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Full Length Research Paper

Molecular identification of probiotics lactobacillus strain isolates by amplified ribosomal DNA restriction analysis (ARDRA)

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In recent years, interest in the probiotic lactobacilli has been stimulated by the use of these bacteria in products that are claimed to confer health benefits on the consumer. The objective of this study is to characterize probiotic *Lactobacillus* sp isolated from fermented millet drink, fresh milk and raw cow milk. All isolates are screened for their probiotic potential activities, including biochemical characterization and antagonistic activity. A total of seven colonies of lactobacilli isolated from twenty samples of fermented millet drink, fresh and raw cow milk were obtained based on their colonial/ morphological and biochemical characteristics. The *Lactobacillus* isolates obtained from fermented millet drinks are more effective than isolates from fresh milk and cow milk as regards their antagonism or inhibition. The results indicate that microbial identification drastically improved both in quality and effectiveness through the application of molecular methods. Molecular characterization of probiotic strains in phenotypic and physiological characteristics is often with low level of discrimination, probably due to their co-evolution in the same ecological niches. Thus, the nucleotide base techniques provide an accurate basis for phylogenetic analysis and identification. The other specific aim of the study is to analyze a probiotic *Lactobacillus* sp isolates. The seven selected isolates were identify to species level as *Lactobacillus plantarum, Lactobacillus lactis*, *Lactobacillus acidophilu and Lactobacillus helveticus* using API 50CH Kits. Amplified ribosomal DNA restriction analysis (ARDRA) using Alu I (AGCT), Mbo I (GATC) and Msp I (CCGG) restriction enzymes and 16S rDNA gene sequencing was identified *Lactobacillus* isolates under study. ARDRA screening revealed unique patterns among seven isolates, with the same pattern for some of the isolates. Gene fragments of 16S rDNA of strains representing specific patterns that needed to be sequence to confirm the identification of these species. These results confirmed that ARDRA is a good tool for identification and discrimination of bacterial species isolated from complex ecosystem and between closely related groups.

Key words: *Lactobacillus* species, probiotic, antagonistic activity*, Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus cereus*, and *Pseudomonas aeruginosa*, phylogenetic.

INTRODUCTION

The members of the genus *Lactobacillus* are Gram-positive organisms that belong to the general category of lactic acid bacteria. The *Lactobacillus* genus consists of a genetically and physiologically diverse group of rod-shaped Gram-positive, non-spore forming, nonpigmenetd (Hasan and Frank, 2001), catalase negative and microaerophilic to strictly anaerobic (Vernoux et al., 2003), lactic acid bacteria (LAB) that have widespread use in fermented food production (Azcarate-Peril and Raya, 2001), and are considered as generally recognized as safe (GRAS) organisms and can be safely used for medical and veterinary applications (Fuller, 1989). In the food industry, LAB is widely used as starter cultures and has been cited to be part of human microbiota (Fuller, 1992; Holzapfel et al., 2001). In raw milk and dairy products such as cheeses, yoghurts and fermented milks, lactobacilli are naturally present or added intentionally, for technological reasons or to generate a health benefit for

the consumer (Vernoux et al., 2003). They inhabit a wide variety of habitats, including the gastrointestinal tracts of animals and vegetation, and are used in the manufacture of fermented foods (Oskar et al., 2004). Interest in the lactobacilli has been stimulated in recent years by the use of these bacteria in products that are claimed to confer health benefits on the consumer (probiotics) (Goldin and Gorbach, 1992). The identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond those of the common fermentation tests (for example, cell wall analysis and electrophoretic mobility of lactate dehydrogenase) (Kandler and Weiss, 1986). In general, about 17 phenotypic tests are required to identify a *Lactobacillus* isolate accurately to the species level (Hammes and Vogel, 1995). The derivation of simple, yet rapid identification methods is therefore required in order to deal with the large numbers of *Lactobacillus* isolates obtained during microbial ecological studies of eco-systems such as the intestinal tract, silage, and food products. Nucleotide base sequences of *Lactobacillus* 16S ribosomal DNA (rDNA) provide an accurate basis for phylogenetic analysis and identification (Amann et al., 1995); Nikolova et al., 2009; Tannock et al., 1999).

