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Protein Pattern and Plasmid Profile of Lactic Acid Bacteria Isolated from Dahi, A Traditional Fermented Milk Product of Pakistan

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Summary

A total of 116 isolates were identified from randomly collected market dahi samples from Rawalpindi, Pakistan. Lactic acid bacteria dominated the microbial population of dahi and were identified according to their morphological and physiological characteristics. Among these lactobacilli were frequently occurring organisms. The phenotypic and biochemical analyses gave a diversity of species (8 presumptive species). The most abundant species were *Lactobacillus delbrueckii* subsp. *bulgaricus* (28 isolates) and *Streptococcus thermophilus* (25 isolates). Some contaminants such as *Staphylococcus*, *Micrococcus* and *Saccharomyces* spp. were also observed. The whole cell protein profiles of selected strains of lactic acid bacteria were examined by SDS-PAGE. It was observed that each species yielded a different electrophoretic pattern. It was further observed that among the strains investigated for the analysis of plasmid DNA 22 strains were found positive, 8 strains of *L. delbrueckii* subsp. *bulgaricus* followed by 5 of *L. acidophilus*, 4 of *L. casei*, 3 of *L. helveticus* and one of each *L. delbrueckii* subsp. *delbrueckii* and *L. delbrueckii* subsp. *lactis*, whereas no plasmid was observed in *S. thermophilus* and *L. lactis* strains investigated during the study. All the plasmids isolated were mostly large size plasmids and ranged from 20 to 25 kb in size.

Key words: isolation, identification, lactic acid bacteria, fermented milk product, dahi, SDS-PAGE, protein profile, plasmid DNA

Introduction

Lactic acid bacteria (LAB) form a phylogenetically diverse group and are defined as Gram-positive, non-sporing, catalase-negative, fastidious, acid tolerant and strictly fermentative bacteria that secrete lactic acid as the major end product of sugar fermentation (*1*). The species of genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus* and *Enterococcus* play an important role in the food industry as a result of their fermentative capacities. Historically, lactic acid-producing bacterial starter cultures are used in the production of fermented dairy, meat and plant products, and the fermentation results in products with improved shelf life, flavour, aroma and texture. Yoghurt and a variety of similar fermented milk products have been given traditional names; the nature of these products is different from region to region depending on the indigenous microflora, which in turn reflects the climatic conditions of the area. Dahi is a traditional fermented milk product popular in Pakistan; it contains a mixed culture of lactic *Streptococcus* and *Lactobacillus* in addition to the well known yoghurt organisms such as *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (*2*,*3*). Bacterial species currently used in the dairy industry belong to the genera *Lactobacillus* and *Streptococcus*. Therefore, the isolation and identification of new

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strains from the indigenous fermented milk products is necessary in order to bring novel strains to the industry. Taxonomic methods relying on morphological, physiological or biochemical criteria often involve multiple subculture and long-term tests; these methods are ambiguous and time-consuming. However, new approaches for identification of LAB by physicochemical methods such as SDS-PAGE protein patterns (*4*) have found wide applications.

Plasmids are extrachromosomal DNA elements with characteristic copy number. Plasmids may constitute a substantial amount of the total genetic content of an organism. The wealth of genetic information carried by plasmids, their impact in the microbial communities and the potential of these elements to act as natural cloning vectors have stimulated research into plasmids not only from the fundamental but also from the clinical, biotechnological and environmental points of view. Some of the industrially significant traits including lactose fermentation, citrate utilization, antiphage mechanism, exopolysaccharide production, aroma production, antibiotic resistance and bacteriocin production are often associated with the specificity of plasmids. Since dahi is prepared from undefined heterogeneous mixture of LAB strains, the determination of plasmid profile offers new possibilities for differentiating the isolates on the strain level (*5*).

As no such study has been carried out on lactic acid bacteria in this region, the aim of this study is to isolate indigenous strains of lactic acid bacteria from dahi for further characterization by using SDS-PAGE of whole cell protein and plasmid DNA analysis as an advanced tool for the identification of these isolates.

