, 4, 17, 22	19, 20	2, 3, 5-14, 18

The levels were defined as formation of a red ring at the top of the tubes in 3 minutes<High, 3-7minutes<Medium, 7-15 minutes<Low, 15-60 minutes<Very low and no ring formation as None

Table 3. Odour intensity of cheeses made from combinations of LAB isolates

Panelists	4 + 23	4 + 24	4 + 25	4 + 26	4 + 8 + 23	4 + 8 + 24	4 + 8 + 25	4 + 8 + 26
1	–	–	–	++	++	–	++	++
2	++	+	++	+	++	–	+	+
3	+	++	+	++	+	+	++	+
4	–	+	+	+	–	–	++	–
5	+	–	+	–	–	–	++	–
6	++	–	++	+	–	+	++	+
7	+	–	+	++	–	+	++	–
8	+	++	+	+	–	–	++	+
9	+	+	+	++	–	++	–	–
10	–	++	++	+	+	+	+	+
11	+	+	+	–	–	–	+	–
12	+	++	++	+	–	–	+	–
13	–	–	+	+	–	+	++	++
14	+	++	–	–	–	–	++	–

– Unpleasant
\+ Moderate
++ Pleasant

2009). *Lactobacillus* strains have been reported to differ in their ability to reduce the pH of milk initially and there were strains that did not change the pH of milk at 6 h. The ΔpH (5 h) of enterococci was shown, in general, higher than that of lactobacilli at 6 h, but there was a tendency for the strains to become slow after 5 h (Hassaïne *et al.*, 2007). *Lactococcus* and *S.* thermophilus strains had the highest acid producing ability at 6 h, but *Lb. helveticus* and some strains were able to cause the largest decrease in pH after 24 h (Piraino *et al*., 2008). A relatively large range in acid production was observed for enterococci and lactococci at 6 h, and for enterococci at 24 h. Reducing the pH value in cheeses during the first 60 days of ripening could be attributed to the continued production of lactic acid by live cells of lactobacilli that could survive much longer in cheese than lactococci. (Awad *et al*., 2007). Figure 1 shows the pH value in the first and third tests decreased to a mean of 3.4 by isolate No. 25. pH value below 4 is usually enough to suppress the growth of most food pathogens (Gadaga *et al*., 2004). Acid production is affected by temperature and it is probable that the greatest acid production occurs at the temperature other than optimal growth temperature (Vukašinović *et al*., 2001). The fast acidifying strains are good candidates in the dairy fermentation process as primary starter organisms, whereas, the poor acidifiers strains can be used as adjunct cultures depending on their other important properties, e. g., proteolytic and autolytic activity (Hassaïne *et al.*, 2007).

The conversion of caseins is undoubtedly the most important biochemical pathway for flavour formation in cheese (Smit *et al*., 2005). The physical properties of cheese are influenced by initial cheese milk composition, manufacturing procedures, and maturation conditions. Two of the most important factors influencing these properties are the condition of the casein particles in cheese and the extent of proteolysis (Lucey *et al*., 2003). In cheese, the activities of proteolytic enzymes from LAB yield small peptides and free amino acids from casein. The small peptides and amino acids are responsible for the background flavours in a matured cheese (Engels, 2014). A few isolates showed proteolytic activity and the majority of the strains showed low or no

proteolytic activity. This is in support of the results obtained on the proteolytic activity of the strains tested in which the thermophilic lactobacilli exhibited by far the highest release of amino groups while the majority of the remaining strains showed low extracellular proteolytic activity (Asteri *et al.*, 2009). By focusing on the importance of autolysis activity in improvement of curd odour and flavour, one can look for new species with higher autolytic activity. This activity was effectively performed by the isolate No. 8 i.e. *Enterococcus faecium* in this study (Figure 2). Hence, the *Enterococcus* genus is the most effective LAB for autolytic activity (Franciosi *et al*., 2009).