The sequence obtained from an isolate can be compared to those of *Lactobacillus* species held in data banks. Although, the species-specific sequences are contained in the first half of the 16S rRNA gene (V1 to V3 region), identification is more accurate if the whole gene is sequenced (Stackebrandt and Goebel, 1994; Lorena et al., 2010). This means that about 1.5 kb of DNA would have to be sequenced. Studies by Tilsala-Timisjarvi and Alatossava (1997), Berthier and Ehrlich (1989), Blaiotta et al. (2008), Nour (1998), Nakagawa et al. (1994) and Nikolova et al. (2007), have demonstrated that the DNA sequence between the 16S and 23S genes of lactobacilli is hypervariable. This intergenic spacer region is about 200 bases in length if tRNA genes are absent (small spacer sequence) (Nakagawa et al., 1994). The 16S to 23S spacer sequences of lactobacilli are sufficiently species specific for the derivation of PCR primers that can be used to identify *Lactobacillus* species (Lorena et al., 2010; Tilsala-Timisjarvi and Alatossava, 1997). Because a relatively large number of different species (at least 18 from monogastric animals) have been described as intestinal inhabitants (Berthier and Ehrlich, 1989), identification of lactobacilli by PCR using sets of specific primers is daunting logistically.

MATERIALS AND METHODS

Isolation of bacteria

Twenty samples were collected from different sources in Riyadh region, KSA. Samples were incubated at 37°C until coagulation. Coagulated samples were then activated in MRS broth (Master Recording Supply 510 E. Goetz Ave - Santa Ana, CA 92707) at 37°C for 24 h in order to obtain enriched cultures. These cultures were streaked on MRS agar medium and incubated under anaerobic condition using a candle extinction jar with a moistened filter paper to provide a CO_2-enriched, water-vapor saturated atmosphere at 37°C for 48 h. Single colonies picked off the plates were sub-cultured in MRS broth at 37°C for 24 h before microscopic examination. The cultures of rod-shaped bacteria were streaked on MRS agar medium for purification. Purified strains were stores at -20°C in sterile MRS broth supplemented with 20% glycerol. Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of *Lactobacillus* (Hartemink et al., 1997).

Biochemical characterization

Identification of the isolates at genus level was carried out following the criteria of Sharpe (1979). Biochemical tests were performed on the isolates according to the scheme of Cowan and Steel (1974).

Detection of antagonistic activity

The antagonistic activity of the isolated *Lactobacillus* cultures was performed using the agar well diffusion assay described by Schillinger and Lucke (1989), against the selected test organisms namely *Escherichia coli* ATCC 9637™, *Staphylococcus aureus* ATCC 9763™, *Klebsiella pneumoniae* ATCC 10031™, *Bacillus cereus* ATCC 11774™, and *Pseudomonas aeruginosa* ATCC 9027™.

Phenotypic characterization

Carbohydrate fermentation profile was obtained by using of commercial API 50 CHL tests according to the manufacturer specification (bioMérueux, France). The apiweb^R identification software was used to interpretation of the carbohydrates fermentation results.

DNA isolation

An aliquot of 2 ml of each 24 h culture was centrifuged at 14000 g for 5 min. The sediment was frozen at −20◦C for 24 h to facilitate the breaking of the cells. The DNA was extracted according to Marmur (1961), modified by Kurzak et al. (1998), and then resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 Mm EDTA, pH 8). An aliquot of 5 µl of this template DNA was added directly to the PCR tube. The amount of DNA obtained was quantified by measuring it in an UV spectrum (260 nm) and its integrity was visualized by agarose gel electrophoresis to 0.7% w/v, by staining with ethidium bromide and visualizing under UV light.

