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

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MM, SM and PM designed the study, performed the statistical analysis and wrote the protocol. Authors MM, SM, GP, RT and TA wrote the first draft of the manuscript. Authors SM and PM managed the analyses of the study. Author GP managed the literature searches. All authors read and approved the final manuscript.

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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 Alul, and TaqI restriction enzymes, have been utilized to distinguish the animal or human source of 64 strains belonging to 13

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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: Alul, TaqI and MaeII, 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. subtilis 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.

Keywords: Bifidobacterium spp.; computer simulated RFLP; Alul; TaqI; MaeII; host specificity; fecal contamination indicator.

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 non-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 subsp. pseudolongum (chicken, rabbit, pig, calf and bovine rumen, rat). Bifidobacteria typically found in insects are: B. actinocoloniforme, B. bohemicum, B. bombi, B. asteroides, B. coryneform 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. subtilis 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 AluI enzyme. This restriction allowed them to obtain seven different groups. However, because two groups contained both animal and human strains, the TaqI 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 55 bifidobacterial 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 Alul. 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. bourn               | 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. crudiactis          | Animal   | DSM 20435      | NR.115342             | 1050                        |
| B. cunicul             | 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                        |
| *(Reference Strain)*      |        |                |                       |                             |
| *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              | 1055                        |
| *B. longum subsp. infantis*| Human | ATCC 15697     | D86184                | 1051                        |
| *B. longum subsp. longum* | Human | ATCC 5813      | 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. mokulabense*          | Animal | JCM 18751      | AB821293              | 1059                        |
| *B. myosotis*             | Animal | JCM 30796      | KP718941              | 1051                        |
| *(Reference Strain)*      |        |                |                       |                             |
| *B. pseudocatenulatum*    | Human  | JCM 1200       | D86187                | 1054                        |
| *B. pseudodongum subsp. globosum* | Animal | DSM 20092 | M58736                | 1065                        |
| *B. pseudodongum subsp. pseudodongum* | 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         | KP7189459             | 1054                        |
| *(Reference Strain)*      |        |                |                       |                             |
| *B. stellenboschense*     | Animal | DSM 23958      | AB559505              | 1060                        |
| *B. subtil*               | Animal | DSM 20096      | D89378                | 1052                        |
| *B. thermacidophilum subsp. porcinum* | Animal | DSM 17755 | AB437361               | 1054                        |
| *(Reference Strain)*      |        |                |                       |                             |
| *B. tsuruimense*          | Animal | DSM 17777      | AB241106              | 1056                        |

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 Alul 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 Alul (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. stellensboscense, 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. biavati, 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. callirichos; 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. subtilis, B. crudlactis, 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. saequinui, 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 I1-NP (590/600-206-145-60-35-6 bp) included B. longum subsp. suis, B. magnum, B. pullorum, B. saeculare, B. longum subsp. infantis, B. longum subsp. longum and B. gallinarum, then resulting heterogeneous. Therefore, restriction with Alul 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 subsp. animalis, B. animalis subsp. lactis, B. choerinum, B. cuniculi, B. pseudolongum subsp. pseudolongum, and 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. callirichos; 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. subtilis, B. crudlactis, 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. saequinui, 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 I1-NP (590/600-206-145-60-35-6 bp) included B. longum subsp. suis, B. magnum, B. pullorum, B. saeculare, B. longum subsp. infantis, B. longum subsp. longum and B. gallinarum, then resulting heterogeneous. Therefore, restriction with Alul 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 subsp. animalis, B. animalis subsp. lactis, B. choerinum, B. cuniculi, B. pseudolongum subsp. pseudolongum, and B. pseudolongum subsp. pseudolongum, 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. magnus. 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 Maell3 resulted able to distinguish human from animal borne
bifidobacteria and five homogenous groups were obtained: group X (372-275-185-157-61) including \textit{B. biavatii}, group XI (468-364-157-61) including \textit{B. actinocoloniforme}, group XII (471-405-113-61) containing \textit{B. longum} subsp. \textit{longum} and \textit{B. longum} subsp. \textit{infantis}, group XIII (518-472-61) containing \textit{B. scardovii}, \textit{B. gallinarum}, \textit{B. pullorum} and \textit{B. saeculare}, group XIV (532-405-113) only containing \textit{B. longum} subsp. \textit{suis} of animal origin (Table 4). Table 5 summarized results obtained from the restriction analysis with the three enzymes used sequentially.

