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Discrimination between *Bifidobacterium* Species from Human and Animal Origin by PCR-Restriction Fragment Length Polymorphism

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ABSTRACT

Bifidobacteria are normal intestinal flora in humans and animals. The genus *Bifidobacterium* includes 31 species of significant host specificity. Taking into account their properties, we proposed to use bifidobacteria as fecal contamination indicators. PCR-restriction fragment length polymorphism on the 16S rDNA gene was used to distinguish the different *Bifidobacterium* species. Sixty-four strains belonging to 13 different species were differentiated from animal or human origin using one or two restriction enzymes. Moreover, the primers used were specifics of the *Bifidobacterium* genus. Therefore, this method made it possible to determine both the presence of bifidobacteria in a sample and its origin of contamination.

Members of the genus *Bifidobacterium* are generally nonpathogenic, gram-positive, anaerobic, nonmotile, and non-spore-forming bacteria *(20).* They possess fructose-6-phosphate phosphoketolase and produce acetic acid and lactic acid as end products of glucose metabolism *(18).* The habitats of bifidobacteria range from sewage to the intestines of humans, animals, and honeybees *(4, 21).* Although 31 species are currently known in the genus *Bifidobacterium,* the taxonomy is sometimes confusing because of existing conflicts between genetic and phenotypic characteristics *(11).* A phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences has been done *(17).* The *Bifidobacterium* species form an independent phylogenic cluster that can be divided into two subclusters: subcluster 1, which is composed of most *Bifidobacterium* species, and subcluster 2, which consists of two species, *B. denticolens* and *B. inopinatum.* Both of these were isolated from human dental caries. Because of the limitations of 16S rDNA analysis in the phylogenetic study of closely related bacterial taxa, a more recent phylogenic analysis of the genus *Bifidobacterium* was based on *Hsp60* gene sequences *(9).*

Bifldobacteria are well known for their probiotic effects *(5)* and are incorporated in many food products *(23).* Nevertheless, because bifldobacteria are also isolated from the feces of humans and many animals-such as ruminants, pigs, poultry, rodents, and rabbits *(18)*-they represent a potential indicator of the fecal contamination of food products. Moreover, these bacteria are strictly anaerobic *(2),* and, in the presence of oxygen, they stop growing but remain cultivable *(2).* It is therefore possible to estimate the initial amount of bifldobacteria present in the food product. Finally, another advantage of bifldobacteria over other fecal contamination indicators, such as *Escherichia coli,* is the host specificity of the *Bifidobacterium* species. For example, *B. pseudolongum* subsp. *globosum, B. thermophilum,* and *B. bourn* are present in ruminant feces *(10); B. suis* in swine; *B. cuniculi* and *B. magnum* in rabbit; *B. pullorum* in chicken; *B. adolescentis, B. dentium, B. bifidum, B. breve, B. catenulatum, B. infantis,* and *B. longum* are present in the human intestine *(13).* Thus, by determining the *Bifidobacterium* species, one can also determine the origin of the contamination (human and/or animal). The identification of *Bifidobacterium* by molecular methods was performed using several strategies: the 16S rDNA is a common target for the identification of bifidobacteria species by PCR. The amplicon is generated either by using species-specific primers *(15, 22)* or by using genus-specific primers followed by either sequencing *(17)* or hybridization with species-specific probes *(12)* or a restriction analysis *(13, 14, 19, 22).* This last method, PCR-restriction fragment length polymorphism (RFLP) based on 16S rDNA, although already described, was applied using a new set of enzymes, to distinguish human- and animalborne strains.

MATERIALS AND METHODS

Bacterial strains. The *Bifidobacterium* strains used in the present study are listed on Table 1. Before testing, the strains were withdrawn from frozen storage on Rosenow medium (Sanofi-Syn-thelabo, Marnes-la-Coquette, France) and grown on brain heart infusion (BHI; Bio-Rad, Marnes-la-Coquette, France) at 37°C for 48 to 72 h under anaerobic conditions, using an anaerobic cabinet (Ruskinn Technology Limited, Leeds, UK).

Other bacteria tested were five *E. coli* strains, one *Salmonella* Typhimurium strain, one *Campylobacterjejuni* strain, one *Yersin- ia enterocolitica* strain, one *Listeria monocytogenes* strain, five *Clostridium prefringens* strains, five enterobacteria strains, six *Lactobacillus* strains, five *Staphylococcus* strains, five *Bacillus ce-reus* strains, and five *Pseudomonas* strains.