16S rDNA amplification

The 16S rDNA gene was amplified by PCR with a thermal cycler (MJ Research). DNA fragments of approximately 1.5 kpb were amplified using the primers 27F (5_-AGAGTTTGATCCTGGCTCAG-3_) and 1492R (5_GGYTACCTTGTTACGACTT-3_). Each PCR tube (50 µl) contained a reaction mix of 10 µl 5X PCR buffer for *Taq* polymerase (Cat. No. N808 – 0152; Applied Biosystems, CA, USA), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Cat. No. 27 – 2094 – 01; Amersham Biosciences, NJ, USA) 0.4 µM of each primer and 2U of *Taq* Polymerase (Cat. No. N808 – 0152; Applied Biosystems, CA, USA), and 5 µl of template DNA. The termocycle

programme was as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; and a final extension step at 72°C for 7 min. After cycling, the PCR products were visualised by electrophoresis on a 1% w/v agarose gel (40 min, 75 V), by staining with ethidium bromide (0.5 µg/ml) and visualising under UV light (DyNA Light UV Transilluminator, LabNet, UV light source wavelength 302 nm).

Amplified ribosomal DNA restriction analysis (ARDRA)

In order to achieve complete digestion, restriction mixes (20 µl of final volume) were carried out for 4 h at 37°C. Each reaction tube contained 2 µl of 10X incubation buffer, 0.2 µl of bovine serum albumin, 6U of the respective restriction enzyme, 2.5 µl of bidistilled water and 15 µl of PCR product. Three restriction enzymes were used: *Alu I* (AGCT) (Cat. No. R0137L; New England Biolabs, USA), *Mbo I* (GATC) (Cat No. R0147S; New England Biolabs, USA) and *Msp I* (CCGG) (Cat. No. R0106L; New England Biolabs, USA) in appropriate restriction enzyme buffer. The resulting digestion products were visualised under UV-light (LabNet Transilluminator, UV light source wavelength 302 nm), after agarose gel electrophoresis 3% w/v (90 min, 75 V) by staining with ethidium bromide (0.5 µg/ml). Restriction patterns identical to the sequenced strains led to the identification of the corresponding species (Lorena et al., 2010).

DNA sequencing

The PCR products of seven representative isolates of each restriction enzyme were purified with PCR Clean-Up System kit (Amersham Biosciences, NJ, USA) and sequenced. The sequences were compared with the sequences deposited in the GenBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/; 1).

Nucleotide sequence accession numbers

The sequences were deposited in the GenBank database using the web-based data submission tool, BankIt (http://www.ncbi.nlm.nih.gov/BankIt).

RESULTS

Inhibition of the test organisms

The zones of inhibition were measured after incubating the isolates alongside the test organisms for 24 h to observe their inhibitory effect (Table 3). It was observed that there were larger zones of inhibition in the plates containing *Lactobacillus* isolates from fermented millet drink especially those of F1 (40 mm), which inhibited *S. aureus* and that of isolate F3 (20 mm) which has the next larger diameter of zone of inhibition active against *E. coli*. Isolate F3 obtained from fermented millet drink was able to inhibit the entire indicator organisms provided, though the lowest diameter of zone of inhibition was observed as 5 mm against *K. pneumoniae*. For the isolate F2 also obtained from fermented millet drink, it exhibited no antagonistic effect on any of the indicator organisms. Likewise, the zone of inhibition (8 mm) from R2, R4 isolate from fresh milk and cow milk were observed only in *aureus* out of all the indicator organisms. A clear zone of inhibition of 18 mm was observes against *B. cereus* as exhibited by isolate F1 from fermented millet drink. This shows that the *Lactobacillus* isolates obtained from fermented millet drink are more effective than isolates from cow milk as regards their antagonism or inhibition (Table 3). A total of 7 isolates were obtained based on their colonial/morphological and biochemical characteristics. These are shown in Tables 1 and 2.

ARDRA analysis

The 16S rRNA gene of each of the seven *Lactobacillus* isolates strains was amplified and restricted with the *Alu I*, *Mbo I* and *Msp I*. Figures 1, 2 and 3 showed fragments after digestion with *Alu I* (Source: an *E. coli* strain that carries the cloned *Alu I* gene from *Arthrobacter luteus* ATCC 21606). Figure 2 showed fragments after digestion with *Msp I* (Source: an *E. coli* strain that carries the cloned *Msp I* gene from *Moraxella* species ATCC 49670).

