In silico RFLP Analysis of 16S rRNA Genes: A Helpful Application for Distinguishing Bifidobacteria from Human and Animal Source

Monica Modesto[1], Samanta Michelini[1], Giorgia Perpetuini[1,2], Rosanna Tofalo[2], Thomas Andlid[3] and Paola Mattarelli[1*]

[1]*Department of Agricultural Sciences, University of Bologna, Bologna, Italy.*
[2]*Faculty of Bioscience and Agro-Food and Environmental Technology, Campus Universitario di Coste Sant' Agostino, University of Teramo, Teramo, Italy.*
[3]*Department of Chemical and Biological Engineering and Food Science, Chalmers University of Technology, Gothenburg, Sweden.*

Authors' contributions

Article Information

Original Research Article

ABSTRACT

Bifidobacterial species are widespread in gastrointestinal tracts of mammalian and other animals; they can be found in extra body environment only after a fecal contamination or human intentional addition (as the case of probiotics). Interestingly their occurrence is strictly linked to their hosts with a clear demarcation between animal and human species. PCR-restriction fragment length polymorphism (PCR-RFLP) on the 16S rRNA gene, using *AluI*, and *TaqI* restriction enzymes, have been utilized to distinguish the animal or human source of 64 strains belonging to 13

_____

Corresponding author: E-mail: paola.mattarelli@unibo.it;

Bifidobacterium species (Delcenserie et al. [15]). Our aim was to test this method updating an *in silico* restriction analysis on the available 16S rRNA gene sequences of all 55 currently described taxa of *Bifidobacterium* genus. Our results confirmed the reliability of this method, optimized with the use of three restriction enzymes: *AluI, TaqI* and *MaeIII*, as a fast and simple strategy to determine the origin (human or animal) of bifidobacteria. Interestingly, the bifidobacterial species recently isolated from non-human primates cluster in the group of animal source except the bifidobacterial species isolated from higher non-human primates closest to humans such as apes (chimpanzee, orangutan and gorilla) that clusters with human group. Moreover, *B. minimum, B. subtile* and *B. mongoliense* isolated only from extrabody environment of which the source is unknown clustered with animal species. The *in silico* RFLP-PCR confirmed its powerful ability to attribute the primary source of occurrence (human or animal) for bifidobacterial species to the human or animal habitat.

1. INTRODUCTION

Bifidobacteria are an important group of intestinal commensals that exert a number of beneficial effects on their hosts such as prevention of diarrhea and microbial infection, alleviation of lactose intolerance and modulation of immune system [1]. Bifidobacteria are considered host-species-specific bacteria as validated by numerous studies [1]. In human beings the following bifidobacterial species have been found*: B. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B. dentium, B. gallicum, B. longum* subsp. *longum*, *B. longum* subsp. *infantis, B. pseudocatenulatum* and *B. scardovii* [2]. Generally, the species present in humans are not present in the other vertebrates, except for the group described as *B. longum-infantis* and for B. *pseudocatenulatum*, which are present in infants and suckling calves. Bifidobacteria have been isolated from many not-human vertebrates, especially mammalian. Of the 55 bifidobacterial taxa described to date, 42 have been isolated from not human vertebrates. [3]. Most of these species have a single host, e.g. *B. magnum* in rabbit, *B. choerinum* in pig and *B. pullorum* in chicken. Moreover, some species are present in more than one host, like the *B. animalis* subsp. *lactis* isolated from rabbit and chicken, *B. thermophilum* from chicken, pig, calf and bovine rumen and *B. pseudolongum* subsp. *globosum* and subp. *pseudolongum* (chicken, rabbit, pig, calf and bovine rumen, rat). Bifidobacteria typically found in insects are: *B. actinocoloniforme, B. bohemicum, B. bombi*, *B. asteroides, B. coryneofrme* and *B. indicum.* Finally in non human primates, except Apes, the following species have been found: *B. aesculapii, B. biavati, B. callitrichos, B. eulemuris, B. lemurum, B. reuteri, B. stellenboschense, B. myosotis, B. tissieri, B. hapali, B. moukalabense* [4–10]. On the other hand, in Apes, such as orangutan and chimpanzee, the bifidobacteria typical of human habitat "*Bifidobacterium angulatum like", B. dentium* and *B. adolescentis* have been found [11,12,13].

