International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (4), pp. 001-004, April, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Amplified ribosomal DNA restriction analysis (ARDRA) of new isolated strains of bifidobacteria from newborn babies microbiota

J. Z. Mazo, A. Z. Dinon, C. Tagliari, E. C. Ilha, E. S. Sant'Anna and A. C. M. Arisi*

Departamento de Ciência e Tecnologia de Alimentos, Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, Brazil.

Accepted 24 February, 2018

Bifidobacterium **species are known for their beneficial effects on health and their wide use as probiotics. Molecular methods applied to accurately and rapidly identify these micro-organisms are necessary. This study was conducted in order to isolate *Bifidobacterium* from newborn babies' microbiota and also to identify the new isolated strains by PCR and ARDRA. The bile and the low pH tolerance for the new isolates were evaluated as evidence of possible probiotic potencial. A total of 30 strains were isolated from four newborn babies and three of them (I6, I17 and I20) were identified as *Bifidobacterium* after PCR using two genus-specific primer pairs. Digestion patterns of I17 and I20 by ARDRA were similar to *B. longum* and *B. breve* and were different to *B. bifidum* patterns. The 30 isolated strains were bile and low pH tolerant. Therefore, this study identified three *Bifidobacterium* isolated strains as good candidates for further investigation to elucidate their potential as probiotic.**

Key words: *Bifidobacterium*, ARDRA, PCR, newborn babies, restriction digestion.

INTRODUCTION

Bifidobacterium species are frequently associated with health-promoting effect in human and animal intestines (Parvez et al., 2005, Musikasang et al 2009). In recent years, growing interest for application of *Bifidobacterium* in many fermented dairy foods has prompted starter industry to screen for new isolates from culture collections or human colonic flora (Mayer et al. 2007). Methods to accurately and rapidly identify these micro-organisms remain largely insufficient and it is difficult to discriminate among different *Bifidobacterium* species (Youn et al. 2008). For the efficient identification of *Bifidobacterium*, molecular techniques such as polymerase chain reaction (PCR) and amplified ribosomal DNA restriction analysis (ARDRA) were recently proposed (Krizovà et al. 2006, Youn et al. 2008).

The aims of this work were the isolation of *Bifidobacterium* from newborn babies' microbiota and the molecular characterization of the new isolates by PCR and ARDRA as the first step to study their possible probiotic function.

MATERIAL AND METHODS

Strain sources

The reference strains of *Bifidobacterium* used in this study were derived from the American Type Culture Collection, *B. bifidum* ATCC 29521, *B. breve* ATCC 15700, *B. longum* ATCC15707, *L. plantarum* ATCC 8014. ATCC strains and *Bifidobacterium* spp. isolates B1 (*B. animalis* subsp. lactis); and B3 (*B. adolescentis*) were kindly provided from Dr. Jacques Robert Nicoli, Federal University of Minas Gerais, Brazil. Strains were routinely grown in MRS broth (De Man, Rogosa and Sharp Broth, Oxoid, England) under anaerobic conditions at 37°C for 24 - 48 h. The anaerobic conditions were achieved through the use of anaerobic jars (Permution, Brazil) and anaerobic generators (AnaeroGen, Oxoid, England).

Bacterial isolation

Fecal samples from four newborn infants from Florianópolis, SC, Brazil, four-months old and breast-fed, were collected from the

*Corresponding author. E-mail: arisi@cca.ufsc.br. Tel: +55 48 37215382. Fax: +55 48 37219943.

Table 1. PCR conditions.