Figure 3 showed fragments after digestion with *Mbo I* (Source: an *E. coli* strain that carries the cloned *Mbo I* gene from *Moraxella bovis* ATCC 10900) Numerical Index of the Discriminatory Ability of Typing System was calculated using the Simpson's Index of diversity (Nour, 1998). Table 5 shows the discriminating indices and Typability (%) for typing methods used in this study. The restriction of the amplified fragment of the 16S rDNA gene with *Alu I* generated two different profiles. *Lactobacillus plantarum, Lactobacillus acidophilu* presented specific profiles for each of these species (Figure 1). The enzyme *Mbo I* also showed four different restriction profiles. Species that showed characteristic profiles were: *L. plantarum, L. acidophilu, Lactobacillus helveticus* and *Lactobacillus lactis* (Figure 2). *Msp* I I produced three restriction profiles, two of which were typical of *L. plantarum, L. acidophilu,* The restriction profiles produced by the species *L. helveticus* and *L. lactis* were not able to distinguish between them (Figure 3).

Identification by sequencing of the 16S rRNA Gene

Seven representative clones of the ARDRA profiles observed were selected for sequencing. The sequences of the gene fragments obtained from the 16S rDNA were aligned with those from GenBank using the BLAST algorithm.

DISCUSSION

Three of the isolates designated F1, F2 and F3 were obtained from fermented millet drink while four isolates designated R1, R2, R3 and R4 were obtained from the

Table 1. Morphological characteristics of isolates from fermented millet drink fresh milk and cow milk.

Isolates	Color on MRS agar	Shape formed as seen under microscope	Morphology arrangement
F1	Yellowish white color on medium surface	Rods arranged in chains	Clustered, straight rods
F2	Yellowish white color on medium surface	Rods arranged in chains	Clustered, straight rods
F3	Yellowish white color on medium surface	Rods arranged in chains	Clustered, straight rods
R1	Cream color on medium surface	Rods arranged in chains	Thick, short rods
R2	Cream color on medium surface	Rods arranged in chains	Thick, short rods
R3	Cream color on medium surface	Rods arranged in chains	Thick, short rods
R4	Cream color on medium surface	Rods arranged in chains	Thick, short rods

Table 2. Biochemical characteristics of *Lactobacillus* isolates.

Isolates	Gram strain	Catalase	Coagulase	Indole	Tentative identity of isolate
F1	+	-	-	-	*Lactobacillus* sp.
F2	+	-	-	-	*Lactobacillus* sp.
F3	+	-	-	-	*Lactobacillus* sp.
R1	+	-	-	-	*Lactobacillus* sp.
R2	+	-	-	-	*Lactobacillus* sp.
R3	+	-	-	-	*Lactobacillus* sp.
R4	+	-	-	-	*Lactobacillus* sp.

Table 3. Inhibition of indicator bacteria by *Lactobacilli* isolated from fermented millet drink fresh milk and cow milk.

Test organisms	F1	F2	F3 (mm)	R1	R2	R3	R4
Bacillus cereus	18 mm	NI	4	NI	NI	NI	NI
Escherichia coli	12 mm	NI	20	6 mm	NI	NI	5 mm
Pseudomonas aeruginosa	NI	NI	4	15 mm	NI	NI	NI
Klebsiella pneumonia	NI	NI	5	NI	NI	6 mm	NI
Staphylococcus aureus	40 mm	NI	4	NI	8 mm	NI	8 mm