The results show the beneficial role of enterococci specially *Enterococcus faecium* in development of aroma. Many authors claim that enterococci may have a desirable role in cheese production (Poznanski *et al*., 2004; Awad *et al*., 2007; Malek *et al*., 2011). *Enterococcus* genus seems to play an important role in ripening of cheeses through proteolysis and lipolysis and contribute to typical taste and flavour (Foulquié *et al*., 2006). Enterococci are ubiquitous bacteria which frequently occur in large numbers in dairy and other food products. Although they share a number of biotechnological traits such as bacteriocin production, probiotic characteristics, usefulness in dairy technology, there is no consensus on whether enterococci pose a threat as foodborne pathogens. The potential pathogenicity of LAB, including enterococci, in human clinical infections have recently become a matter of controversy, in spite of the fact that foods containing enterococci have a long history of safe use (Giraffa *et al*., 1997). Despite the concern about pathogenicity of enterococci, recent studies point out that food and meat enterococci, especially *Enterococcus faecium* has a much lower pathogenicity potential than clinical strains (Hugas *et al*., 2003). Apparently cheese aroma is not affected by the presence of the *Lactococcus* and *Lactobacillus* species in raw milk. Mesophilic species of *Lactococcus* and *Leuconostoc* in raw milk were not metabolically active and may not affect typical flavour in dairy products (Randazzo *et al*., 2002). The initial high mesophilic *Lactobacillus* counts decreased during the cooking process but increased again during ripening. Lactococci with extended temperature range may have been selected by the ecological conditions prevailing during the manufacture of artisanal pasta filata cheeses, in which cooking temperature reaches 45ºC, but curd ripening is performed without strict temperature control, with temperatures decreasing from 45 to <30 ºC (Piraino *et al.*, 2008). The levels of free amino acids and free fatty acids were correlated well with flavour development in Ras cheese (Awad *et al*., 2007). The highest overall score of flavour intensity, flavour and texture acceptability were in cheese made using YY47 lactic culture in addition to adjunct culture of *Lactobacillus helveticus, Lactobacillus paracasei* subsp. p*aracasei, Lactobacillus delbrueckii* subsp. *lactis* and *Enterococcus faecium*. This culture can be recommended for Ras cheese manufacture using pasteurized milk (Awad *et al*., 2007).

Table 3 shows that different strains could influence each other in formation of flavour components and flavour cheese (Ayad *et al.*, 2001). The cheese type with a single isolate i.e. No. 4, was unpleasant. However this isolate produced acceptable cheese when mixed with isolate No. 26. It was demonstrated that different strains affect each other's activity in flavour production. It also appears that not all the enzymes have necessarily to be present in one strain, making it feasible to combine a number of strains in defined starter systems (Smit *et al*., 2005). The isolate No. 4 had the highest proteolytic property (Table 2). Combination of isolate No. 4 with isolates No. 8 and 25, the strong acidifiers, brought about pleasant flavour indicating importance of acid producers in cheese industry. The data reported here on acidification and proteolytic activity showed that the isolates presented the highest acidifying activity were not the most proteolytic, this suggest that no relationship exists between the proteolytic and acidifying activities of the strains (Durlu-Ozkaya *et al.*, 2001; Piraino *et al.*, 2008) This indicates the important role of acidification, autolysis, diacetyl production and proteolytic activity in curd or cheese flavour.

Diacetyl has a strong, buttery flavour and is essential at low concentrations in many dairy products, such as butter, buttermilk, and fresh cheese (Hugenholtz *et al.*, 2000). The selection of LAB isolates in relation to production of diacetyl should be considered with care, as this compound is not usually present at high concentrations in raw-milk cheeses (Smit *et al*., 2005; Menéndez *et al.*, 2001). This proto-cooperation between strains as it is called offers new possibilities for the construction of tailor made starter cultures, because it makes it clear that not all the desired enzyme activities in a certain flavour pathway leading to flavour need to be present in one strain (Smit *et al*., 2005). In conclusion local and native isolates are widely available in natural environments and rich biodiversity of natural dairy products can be considered as good sources of new LAB species or strains. With these in mind we can focus on pleasant local dairy products for new LAB strains. The study indicated that the different

combinations of strains for cheese making can positively influence dairy products flavour. Several properties of LAB i.e. acid production, proteolysis, production of diacetyl and autolysis can be explored for their use as starters or adjuncts.

Conclusions

Local and native isolates are widely available in natural environments and rich biodiversity of natural dairy products can be considered as good sources of new LAB species or strains. With these in mind we can focus on pleasant local dairy products for new LAB strains. The study indicated that the different combinations of strains for cheese making can positively influence cheese flavour. Several properties of LAB i.e. acid production, proteolysis, production of diacetyl and autolysis can be explored for their use as starters or adjuncts.

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