Eventually *B. minimum* and *B. subtile* being isolated from sewage cannot be attributed to a specific animal or human host source. The very peculiar feature of species specific bifidobacterial distribution in gastrointestinal tracts of their hosts can be a valuable tool for individuating the source of faecal contamination in water environments or along the meat processing chain [14]. For these applications the development and implementation of tools which can attribute the primary source of bifidobacterial species of unknown origin is of great importance in order to determine the original source of bifidobacterial species. The discriminatory power of rRNA gene analysis utilized for species identification can be improved by digestion of PCR products, and analysis of restriction fragment length polymorphism (PCR – RFLP) after gel electrophoresis [15] in order to identify the human or animal origin of the strains. For the first time Delcenserie et al. [16] after designing specific PCR primers matching the 16S rDNA region performed a study of sixty-four strains belonging to thirteen *Bifidobacterium* species by means of the *Alu*I enzyme. This restriction allowed them to obtain seven different groups. However, because two groups contained both animal and human strains, the *Taq*I enzyme was then used to correctly differentiate the origin of those strains. The current availability of a large number of bifidobacterial 16S rRNA sequences makes possible to simulate restriction digestions *in silico* and to generate virtual RFLP patterns for high throughput study of these bacteria. Here, we report the exploitation of a computer-simulated

RFLP analysis method, performing an *in silico* restriction analysis on the available 16S rRNA gene sequences, with the aim to verify the reliability of this method in differentiating from animal or human origin of the currently described 55 bifidobacterial taxa.

2. MATERIALS AND METHODS

2.1 *Bifidobacterium* 16S rRNA Partial Gene Sequences

The 16S rRNA gene sequences of the 55bifidobacterial taxa were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) and listed in Table 1. We also included the 16S rRNA gene sequences of three novel bifidobacterial taxa recently isolated from non-human primates. All the sequences were first aligned in CLC_Sequence Viewer version 7.5, for Mac OS (CLC, Inc., Aarhus, Denmark) using Clustal Omega and then edited in the region flanked by primers 16S direct, 5' – AAT AGC TCC TGG AAA CGG GT – 3', and 16S reverse, 5' - CGT AAG GGG CAT GAT GAT CT – 3' [14].

Final sequences of about 1050 bp were obtained.

2.2 Restriction Enzyme Analysis

All restriction analyses were performed *in silico* using the tool Restriction Site Analysis available in CLC_Sequence Viewer version 7.5, for Mac OS (CLC, Inc., Aarhus, Denmark). According to the method proposed by Delcenserie et al. [15], as first step, the 16S rRNA partial gene sequences were digested with *AluI*. Each pattern was analysed and compared to the groups previously described and associated with the different origin by Delcenserie et al. [16], using a script written in Python (version 2.7.8) (https://www.python.org/) (Supplementary File 1) for this study. If an unknown restriction profile was obtained, it was labelled as New Profile (NP) and the origin of the corresponding bifidobacterial species was recognized. When a heterogeneous pattern was obtained, the corresponding 16S rRNA gene sequences were restricted with *TaqI* and successively, if necessary, with *MaeIII* until a correct origin was obtained.

Table 1. List of species (all type strains if not specified), origin, international collection and GenBank accession number and fragment size (in bp) for each partial 16S rRNA gene sequence used in this study

Species	Origin	Collection Nr.	GenBank accession Nr.	16S rRNA fragment size (bp)
B. actinocoloniforme	Animal	DSM 22766	FD858731	1054
B. adolescentis	Human	DSM 20089	AB437355	1056
B. aesculapii	Animal	DSM 26737	KC807989	1055
B. angulatum	Animal	AATCC 27535	D86182	1054
B. animalis subsp. *animalis*	Animal	JCM 1190	D86185	1066
B. animalis subsp. *lactis*	Animal	DSM 10140	AB050136	1064
B. asteroides	Animal	DSM 20089	EF187235	1052
B. biavatii	Animal	DSM 23969	AB559506	1062
B. bifidum	Human	DSM 20456	AB437356	1054
B. bohemicum	Animal	DSM 22767	FD858736	1053
B. bombi	Animal	DSM 19703	HE582780	1051
B. boum	Animal	JCM 1211	D86190	1054
B. breve	Human	AATCC 15700	AB006658	1056
B. callitrichos	Animal	DSM 23973	AB559503	1051
B. catenulatum	Animal	DSM 16992	AB437357	1054
B. choerinum	Animal	AATCC 27686	D86186	1064
B. commune	Animal	DSM 28792	LK054489	1051
B. coryneforme	Animal	DSM 20216	AB437358	1052
B. crudilactis	Animal	DSM 20435	NR_115342	1050
B. cuniculi	Animal	DSM 20435	AB438223	1065
B. dentium	Human	AATCC 27534	D86183	1056
B. faecal	Animal	JCM 19861	KF990498	1055
B. gallicum	Animal	JCM 8224	D86189	1064