Materials and Methods

Collection of samples

Fifty samples of dahi, a traditional fermented milk product, were collected randomly from different local markets of Rawalpindi, in sterilized screw capped bottles and were brought to the laboratory of the Department of Food Technology for further analysis.

Isolation of bacterial strains

The isolation was performed on solid selective media. The streak plate method was used to isolate the lactic acid bacteria. For this purpose a loopful of each sample was streaked on MRS agar and M17 agar (Oxoid, UK) plates with 10 % lactose solution and the plates were incubated at 37 °C for 24 h. After incubation, the culture was observed for growth, single and isolated colonies were picked and subcultured on MRS agar and M17 agar media and incubated at 37 °C for 24 h to obtain pure culture of the isolates. Simultaneously the smears were prepared and stained with Gram's stain and examined under microscope for the staining characteristics and morphology of the isolates.

Identification of lactic acid bacteria

The identification of LAB was performed according to their morphological, cultural and biochemical characteristics as described by Collins and Lyne (*6*).

Protein profile

For the determination of protein profile of the lactic acid bacteria, strains previously identified by their phenotypic characteristics were submitted to SDS-PAGE as described by Laemmli (*7*).

Preparation of bacterial sample

The LAB were grown in their respective broth at 37 °C for 18 h. The cells were harvested by centrifugation at 8000 rpm for 1 min. The pellet was washed with 1 M Tris HCl buffer (pH=6.8) and resuspended in 10 μL of the same buffer and vortexed. Then 80 μL of the sample buffer (1 M Tris HCl, pH=6.8, 20 % SDS, 20 % glycerol, 10 % β-mercaptoethanol and 0.005 % Bromophenol blue) and 6.5 μL of β-mercaptoethanol were added to the preparations and boiled immediately in water bath at 100 °C for 5 min. After boiling the samples were placed on ice for 5 min and centrifuged at 8000 rpm for 1 min.

Preparation of SDS-PAGE

For electrophoresis 12.5 % separating gel and 6.5 % resolving gel were prepared. A volume of 15 μL of each sample was loaded on gel and was run on mini gel electrophoresis at 100 V for 2 h and stained in a solution containing 0.1 % (by mass per volume) Coomassie blue, 10 % (by volume) acetic acid and 40 % (by volume) methanol. Destaining was performed in a solution containing 10 % (by volume) acetic acid and 45 % (by volume) methanol. The protein molecular mass marker (116 to 14.4 kDa; Fermentas, USA) was used as standard.

Plasmid DNA isolation

Plasmid DNA of the selected strains was isolated by the method of Anderson and McKay (*8*) with some modifications. The samples of *Lactococcus* and *Lactobacillus* species for plasmid isolation were prepared. The preparation protocol was designed to be performed in a 1.5-mL Eppendorf tube. A volume of 2 mL of overnight culture was inoculated in 10 mL of fresh broth and incubated at 37 °C for 2 h. The cells were harvested by centrifugation at 8000 rpm for 5 min and washed once with STE buffer (8 % sucrose, 50 mM Tris HCL, 1 mM EDTA, pH=8.0) and then resuspended in 500 μL of the same buffer followed by the addition of 100 μL of lysozyme (20 mg/mL) solution and incubated for 1 h in ice. The samples were then centrifuged and the pellet was resuspended in 500 μL of freshly prepared lysis solution of 3 % SDS, 50 mM Tris HCl, 5 mM EDTA and pH=12.35 adjusted with 5 M NaOH mixed by slow and gentle inversion and incubated at 65 °C for 1 h. The samples were then cooled at room temperature and 50 μL of 2 M Tris HCl, pH=7, and 150 μL of 5 M NaCl were added to the tubes, which were then mixed gently and centrifuged at 12 000 rpm for 10 min. The supernatant was transferred to new microfuge tubes and 800 μL of ice cold ethanol were added, mixed by inversion and placed on ice for 1 h. The tubes were then centrifuged at 12 000 rpm for 10 min and the pellet was washed with 70 % ethanol, dried and dissolved in 15 μL of TE buffer (50 mM Tris and 10 mM EDTA, pH=7) containing RNase A and incubated at

37 °C for 30 min. The preparations were incubated overnight at 4 °C for solubilization of the DNA.

