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Isolation and Identification of *Bifidobacterium* sp. in Iranian Traditional Dairy Products

[1,2]M. Heidarpour, [2]F. Mokhtari, [3]S. Mirdamadi and [4]A. Gharashi
[1]Islamic Azad University of Karaj, Iran
[2]Institute of Standards and Industrial Researches of Iran (ISIRI), Iran
[3]Iranian Research Organization of Science and Technology (IROST), Iran
[4]National Institute for Genetic Engineering and Biotechnology (NIGEB), Iran

Abstract: The probiotic effect of *Bifidobacterium* explains the popularity of these bacteria in different commercial products. Fermented dairy products are generally considered to be one of the most suitable vehicles for the administration of an adequate number of probiotic bacteria to the consumer. In this study the aim was focused to isolate the strains which may exist in traditional dairies for further use as probiotic strains. In order to achieve our goal and find local strains 100 samples were taken from a wide area of Iran. Total 43 isolated colonies which have been identified by selective culture media, biochemical tests and antimicrobial resistance to mupirocin and confirmed by enzymatic assay with F6PPK, were exposed to PCR assay using Bif164/Bif662 primers which amplified a 523 bp region of the 16SrRNA gene. 30 isolates out of 43 primarily isolated colonies, which match all of microbiological and molecular assays reported as species belonging to *Bifidobacterium* genus.

Key words: *Bifidobacterium*, F6PK, identification, PCR, mupirocin, probiotic

INTRODUCTION

Bifidobacteria are gram-positive, pleomorphic rods, ranging from uniform to branched, bifurcated Y and V forms, spatulate or club shaped. They are strictly anaerobic (although some strains can tolerate oxygen in the presence of carbon dioxide); non-motile and non-spore-forming which constitute an important class of organisms in the intestinal microflora of healthy children and adults (George, 2005). They have different health-promoting properties such as stimulation of immune responses, reduction of growth of many potential pathogens and prevention of constipation, diarrhea and intestinal infections. They colonize the neonatal intestine from the first week after birth. Most studies are focused on identification of new strains which may be useful as probiotics.

In recent years, different methods based on microbiological culture media, enzymatic and molecular assays have been developed for identification of *Bifidobacterium* sp. Several selective culture media were investigated to isolate bifidobacteria from feces and environment. An enzymatic assay (Vlkovaa *et al*., 2005) based on the following reaction has been known as the best specific method for identification and distinguishing of *Bifidobacterium* which is just limited to this genus. Bifidobacteria ferment hexoses by a fructose-6-phosphate phosphoketolase (F6PPK) shunt and are the only intestinal bacteria known to utilize this fermentation pathway. It is often termed the “bifidus pathway”. Fructose-6-phosphate phosphoketolase catalyses the splitting of fructose-6-phosphate to erythrose-4-phosphate and acetyl-phosphate. Demonstration of F6PPK activity in cellular extracts has been a useful method for differentiating bifidobacteria from morphologically similar bacteria.

Biochemical tests for the identification of members of the genus *Bifidobacterium* are now largely superseded by the use of the genus-specific PCR primers described by Kok *et al*. (1996) and Pfefferkow *et al*. (1997). These primers amplify a 523 bp or 1.35 kbp region, respectively, of the 16SrRNA gene (Table 1). Genus-specific probes, Bif164 and Im3, have been proved to be useful in the enumeration of total populations of bifidobacteria in faecal and food samples, respectively. The use of primers targeting different regions of the 16SrDNA led to simultaneous detection of several isolates of *Bifidobacterium* sp. (Tannock, 1999).

Corresponding Author: H. Mojgan, Institute of Standards and Industrial Researches of Iran (ISIRI), Iran

Table 1: Currently used genus-specific primers for *Bifidobacterium*

Primers	Sequence	Region	Product size (bp)	References
Bif164-PCR	GGGTGGTAATGCCGGATG			
Bif662-PCR	CCACCGTTACACCGGGAA	16SrDNA	523	Kok *et al.* (1996)
lm26	GATTCTGGCTCAGGATGAACG			
Lm3r	CGGGTGCTICCCACTTTCATG	16SrDNA	1.35k	Kaufmann *et al.* (1997)