fresh milk and raw cow milk. Their antagonistic effect was tested against five selected bacteria namely *Escherichia coli* ATCC 9637™, *Staphylococcus aureus* ATCC 9763™, *Klebsiella pneumoniae* ATCC 10031™, *Bacillus cereus* ATCC 11774™, and *Pseudomonas aeruginosa* ATCC 9027™. The results showed that the isolates were able to inhibit the growth of some of the selected indicator organisms in varying degrees. Isolate F1 was found to be the most effective with a zone of inhibition of 30 mm recorded against *S. aureus* (Petrova et al., 2009). Also observed to be next in effectiveness is isolate F3 obtained from the same source as isolate F1 with a zone of inhibition of 20 mm against *E. coli* (Table 3). However, the least level of inhibition, 3 mm, was recorded against *K. pneumoniae* by isolate F3 (Table 3). Isolate F2 was also found not to have inhibitory effect on any of the indicator organisms (Table 3). The inhibition recorded in the case of the isolates that have antagonistic effect may be due to the production of organic acids, bacteriocins and hydrogen peroxide (Dimitonova, 2007). In the present study, *Lactobacillus* isolates, isolated from fermented millet drink, fresh milk and raw cow milk were identified, according to polyphasic taxonomy. As a first step classical phenotypic characterization was done. Basis on the of results from carbohydrate utilization, estimated by API 50 CHL system, *Lactobacillus* was classified as species *L. lactis, L. helveticus, L. plantarum* and *L. acidophilu* with low Similarity 77.5% for *L. helveticus* and 99% for the other isolates (Table 4).

The API tests are fast and widely used system for physiological characterization and grouping of LAB isolates. The reliability of these tests in the case of philogenetically closely related lactobacilli have been questioned and some controversial results were achieved (Amarela et al., 2009). Thus, the application of more reliable and discriminative method was necessary. ARDRA fingerprinting technique was shown to be most suitable method for differentiating, indicating high

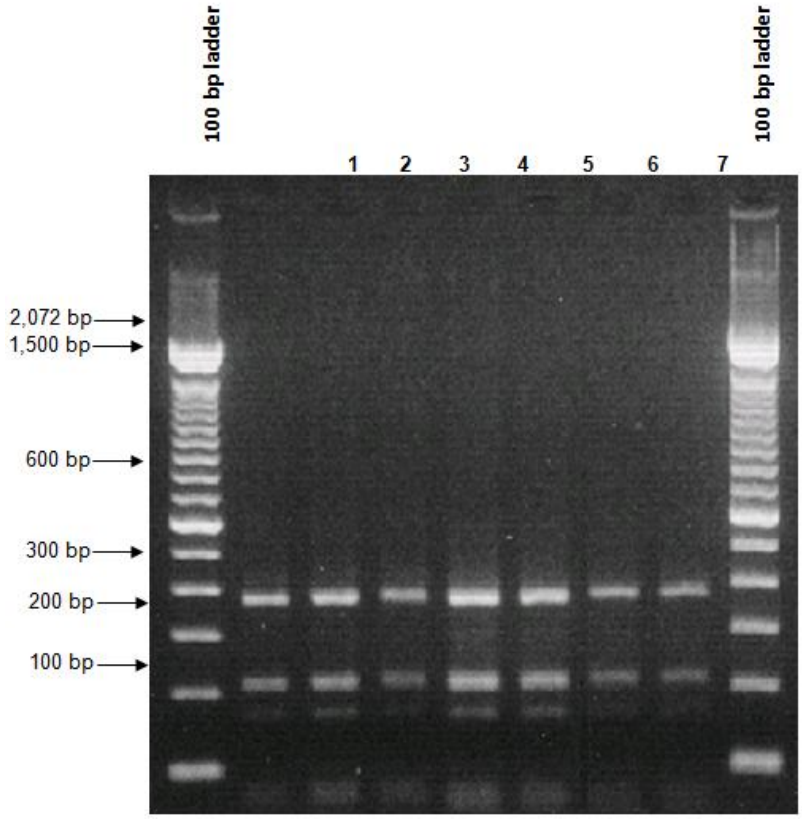

Figure 1. Restriction patterns obtained after digestion with 5 units Alu I for amplified 16S rDNA of Lactobacillus sp. after running in 2% agarose gel. 100 bp ladder was used as a standard size marker (F1, F2, F3, R1, R2, R3 and R4 are 1 to 7, respectively).

typability percentage discriminatory power for the method. In this study, evaluation of genomic ARDRA fingerprinting methods performed by computerized comparison of digitized fingerprinting patterns gives an accurate analysis. Data analysis by computer offers the possibility of comparison of large numbers of patterns, formation of databases, and cluster analysis. In this study, to give an assessment in which typing method is the most efficient several factors must be considered that includes: reproducibility, typability and discrimination. Reproducibility is the percentage of strains that give the same result on repeated testing.