Agarose gel electrophoresis

Agarose gel electrophoresis was conducted on 0.7 % agarose in a horizontal mini electrophoresis (Wealtec, USA) in TBE buffer (Tris, boric acid and EDTA, pH=8.0) at 70 V for 1.5 h. A volume of 12 μL of DNA sample mixed with 3 μL of Bromophenol blue tracking dye was poured in wells. Lambda HindIII DNA molecular mass marker (23130-564 bp) was used as a standard for molecular mass determination. The gel was stained with ethidium bromide 0.5 μg/mL and observed under UV transilluminator for the presence of plasmid band.

Results and Discussion

One hundred and sixteen isolates from dahi samples were identified phenotypically, and the majority of isolates were Gram-positive and catalase-negative. These were subdivided into four groups: (*i*) small, white, gray white, smooth and rough colonies with maroon convex centre on MRS agar; rolled up, short or filamentous long rods, alone or in chains; homofermentative (presumptive thermophilic lactobacilli: 64 isolates); (*ii*) small round or lenticular white colonies on MRS agar, small rods in chains; homofermentative (presumptive mesophilic lactobacilli: 15 isolates); (*iii*) gray white, small, round pin point colonies on M17 agar; cocci, single and in chain cells; thermophilic and homofermentative (presumptive *S. thermophilus*: 25 isolates); (*iv*) gray white round pin point colonies on M17 agar; cocci, single and in chains; mesophilic and homofermentative (presumptive lactococci: 12 isolates). Some of the contaminants such as *Staphylococcus*, *Micrococcus* and *Saccharomyces* were also observed during the investigation. The data on the total number of different organisms isolated from 50 dahi samples are listed in Table 1.

Table 1. Incidence of lactic acid bacteria isolated from dahi

Name of species	No. of isolates
Lactobacillus delbrueckii subsp. *bulgaricus*	28
Lactobacillus delbrueckii subsp. *delbrueckii*	10
Lactobacillus delbrueckii subsp. *lactis*	8
Lactobacillus casei	15
Lactobacillus acidophilus	14
Lactobacillus helveticus	4
Streptococcus thermophilus	25
Lactococcus lactis	12

The dominant lactic acid bacteria identified are the genera of *Lactobacillus* (79) followed by *Streptococcus* (25) and *Lactococcus* (12). The dominance of *Lactobacillus* among the isolated strains is consistent with the findings of Raquib *et al.* (*3*), as the dahi is the heterogeneous mixture of different microorganisms. Therefore, this could be attributed to the use of old inoculum having high proportion of *Lactobacillus*. Moreover, the buffalo milk, which is commonly used for the preparation of this fermented milk, might favour the growth of these species. *Lactobacillus* was also observed as the dominant microflora in Egyptian fermented milk product laban rayeb (*9*). *Lactobacillus* is able to survive in highly acidic environment of pH=4 to 5 or even lower, and due to these properties, *Lactobacillus* is responsible for final stages of fermentation in the products. This further showed that low pH conditions favour the growth of *Lactobacillus*.

The nature of fermented milk products is different from one region to another, depending on the local indigenous microflora, which in turn reflects the climatic conditions of the area. Thus, traditionally fermented milk products in regions with a cold climate contained mesophilic bacteria such as *Lactococcus* and *Leuconostoc* spp., whilst thermophilic bacteria, which include mostly *Lactobacillus* and *Streptococcus*, prevailed in regions with a hot, subtropical or tropical climate (*10*). These findings support the theory that traditionally fermented milk products depend on the microorganisms found in a particular climatic region and the distribution of lactic acid bacteria depends on the nature of fermented milk product.

In the present investigation, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were observed as dominant microflora in the market dahi samples (*2*). Besides the presence of different LAB these two species, *L. bulgaricus* and *S. thermophilus*, exhibit a symbiotic relationship during the processing of yoghurt, with the ratio between the species changing constantly.