Primer pair	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final extension
Bif164	95°C	30	95°C	55°C	72°C	72°C
Bif662	5 min		1 min	1 min	2 min	5 min
PbiR2	95°C	35	95°C	50°C	72°C	72°C
PbiF1	5 min		1 min	1 min	2 min	5 min
16	96°C	40	95°C	55°C	72°C	72°C
Lpl	3 min		30 s	1 min	30 s	5 min

diapers, using the swab technique. The swab was placed in a test tube containing 10 ml of peptoned water, which was the only dilution, since the goal was only the bifidobacteria isolation and not counting. Samples diluted were transferred to LP-MRS Agar (De Man, Rogosa and Sharp Agar added of lithium chloride 0,2% w/v and sodium propionate 0,3% w/v) plates (Vinderola and Reinheimer 1999). Plate cultures were grown in anaerobic conditions at 37°C for 72 h. Isolated colonies were transferred to MRS broth and anaerobic grown at 37°C for 24 - 48 h and then purified by streaking onto MRS agar and grown in anaerobic conditions at 37°C for 48h. The isolated colonies were subjected to Gram staining and catalase test. The Gram-positive and catalase negative isolates were tested for fructose-6-phosphate phosphoketolase enzyme (F6PPK), using as positive control *Bifidobacterium longum* ATCC 15707 and as negative control *Lactobacillus plantarum* ATCC 8014, according to the modified method by Orban and Patterson (2000).

DNA extraction

One milliliter of each culture in MRS broth (36 h at 37°C) was centrifuged at 13,000 *g* for 2 minutes. The pellet was washed twice with deionized distilled water to remove PCR inhibitors. DNA isolation was carried out from pellet cells by using the Wizard Genomic DNA Purification Kit (Promega) with the addition of lisozime (10 mg/ml), as recommended for Gram positive bacteria (Delcenserie et al., 2005).

PCR and ARDRA analysis

Amplifications by PCR were performed with primer pairs Bif164/Bif662 (Kok et al., 1996) and PbiR2/PbiF1 (Roy and Sirois, 2000) allowing the identification of the *Bifidobacterium* genus and primer pair Lpl/16F allowing the identification of the *Lactobacillus plantarum* species (Berthier and Ehrlic, 1998). PCR reactions were carried out in a final volume of 25 µl containing 2 µl of template DNA, 1.5 mM $MgCl_2$, 0.2 µM of each primer (IDT), 200 µM of each dNTP and 1.25 U of *Taq* DNA polymerase (Promega) in 1X PCR buffer. Amplifications were performed in a Minicycler™ (MJ Research, Ind. Watertown, MA) or a Mastercycler® gradient 5331 (Eppendorf AG, Hamburg). PCR conditions are listed in Table 1.

The PCR products (10 ∝l reaction + 2 ∝l loading buffer) were separated through electrophoresis at 400 mA and 80V for 50 min in 2.5 % agarose gel, 1X TBE buffer and stained with ethidium bromide. Visualization was performed in UV-transilluminator and images photographed with digital camera (Canon Powershot A70).

Amplicons obtained using genus-specific Bif164/Bif662 (523 bp) and PbiR2/PbiF1 (914 bp) primers have been used for the restriction analysis using *Bam*HI, *Sau*3AI, *Taq*I, *Sau*96I e *Alu*I (Promega) according to Krizovà et al. 2006. The restriction reaction contained 10 U of each enzyme, 2 ∝l of corresponding buffer and 10 ∝l of the PCR product in a final volume of 20 ∝l. Restriction digestion proceeded for 3 h at 37 or 65°C, according to the recommendation of enzyme producer. Restriction products were separated in 2.5% agarose gel, 1X TBE buffer and stained with ethidium bromide.

Bile and pH tolerance

Bile tolerance was determined by growing the isolated strains in MRS broth supplemented with 0.3% dehydrated fresh bile (Oxgall, Difco) according to Lin et al (1991). The tolerance to low pH of the stomach was performed according to Newmann and Ferreira (1995).