Target DNA preparation. Bacterial cultures in BHI broth were centrifuged at 12,000 X *g* for 2 min using a bench-top centrifuge. The pellets were resuspendedin sterile, demineralized water, and the DNA was extracted using a Wizard Genomic DNA purification kit (Promega, Madison, Wis.). The purity and concentration of DNA were spectrophotometrically estimated (Ge-nequant-plus, Amersham Pharmacia Biotech, Amersham, UK).

PCR. To detect *Bifidobacterium,* we used the following primers, chosen using Oligo 6 software (Molecular Biology Insights, Cascade, Colo.): 16S direct, 5'-aat agc tcc tgg aaa cgg gt-3' and 16S reverse, 5'-cgt aag ggg cat gat gat ct-3' (Eurogentec, Seraing, Belgium), which corresponds to a fragment of ~1,050 bp from 16S rDNA sequence. The PCR mix was 0.2 mM dNTPs, 400 pmoles l^{-1} each primer, 4 U of Dap Goldstar (Eurogentec), 1X buffer (20 mM Tris-HCl [pH 8.0], 100 mM KC1, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol, 0.5% Nonidet P-40, and 0.5% Tween 20; Eurogentec), 5% dimethylsulfoxide (1.1 kg/L; Merck Eurolab, Leuven, Belgium), 20 µl DNA (40 to 200 ng), and H_2O in a total volume of 80 µl. The following cycles were applied: 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min 30 s. Amplified PCR products were then analyzed by gel electrophoresis using 1% gel agarose (20 by 10 cm) and 1 X Tris, acetic acid, and EDTA buffer (TAE; Bio-Rad). The voltage used was 10 V CM^{-1} for 1 h. After electrophoresis, gels were stained with ethidium bromide (1 mg ml^{-1}) and photographed under UV light (302 nm).

Restriction enzyme analysis. For restriction analysis of the PCR products, we used two enzymes, *Alul* (MBI Fermentas, St. Leon-Rot, Germany) and *TaqI* (Roche, Basel, Switzerland), according to the recommendations of the manufacturer Twenty mi-croliters of the PCR product was restricted by 1 U of enzyme in 1X buffer at 37°C for 3 h with *Alu*I and at 65°C for 3 h with *Taql* in a total volume of 30 µl. After digestion, the products were analyzed by gel electrophoresis using 2.5% agarose gel in 1X TAE buffer under 10 V cm^{-1} constant voltage. After electrophoresis, gels were stained with ethidium bromide (1 mg ml^{-1}) and photographed under UV light (302 nm). The size of the bands was determined using Kodak 1D software (Thermolabsystems, Brussels, Belgium).

FIGURE 1*. Alu/ restriction digest patterns. B, blanc; M, 5 µl molecular-weight marker (1,000-800-700-600-500-400-300-200-100 bp). (a) Strains A4, A5, and A6 (B. animalis) show pattern I (800-150-100 bp), and strains B1, B2, and B3 (B. thermophilum) show pattern VII (800-150-50-30 bp). (b) Strains Dl, D2, and D3 (B. pseudolongum subsp. globosum) show pattern II (600-200-150-100 bp). (c) Strains El, E2, and E3 (B. pseudolongum subsp. pseudolongum) show pattern II (600-200-150-100 bp). (d) Strain F1 (B. merycicum) shows pattern III (400-300-200-150 bp), strain G1 (B. ruminantium) shows pattern IV (900-150 bp), strains H1, H2, and H3 (B. minimum) show pattern V (310-290-200-150-100 bp), and strain II (B. cuniculi) and strains J1, J2, and J3 (B. adolescentis) show pattern I (800-150-100 bp). (e) Strains Kl (B. bifidum) and LI (B. breve) show pattern II (600-200-150-100 bp). (f) Strain Ml (B. dentium) shows pattern II (600-200-150-100 bp), and strains Ol, 02, and 03 (B. pseudocatenulatum) show pattern VI (700-200-150 bp).*