The presence of *L. acidophilus* in dahi samples is beneficial and could be used in combination with *L. delbrueckii* subsp. *bulgaricus* in the preparation of yoghurt as probiotic culture with improved organoleptic characteristics and enhanced therapeutic benefits.

Furthermore, *Lactobacillus acidophilus* is a natural inhabitant of mammalian gastrointestinal systems. This species is of considerable industrial and medical interest, because *L. acidophilus* is believed to play an important role in human health and nutrition by its influence on the intestinal flora (*11*).

The presence of *L. casei* and *L. helveticus* also favours the production of different fermented products as these are mostly used as cheese starter culture. However, their role in dahi is unknown. All the *Lactococcus* isolates were identified as *Lactococcus lactis*. Several studies elsewhere reported that *Lactococcus lactis* was more frequently isolated from raw milk samples (*12*).

Moreover, the *Staphylococcus, Micrococcus* and *Saccharomyces* were not identified up to the species level. The presence of *Staphylococcus, Micrococcus* and *Saccharomyces* shows the poor conditions of production, handling and postproduction contaminations. It is further added that the use of contaminated raw materials, lack of pasteurization, use of poorly controlled natural fermentations and inadequate storage and maturation conditions are the major risk enhancing factors (*13*). The presence of yeasts may be influenced by the age of the product as well as the containers and processing methods used (*14*).

The analysis of cell protein extracts of the selected strains of LAB isolated from dahi by SDS-PAGE showed different profiles. In this regard 6 strains of *L. delbrueckii* subsp. *bulgaricus* showed a total of 10 bands with 4 pro-

minent protein bands at 60, 50, 48 and 37 kDa. The SDS--PAGE of all strains of *L. delbrueckii* subsp. *bulgaricus* produced protein bands at 69, 65, 56, 50, 48, 43, 38 and 37 kDa, which is characteristic of genus *Lactobacillus* (*5*,*15*). While 4 strains of *L. delbrueckii* subsp. *delbrueckii* showed 12 bands with 4 major bands at 90, 62, 55 and 39 kDa, 2 strains of *L. delbrueckii* subsp. *lactis* showed 11 bands with three major protein bands observed at 60, 50 and 37 kDa. This difference in protein profile may be due to their genetic variation. Similarly, 9 strains of *L. acidophilus* showed 4 prominent bands of molecular mass of 60, 50, 45 and 35 kDa, while the minor bands were at the positions of 39, 32, 25 and 18 kDa (Fig. 1). These results are comparable with the findings of Xanthopoulos *et al*. (*16*); they also reported a single prominent band of 45 kDa in the *L. acidophilus* JCM 1132T. The band of 45 kDa is common in almost all the examined strains, while other minor differences in the protein pattern may be due to the nature of the fermented product and environmental factors involved in the isolation of these strains.

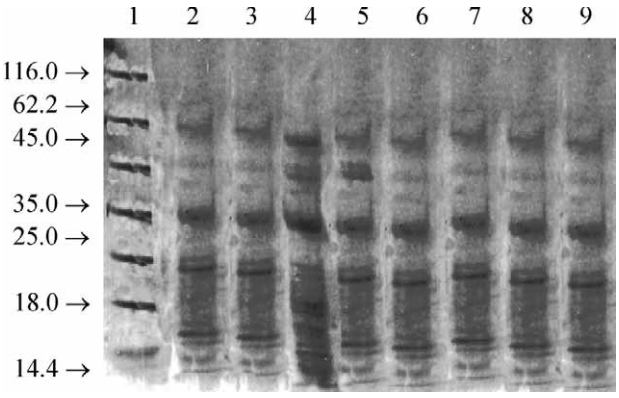

Fig. 1. SDS-PAGE of cell-free protein extracts of *L. acidophilus* strains: lane 1, molecular mass marker; lane 2 to 9, *L. acidophilus* Z2, M4, ID3, ID8, L15, L18, J2 and F11, respectively

The 7 strains of *L. casei* have 11 different bands. The major bands were observed at 60, 45 and 35 kDa, whereas other minor bands were observed at 50, 47, 32, 30, 25, 18, 16 and 14.4 kDa. Other workers reported bands in *L. casei* strains at 66, 62, 45 and 35 kDa (*17*).