RESULTS AND DISCUSSION

DNA was extracted in triplicate from 30 isolated strains and from ATCC strains *B. bifidum*, *B. breve*, *B. longum* and *L. plantarum*. Three out of thirty isolated strains and the three reference *Bifidobacterium* strains presented the expected 523 bp fragment when Bif164/Bif662 primers were used and the expected 914 bp fragment when PbiF1/PbiR2 primers were used, confirming that these three new isolated strains (I6, I17 and I20) belonged to *Bifidobacterium* genus. These PCR products were not observed for the negative control *L. plantarum* ATCC strain (Figure 1a and b). No PCR products were observed when the isolated strains and the *Bifidobacterium* reference strains were submitted to PCR using *L. plantarum* species-specific 16/Lpl primers (data not shown).

The *Alu*I digestion of PCR product amplified using Bif164/Bif662 primers were carried out and it was possible to observe the same profile for all isolated samples and reference strains (Figure 1c). The same was observed after digestion with *Sau*3AI, *Taq*I, *Sau*96I (data not shown). These enzymes were not helpful for differentiation among these specific species. The amplicon of *B. bifidum* reference strain were not digested when *Bam*HI were used to digest PCR product obtained with Bif164/Bif662 and PbiF1/PbiR2 primers (Figure 1c). It is in agreement with results obtained by Krizovà et al. (2006) and it is useful to differentiate *B. bifidum* from the other two reference ATCC strains (*B. longum* and *B. breve*). Digestion patterns of the two new isolates I17 and I20 differed from *B. bifidum* and were similar to *B. longum* and *B. breve* (Figure 1c). In this case, it was difficult to

