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Identification of *Lactobacilli* using PIB Software

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Lactobacilli **are major part of microflora of the human gut and of many fermented dairy products and are found in variety of environments. A total of 30 strains of probiotic lactobacilli (previously isolated from milk and curd samples) were tentatively identified as *Lactobacillus* on the basis of their cultural, morphological and biochemical characteristics. Among the 30 isolates, five isolates namely CM25, CM27, CM28, CM33 and CM34 were identified as *Lactobacillus* when subjected to genus specific PCR. Species level identification of isolates was done using PIB Bryant software. Isolates CM25 was identified as *Lactobacillus plantarum*, CM27 as *L. lactics*, CM28 as *L. fermentum*, CM33 as *L. casei* subsp. *rhamnosus* and CM34 as *L. casei* subsp.*casei*.**

Key words: *Lactobacillus,* PCR, PIB software.

Lactobacilli are ubiquitous and widespread communally bacteria found in the human body which are useful as adjuvant against gastrointestinal disorders, as dietary supplements, and as biological food processors (Aguire, *et. al.,* 1993; Gasser, *et., al.,* 1994). Lactobacilli are also used as health promoting probiotic ingredients in food and pharmaceuticals preparations. Probiotics are viable organisms and supportive substances that improve intestinal microbial balance (Fuller, 1991).

Conventional cultural, morphological and biochemical tests clearly have some limitations in discriminating large number of isolates showing similar physiological characters. Therefore the application of molecular biology techniques for the rapid identification of *Lactobacillus* genus using specific PCR primer that target the 16S-23S rRNA spacer regions is important (Verthier, *et. al.,* 1994; Andrighetto, *et. al.,* 1998).

16S-23S rRNA sequence has also a reliable method for identification of several bacterial species (Kwon, *et. al.,* 2004; Song, *et. al.,*2002). But molecular methods have some drawbacks that they are too expensive specially species specific PCR and some time it gives false

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results due to mutations and some other changes. In contrast identification of *Lactobacillus* up to the species level via biochemical methods aided with software programmes such as PIB Bryant software (Bryant, 1993), is cheaper, less time consuming and more reliable.

The aim of this study was focused on the previously isolated strains of probiotic lactobacilli for conforming their genus by PCR specific primers and identification up to species level using PIB Bryant software.

MATERIAL AND METHODS

Isolation of lactobacilli

A total of 4 samples of milk and 4 samples of curd were incubated in MRS broth at 37°C for 24 hr. and then plated on MRS agar medium (De Man, *et. al.,* 1959). A total of 30 isolates were randomly picked up for identification using PCR.

Preparation of DNA templates for PCR assay

Bacterial genomic DNA was extracted from pure cultures by the method of Pospiech and Neikmann, 1995.

PCR primer for identification of the genus *Lactobacillus*

Primer pair, R16-1 (5'- CTTGTACA CACCGCCCGTCA-3') and LbLMA1-rev (5'-CTCAAAACTAAACAAAGTTTC-3'), corresponding to position of 1400-1419 and 1597-1617 of the 16S rRNA and its flanking ISR region of *L. casei*, respectively (Nakatawa, *et., al.,* 1994; Tanncok, , *et., al.,* 1994; Torriani, *et., al.,* 1994) was also used to verify the *Lactobacillus* genus-specificity (Dubernet *et. al.*, 2002;, Moreira, *et. al.*, 2005) used in this study for identification of lactobacilli isolates at the genus level.

PCR assay

The reaction mixture (25µl) contained 0.5µl Taq DNA polymerase, 1µl forward primer, 1µl reverse primer, 2µl deoxyribonucleotide triphoshates, 2.5µl 1X PCR buffer and 17µl milli Q water, and 1.2µl DNA template is prepared as described above. PCR amplification was performed with a thermocycler (Eppendrof master cycle gradient, 5331, Germany) and DNA fragments were amplified as follows: initial heating at 94°C for 2 min., followed by 35 cycles consisting of denaturation at 94°C for 20 sec., annealing at 15^0C for 40 sec., extension at 68°C for 30 sec. and a 7 min. final extension step at 68 °C and after that hold at 4°C for 5-10 min. Amplicons were separated on 2.0 % agarose gel along with known molecular marker (100 bp ladder procured from Bangalore Genie) by electrophoresis and analyzed by ethidium bromide staining.

Biochemical tests for species level identification

The isolates were subjected to biochemical tests and characterization by PIB Bryant software. The biochemical tests including Gram reaction, catalase test, growth at different temperature like 15°C and 45°C for 24 hr., growth in litmus milk, gas production from glucose, streaking on BCP- agar medium were done. For nitrate reduction, nitrate medium was used and arginine hydrolysis was observed in MRS containing 0.3 % arginine and incubated at 37°C for 24 hr. (Gibson, *et. al.*, 1945).