Table 2. \textit{Alul} \textit{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 | \textit{Alul} \textit{I} pattern | Origin                  |
|---------------------------------|--------|--------|--------|--------|--------|--------|----------------------------------|-------------------------|
| \textit{B. animalis} subsp. \textit{lactis} | 814    | 134    | 96     | 6      |        |        | I                                | Human or Animal         |
| \textit{B. cuniculi}            | 814    | 133    | 97     | 6      |        |        | I                                | Human or Animal         |
| \textit{B. animalis} subsp. \textit{animalis} | 815    | 132    | 97     | 6      |        |        | I                                | Human or Animal         |
| \textit{B. stellenboschense}    | 810    | 137    | 97     | 6      |        |        | I                                | Human or Animal         |
| \textit{B. aesculapii}          | 806    | 142    | 96     | 6      |        |        | I                                | Human or Animal         |
| \textit{B. adolescentis}        | 806    | 142    | 96     | 6      |        |        | I                                | Human or Animal         |
| \textit{B. reuteri}             | 804    | 144    | 96     | 6      |        |        | I                                | Human or Animal         |
| \textit{B. myosotis}            | 492    | 406    | 146    | 6      |        |        | I-NP                             | Animal (Common marmoset) |
| \textit{B. myosotis} (reference strain) | 492    | 406    | 146    | 6      |        |        | I-NP                             | Animal (Common marmoset) |
| \textit{B. asteroides}          | 596    | 206    | 146    | 96     | 6      |        | II                               | Human or Animal         |
| \textit{B. scardovi}            | 598    | 206    | 145    | 95     | 6      |        | II                               | Human or Animal         |
| \textit{Bifidobacterium spp. MRM \_8.19} | 598    | 206    | 144    | 96     | 6      |        | II                               | Human or Animal         |
| \textit{B. actinocoloniforme}   | 598    | 206    | 144    | 102    | 0      |        | II                               | Human or Animal         |
| \textit{B. tsurumienne}         | 600    | 206    | 142    | 96     | 6      |        | II                               | Human or Animal         |
| \textit{Bifidobacterium spp. MRM \_9.3} | 597    | 206    | 144    | 97     | 6      |        | II                               | Human or Animal         |
| \textit{B. tissieri}            | 597    | 206    | 145    | 96     | 6      |        | II                               | Human or Animal         |
| \textit{B. tissieri} (reference strain) | 597    | 206    | 145    | 96     | 6      |        | II                               | Human or Animal         |
| \textit{B. dentium}             | 600    | 206    | 142    | 96     | 6      |        | II                               | Human or Animal         |
| \textit{B. bifidum}             | 598    | 206    | 144    | 96     | 6      |        | II                               | Human or Animal         |
| \textit{B. kashiwanohense}      | 598    | 206    | 145    | 95     | 6      |        | II                               | Human or Animal         |
| \textit{B. biavati}             | 606    | 206    | 136    | 96     | 6      |        | II                               | Human or Animal         |
| Sequence                      | Frag 1 | Frag 2 | Frag 3 | Frag 4 | Frag 5 | Frag 6 | Alul_I pattern | Origin        |
|------------------------------|--------|--------|--------|--------|--------|--------|----------------|---------------|
| B. pseudolongum subsp.       | 608    | 206    | 133    | 97     | 6      | II     | Human or Animal |
| globosum                     |        |        |        |        |        |        |                |               |
| B. choerinum                 | 608    | 206    | 134    | 96     | 6      | II     | Human or Animal |
| B. pseudolongum subsp.       | 608    | 206    | 134    | 96     | 6      | II     | Human or Animal |
| pseudolongum                 |        |        |        |        |        |        |                |               |
| 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. magnus                    | 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-NP          | Animal         |
| B. bohemicum                 | 310    | 246    | 207    | 145    | 102    | 40     | IV-NP         | Animal         |
| B. mongoliense               | 310    | 286    | 206    | 147    | 95     | 6      | V-NP          | Animal         |
| B. psychraerophilum          | 310    | 285    | 206    | 148    | 95     | 6      | V-NP          | Animal         |
| B. crudilactis               | 310    | 285    | 206    | 148    | 95     | 6      | V-NP          | Animal         |
| B. subtilis                  | 310    | 286    | 206    | 146    | 96     | 6      | V-NP          | Animal         |
| B. commune                   | 310    | 286    | 206    | 147    | 95     | 6      | V-NP          | Animal         |
| B. coryneformae              | 310    | 286    | 206    | 146    | 96     | 6      | V-NP          | Animal         |
| B. minimum                   | 310    | 286    | 206    | 147    | 95     | 6      | V-NP          | Animal         |
| B. indicum                   | 310    | 286    | 206    | 146    | 96     | 6      | V-NP          | Animal         |
| B. lemurum                   | 352    | 311    | 286    | 60     | 35     | 6      | V-NP          | Animal         |
| B. moukalabense              | 699    | 206    | 139    | 6      |        |        | V-NP           | Animal         |
| B. pseudocatenulatum         | 694    | 206    | 144    | 6      |        |        | VI-NP          | Human          |
| B. catenulatum               | 694    | 206    | 144    | 6      |        |        | VI-NP          | Human          |
| B. gallicum                  | 375    | 233    | 206    | 134    | 96     |        | VI-NP          | Human          |
| B. thermophilum              | 805    | 144    | 60     | 35     | 6      |        | VII-NP         | Animal         |
| B. boum                      | 805    | 144    | 60     | 35     | 6      |        | VII-NP         | Animal         |
| B. thermacidophilum subsp.   | 805    | 144    | 60     | 35     | 6      |        | VII-NP         | Animal         |
| thermacidophilum subsp.      | 805    | 144    | 60     | 35     | 6      |        | VII-NP         | Animal         |
| porcinum                     | 805    | 144    | 60     | 35     | 6      |        | VII-NP         | Animal         |
| B. saguini                   | 804    | 145    | 60     | 35     | 6      |        | VII-NP         | Animal         |
| B. faecale                   | 806    | 143    | 60     | 35     | 6      |        | VII-NP         | 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. kashiwanoense*                    | 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 | Frag 5 | MaelliI pattern | Origin         |
|-------------------------------|--------|--------|--------|--------|--------|----------------|----------------|
| B. reuteri                    | 0      | 0      | 250    | 800    |        | X-NP           | Animal         |
| 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. *Maelli* restriction profiles obtained for each species with information about the pattern attribution and the origin.
Table 5. Summary of the results obtained from the restriction analysis with the three enzymes