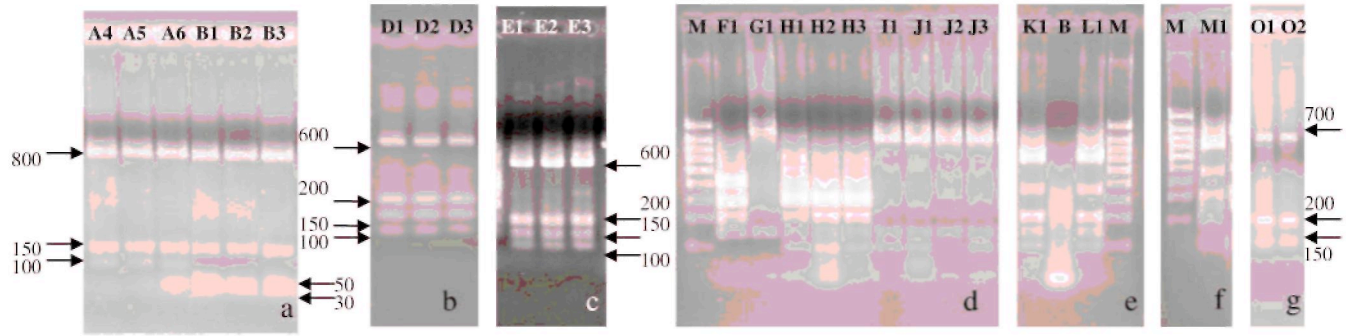

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TABLE 1. *Bacterial strains used in the study*

International or INRA internal reference	Laboratory's strains number	Species	Origin
ATCC 27672	A1	*B. animalis*	Animal
Biavati PI6	A2	*B. animalis*	Animal
Biavati F434	A3	*B. animalis*	Animal
Biavati RA16	A4	*B. animalis*	Animal
Biavati RA20	A5	*B. animalis*	Animal
NCFB 2242T	A6	*B. animalis*	Animal
Cheval 1/1	Bl	*B. thermophilum*	Animal
Pigeon 1/2	B2	*B. thermophilum*	Animal
LC294/2	B3	*B. thermophilum*	Animal
LC103/1	B4	*B. thermophilum*	Animal
B39/3	B5	*B. thermophilum*	Animal
B105/5	B6	*B. thermophilum*	Animal
LC288/1	B7	*B. thermophilum*	Animal
Porc 3/1	B8	*B. thermophilum*	Animal
B42/1	B9	*B. thermophilum*	Animal
LCI 10/1	BIO	*B. thermophilum*	Animal
T585/1/2	Bll	*B. thermophilum*	Animal
Pigeon 1/1	B12	*B. thermophilum*	Animal
Cheval 5/1	B13	*B. thermophilum*	Animal
T528/4	B14	*B. thermophilum*	Animal
B79/3	B15	*B. thermophilum*	Animal
Internal	Dl	*B. pseudolongum* subsp. *globosum*	Animal
Internal	D2	*B. pseudolongum* subsp. *globosum*	Animal
Biavati RU224	D3	*B. pseudolongum* subsp. *globosum*	Animal
Internal	El	*B. pseudolongum* subsp. *pseudolongum*	Animal
Biavati MB7	E2	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC287/2	E3	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC289/2	E4	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC302/2	E5	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC407/1/1	E6	*B. pseudolongum* subsp. *pseudolongum*	Animal
B81/1	E7	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC312/2	E8	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC317/2	E9	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC405/3	E10	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC290/1	Ell	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC464/3	E12	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC287/1	E13	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC305/2	E14	*B. pseudolongum* subsp. *pseudolongum*	Animal
B81/1	E15	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC304/1	E16	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC323/1	E17	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC324/2	E18	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC340/3	E19	*B. pseudolongum* subsp. *pseudolongum*	Animal
LC306/1	E20	*B. pseudolongum* subsp. *pseudolongum*	Animal
Bs82	E21	*B. pseudolongum* subsp. *pseudolongum*	Animal
RU915BT	Fl	*B. merycicum*	Animal
RU687T	Gl	*B. ruminantium*	Animal
DSM20102T	HI	*B. minimum*	Animal
LC396/4	H2	*B. minimum*	Animal
LC300/1	H3	*B. minimum*	Animal
Internal	11	*B. cuniculi*	Animal
Internal	Jl	*B. adolescentis*	Human
Bs3	J2	*B. adolescentis*	Human
CCUG18363T	J3	*B. adolescentis*	Human
2061a	J4	*B. adolescentis*	Human

5031e	J5	*B. adolescentis*	Human
DSM20082	Kl	*B. bifidum*	Human
NCFB2257T	LI	*B. breve*	Human
CCUG18363T	Ml	*B. dentium*	Human
DSM20438T	Ol	*B. pseudocatenulatun*	Human
B2B	02	*B. pseudocatenulatun*	Human
BS40	03	*B. pseudocatenulatun*	Human
C19I	04	*B. pseudocatenulatun*	Human
C20B	05	*B. pseudocatenulatun*	Human