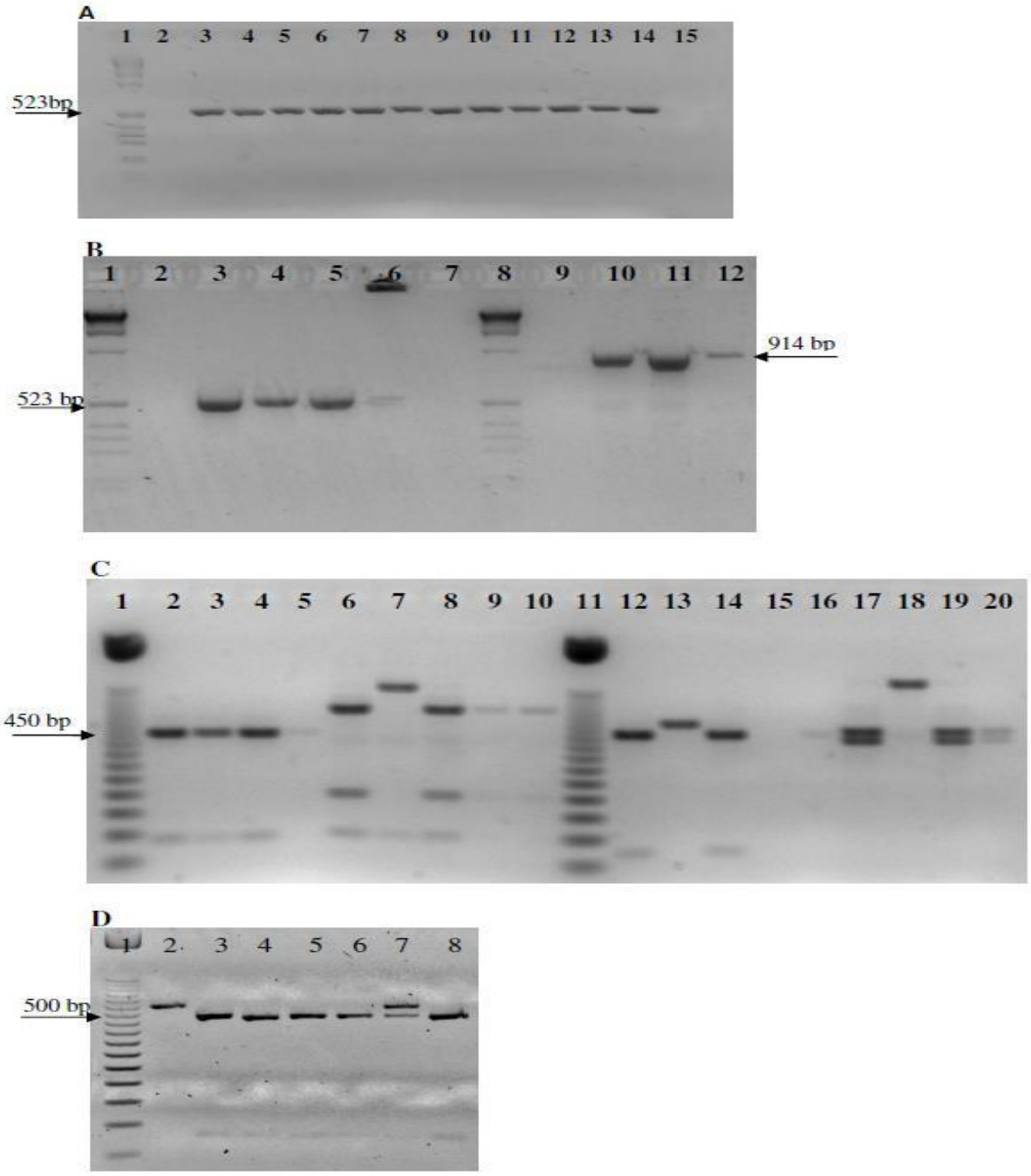

Figure 1A. PCR for *Bifidobacterium* genus identification using Bif164/Bif662 primers 1: 1Kb ladder; 2: water ; 3: *B. longum* ATCC15707 ; 4: isolate B1 (*B. animalis* subsp. *lactis*); 5: *B. bifidum* ATCC 29521; 6: *B. breve* ATCC 15700 ; 7: isolate I6; 8: isolate B3 (*B. adolescentis*); 9: isolate B1(*B. animalis* subsp. *lactis*); 10: *B. bifidum* ATCC 29521 ; 11: *B. breve* ATCC 15700 ; 12: *B. longum* ATCC15707 ; 13: *B. bifidum* ATCC 29521 ; 14: isolate I17, 15: *L. plantarum*. **B.** PCR for *Bifidobacterium* genus identification using Bif164/Bif662 (2-6) and PbiF1/PbiR2 (8-12) primers: 1: 1 Kb ladder, 2: water, 3: *B. longum* ATCC15707, 4: *B. bifidum* ATCC 29521, 5: *B. breve* ATCC 15700, 6: sample I20, 7: gap, 8: 1Kb ladder, 9: water, 10: *B. longum* ATCC15707, 11: *B. bifidum* ATCC 29521, 12: sample I20. **C.** ARDRA *Alu*I after Bif164/Bif662 amplification (1-5): 1: 50 bp ladder; 2: *B. longum* ATCC15707; 3: *B. bifidum* ATCC 29521; 4: *B. breve* ATCC 15700; 5: sample I20; ARDRA *Alu*I after PbiF1/PbiR2 amplification (6-10): 6: *B. longum* ATCC15707; 7: *B. bifidum* ATCC 29521; 8: *B. breve* ATCC 15700; 9-10: sample I20; ARDRA *Bam*HI after Bif164/Bif662 amplifciation (11-16): 11: 50 bp ladder; 12: *B. longum* ATCC15707; 13: *B. bifidum* ATCC 29521; 14: *B. breve* ATCC 15700; 15: sample I20, 16: sample I17; ARDRA *Bam*HI after PbiF1/PbiR2 amplification (17-20): 17: *B. longum* ATCC15707; 18: *B. bifidum* ATCC 29521; 19: *B. breve* ATCC 15700; 20: sample I20. **D.** ARDRA *Bam*HI after Bif164/Bif662 amplification 1:50bp ladder; 2:isolate B1 (*B. animalis* subsp. *lactis*); 3: *B. longum* ATCC15707; 4:isolate B3 (*B. adolescentis*); 5: *B. bifidum* ATCC 29521; 6: *B. breve* ATCC 15700; 7:isolate I6; 8: isolate I17.

discriminate between different species, because the resulting band patterns were common to more than one *Bifidobacterium* species. Digestion pattern of the isolate I6 differed from the reference ATCC strains *B. bifidum, B. longum* and *B. breve* (Figure 1d).

