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Evaluation of amplified ribosomal DNA restriction analysis (ARDRA) and species-specific PCR for identification of *Bifidobacterium* species

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Abstract

Molecular biological methods based on genus-specific PCR, species-specific PCR, and amplified ribosomal DNA restriction analysis (ARDRA) of two PCR amplicons (523 and 914 bp) using six restriction enzymes were used to differentiate among species of *Bifidobacterium.* The techniques were established using DNA from 16 type and reference strains of bifidobacteria of 11 species. The discrimination power of 914 bp amplicon digestion was higher than that of 523 bp amplicon digestion. The 914 bp amplicon digestion by six restrictases provided unique patterns for nine species; *B. catenulatum* and *B. pseudocatenulatum* were not differentiated yet. The *Nci*I digestion of the 914 bp PCR product enabled to discriminate between each of *B. animalis*, *B. lactis,* and *B. gallicum*. The reference strain *B. adolescentis* CCM 3761 was reclassified as a member of the *B. catenulatum*/*B. pseudocatenulatum* group. The above-mentioned methods were applied for the identification of seven strains of *Bifidobacterium* spp. collected in the Culture Collection of Dairy Microorganisms (CCDM). The strains collected in CCDM were differentiated to the species level. Six strains were identified as *B. lactis*, one strain as *B. adolescentis*.

Keywords: *Bifidobacterium* spp.; Identification; PCR; ARDRA

Introduction

Bifidobacteria were first isolated by Tissier from the faeces of breast-fed infants in 1900, termed *Bacillus bifidus,* and it was not until 1924 that the new genus *Bifidobacterium* was proposed for classification by Orla-Jensen [32,33]. These bacteria are generally considered to be non-pathogenic [2,32]. From among the total number of *Bifidobacterium* species, *B. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B. pseudocatenulatum, B. longum, B. infantis, B. gallicum,* and *B. dentium* have been reported to be the human intestinal bifidobacteria [9,16,23,24].

Already at the beginning of the 20th century, Metchnikoff proposed the concept that bifidobacteria and other lactic acid bacteria (LAB) in the intestine might be associated with good health and longevity of humans [25]. Many studies and experiments postulated that human-specific species of *Bifidobacterium* have many favourable effects on the host [4,5,11,15,22,25]. Therefore some species of bifidobacteria have been widely used in the food industry as cultures enriching fermented products [40] and dietary supplements [8].

The use of probiotic bifidobacterial cultures necessitates the correct identification of bacterial species, including their characteristics. In some cases the traditional phenotypic identification methods provide unreliable results [19,28,41]. The recently developed genotypic traits-based technologies have been rapidly

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replacing the conventional methods [3]. Many molecular techniques have been reported as the tools providing significant information of the genera, species, and strains of bifidobacterial cultures [1,8,14,17,18,26,30,34]. The rapid, reliable, and sensitive PCR method with specific 16S rDNA-based oligonucleotide primers has frequently been used in the detection of the genus [12,13,28] and the species of bifidobacteria [20,21,41]. Another method applied for differentiation of the strains is the amplified ribosomal DNA restriction analysis (ARDRA). This method involves amplification of 16S rDNA region, 16S–23S rDNA spacer region or 23S rDNA followed by digestion with one or more selected restriction enzymes, having a potential to discriminate bacteria to the species level [10,27,36].

The aim of this study was to evaluate the species-specific PCR and ARDRA method for the identification and characterisation of the type and reference strains of human species of *Bifidobacterium* together with *B. lactis* and *B. animalis* (species commonly added into dairy products). Subsequently, these techniques were applied to determine the identity of the strains collected in the Culture Collection of Dairy Microorganisms (CCDM).