For carbohydrate fermentation test CHL medium was used with different sugar disks. Overnight grown culture were inoculated in CHL medium and incubated at 37°C for 24 hr. to 48 hr. (Crittenden, *et. al.*, 2002).

PIB Bryant Software

PIB Bryant software programme was used for the probabilistic identification of unknown bacteria up to species level. This program has *Lactobacillus* identification matrices, in which the results of all biochemical tests including carbohydrate fermentation are fed and it gives result in percentage form closely similar to different species of *Lactobacillus*.

RESULTS AND DISSCUSION

A total 30 strains were isolated from 4 cow milk and 4 curd samples and initially tested for cultural, morphological characteristics and then they were subjected to genus specific PCR. All the organisms were found to be rod shaped. Among 30 isolates, five isolates showed 250 base pair product confirming that they belong to the *Lactobacillus* genus. These results are complete agreement with Dubernet *et. al.*, 2002, who also demonstrated amplification of 250 bp product in the PCR assay (Fig.1). These five *Lactobacillus* isolates namely CM 25, CM 27, CM 28, CM 33 and CM 34, subjected to biochemical tests. Results

produced acid and color change was observed (Fig. 2 A, B, C).

Above results of cultural, morphological and biochemical tests including carbohydrate fermentation tests were fed into *Lactobacillus* matrice of the PIB Bryant software and the following results were obtained from the programme (Table 3) CM 25 identified as a *Lactobacillus plantarum*, its output reached to 0.9800, similarly output percentile for CM 27 was 0.9690and it was identified as *L. lactics* , output percentile of CM 28 was 0.7695 and it was identified as *Lactobacillus plantarum*, CM 33, with output percentile of 0.9800 was identified as *L. casei* subsp. *rhamnosus*, CM 34 was identified as *L. casei* subsp.*casei*. Its identification scores was 0.8953. The results further match with Bergey's manual (Buchanan and Gibbons, 1974).

Table 1. Cultural, morphological and biochemical activities of the *Lactobacilli* isolates

S. No.	Tests	*Lactobacilli* isolates				
		CM 25	CM 27	CM 28	CM 33	CM 34
1	Colonies on MRS Agar	Off white in color	Off white in color	Off white in color	Off white in color	Off white in color
2	Gram Reaction & Cell Shape	Gram positive Long rods	Gram positive Short rods	Gram positive Short rods	Gram positive Short rods	Gram positive Short rods
3	Catalase test	-	-	-	-	-
4	Growth at 45°C	-	+	-	+	-
	15°C	+	-	+	+	+
5	Gas from Glucose	No gas production	No gas production	No gas production	No gas production	No gas production
6	Litmus Milk Coagulation	In 48 hours	In 48 hours	In 48 hours	In 48 hours	In 48 hours
7	Growth on BCP	Yellowish Colonies	Yellowish Colonies	Yellowish Colonies	Yellowish Colonies	Yellowish Colonies
8	Nitrate Reduction	-	-	-	-	-
9	Arginine Hydrolysis	-	-	-	-	-

Table 2. Fermentation of different sugar by *Lactobacilli* isolates

S. No	Lactobacilli Isolates	Ar	Ce	Ga	Mo	Me	Ma	Rf	Rh	Sa	So	Su	La	Is	Te	Xy	Fc
1	CM 25	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+
2	CM 27	-	-	+	+	-	+	-	-	+	-	+	+	-	-	-	+
3	CM 28	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+
4	CM 33	+	+	+	+	-	+	-	+	+	+	+	+	-	+	-	+
5	CM 34	-	+	+	+	-	+	-	-	+	+	+	+	-	+	-	+

Ar = Arabinose Sa= Salicin Ce = Cellobiose So= Sorbitol Ga = Galactose Su= Sucrose
Mo = Maltose La= Lactose Me = Melibiose Is = Inositol Ma = Mannose Te= Trehalose
Rf = Raffinose Xy= Xylose Rh = Rhamanose Fc= Fructose

Table 3. Identification of *Lactobacilli* using PIB Bryant Software

S.No	*Lactobacilli* Isolate	Possible Strains	Identification Score
1	CM 25	*Lactobacillus plantarum*	0.9800
2	CM 27	*Lactobacillus lactics*	0.9690
		Lactobacillus leichmanii	0.0253
		Lactobacillus casei subsp.*casei*	0.0037
		Lactobacillus helveticus	0.0010
3	CM 28	*Lactobacillus plantarum*	0.7695
		Lactobacillus fermentum	0.1943
		Lactobacillus lactics	0.330
		Lactobacillus leichmanii	0.0626
4	CM 33	*Lactobacillus casei* subsp. *rhamnosus*	0.9800
5	CM 34	*Lactobacillus casei* subsp.*casei*	0.8953
		Lactobacillus alact	0.0416
		Lactobacillus casei subsp. *rhamnosus*	0.0416
		Lactobacillus leichmanii	0.0208