Species	Origin	Collection Nr.	GenBank accession Nr.	16S rRNA fragment size (bp)
B. gallinarum	Animal	JCM 6291	D86191	1050
B. hapali	Animal	JCM 30799	KP7189460	1057
B. hapali	Animal	JCM 30800 (Reference Strain)	KP7189462	1055
B. indicum	Animal	JCM 1302	D86188	1052
B. kashiwanohense	Animal	DSM 21854	NR_112779	1053
B. lemurum	Animal	DSM 28807	KJ658281	1052
B. eulemuris	Animal	JCM 30801	KP979748	1051
B. longum subsp. *infantis*	Human	ATCC 15697	D86184	1051
B. longum subsp. *longum*	Human	ATCC 55813	DB437359	1051
B. longum subsp. *suis*	Animal	ATCC 27533	M58743	1051
B. magnum	Animal	JCM 1218	D86193	1062
B. merycicum	Animal	JCM 8219	D86192	1054
B. minimum	Animal	DSM 20102	AB437350	1051
B. mongoliense	Animal	DSM 21395	AB433856	1051
B. moukalabense	Animal	JCM 18751	AB821293	1059
B. myosotis	Animal	JCM 30796	KP718941	1051
B. myosotis	Animal	JCM 30797 (Reference Strain)	KP718942	1051
B. pseudocatenulatum	Human	JCM 1200	D86187	1054
B. pseudolongum subsp. *globosum*	Animal	DSM 20092	M58736	1065
B. pseudolongum subsp. *pseudolongum*	Animal	JCM 1205	D86195	1064
B. psychraerophilum	Animal	DSM 22366	AB437351	1050
B. pullorum	Animal	JCM 1214	D86196	1051
B. reuteri	Animal	DSM 23975	AB613259	1054
B. ruminantium	Animal	JCM 8222	D86197	1056
B. saeculare	Animal	DSM 6531	D89328	1051
B. saguini	Animal	DSM 23967	AB559504	1052
B. scardovii	Human	DSM 13734	N180852	1052
Bifidobacterium spp.	Animal	BUSCOB MRM_8.19	KP7189459	1054
Bifidobacterium spp.	Animal	BUSCOB MRM_9.3	KP7189460	1053
B. stellenboschense	Animal	DSM 23968	AB559505	1060
B. subtile	Animal	DSM 20096	D89378	1052
B. thermacidophilum subsp. *porcinum*	Animal	DSM 17755	AB437361	1054
B. thermacidophilum subsp. *thermacidophilum*	Animal	DSM 15837	AB437362	1054
B. thermophilum	Animal	DSM 20210	AB437364	1054
B. tissieri	Animal	JCM 30798[T]	KP7189451	1053
B. tissieri	Animal	JCM 30803 (Reference Strain)	KP7189457	1053
B. tsurumiense	Animal	DSM 17777	AB241106	1056

DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM, Japan Collection of Microorganisms; BUSCOB, Bologna University Scardovi Collection of Bifidobacteria

3. RESULTS

Computer-simulated analysis is revolutionising some of the manners in which microbiological research is carried out. *In silico* approaches do not require any expensive materials (i.e., chemicals and enzymes) and analytical instruments and facilitate and speed up the screening of several strains rapidly and provide a better vision of microbial ecology [17]. The

restriction analysis of the 16S rRNA partial gene sequence with *AluI* and *TaqI* had been described as an easy way to distinguish human from animal borne bifidobacteria [15]. The method was previously tested on sixty-four strains belonging to only 13 *Bifidobacterium* species [15], but nowadays, 50 bifidobacterial species and 6 subspecies have been validated. To verify the reliability of this method, an *in silico* restriction analysis was performed on the 16S rRNA partial gene sequences of the 55 validated type strains. We also included two strains belonging to two putative novels *Bifidobacterium* species isolated from baby common marmosets [8]. All the aligned 1050 bp sequences, belonging to the 57 bifidobacterial type strain, were firstly digested with the enzyme *AluI* (Table 2) and thirteen different patterns were obtained. Seven pattern out of the thirteen described were found also by Delcenserie et al. [15] while for the other ones they were labelled as New Profile and added in the Python script, which resulted able to rapidly calculate each restriction profile and to associate them to the respective group (Table 2). The following pattern are shown: pattern I (800–150–100 bp) included *B. animalis* subsp. *animalis, B. animalis* subsp. *lactis*, *B. cuniculi*, *B. stellenboschense*, *B. aesculapii*, *B. reuteri* and *B. adolescentis*; pattern II (600–200–150–100 bp) included *B. asteroides, B. scardovii, B. acticolooniforme, B. tsurumiense, B. tissieri, B. kashiwanohense, B. biavatii, B. choerinum, B. pseudolongum* subsp. *globosum*, *B. pseudolongum* subsp. *pseudolongum*, *B. bifidum*, *B. breve*, and *B. dentium,* strains *Bifidobacterium* spp. MRM 8.19 and *Bifidobacterium* spp. MRM 9.3; pattern III (400–300–200–150 bp) included*B. merycicum, B. angulatum* and *B. callitrichos*; pattern IV (900–150 bp) included *B. ruminantium;* pattern V (310–290–200–150– 100 bp) included *B. minimum, B. indicum, B. coryneforme, B. commune, B. subtile, B. crudilactis, B. psychraerophilum, B. mongoliense*; pattern VI (700–200–150 bp) included *B. pseudocatenulatum, B. catenulatum* and *B. moukalabense*; and pattern VII (800–150–50–30) included *B. thermophilum, B. boum, B. thermoacidophilum* subsp. *thermoacidophilum, B. thermoacidophilum* subsp. *porcinum, B. saguini, B. faecale.* The following new patterns were shown: pattern I-NP (492-406-146-6), IV-NP (310-246-107-145-102-40), V-NP (352-311-286-60-35-6) and VI-NP (375-233-206-134-96) each included one species: *B. myosotis* (type and reference strains), *B. bohemicum*, *B. lemurum* and *B. gallicum,* respectively. Furthermore, group III-NP (315-286-206-140-60-