| Species                        | Origin  | First digestion: *AluI* 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   | I                               | 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. tsuriumiense*              | Animal  | II                              | IX                               | XII                              |
| *B. asteroides*                | Animal  | II                              | IX                               | XII                              |
| *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                            | XII                              |
| *B. scardovii*                 | Animal  | II                              | XI-NP                            | XII                              |
| *B. saeculare*                 | Animal  | II-NP                           | XI-NP                            | XII                              |
| *B. pullorum*                  | Animal  | II-NP                           | XI-NP                            | XII                              |
| *B. gallinarum*                | Animal  | II-NP                           | XI-NP                            | XII                              |
| *B. magnum*                    | Animal  | II-NP                           | IX                               | XIV                              |
| *B. longum* subsp. *suis*      | Animal  | II-NP                           | XI-NP                            | XII                              |
| *B. cuniculi*                  | Animal  | I                               | VIII                             |
| *B. animalis* subsp. *lactis*  | Animal  | I                               | VIII                             |
| *B. animalis* subsp. *animalis* | Animal  | I                               | VIII                             |
| *B. tissier*                   | Animal  | II                              | VIII                             |
| *B. tissier*<sup>+</sup>       | 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  | I                               | 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*<sup>+</sup>      | Animal  | I-NP                            |
| *B. myosotis* MRM_5.10         | Animal  | I-NP                            |
| *B. meryicum*                  | Animal  | III                             |
| *B. callitrichos*              | Animal  | III                             |
| *B. angulatum*                 | Animal  | III                             |
| *B. eulemuris*                 | Animal  | III-NP                          |
| *B. hapalii*                   | Animal  | III-NP                          |
| Species          | Origin    | First digestion: AluI pattern | Second digestion: TaqI pattern | Third digestion: MaeIII pattern |
|------------------|-----------|--------------------------------|--------------------------------|--------------------------------|
| B. hapalii†      | Animal    | III-NP                         |                                |                                |
| B. bombi         | Animal    | III-NP                         |                                |                                |
| B. ruminantium   | Animal    | IV                             |                                |                                |
| B. bohemicum     | Animal    | IV-NP                          |                                |                                |
| B. subtle        | 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 *AluI* then *TaqI* with implementation of a third restriction step with *MaeIII* 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. callithricus* 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. stellenboschense 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.

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