The 4 strains of *L. helveticus* showed 4 major bands at 60, 50, 45 and 36 kDa. These results are further supported by Hebert *et al.* (*18*), who observed a major protein band in *L. helveticus* strains at 36 kDa; the other bands observed were approximately at 65, 50, 45 and 30 kDa.

Eight strains of *S. thermophilus* revealed 21 bands with prominent bands at 100, 97, 64, 49 and 47 kDa, whereas the minor bands were observed at 84, 60, 55, 42, 37, 35, 32, 30, 24, 22, 20, 18, 17, 16, 15 and 14.4 kDa. These results coincide with the findings of Tsakalidou *et al.* (*19*) that *Streptococcus* contained four major proteins of about 100, 49, 47 and 41 kDa. The 4 strains of *L. lactis* showed total of 16 bands with 4 prominent protein bands at 60, 50, 40 and 27 kDa; the minor bands were observed at 97, 84, 42, 39, 37, 24, 20, 17 and 16 kDa. The three bands of major intensity at 50, 40 and about 30 kDa in *L. lactis* from Tenerife cheese were also reported (*17*). *L. lactis* isolated from human infections has a prominent band at 40.2 kDa and the other bands reported were at 66, 55, 50 and 30 kDa (*20*).

These results suggest that each species yielded a different electrophoretic pattern. However, a slight variation among the examined strains may be due to different growth conditions used for propagation along with the type of media used. It was also reported that the growth medium but not growth phase can modify protein pattern of *Enterobacteriaceae* (*21*). It is furthermore added that these slight dissimilarities may be due to the difference in the origin of the strains. Similar views have been expressed by Samelis *et al.* (*22*) when identifying *Lactobacillus* species isolated from naturally fermented Greek dry salami.

Later on these wild strains were subjected to plasmid isolation to assess their copy number. On the basis of the results obtained, the number and size of the plasmids from various isolates was variable, as shown in Table 2. The presence of plasmids was observed in 22 strains of LAB. Eight were from *L. delbrueckii* subsp. *bulgaricus*, followed by *L. acidophilus* (5), *L. casei* (4), *L. helveticus* (3), and one in each *L. delbrueckii* subsp. *delbrueckii* and *L. delbrueckii* subsp. *lactis*. No plasmid was observed in *S. thermophilus* and *L. lactis* strains investigated during the study. The majority of the plasmids isolated was large size and ranged from 20 to 25 kb.

The plasmids isolated from three subspecies of *L. delbrueckii* ranged from 4.0 to 25 kb in size (Fig. 2). Three strains of *L. delbrueckii* subsp. *bulgaricus* carried two plasmids of varying sizes and 4 strains had a single plasmid of the same size. These results are supported by Miteva *et al.* (*23*), who observed that two strains of *L. bulgaricus* harboured one and the same plasmid of 1.5 MDa, while *L. bulgaricus* 144 showed a large plasmid but the result was not reproducible due to the low copy number or the instability of the plasmid in the conditions used.

It was further observed that 3 strains of *L. delbrueckii* subsp. *delbrueckii* and one strain of *L. delbrueckii* subsp. *lactis* showed large and single plasmids (Fig. 2). In a different study, quite small size low copy number plasmids ranging between 7.5 and 11 kb were reported from the strains of *L. delbrueckii* subsp. *lactis* (*24*), whereas the strains of *L. delbrueckii* subsp. *lactis* CRL 581 and CRL 564 were without a plasmid (*18*). This difference may be due to the procedure used coupled with the nature of the strain isolated from this region.