37) included three animal species: *B. bombi*, isolates from insects, *B. hapali* (type and reference strains), recently isolated from baby common marmosets and *B. eulemuris*, a novel species isolated from the black lemur. However, pattern II-NP (590/600-206-145-60-35-6 bp) included *B. longum* subsp. *suis*, *B. magnum*, *B. pullorum*, *B. saeculare*, *B. longum* subp. *infantis, B. longum* subsp. *longum* and *B. gallinarum,* then resulting heterogeneous. Therefore, restriction with *AluI* generated four heterogeneous groups (I, II, the new II-NP and the VII pattern, previously described by Delcenserie et al. [15] as homogenous differently from this study) as including species of both human- and animal borne bifidobacteria. As the aim of the present study was to set up a method to distinguish bifidobacteria with respect to their origin, the second enzyme *TaqI* has been utilized for restriction of the 16S rRNA sequences clustered in all heterogeneous profiles. Resulting profiles were elaborated with the Python script: two patterns, VIII and IX, previously identified and associated with the different origin by Delcenserie et al. [15] were retrieved together with the two new profiles X-NP and XI-NP (Table 3). Based on the new fragment length profiles calculated, species of group I, II and II-NP were reassigned to groups VIII and IX, X-NP and XI-NP (Table 3). Groups VIII and X-NP resulted homogeneous as including only species of animal origin: group VIII (471-340-240) included *B. animalis* susbp. *animalis*, *B. animalis* subsp. *lactis*, *B. choerinum*, *B. cuniculi*, *B. pseudolongum* subsp. *pseudolongum*, and *B. pseudolongum* subsp. *pseudolongum* while group X-NP (800-250 bp) included two animal species recently described in common marmoset, *B. reuteri* and *B. aesculapii*. Group IX (471-250-198-134) resulted heterogeneous as containing bifidobacterial species from human and animal origin such as *B. asteroides, B. adolescentis, B. breve, B. kashiwanohense, B. bifidum, B. dentium,* and *B. magnum*. The same for the group XI-NP (666-253-133) including *B. gallinarum, B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *suis*, *B. saeculare*, *B. pullorum*, *B. actinocoloniforme, B. biavati*. Analysing all the currently described species of bifidobacteria, *TaqI* was not able to correctly differentiate origin of some species in the groups II and II-NP (Table 3). Therefore, the 16S rRNA sequences in those groups were further restricted by means of other several enzymes available in the CLC_Sequence Viewer database. Only the enzyme *MaeIII* resulted able to distinguish human from animal borne

bifidobacteria and five homogenous groups were obtained: group X (372-275-185-157-61) including *B. biavatii*, group XI (468-364-157-61) including *B. actinocoloniforme*, group XII (471-405-113-61) containing *B. longum* subsp*. longum* and *B. longum* subsp*. infantis,* group XIII (518-472-61) containing *B. scardovii*, *B. gallinarum*, *B. pullorum* and *B. saeculare*, group XIV (532-405-113) only containing *B. longum* subsp. *suis* of animal origin (Table 4). Table 5 summarized results obtained from the restriction analysis with the three enzymes used sequentially.

Table 2. *AluI_I* restriction profiles obtained for each species with information about the pattern attribution and the origin

Sequence	Frag 1	Frag 2	Frag 3	Frag 4	Frag 5	Frag 6	*AluI_I* pattern	Origin
B. animalis subsp. *lactis*	814	134	96	6			I	Human or Animal
B. cuniculi	814	133	97	6			I	Human or Animal
B. animalis subsp. *animalis*	815	132	97	6			I	Human or Animal
B. stellenboschense	810	137	97	6			I	Human or Animal
B. aesculapii	806	142	96	6			I	Human or Animal
B. adolescentis	806	142	96	6			I	Human or Animal
B. reuteri	804	144	96	6			I	Human or Animal
B. myosotis	492	406	146	6			I-NP	Animal (Common marmoset)
B. myosotis (reference strain)	492	406	146	6			I-NP	Animal (Common marmoset)
B. asteroides	596	206	146	96	6		II	Human or Animal
B. scardovi	598	206	145	95	6		II	Human or Animal
Bifidobacterium spp. MRM_8.19	598	206	144	96	6		II	Human or Animal
B. actinocoloniforme	598	206	144	102	0		II	Human or Animal
B. tsurumiense	600	206	142	96	6		II	Human or Animal
Bifidobacterium spp. MRM_9.3	597	206	144	97	6		II	Human or Animal
B. tissieri	597	206	145	96	6		II	Human or Animal
B. tissieri (reference strain)	597	206	145	96	6		II	Human or Animal
B. dentium	600	206	142	96	6		II	Human or Animal
B. bifidum	598	206	144	96	6		II	Human or Animal
B. kashiwanohense	598	206	145	95	6		II	Human or Animal
B. biavati	606	206	136	96	6		II	Human or Animal