MATERIALS AND METHODS

Samples: Samples containing raw and cooked milk, traditional yogurt, cheese and Iranian dough were collected from farms in western provinces of Iran. Immediately about 2 g of each sample was inoculated in 18 mL m-MRS broth as a transport and enrichment medium. 500 mg L^{-1} L-cystein hydrochloride has been added to MRS broth to reduce the ox-re potential. The inoculated media were incubated at 37°C for 48 h in anaerobic conditions. The procedure was performed at the same time on 3 positive strains (*Bifidobacterium bifidum,* PTCC 1644, *Bifidobacterium angulatum,* PTCC 1366; and *Bifidobacterium animalis,* PTCC 1631) and 3 negative strains (*Lactobacillus casei subsp.casei* PTCC 1608; *Lactobacillus acidophilus* PTCC 1643 and *Lactococcus lactis subsp. Lactis* PTCC 1336).

Isolation by selective media: Three media used for the isolation of bifidobacteria. Dicloxacillin–Propionic acid medium (DP) (Roy, 2001), *Bifidobacterium* medium (BFM) (Nebra and Blanch, 1999) and MRS-NPLN medium prepared according to Dave and Shah (1996) in Van de Casteelea *et al.* (2006).

All of media consisted a base medium which have been modified by using antibiotics, sugars, lithium chloride, L-cystein hydrochloride and propionic acid to make selective for isolation of bifidobacteria (Fachin *et al*., 2006).

In order to obtain single colonies, the cultured m-MRS broths were inoculated on the surface of the selective media and incubated at 37°C for 72 h in anaerobic condition.

Typical colonies were used for further investigations.

Identification: All typical isolated colonies on selective agars were submitted to Gram staining, the catalase and oxidase test, coagulation in skimmed milk, gas generation (Benama *et al*., 2005) antimicrobial resistance to mupirocin and enzymatic assays by F6PP as well as molecular assay by species specific PCR. Colonies and cells morphology on the selective agars were also examined.

Enzymatic assay by F6PP: The isolates considering belong to the genus *Bifidobacterium* were identified by the detection of fructose-6-phosphate phosphoketolase (F6PPK) enzyme in cellular extracts as described by Scardovi and Trovatelli (1965).

Cells were grown in 10 mL of TPY broth at 37°C for 18 h and harvested by centrifugation at 5000 g for 10 min. The pellet was washed twice with 5 mL of 0.5 g L^{-1} phosphate cysteine buffer. After centrifugation, the pellet was collected in 1 mL buffer, 50 μL lysozyme added and after incubation for 1 h cells were disrupted by 0.4 mL hexadecyltrimethylammonium bromide (CTAB 0.45 mg mL^{-1}) for obtaining crud cells extract. Use of CTAB to disrupt cell membranes is an effective alternative to the time consuming traditional cell disruption procedures and increases the number of cultures that can be simultaneously assayed and presumptively identified using the phosphoketolase assay. 0.25 mL of reagents (6 mg mL^{-1} NaF, 10 mg mL^{-1} sodium iodoacetate and 80 mg mL^{-1} fructose-6-phosphate) was added to the cells extract. The reaction was started by incubation 30 min at 37°C and stopped by adding 1.5 mL of hydroxylamine-HCl (13.9%). After 10 min, 1 mL of trichloroacetic acid (15%) and 1 mL of $FeCl_3.6H_2O$ (5%) were added. A negative control consisting of all reagents except the substance (fructose-6-phosphate) as well as a control strain used to verify the color. The presence of fructose-6- phosphate phosphoketolase enzyme was revealed by the appearance of red and purple colors (Orban, 2000).

Antimicrobial resistance to mupirocin: The isolated colonies and control strains were grown in the m- MRS broth at 37°C for 20 h. Twenty mL of modified MRS agar was seeded with 1 mL grown culture and poured to Petri dishes. Afterwards, the paper discs contained 200 mg of mupirocin were applied on the agar surface (Rada, 1997). Plates were incubated in anaerobic condition at 37°C for 30 h. Thereafter, the sensitivity was observed as clear zones surrounding discs. The diameters of inhibition zones were measured in mL, less than 10 mm was considered resistant, equal to or greater than 10 mm considered sensitive.