Five strains of *L. acidophilus* carried plasmids (Fig. 2). The strain F11 harboured 2 plasmids of 23 and 2.3 kb, whereas other strains carried single and large plasmids of 20 to 23 kb in size (Table 2). The presence of plasmids in *L. acidophilus* was also reported by Klaenhammer and Sutherland (*25*), with two plasmids of 13.7 and 6.3 MDa in 1 out of 8 strains. However, plasmid-free strains of *L. acidophilus* were also reported (*5*).

Among the four positive strains of *L. casei*, two strains F6 and ID4 showed a single plasmid of different sizes, 20 and 6.5 kb, respectively, while J8 and F13 showed two small plasmids (Fig. 3). This variation in size as well as copy number is also reported (*5*). The positive strains of *L. helveticus* ID2 and J6 have single plasmids of 23 kb, whereas the strain L5 harboured a plasmid of 20 kb (Fig. 3).

Table 2. Profile of plasmids in lactic acid bacteria isolated from dahi

Species	No. of strains tested	No. of strains positive	No. of plasmids	Approximate size in kb
L. delbrueckii subsp. *bulgaricus*	18	8		
Lb1			1	23
Lb3			1	23
Lb4			1	23
Lb5			1	10
Lb7			2	23, 4.0
Lb8			1	23
Lb9			2	6.5, 4.3
Lb10			2	25, 23
L. acidophilus	14	5		
J1			1	16
M3			1	20
M1			1	20
ID8			1	23
F11			2	23, 2.3
L. casei	15	4		
F6			1	20
ID4			1	6.5
J8			2	6.2, 2.0
F13			2	6.5, 3.0
L. helveticus	4	3		
L5			1	20
ID2			1	23
J6			1	23
L. delbrueckii subsp. *delbrueckii* ID6	3	1	1	23
L. delbrueckii subsp. *lactis* M2	1	1	1	23
S. thermophilus	8	none		
L. lactis	4	none		

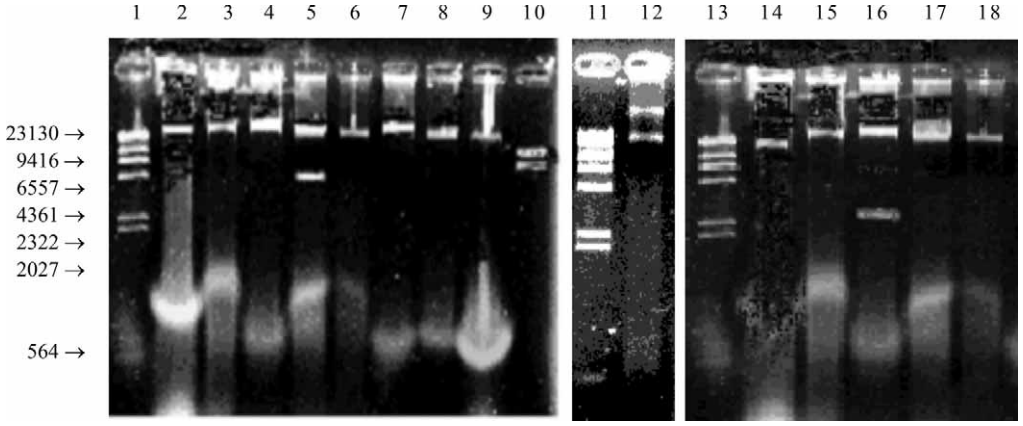

Fig. 2. Plasmid profile of *Lactobacillus*: lane 1 molecular mass marker Lambda DNA HindIII from 23130 to 564 bp, lane 2 *L. delbrueckii* subsp. *bulgaricus* Lb4, lane 3 *L. delbrueckii* subsp. *bulgaricus* Lb3, lane 4 *L. delbrueckii* subsp. *delbrueckii* ID6, lane 5 *L. delbrueckii* subsp. *bulgaricus* Lb7, lane 6 *L. delbrueckii* subsp. *lactis* M2, lane 7 *L. delbrueckii* subsp. *bulgaricus* Lb5, lane 8 *L. delbrueckii* subsp. *bulgaricus* Lb1, lane 9 *L. delbrueckii* subsp. *bulgaricus* Lb8, lane 10 *L. delbrueckii* subsp. *bulgaricus* Lb9, lane 11 molecular mass marker Lambda DNA HindIII from 23130 to 564 bp, lane 12 *L. delbrueckii* subsp. *bulgaricus* Lb10, lane 13 molecular mass marker Lambda DNA HindIII from 23130 to 564 bp, lane 14 *L. acidophilus* J1, lane 15 *L. acidophilus* ID8, lane 16 *L. acidophilus* F11, lane 17 *L. acidophilus* M3, and lane 18 *L. acidophilus* M1