CONCLUSION

The present study reveals the identification of five different probiotic *Lactobacillus* strains. Correct identification of them by PCR assay and biochemical tests aided with PIB Bryant software may prove useful to study their exact role in intestine to reduce serum cholesterol.

REFERENCES

1. Aguire, M. and Collins, M.D. Lactic acid bacteria and clinical infection. *J. Appl. Bacteriol*., 1993; **75**: 95-107.
2. Gasser, F. Safety of lactic acid bacteria and their occurrence in human clinical infection. *Bull. Inst. Pastur*. 1994; **92**: 45-67.
3. Fuller, R. Probiotics in human medicine. *Gut*.1991; **32**: 439-42.
4. Verthier, F. and Ehrlich, S.D. Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design PCR primer for its detection using randomly amplified polymorphic DNA. *J. Syst. Bacteriol*.1999; **49**: 997-1007.
5. Andrighetto, L., de Ded, P., Lambardi, A., Neviane, E., Rossetti, L. and Giraffa, G. Molecular identification and cluster analysis of homofermentative thermophilic lactobacilli isolated from dairy products. *Res. Microbiol*.1998; **149**: 631-643.
6. Kwon, H.S., Yang, E.H., Yeon, S.W., Kang, B.H. and Kim, T.Y. Rapid identification of probiotics *Lactobacillus* species by multiplex PCR using species- specific primer based on the region extending from 16S rRNA. *FEMS Microbiol. Letter*. 2004; **239**: 267-275.
7. Song, Y.L., Kato, N. Liu, C.X., Matsumiya, Y., Kato, H. and Watanabe, K. Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assay using group and species -specific primer derived from the 16S- 23S rRNA integenic spacer region and its flanking 23S rRNA. *FEMS Microbiol. Letter*. 2002; **187**: 167-173.
8. Bryant, T.N. A compilation of probabilistic bacterial identification marices. 1993; **5**: 207-210.
9. De Man, J.C., Rogosa, M., and Sharpe, M.E. A medium for cultivation of lactobacilli. *J. Appl. Bacteriol.*, 1959; **22**: 329.
10. Pospiech, A. and Neikmann, B. *T I G*. 1995; **11**: 217-218.
11. Nakatawa, T., Shimada, M., Mukai, H., Asada, K., Kato,. And Sato,. T. Detection of alcohol – tolerant bacteria by PCR. *Appl. Enviro. Microbiol*. 1994; **60**: 637-640.
12. Tanncok, G.W., Timisjasrvi, A.T., Rodtong, S.NG.J., Munro, K. Alatossva, T. Identification of *Lactobacillus* isolates from gastrointestinal tract, silage and yoghurt by 16S-23S rRNA gene antigenic spacer region sequence compressions. *Appl. Enviro. Microbiol*. 1994; **65**: 4264-4267.
13. Torriani, S., Zapparoli, G., Dellaglio, F. Use of

PCR-based method for rapid differention of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactic*. *Appl. Enviro. Microbiol*. 1994; **65**: 4351-4356.

14. Dubernet, S., Desemasures, N. and Gueguen, M. A PCR- based method for identification of lactobacilli at genus level. *FEMS Microbiol. Letter*. . 2002; **214**: 271-275.
15. Moreira, J.L.S., Mota, R.M., Hota, M.F., Teixrira, S.M.R., Neumann, E., Nicoli, J.R., Nunes, A.C. Identification to the species level of *Lactobacillus* isolates in probiotic prospecting studies of human, animal or food origin by 16S-23S rRNA resection profiling. *BMC Microbiol*. 2005; **5**: 1-9.
16. Gibson, T. and Malek, T.Y. J. *Dairy Res*. 1945; **14**: 35-42.
17. Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagestrom, R., Matto, J., Saarela, M., Sandholm, T.M., and Poutanen, K. *In-vitro* fermentation of cereal dietary fiber carbohydrates by probioitc and intestinal bacteria. *J. sci. Food Agri*. 2002; **82**: 781-789.
18. Buchanan, R.E. and Gibbons, N.E. Bergey's manual of determinative bacteriology, 8th ed., Williams and Co., Baltimore, MD, U.S.A. 1974.