Materials and methods

Bacterial strains and culture conditions

The type and reference strains of bifidobacteria were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), the American Type Culture Collection (ATCC, Manassas, USA), and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). A list of used strains is given in Table 1. The bifidobacteria strains tested were from the CCDM (Tábor, Czech Republic) – (Table 2). All strains were cultured on MRS medium or MRS agar (Oxoid, Hampshire, UK) supplemented with L-cysteine-hydrochloride (0.5 g/l), pH 6.5–7.0. The cultures were grown anaerobically at 37 °C overnight (18 h) to 48 h. Cellular morphology was assigned microscopically after Gram-staining of the strains. All the strains were maintained at –70 °C in MRS broth with cysteine supplemented with glycerol (15% end concentration).

DNA preparation

Altogether 2 ml of the cells were washed and resuspended in 500 µl lysis buffer (10 mM Tris–HCl, pH 7.8, 5 mM EDTA, pH 8.0, lysozyme 1.0 mg/ml), and incubated at 37 °C overnight (18 h); 10 µl proteinase K (10 mg/ml) and 12.5 µl SDS (20%) were then added and the mixture was incubated at 55 °C for 3 h. DNA was extracted from crude cell lysates by phenol [29]. Chromosomal DNA was precipitated with 96% ethanol and dissolved in 50 µl TE buffer (10 mM Tris–HCl pH 7.8, 1 mM EDTA, pH 8.0). The purity and concentration of nucleic acids was confirmed by gel electrophoresis and UV spectrophotometry.

Genus-specific PCR

Purified bacterial DNA (25 ng/µl) was used as matrix in PCR. Amplification of *Bifidobacterium* spp. DNA was carried out using two different genus-specific primer sets: (i) PbiF1/PbiR2 [28] and (ii) Bif164/Bif662 [13] targeted on 16S rDNA region. The PCR mixture contained 0.5 µl of each 10 mM dNTP, 0.5 µl (10 pmol/µl) of each primer, 0.5 µl of Taq 1.1 polymerase (1 U/µl), 2.5 µl of buffer (1.5 mM), 1–2 µl of DNA matrix, and PCR water was added up to a 25 µl volume. The primers used in this study were supplied by Generi Biotech (Hradec Králové, Czech Republic). The amplification reactions were carried out in an MJ Research Programme Cycler PTC-100 (MJ Research, Watertown, USA) using the following cycle parameters: (i) 5 min of the initial denaturation period at 94 °C (hot start), 60 s of denaturation at 94 °C, 60 s of primer annealing at 50 °C, and 120 s of extension at 72 °C. The final elongation step was prolonged to 10 min, the number of cycles was 30; (ii) hot start at 95 °C for 5 min, followed by 30 cycles of 95 °C for 60 s, 55 °C for 60 s and 72 °C for 120 s concluded by a prolonged polymerisation step to 5 min. The PCR products were separated using electrophoresis in 1.5% agarose gel in TBE buffer (45 mM boric acid, 45 mM Tris-base, 1 mM EDTA, pH 8.0). DNA was stained using ethidium bromide (0.5 µg/ml), the gel was destained in water, and photographed at 305 nm UV light on a TT667 film with a CD 34 Polaroid Camera (Polaroid, Cambridge, USA).

Species-specific PCR

Amplifications of *B. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum and B. pseudocatenulatum, B. gallicum, B. infantis, B. longum* (i), *B. animalis, and B. lactis* (ii) were performed using primers (i) BiADO-1,2; BiANG-1.2; BiBIF-1,2; BiBRE-1,2; BiCATg-1,2; BiGAL-1,2; BiINF-1,2; BiLON-1,2 [20], and (ii) BanF2/PbiR1 [28]. PCR mixtures were prepared as mentioned above. Each PCR cycling profile consisted of an initial denaturation time of (i) 5 min at 94 °C followed by amplification for 40 cycles of denaturation 20 s at 94 °C, annealing 20 s at 55 °C, and extension steps 30 s at 72 °C. The reaction was completed with an elongation period of 10 min at 72 °C; (ii) the amplification programme included one cycle of 94 °C for 5 min, then 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C