Alternatively, by adding the concentrations 50, 75 and 100 mg L^{-1} mupiricin to m-MRS agar (BSM), the susceptibility of isolated colonies and control microorganisms were examined. The grown colonies in MRS broth were inoculated on the surface of BSM. Growth of resistant bacteria was well, while susceptible microorganisms has shown no growth (Simpsona *et al*., 2004; Sampo *et al*., 2006).

Molecular assay by species specific PCR

DNA preparation: Isolated colonies considered to be *Bifidobacterium*, were inoculated in m-MRS broth for overnight at 37°C in anaerobic condition. About 1mL of the cells were washed and resuspended in 500 μL lysis buffer (10 mM Tris-HCl, pH 7.8, 5mM EDTA, pH 8.0, lysozyme 1.0 mg mL^{-1}) and incubated at 37°C for 2-3 h; 10 μL proteinase K (10 mg mL^{-1}) and 12.5 μL SDS (20%) were then added and the mixture was incubated at 55°C for 2-3 h. Proteinase K should be inactivated at 95°C for 5-10 min. DNA was extracted from crude cell lysates by phenol (ISO, 2005). Chromosomal DNA was precipitated with 96% ethanol and after centrifugation; the pellet was dissolved in 70% ethanol, in order to obtain high quality DNA. The pellet remained after centrifugation dissolved in 30 μL deionized water. The purity and concentration of nucleic acids was confirmed by gel electrophoresis and UV spectrophotometry (Spanova and Rittich, 2006).

DNA amplification was carried out using a genus-specific primer set: Bif164/Bif662 (Kok *et al*., 1996) targeted on 16S rDNA region. The amplification reactions (50 μL PCR mixture containing: 1 μL dNTP10 mM, 1.5 μL $MgCl_2$ 50mM, 5 μL 10X buffer, R and F primer each 2 μL from stock 10 μM, 0.2 μL Taq DNA polymerase 5 U μL^{-1} all purchased from Cinnagen) were carried out in a Tochgene Gradient TECHNE Cycler (FTGRAD20) using the following cycle parameters: 5 min of the initial denaturation period at 94°C (hot start), 45 sec of denaturation at 94°C, 50 sec of primer annealing at 56°C and 60 sec of extension at 72°C. The final elongation step was prolonged to 5 min, the number of cycles was 35. The PCR products were separated using electrophoresis in 1% agarose gel in TBE buffer (45mM boric acid, 45mM Tris-base, 1mM EDTA, pH 8.0). DNA was stained using ethidium bromide (0.5 mg mL^{-1}), the gel was destained in water and photographed at 260 nm UV light.

RESULTS

Isolation by selective media: Samples which have grown on all of three selective media (BFM, MRS-NLNP and DP) considered as positive. The samples which have grown on one or two media, have been discarded. 43 samples out of 100 samples grew on the selective media and were submitted for identification. All of control positive strains had good growth on all of three media, while 3 lactobacilli as a negative control had no growth on BFM and DP, but showed rare growth on MRS-NLNP.

Identification: The 43 isolates, have grown on 3 selective media (BFM, MRS-NLNP and DP). The selectivity of BFM did not affect the growth of isolates and most of them showed round, blue colonies approximately 2 mm in diameter a few minutes after removal from the anaerobic condition. Grown colonies on MRS-NLNP were creamy to white. Although MRS-NLNP agar has been suggested for isolation and enumeration of bifidobacteria, in this study we observed that other strains (for example: lactobacilli) can also grow on it. We found that 2 mg L^{-1} of Dicloxacillin which has been suggested by Roy may inhibit the growth of *Bifidobacterium*, since neither isolates no positive strains had grown on the mentioned medium. When the concentration of Dicloxacillin has reduced to 1 mg L^{-1}, both positive strains and isolates were grown on this medium.

All purified isolates were Gram positive bacteria and negative for catalase, oxidase, generated gas in the m-MRS broth and coagulated milk between 6-8 h incubation with a thick and quite firm coagulum. All of 43 isolates which had met the mentioned criteria, considered as *Bifidobacterium* genus.

Mupirocin susceptibility testing was performed to differentiate bifidobacteria from lactobacilli, because cultural and biochemical properties of both genera are largely overlapped. While, bifidobacteria were consistently resistant to mupirocin, all lactobacilli which had been used as negative control strain were susceptible. The isolates as well as positive controls showed mupirocin resistance.