Sequence	Frag 1	Frag 2	Frag 3	Frag 4	Frag 5	Frag 6	*AluI_I* pattern	Origin
B. pseudolongum subsp. *globosum*	608	206	133	97	6		II	Human or Animal
B. choerinum	608	206	134	96	6		II	Human or Animal
B. pseudolongum subsp. *pseudolongum*	608	206	134	96	6		II	Human or Animal
B. breve	601	206	142	95	6		II	Human or Animal
B. longum subsp. *longum*	596	206	147	60	35	6	II-NP	Human or Animal
B. gallinarum	595	206	148	60	35	6	II-NP	Human or Animal
B. saeculare	596	206	147	60	35	6	II-NP	Human or Animal
B. longum subsp.*suis*	596	206	147	60	35	6	II-NP	Human or Animal
B. longum subsp.*infantis*	596	206	147	60	35	6	II-NP	Human or Animal
B. pullorum	596	206	147	60	35	6	II-NP	Human or Animal
B. magnum	607	206	136	60	35	6	II-NP	Human or Animal
B. merycicum	408	286	206	144	6		III	Animal
B. callitrichos	405	286	206	147	6		III	Animal
B. angulatum	408	286	206	144	6		III	Animal
B. hapalii	315	286	206	140	60	37	III-NP	Animal
B. hapalii (reference strain)	313	286	206	142	60	37	III-NP	Animal
B. bombi	310	286	206	147	60	35	III-NP	Animal
B. eulemuris	310	286	206	147	60	35	III-NP	Animal
B. ruminantium	902	142	6				IV	Animal
B. bohemicum	310	246	207	145	102	40	IV-NP	Animal
B. mongoliense	310	286	206	147	95	6	V	Animal
B. psychraerophilum	310	285	206	148	95	6	V	Animal
B. crudilactis	310	285	206	148	95	6	V	Animal
B. subtile	310	286	206	146	96	6	V	Animal
B. commune	310	286	206	147	95	6	V	Animal
B. coryneforme	310	286	206	146	96	6	V	Animal
B. minimum	310	286	206	147	95	6	V	Animal
B. indicum	310	286	206	146	96	6	V	Animal
B. lemurum	352	311	286	60	35	6	V-NP	Animal
B. moukalabense	699	206	139	6			VI	Human
B. pseudocatenulatum	694	206	144	6			VI	Human
B. catenulatum	694	206	144	6			VI	Human
B. gallicum	375	233	206	134	96		VI-NP	Human
B. thermophilum	805	144	60	35	6		VII	Animal
B. boum	805	144	60	35	6		VII	Animal
B.thermacidophilum subsp*. thermacidophilum*	805	144	60	35	6		VII	Animal
B. thermacidophilum subsp. *porcinum*	805	144	60	35	6		VII	Animal
B. saguini	804	145	60	35	6		VII	Animal
B. faecale	806	143	60	35	6		VII	Animal

Table 3. *TaqI* restriction profiles obtained for each species with information about the pattern attribution and the origin. For each species, type strains have been utilized; only for *B. tissieri, B. hapali* and *B. myosotis* also the reference strains have been used

Species	Frag 1	Frag 2	Frag 3	Frag 4	*TaqI* Pattern	Origin
B. animalis subsp. *animalis*	0	238	341	471	VIII	Animal
B. animalis subsp. *lactis*	0	240	339	471	VIII	Animal
B. boum	0	250	330	470	VIII	Animal
B. choerinum	0	240	339	471	VIII	Animal
B. cuniculi	0	239	340	471	VIII	Animal
B. pseudolongum subsp. *globosum*	0	239	340	471	VIII	Animal
B. pseudolongum subsp. *pseudolongum*	0	240	339	471	VIII	Animal
B. saguini	0	251	328	471	VIII	Animal
B. tissieri	0	251	328	471	VIII	Animal
B. tissieri (reference strain)	0	251	328	471	VIII	Animal
Bifidobacterium spp. MRM_8.19	0	250	329	471	VIII	Animal
Bifidobacterium spp. MRM_9.3	0	250	328	472	VIII	Animal
B. asteroides	134	193	252	471	IX	reconsidered as Human or Animal
B. dentium	134	197	248	471	IX	reconsidered as Human or Animal
B. bifidum	134	195	250	471	IX	reconsidered as Human or Animal
B. breve	133	198	248	471	IX	reconsidered as Human or Animal
B. adolescentis	134	197	248	471	IX	reconsidered as Human or Animal
B. faecal	133	197	249	471	IX	reconsidered as Human or Animal
B. kashiwanohense	133	195	251	471	IX	reconsidered as Human or Animal
B. tsurumiense	134	197	248	471	IX	reconsidered as Human or Animal
B. magnum	133	204	242	471	IX	reconsidered as Human or Animal
B. thermophilum	133	197	250	470	IX	reconsidered as Human or Animal
B. thermacidophilum subsp. *thermacidophilum*	133	197	250	470	IX	reconsidered as Human or Animal
B. thermacidophilum subsp. *porcinum*	133	197	250	470	IX	reconsidered as Human or Animal
B. aesculapii	0	0	249	801	X-NP	Animal (Monkey)