The 30 isolated strains presented bile and low pH tolerance. Therefore, the three isolates identified as *Bifidobacterium* are good candidates for further investigation to elucidate their potential as probiotic. In vitro and *in vivo* studies must be conducted to confirm their health-promoting effects and their application in the food industry.

ACKNOWLEDGMENTS

This work was financially supported by CNPq process 506148/2004-1, Ministry of Science and Technology, Brazil. This work was approved by Ethical Comitte of Universidade Federal de Santa Catarina (209/2006). C.T is supported by IC fellowship from CNPq. We would like to express our gratitude to Dr. Jacques Robert Nicoli, Universidade Federal de Minas Gerais, Brazil, for the provision of ATCC strains and *Bifidobacterium* spp. isolates B1 and B3.

REFERENCES

Berthier F, Ehrlic SD (1998). Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. FEMS Microbiol. Let., 161: 97-106.

Delcenserie V, Bechoux N, China B, Daube G, Gavini F (2005). A PCR method for detection of bifidobacteria in raw milk and raw milk cheese: comparison with culture-based methods. J. Microbiol. Meth., 61: 55-67.

Kok RG, De Waal A, Schut F, Welling G, Weenk G, Hellingwerf KJ (1996). Specific detection and Analysis of a Probiotic *Bifidobacterium* strain in infant feces. Appl. Environ. Microbiol., 62(10): 3668-3672.

Krizovà J, Spanovà A, Rittich B (2006). Evaluation of amplified ribosomal DNA restriction analysis (ARDRA) and species-specific PCR for identification of *Bifidobacterium* species. Syst. Appl. Microbiol., 29: 36-44.

Lin MY, Savaiano D, Harlander S (1991). Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. J. Dairy Sci., 74(1): 87-95.

Mayer HK, Amtmann E, Philippi E, Steinegger G, Mayrhofer S, Kneife W (2007). Molecular discrimination of new isolates of *Bifidobacterium animalis* subsp. lactis from reference strains and commercial probiotic strains. Int. Dairy J., 17: 565-573.

Musikasang H, Tani A, H-kittikun A, Maneerat S (2009). Probiotic potential of lactic acid bacteria isolated from chicken gastrointestinal digestive tract. World J. Microbiol. Biotechnol., 25(8): 1357-1345.

Neumann E, Ferreira CLLF (1995). *Lactobacillus acidophilus* as dietary adjunct: in vitro susceptibility to gastric juice, bile salts, lysosyme and chemotherapics. Rev. Microbiol., 26(1): 59-65.

Orban JI, Patterson JA (2000). Modification of the phosphoketolase assay for rapid identification of Bifidobacteria. J. Microbiol. Meth., 40: 221-224.

Parvéz S, Kim HY, Lee HC, Kim DS (2006). Bile salt hydrolase and cholesterol removal effect by *Bifidobacterium bifidum* NRRL 1976. World J. Microbiol. Biotechnol., 22: 455-459.

Roy D, Sirois S (2000). Molecular differentiation of *Bifidobacterium* species with amplified ribosomal DNA restriction analysis and alignment of short regions of the ldh gene. FEMS Microbiol. Lett., 191: 17-24.

Vinderola CG, Reinheimer JA (1999). Culture media for the enumeration of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in the presence of yoghurt bacteria. Int. Dairy J., 9: 497-505.

Youn SY, Seo JM, Ji GE (2008). Evaluation of the PCR method for identification of *Bifidobacterium* species. Lett. Appl. Microbiol., 46: 7-13.