The phosphoketolase assay is commonly used as a definitive criterion for identification of bifidobacteria. The test by F6PPK was performed on all of the isolates which were considered to belong to *Bifidobacterium* genus. All of 43 were F6PP positive. The mentioned test is the most unique and specific non-molecular test for identification of bifidobacteria.

By all these characters, we confirmed the *Bifidobacterium* genus. The majority of the isolates have been identified as belonging to *Bifidobacterium.* Further tests were performed by amplification by a genus specific primer Bif164/662 which targets the 16SrRNA. A PCR product with 523 bp length was suspected. Products with the same length were detected (Fig. 1). The expected region in 16SrDNA was found in 30 out of 43 isolates, among which 19 strains have been isolated from milk and raw milk. For further studies, nucleotide sequencing is in process.

In this study we have not detected bifidobacteria from dairy products with low pH, which is due to the sensitivity of this bacteria to low pH.

- Ladder marker (100-3000).
- Control starin (*Bifidobacterium bifidum,* PTCC 1644).
- Control starin (*Bifidobacterium animalis,* PTCC 1631).

Fig. 1: Genus-specific PCR using Bif164/662 primer-pair

- Isolated bacteria from raw milk.
- Isolated bacteria from raw milk.
- Isolated bacteria from yoghurt.
- Negative control strain (*Lactobacillus casei subsp.casei* PTCC 1608).

DISCUSSION

BFM is antibiotic-free and includes lactulose as the main carbon source as well as propionic acid and the inhibitory agents methylene blue and lithium chloride. BFM was able to sustain growth of all tested strains except for one type strain of B. bifidum and strain LMG 11084 of B. breve (Nebra and Blanch, 1999). Overall, colonies obtained on BFM tended to be very small and hence difficult to pick. BFM did not allow growth of the yoghurt starter cultures and L. acidophilus (Masco *et al*., 2005; Shah, 2003).

The recovery on BFM agar suggests the practical use of BFM for enumeration of *Bifidobacterium* in routine monitoring of fermented dairy products. Two vitamins (riboflavin and thiamine) acted as growth factor promoters. Propionic acid, lithium chloride and methylene blue inhibited the growth of some related bacterial species. The low pH (5.5) of BFM inhibited the growth of *Enterobacteriaceae*. Wide width of physiological of bifidobacteria characterization, suggests that a second media such as MRS-NLNP or DP medium should be used in addition to BFM.

Enzymatic assay by F6PP is the key test for identification of bifidobacteria, since all bacteria other than bifidobacteria show negative F6PP (except *Gardenella vaginalis*). In our study negative control strains were F6PP negative too, while all positive control strains as well as isolated strains were F6PP positive. A limitation of this assay is the time-consuming process of cell disruption by sonication. We have replaced the time consuming cell disruption process with a more rapid cell membrane disruption process by the detergent hexadecyltrimethylammonium bromide (CTAB) (Orban, 2000).

We have found out also that a pretreatment with lysozyme makes the cell disruption more effective.

Antimicrobial resistance to mupirocin together with F6PP assay could act as a key diagnostic way for identification of bifidobacteria. According to our findings it is suggested to use mupirocin as a supplement in the enrichment broth (such as m-MRS broth) used in the first steps of isolation from dairy products to eliminate the related microflora especially lactobacilli which could cover the growth of bifidobacteria.

Molecular assay is an effective diagnostic tool for confirmation of isolates. Amplification of 16S rDNA region, was used for confirmation the isolates which considered as bifidobacteria by cultural and enzymatic methods. Using analysis of 16S rRNA gene (rDNA) probes, specific for the genus *Bifidobacterium* (Tahahiro *et al*., 2003), enabled us to detect Bifidobacterium genus in dairy products exclusively. Another probes targeted 16SrDNA Lm26/Lm3 primers (Kaufmann, 1997) could also been used for detection of Bifidobacterium genus from food, but as well as short length PCR products would be more adequate for subsequent investigations, Bif164/662 were used in our study.

CONCLUSION

Isolation of Bifidobacterium from dairy products demonstrates that these products are good source for probiotic strains, especially *Bifidobacrium* sp. For isolation of local strains of probiotics which are more compatible with the gastrointestinal microflora of people in each area, the conventional products could be used. We are investigating new strains which may be used as industrial probiotics.