Species	Frag 1	Frag 2	Frag 3	Frag 4	*TaqI* Pattern	Origin
B. reuteri	0	0	250	800	X-NP	Animal (Monkey)
B. longum subsp. *suis*	0	133	253	664	XI-NP	Animal or Human
B. longum subsp. *infantis*	0	133	253	664	XI-NP	Animal or Human
B. longum subsp. *longum*	0	133	253	664	XI-NP	Animal or Human
B. actinocolinoforme	0	134	250	666	XI-NP	Animal or Human
B. scardovii	0	133	251	666	XI-NP	Animal or Human
B. stellenboschense	0	135	243	672	XI-NP	Animal or Human
B. biavatii	0	134	242	674	XI-NP	Animal or Human
B. gallinarum	0	133	254	663	XI-NP	Animal or Human
B. pullorum	0	133	253	664	XI-NP	Animal or Human
B. saeculare	0	133	253	664	XI-NP	Animal or Human

Table 4. *MaeIII* restriction profiles obtained for each species with information about the pattern attribution and the origin

Species	Frag 1	Frag 2	Frag 3	Frag 4	Frag 5	Frag 6	*MaeIII* pattern	Origin
B. biavatii	61	157	185	275	372	0	X	Animal
B. actinocolinoforme	0	61	157	364	468	0	XI	Animal
B. adolescentis	0	61	114	409	466	0	XII	Human
B. breve	0	61	113	410	466	0	XII	Human
B. dentium	0	61	114	409	466	0	XII	Human
B. faecal	0	61	113	409	467	0	XII	Human
B. bifidum	0	61	114	407	468	0	XII	Human
B. kashiwanohense	0	61	113	407	469	0	XII	Human
B. longum subsp. *longum*	0	61	113	405	471	0	XII	Human
B. longum subsp. *infantis*	0	61	113	405	471	0	XII	Human
B. stellenboschense	0	0	61	461	528	0	XIII	Animal
B. tsurumiense	0	0	61	466	523	0	XIII	Animal
B. thermacidophilum subsp. *porcinum*	0	0	61	467	522	0	XIII	Animal
B. thermophilum	0	0	61	467	522	0	XIII	Animal
B. thermacidophilum subsp. *thermacidophilum*	0	0	61	467	522	0	XIII	Animal
B. pullorum	0	0	61	471	518	0	XIII	Animal
B. scardovii	0	0	61	469	520	0	XIII	Animal
B. asteroides	0	0	61	470	519	0	XIII	Animal
B. saeculare	0	0	61	471	518	0	XIII	Animal
B. stellenboschense	0	0	61	461	528	0	XIII	Animal
B. gallinarum	0	0	61	472	517	0	XIII	Animal
B. magnum	0	0	113	416	521	0	XIV	Animal
B. longum subsp. *suis*	0	0	113	405	532	0	XIV	Animal

Table 5. Summary of the results obtained from the restriction analysis with the three enzymes

Species	Origin	First digestion: *AluII* pattern	Second digestion: *TaqI* pattern	Third digestion: *MaeIII* pattern
B. biavatii	Animal	II	XI-NP	X
B. actinocolinoforme	Animal	II	XI-NP	XI
B. adolescentis	Human	I	IX	XII
B. kashiwanohense	Human	II	IX	XII
B. dentium	Human	II	IX	XII
B. breve	Human	II	IX	XII
B. bifidum	Human	II	IX	XII
B. faecale	Human	VII	IX	XII
B. longum subsp. *longum*	Human	II-NP	XI-NP	XII
B. longum subsp. *infantis*	Human	II-NP	XI-NP	XII
B. tsurumiense	Animal	II	IX	XIII
B. asteroides	Animal	II	IX	XIII
B. thermophilum	Animal	VII	IX	XIII
B. thermacidophilum subsp. *thermacidophilum*	Animal	VII	IX	XIII
B. thermacidophilum subsp. *porcinum*	Animal	VII	IX	XIII
B. stellenboschense	Animal	I	XI-NP	XIII
B. scardovii	Animal	II	XI-NP	XIII
B. saeculare	Animal	II-NP	XI-NP	XIII
B. pullorum	Animal	II-NP	XI-NP	XIII
B. gallinarum	Animal	II-NP	XI-NP	XIII
B. magnum	Animal	II-NP	IX	XIV
B. longum subsp. *suis*	Animal	II-NP	XI-NP	XIV
B. cuniculi	Animal	I	VIII	
B. animalis subsp. *lactis*	Animal	I	VIII	
B. animalis subsp. *animalis*	Animal	I	VIII	
B. tissieri	Animal	II	VIII	
B. tissieri[T]	Animal	II	VIII	
Bifidobacterium spp. MRM_8.19	Animal	II	VIII	
Bifidobacterium spp. MRM_9.3	Animal	II	VIII	
B. pseudolongum subsp. *pseudolongum*	Animal	II	VIII	
B. pseudolongum subsp.*globosum*	Animal	II	VIII	
B. choerinum	Animal	II	VIII	
B. saguini	Animal	VII	VIII	
B. boum	Animal	VII	VIII	
B. reuteri	Animal	I	X-NP	
B. aesculapii	Animal	I	X-NP	
B. myosotis[T]	Animal	I-NP		
B. myosotis MRM_5.10	Animal	I-NP		
B. merycicum	Animal	III		
B. callitrichos	Animal	III		
B. angulatum	Animal	III		
B. eulemuris	Animal	III-NP		
B. hapalii	Animal	III-NP		