All the examined strains of *S. thermophilus* and *L. lactis* during the present investigation did not harbour any plasmids. Many authors have reported small and single plasmids in *S. thermophilus* with sizes of 3.4 MDa and 3 kb (*23*). Furthermore, this species appears to be naturally poor in the plasmid composition and most of the industrial strains of *S. thermophilus* investigated were plasmid-free (*26*).

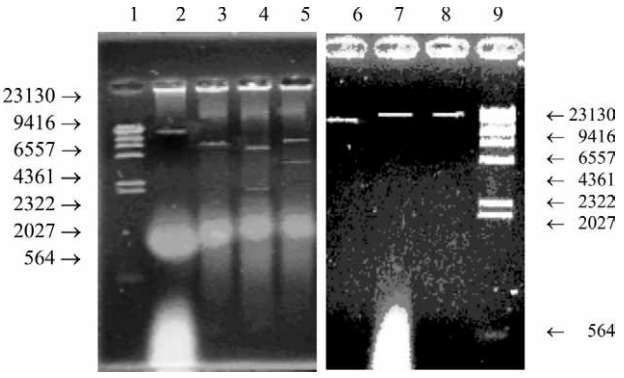

Fig. 3. Plasmid profile of *Lactobacillus*: lane 1 molecular mass marker Lambda DNA HindIII from 23130 to 564 bp, lane 2 *L. casei* F6, lane 3 *L. casei* ID4, lane 4 *L. casei* J8, lane 5 *L. casei* F13, lane 6 *L. helveticus* L5, lane 7 *L. helveticus* ID2, lane 8 *L. helveticus* J6, lane 9 molecular mass marker Lambda DNA HindIII from 23130 to 564 bp

However, the results of the present study were supported by the findings of Sewaki *et al.* (*5*), who also observed none of the plasmids in three strains of *S. thermophilus* they investigated. They further reported the presence of plasmids of different sizes in *Lactococcus lactis* strains. This difference may be due to the different extraction protocols, growth conditions in the laboratory as well as mixed culture conditions prior to isolation. Furthermore, the low recovery of plasmids from the identified strains may also be due to the continuous propagation in MRS and M17 broth.

It is furthermore added that many authors studying the plasmids in lactic acid bacteria, particularly in the genus *Lactobacillus*, emphasized the difficulties in the isolation, explaining them with the poor lysis due to the specific structure of the cell wall, the low copy number of the plasmids, and the action of nucleases (*23*). In the present investigation a number of protocols were applied as such and with different modifications for the isolation of plasmids from the wild strains of lactic acid bacteria, and only the method of Anderson and McKay (*8*) with some modifications was found successful. Similar views have been made that the published extraction methods were not always useful in the detection of plasmids (*24*). Moreover, plasmids larger than 40–50 kb or low-copy plasmids may remain undetectable even with Qiagen Plasmid Protocol Kit (*27*).

Conclusion

This investigation showed that dahi contains heterogeneous mixture of LAB. These species could be used in the production of variety of different products. The analysis of cell-free extracts by SDS-PAGE is a reliable method for molecular characterization. The results indicated that by plasmid profile analysis it is possible to differentiate the strains for presence and absence of plasmids. Based upon these observations it can be suggested that plasmid profile analysis may be a useful tool for evaluation of the stability of different traits in starter culture. Moreover, the strains identified during the present investigation should further be characterized with more useful genotypic methods and could be used as reference strains of this region in the future.

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