***FIGURE 2**. Taq/ restriction digest patterns. B, blanc; M, 5 µl molecular-weight marker (1,000-800-700-600-500-400-300-200-100 bp). (a) Strains Al, A2, and A3 (B. animalis) show pattern VIII (470-330-250 bp). (b) Strain II (B. cuniculi) shows pattern VIII (470-330-250 bp), and strains Jl, J2, and J3 (B. adolescentis) show pattern IX (470-250-210-120 bp). (c) Strains Kl, L1, and Ml (B. bifidum, B. breve, and B. dentium) show pattern IX (470-250-210-120 bp), and strains Ol and 02 (B. pseudocatenulaturn) show pattern VIII (470-330-250 bp). (d) Strains Dl, D2, and D3 show pattern VIII (470-330-250 bp), and strains E18, E19, and E20 show pattern VIII (470-330-250 bp).*

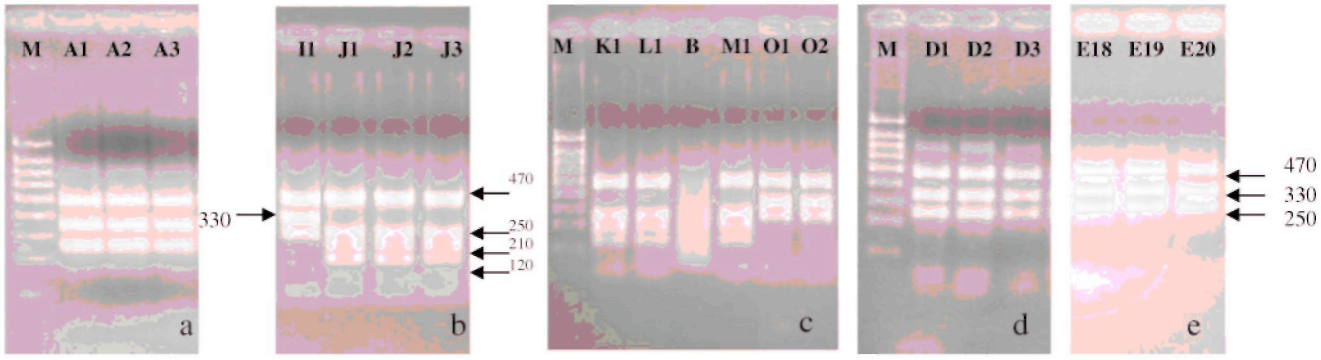

***TABLE 2.** Restriction patterns obtained with Alu/ and Taq/ on 13 Bifidobacterium species or subspecies*

Species	Tested strains	Origin	First digestion: *Alu*I pattern	Second digestion: *Taq*I pattern
B. animal is	6	Animal	I	VIII
B. cuniculi	1	Animal	I	VIII
B. adolescentis	5	Human	I	IX
B. pseudolongum subsp. *globosum*	3	Animal	II	VIII
B. pseudolongum subsp. *pseudolongum*	21	Animal	II	VIII
B. bifidum		Human	II	IX
B. breve		Human	II	IX
B. dentium		Human	II	IX
B. merycicum		Animal	III	
B. ruminantium		Animal	IV	
B. minimum	3	Animal	V	
B. pseudocatenulatun	5	Human	VI	
B. thermophilum	15	Animal	VII	

RESULTS

The selected primers were used on DNA isolated from different bacteria: *E. coli, Salmonella* Typhimurium, *C. je-juni, Y. enterocolitica, L. monocytogenes, C. perfringens,* enterobacteria, *Lactobacillus, Staphylococcus, B. cereus, Pseudomonas,* and *Bifidobacterium* species. The results indicated that an ~l,050-bp product was only detected in *Bifidobacterium* species.

The obtained PCR products were first digested by *AluI.* Seven different patterns were observed: pattern I (800-150-100 bp) included *B. animalis* (A), *B. cuniculi* (I), and *B. adolescentis* (J) (Fig. 1a and 1d); pattern II (600-200-150-100 bp) included *B. pseudolongum* subsp. *globosum* (D), *B. pseudolongum* subsp. *pseudolongum* (E), *B. bifidum* (K), *B. breve* (L), and *B. dentium* (M) (Fig. 1b, 1c, 1e, and 1f); pattern III (400-300-200-150 bp) included *B. mery-cicum* (F) (Fig. 1d); pattern IV (900-150 bp) included *B. ruminantium* (G) (Fig. 1d); pattern V (310-290-200-150-100 bp) included *B. minimum* (H) (Fig. 1d); pattern VI (700-200-150 bp) included *B. pseudocatenulatum* (O) (Fig. 1f); and pattern VII (800-150-50-30) included *B. thermophilum* (B) (Fig. la).