Typability of a method is the percentage of distinct bacterial strains which can be assigned a positive typing marker. ARDRA pattern shows 96.2 to 97.5% typability using different restriction enzymes. The discriminatory power of a typing method is its ability to distinguish between unrelated strains. Numerical Index of discriminatory ability of typing system was calculated using Simpson's index of diversity. It can be seen that the discriminatory power the ARDRA analysis is relatively suitale, which varies between 0.773 (Alu I), 0.438 (Mbo I) and 0.659 (Msp I). In conclusion, our data suggest that in choosing a typing scheme for epidemiological studies, one should aim for as large discriminatory index as possible (Hunter and Gaston, 1988). The acceptable level of discrimination will depend on a number of factors, but an index of greater than 0.90 would seem to be desirable if the typing results are to be interpreted with confidence. The identification of microbial species through the use of phenotypic methods can sometimes be uncertain, complicated and time-consuming. The use of molecular methods has revolutionized their identification, by improving the quality and effectiveness of this identification. Some of these methodologies use either the rDNA spacer region or its target. These techniques are useful both in identification and reliable detection of different bacterial species as well as the monitoring of the species (Guan et al., 2003). The use of species-specific primers or probes is not applicable in environments where there are several *Lactobacillus* species because prior knowledge of them is required. In these cases, more general molecular tools should be applied (Delfederico et al., 2006; Guan et al., 2003). The techniques used to identify *Lactobacillus* species in different environments are the comparison of total or partial sequences of 16S

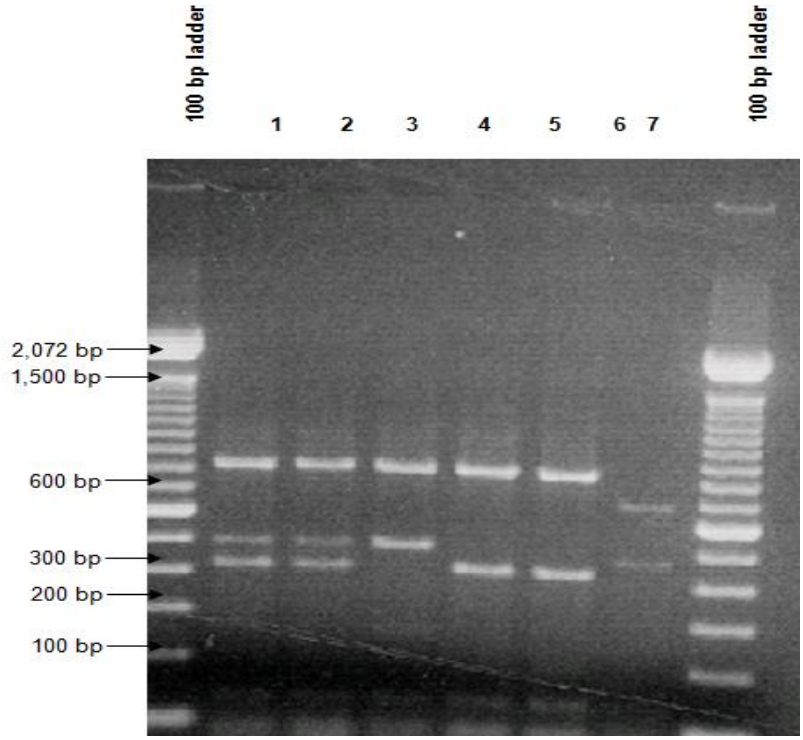

Figure 2. Restriction patterns obtained after digestion with 10 units Mbo I for amplified 16S rDNA of *Lactobacillus* sp. running in 2% agarose gel. 100 bp ladder was used as a standard size marker (F1, F2, F3, R1, R2, R3 and R4 are 1 to 7 respectively).