Species	Origin	First digestion: *AluII* pattern	Second digestion: *TaqI* pattern	Third digestion: *MaeIII* pattern
B. hapalii[T]	Animal	III-NP		
B. bombi	Animal	III-NP		
B. ruminantium	Animal	IV		
B. bohemicum	Animal	IV-NP		
B. subtile	Animal	V		
B. psychraerophilum	Animal	V		
B. mongoliense	Animal	V		
B. minimum	Animal	V		
B. indicum	Animal	V		
B. crudilactis	Animal	V		
B. coryneforme	Animal	V		
B. commune	Animal	V		
B. lemurum	Animal	V-NP		
B. pseudocatenulatum	Human	VI		
B. moukalabense	Human	VI		
B. catenulatum	Human	VI		
B. gallicum	Human	VI-NP		

4. DISCUSSION

Bifidobacteria exert a positive health action towards target hosts such as human beings and other animals and literature supporting their beneficial use [3]. Characteristically *Bifidobacterium* species are characterized by significant host specificity. Based on this hypothesis, PCR methods have been utilized for detecting bifidobacteria as general indicators of faecal human or animal contamination in water pollution, in raw milk and raw milk cheese processes [16].

In the present study the method described by Delcenserie et al. [15] using at first step *Alu*I then *Taq*I with implementation of a third restriction step with *Mae*III was applied to all bifidobacterial species nowadays described and confirmed its ability to differentiate their human or animal origin.

Interestingly *B. angulatum* up to now considered of human origin, in this study clusters with *B. merycicum* and *B. callithricos* both from animal origin. This supports the hypothesis that finding a species with only one strain in one single habitat is not sufficient to ascribe this habitat to that species. Infact this species has been isolated from human faeces but probably derived from another source. Similarly *B. scardovii* which has been isolated from female adult patients, viz. from 50-year-old female's blood sample in Sweden, from two elderly Swedish patients' urine

sample, and from a 44- year-old female patient's hip [18], cluster with other animal species: also the source of this species probably need to be revised. Another interesting finding is the clustering of *B. moukalabense,* isolated from gorilla, a primate very close to humans, to human bifidobacterial group: the occurrence of human bifidobacterial species in apes has been yet described with the presence of *B. adolescentis* and *B. dentium* in chimpanzee, orangutan and gorilla [11,12]; on the other hand all the other recently describe species from primates, belonging to Old and New World monkeys and to Prosimians, which are at evolutionary level more distant from humans, cluster in the animal group of bifidobacteria.

The use of bifidobacteria as indicators could be a powerful potential tool for the detection of antropic or livestock faecal contamination.RFLP-PCR beside confirming the ecological habitat of the species that have been isolated from different animals and humans, is also able to attribute the niche to species of unknown origin: infact in the present work *B. minimum* and *B. subtile* isolated from sewage and *B. mongoliense* from fermented milk have been associated to animal source. Moreover, bifidobacteria isolated from primates are divided in two groups, where the species isolated from apes (orangutan, gorilla and chimpanzee) are associated to human source (*B. moukalabense* for instance cluster with human species) differently from all other bifidobacterial primate species (*B. aerophilum, B.*

avesanii, B. biavatii, B. callithricos, B. hapali, B. myosotis, B. ramosum, B. saguini, B. stellenboshense and *B. tissieri*), which cluster with animal sources.

In silico RFLP analysis is very efficient also to discriminate bifidobacterial subspecies origin confirming *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* typically found in humans different from *B. longum* subsp. *suis* and *B. longum* subsp. *suillum* typically found in animals.

Further investigation by comparative genomics could probably better explain the link between genetic restriction profiles and specific niche distribution of bifidobacteria.