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REFERENCES

Benama, H.M., R.N. Saidi, D.E. Henni and M. Kihal, 2005. Identification of cultivable *Bifidobacterium* sp. isolated from breast-fed infants feces in West-Algeria, Afr. J. Biotechnol., 4 (5): 422-430.

Fachin, M.J., L.A.L.N. Gândara1 and W.H. Viotto, 2006. Evaluation of culture media for counts of *Bifidobacterium* animalis in the presence of yoghurt bacteria. Brazilian J. Microbiol., 37: 516-520.

George, G.M., 2005. Bergey's Manual of Systematic Bacteriology. 2nd Edn. LXXXII.

ISO 21571, 2005. Foodstuffs-Methods of analysis for the detection of genetically modified organisms and derived products- Nucleic acid extraction.

Kok, A. de Waal, F. Schut, G.W. Welling, G. Weenk and K.J. Hellingwerf, 1996. Specific detection and analysis of a probiotic *Bifidobacterium* strain in infant feces. Applied Environ. Microbiol., 62 (10): 3668-3672.

Masco, G.L., Huys, E. De Brandt, R. Temmerman and J. Swings, 2005. Culture-dependent and Culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria. Int. J. Food Microbiol., 102: 221-230.

Nebra and A.R. Blanch, 1999. A new selective medium for *Bifidobacterium* sp. Applied and Environ. Microbiol., pp: 5173-5176.

Orban, J.A. Patterson, 2000. Modification of the phosphoketolase assay for rapid identification of bifidobacteria. J. Microbiol. Methods, 40: 221-224.

Pfefferkorn, K.A., M. Teuber and L. Meile, 1997. Identification and Quantification of *Bifidobacterium* sp. isolated from Food with Genus-Specific 16SrRNA-Targeted Probes by Colony Hybridization and PCR, Applied and Environ. Microbiol., 63 (4): 1268-1273.

Rada, V., 1997. Department of Microbiology and Biotechnology, Detection of *Bifidobacterium* sp. by enzymatic methods and antimicrobial susceptibility testing. Biotechnol. Techniques, 11 (12): 909-912.

Roy, 2001. Media for the isolation and enumeration of bifidobacteria in dairy products. Int. J. Food Microbiol., 69: 167-182.

Sampo J., L.M. Gueimonde, A.C. Ouwehand, J.P. Reinikainen and S.J. Salminen, 2006. Comparison of 4 methods to enumerate probiotic bifidobacteria in a fermented food product. Food Microbiol., 23: 571-577.

Scardovi, V. and L.D. Trovatelli, 1965. The fructose-6-phosphate shunt as a peculiar pattern of hexose degradation in thegenus*Bifidobacterium*. Annali di Microb´ıologia ed Enzimologia, 15: 19-29.

Shah.N.P. (2003): Selective enumeration of *Lactobacillus delbruekii* sbsp. bulgaricus, *Sterptococcs thermophilus*, *Lactobacillus acidophilus*, *Bifidobacteria*, *Lactobacillus casie*. J. Dairy Sci., 86: 2288-2296.

Simpsona, P.J., G.F. Fitzgeraldb, C. Stantona and C.R.P. Ross, 2004. The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria from probiotic animal feed. J. Microbiol. Methods, 57 (1): 9-16.

Spanova, K.J., A.B. Rittich, 2006. Evaluation of amplified ribosomal DNA restriction analysis (ARDRA) and species-specific PCR for identification of *Bifidobacterium* sp., Systematic and Applied Microbiol., 29: 36-44.

Tahahiro, M., W. Koichi and R. Tanaka, 2003. Genus- and Species-Specific PCR Primers for the Detection and Identification of Bifidobacteria. Curr. Issues. Intest. Microbiol., 4: 61-69.

Tannock, G.W., 1999. Identification of Lactobacilli and Bifidobacteria, Current Issues Molec. Biol., 1 (1): 53-64.

Van de Casteelea, T. Vanheuverzwijna, T. Ruyssena, P. Van Asschea, J. Swingsb and C.G. Huys, 2006. Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. Int. Dairy J., 16: 1470-1476.

Vlkova´a, E., Nevoralb, J. Jencikovac, B.J. Kopecnyd, J. Godefrooije, I. Trojanova´a and V. Radaa, 2005. Detection of infant faecal bifidobacteria by enzymatic methods. J. Microbiol. Methods, 60: 365-373.