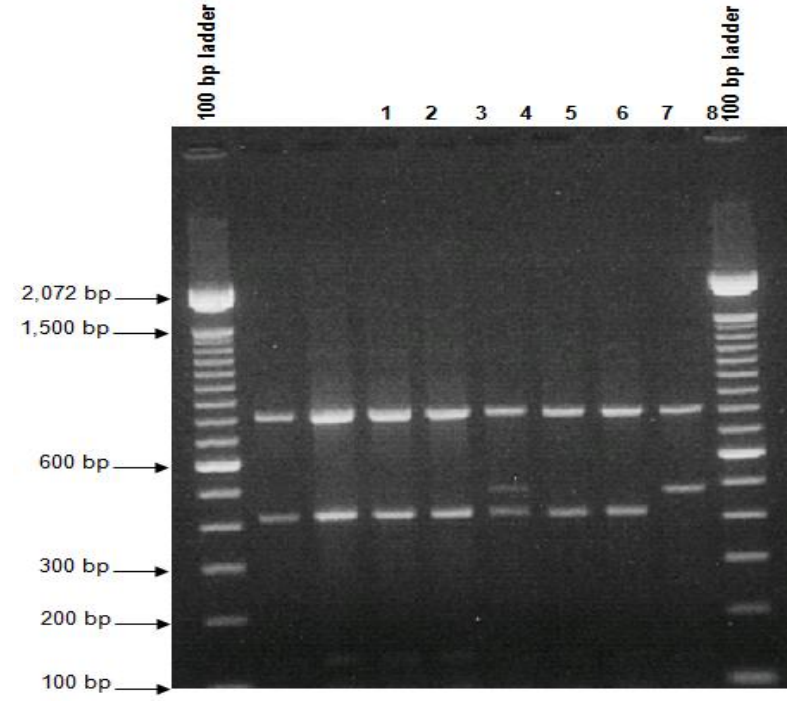

Figure 3. Restriction patterns obtained after digestion with 10 units Msp I for amplified 16S rDNA of *Lactobacillus sp* after running in 2% agarose gel. 100 bp ladder was used as a standard size marker (F1, F2, F3, R1, R2, R3 and R4 are 1-7, respectively).

Table 4. Origin and identification of *Lactobacilli* isolates using API system.

Isolates	Origin of the isolates	API 5OCH identification (% similarity)*
F1	Fermented millet drink	*L. lactis* (99.9%)
F2	Fermented millet drink	*L. plantarum* (99.9%)
F3	Fermented millet drink	*L. acidophilu* (99.9%)
R1	Fresh milk	*L. acidophilu* (99.9%)
R2	Fresh milk	*L. plantarum* (99.9%)
R3	Raw cow milk	*L. plantarum* (99.9%)
R4	Raw cow milk	*L. helveticus* (77.5%)

*****, The percentages following the scientific names of strains represent the similarities from the computer-aided database of the ApiwebTM API 50 CH V5.1 software.

Table 5. The discriminating indices and typability (%) of ARDRA analysis for *Lactobacillus* isolates.

Methods	No. of types		Discrimination index	Typability (%)
ARDRA analysis	Alu 1	4	0.773	97. 5
	Mbo 1	8	0.439	97.5
	MSP1	6	0.679	96.2

of 16S rDNA or the intergenic region of the 16S to 23S rDNA (Stackebrandt and Goebel., 1994; Ziemer et al., 2004). While the use of 16S DNA sequencing methods gives a high resolution of the diversity of microbial species in an environment, it is very time-consuming and too costly to be used for routine screening of samples. Methods for the initial analysis of samples should be rapid and able to give a broad view of the microbial ecology. ARDRA has been used to compare bacterial isolates within a wide range of microbial communities. The advantages of ARDRA are that it is rapid, reproducible, relates to microbial diversity, and will be invaluable in analyzing a greater number of samples together with experimental objectives such as dietary interventions (Stackebrandt and Goebel, 1994). In the present work, ARDRA allowed us to differentiate the *Lactobacillus* spp isolates. This differentiation was observed by restricting with any of the three enzymes used.

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