5. CONCLUSION

In the present work the application of PCR-RFLP for a rapid molecular recognition of natural habitat of bifidobacteria has been shown. This method with restriction databases of other strains belonging to *Bifidobacteriaceae* family would be an extremely useful and practical tool for application in microbial ecology studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mattarelli P, Biavati B. The genera *Bifidobacterium, Parascardovia* and *Scardovia*. In: Wood B, Holzapfel W, editors. The Lactic Acid Bacteria: Biodiversity and Taxonomy. John Wiley & Sons (UK); 2014.
2. Biavati B, Mattarelli P. Genus *Bifidobacterium*. In: Goodfellow M, Kampfer P, Busse HJ, Suzuki KI, Ludwig W, Whitman WB, editors. The Actinobacteria. Springer; 2012.
3. Yanokura E, Oki K, Makino H, Modesto M, Pot B, Mattarelli P, et al. Subspeciation of *Bifidobacterium longum* by multilocus approaches and amplified fragment length polymorphism: Description of *Bifidobacterium longum* subsp. *suillum* subsp. nov., isolated from the faeces of piglets. Syst Appl Microbiol. 2015;38(5): 305–14.
4. Michelini S, Oki K, Yanokura E, Shimakawa Y, Modesto M, Mattarelli P, et al. *Bifidobacterium myosotis* sp. nov. *Bifidobacterium tissieri* sp. nov. and *Bifidobacterium hapali* sp. nov.: Three novel taxa from the faeces of baby common marmoset (*Callithrix jacchus* L.). Int J Syst Evol Microbiol. 2016;6(1):255–65.
5. Modesto M, Michelini S, Stefanini I, Ferrara A, Tacconi S, Biavati B, et al. *Bifidobacterium aesculapii* sp. nov., from the faeces of the baby common marmoset (*Callithrix jacchus*). Int J Syst Evol Microbiol. 2014;64(8):2819–27.
6. Endo A, Futagawa-Endo Y, Schumann P, Pukall RR, Dicks LMT. *Bifidobacterium reuteri* sp. nov., *Bifidobacterium callitrichos* sp. nov., *Bifidobacterium saguini* sp. nov., *Bifidobacterium stellenboschense* sp. nov. and *Bifidobacterium biavatii* sp. nov. isolated from faeces of common marmoset (*Callithrix jacchus*) and red. Syst Appl Microbiol. 2012;35(2):92–7.
7. Tsuchida S, Takahashi S, Mbehang Nguema PP, Fujita S, Kitahara M, Yamagiwa J, et al. *Bifidobacterium moukalabense* sp. nov. isolated from the faeces of wild west lowland gorilla (*Gorilla gorilla gorilla*) in Gabon. Int J Syst Evol Microbiol. 2014;64(Pt 2):449–55.
8. Michelini S, Modesto M, Oki K, Stenico V, Stefanini I, Biavati B, et al. Isolation and identification of cultivable *Bifidobacterium* spp. from the faeces of 5 baby common marmosets (*Callithrix jacchus* L.). Anaerobe. 2015;33:101–4.
9. Modesto M, Michelini S, Stefanini I, Sandri C, Spiezio C, Pisi A, et al. *Bifidobacterium lemurum* sp. nov., from faeces of the ring-tailed lemur (*Lemur catta*). Int J Syst Evol Microbiol. 2015;65(6):1726–34.
10. Michelini S, Modesto M, Pisi A, Filippini G, Sandri C, Spiezio C, et al. *Bifidobacterium eulemuris* sp. nov. isolated from the faeces of the black lemur (*Eulemur macaco*). Int J Syst Evol Microbiol. 2016;66:1567–1576.
11. Ushida K, Uwatoko Y, Adachi Y, Soumah AG, Matsuzawa T. Isolation of bifidobacteria from feces of chimpanzees in the wild. J Gen Appl Microbiol. 2010;56(1):57–60.
12. D'Aimmo MR, Modesto M, Mattarelli P, Biavati B, Andlid T. Biosynthesis and cellular content of folate in bifidobacteria across host species with different diets. Anaerobe. 2014;30:169–77.

13. Moeller AH, Caro-Quintero A, Mjungu D, Georgiev AV, Lonsdorf EV, Muller MN, et al. Cospeciation of gut microbiota with hominids. Science. 2016:353(6297):380–2.

14. Bonjoch X, Lucena F, Blanch AR. The persistence of bifidobacteria populations in a river measured by molecular and culture techniques. J Appl Microbiol. 2009;107(4): 1178–85.

15. Delcenserie V, Bechoux N, Leonard T, Daube G. Discrimination between *Bifidobacterium* species from human and animal origin by PCR-restriction fragment length polymorphism. J Food Prot. 2004;67:1284–8.

16. Delcenserie V, Bechoux N, China B, Daube G, Gavini F. A PCR method for detection of bifidobacteria in raw milk and raw milk cheese: Comparison with culture-based methods. J Microbiol Methods. 2005;61(1):55–67.

17. Escobar-Zepeda A, de Leon VP, Sanchez-Flores A. The road to metagenomics: From microbiology to DNA sequencing technologies and bioinformatics. Front Genet. 2015;6:348.

18. Hoyles L, Inganäs E, Falsen E, Drancourt M, Weiss N, McCartney AL, et al. *Bifidobacterium scardovii* sp. nov., from human sources. Int J Syst Evol Microbiol. 2002;52(Pt 3):995–9.

_____

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/19680