Because the aim of the present study was to be able to distinguish bifidobacteria of animal origin from those of human origin as well as to identify the species, we had to use a second restriction enzyme. Indeed, group I and group II were heterogeneous. In group I, *B. adolescentis* was of human origin, and *B. animalis* and *B. cuniculi* were of animal origin. We used then the enzyme *Taq*I to differentiate the different species. Species of human origin can be distinguished from species of animal origin (Fig. 2a and 2b). In group II, *B. bifidum, B. dentium,* and *B. breve* were of human origin, and *B. pseudolongum* subsp. *globosum* and *B. pseudolongum* subsp. *pseudolongum* were of animal origin. It was also possible to distinguish animal from human strains by using *TaqI* (Fig. 2c through 2e). The other groups were homogeneous: groups III and IV regrouped strains ofanimal origin, groups V and VII contained strains of animal origin, and group VI contained strains of human origin. Table 2 shows the different restriction patterns obtained with the two enzymes on 13 *Bifidobacterium* species or subspecies.

DISCUSSION

Food quality is a priority in our modern society, because the food chain is frequently the subject of periodic crises. Therefore, it is important to have objective tests to control the hygienic quality of food. Microbiological control is of major importance. It is important to be able to detect pathogens *(16)* and to detect fecal contamination *(6-8).* Indeed, that kind of contamination is the signature of a hygiene problem during foodstuff preparation. Classically, the bacterium used for this purpose is *E. coli (6).* However, because this species is present both in animal and humans, it is difficult to determine the origin of the contamination. Moreover, these bacteria continue to multiply after contamination, giving a false idea of the initial contamination level. To bypass these drawbacks, the use of *Bifidobacterium* species was proposed *(1).* Indeed, the *Bifidobacterium* species have good host specificity; therefore, it seems possible to identify the origin of the contamination. Moreover, bifidobacteria are strictly anaerobic, giving a better idea of the contamination level because there is no bacterial multiplication after the initial contamination in aerobic conditions. Beerens *(1)* compared *E. coli* and *Bifidobacterium* as indicators of fecal contamination in meat and meat products by classical microbiological methods and found that there was good correlation in the presence or absence of both bacteria. Moreover, most of the isolated bifidobacteria were of animal origin. The sequence of the gene encoding 16S rRNA (16S rDNA) is a common taxonomic tool to identify bacterial species. This gene was sequenced in 21 *Bifidobacterium* species, but the deduced dendrogram did not allow discrimination of the species regarding their host (human or animal) *(17).* Therefore, identifying the *Bifidobacterium* species origin was challenging. To distinguish the *Bifidobacterium* species, we chose the PCR-RFLP technique *(13, 14, 19, 22).* In previous studies, the 16 rDNA amplicon was digested by *Hae*III or *Taq*I, which allowed the distinction between human and cow strains *(3),* but this approach was incomplete, because only contamination by cows was investigated. The most extensive PCR-RFLP study on *Bifidobacterium* species using 16 rDNA was done by Ventura et al. *(22).* Sixteen species were investigated using the enzymes *Sau*3AI and *Bam*HI. However, some species were not investigated *(B. merycicum, B. ruminan-tium, B. minimum,* and B. *thermophilum).* In our study, PCR primers were selected in the 16S rDNA region, because this sequence is available for most *Bifidobacterium* species. The specificity of the chosen primers was good-the other bacteria tested were negative in amplification. The strategy used was as follows: the *AluI* enzyme was used to perform a first classification, which allowed us to obtain seven different groups. Because some of these groups contained both animal and human strains, the *TaqI* enzyme was then used to distinguish strains of human or animal origin. Finally, a fast and simple strategy to determine both the presence and origin (human or animal) of bifidobacteria was obtained. The next step will be to apply this method to artificial and natural contaminated food samples. Among possible PCR templates, *Hsp60* also seems to be a good candidate *(9),* because it was sequenced in most *Bifidobacterium* species and it is more variable between species than the 16S rDNA sequence. For the quantification of *Bifidobacterium* in feces by real-time PCR, the *hsp60* gene can be used opposite to 16SrDNA, because it is present in a single copy.

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