Bifidobacterial Diversity and the Development of New Microbial Source Tracking Indicators[▽]

Elisenda Ballesté and Anicet R. Blanch*

Department of Microbiology, University of Barcelona, Avda. Diagonal 645, E-08028 Barcelona, Spain

Received 16 September 2010/Accepted 22 March 2011

Many studies suggest a close relationship between species of *Bifidobacterium* and their hosts. Thus, species such as *B. adolescentis* and *B. thermacidophilum* subsp. *porcinum* have been proposed as potential indicators of human and porcine fecal pollution. The diversity of bifidobacteria in wastewaters (human and animal) and slurries is analyzed using nested PCR followed by denaturant gradient gel electrophoresis (DGGE). The sewage samples showed similar DGGE patterns. The predominant bands were recognized as *B. adolescentis*, *B. longum*, and two unidentified species related to *B. adolescentis*. A single band detected in poultry samples was identified as *B. saeculare*. Bifidobacterial diversity was higher within porcine and bovine samples. The main bands in porcine samples were identified as *B. minimum*, an unknown species, and *B. thermophilum*/*B. thermacidophilum* subsp. *porcinum*. The latter species was also identified among the main bands in bovine samples together with *B. pseudolongum* and *B. ruminantium*. We then attempted to isolate the host-specific strains. DGGE bands were examined to develop specific probes to screen environmental samples by colony hybridization and further isolation of strains from positively hybridized colonies. Bifidobacterial strains that are host associated by DGGE bands to human and pig were successfully isolated from the environment: *B. adolescentis* from human sewage samples and the unidentified species related to pig from slurries and slaughterhouse wastewater. Neither the poultry-associated *B. saeculare* nor the ruminant-associated *B. pseudolongum* could be isolated with the current methodology, suggesting either a low prevalence in the samples or failure of the culture to grow in the media used.

The digestive tract of mammalian species is a complex ecosystem that harbors an elaborate bacterial community containing as many as 400 different species (62). However, these species belong mainly to three bacterial groups: *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (1, 15, 47). Some species of these groups show a high abundance in feces (15, 59) and an anaerobic physiology which hampers their growth in the environment, thereby preventing horizontal transfer between hosts (6, 17, 28). On the basis of such features, they have been proposed as microbial indicators to detect the source of fecal pollution in water (5, 13, 25, 31, 45, 48, 60). *Bifidobacterium* species are anaerobic Gram-positive bacteria belonging to the *Actinobacteria* group. Although they share these characteristics, only a few species have been proposed for use as microbial source tracking (MST) indicators (10, 32, 34, 35, 42, 50). They are the first to colonize the infant digestive tract, providing many benefits for the host, such as regulation effect of the gut microflora, enhancement of the immune system, and production of antimicrobial substances and vitamins (7). Studies of bifidobacterial diversity have focused mainly on the human intestine and on the selection of probiotics for use in milk-based products (16, 56). Currently, the *Bifidobacterium* genus harbors 32 species, and strong host specificity has been described. Several *Bifidobacterium* species are believed to be human host specific, such as *B. adolescentis*, *B. angulatum*, *B.*

longum, and *B. catenulatum*. Other species have been linked to certain animals, such as *B. pullorum* and *B. gallinarum* to chickens; *B. choerinum*, *B. longum* subsp. *suis*, *B. pseudolongum* subsp. *pseudolongum*, *B. thermacidophilum* subsp. *porcinum*, and *B. thermophilum* to swine; and *B. ruminantium*, *B. boum*, *B. pseudolongum* subsp. *globosum*, and *B. thermophilum* to cows (7, 19, 56).

Bifidobacterium spp. were first proposed as MST indicators 20 years ago, when sorbitol-fermenting species were related to a possible human source (34). Later, molecular tools enabled the detection of host-specific species to track the source of fecal pollution. *B. adolescentis* and *B. dentium* were described as potential MST indicators for human fecal pollution in water (10, 14, 32, 42). These bacterial species show high human specificity, and they are frequently included in the set of best indicators when MST predictive models are developed (3, 9). However, although many *Bifidobacterium* spp. have been isolated and a host relation has been suggested, only the amplification of the internal transcribed spacer of *B. thermacidophilum* subsp. *porcinum* has been proposed as an MST marker for animal species (35). Some of these *Bifidobacterium* markers have been successfully applied in Europe and some parts of the United States (2, 8, 14, 21, 26, 38).

In microbial ecology, fingerprinting techniques to explore the diversity of 16S rRNA genes have been used to study bacterial communities, and they appear to be ideal for monitoring community shifts and comparing communities. Denaturing gradient gel electrophoresis (DGGE) is based on sequence-specific melting behavior and allows discrimination between PCR amplicons of identical sizes but with different DNA sequences (39, 41). The resulting banding profile pro-

* Corresponding author. Mailing address: Department of Microbiology, University of Barcelona, Avda. Diagonal 645, E-08028 Barcelona, Spain. Phone: 34-934029012. Fax: 34-934039047. E-mail: ablanch@ub.edu.

[▽] Published ahead of print on 1 April 2011.

vides information on the bacterial diversity of each sample. DGGE analyses of 16S rRNA genes have been successfully used to characterize and monitor gastrointestinal bacterial communities in humans (44, 49, 63), pigs (46, 52, 53), cattle (27), and chickens (55).

The aim of this study was to analyze the diversity of bifidobacteria in wastewaters (human and animal) and slurries and to assess whether the distribution of species is related to fecal sources. We examined the DGGE patterns of bacteria from the various sources with the aim of identifying host-specific species that could be used as MST markers for animal fecal pollution. We then designed molecular probes to allow the isolation of host-specific *Bifidobacterium* species from the environment. Their isolation could facilitate the development of culture-dependent or molecular methods to provide new MST indicators that improve predictive models on fecal source tracking. Later on, the persistence in the environment of these new indicators should be evaluated by using their respective established methods of enumeration.

MATERIALS AND METHODS

Bacterial strains. The following reference strains were used to establish the proper DGGE gradient and conditions for further analysis: *Bifidobacterium adolescentis* (DSM 20083^T), *B. angulatum* (DSM 20098^T), *B. animalis* subsp. *animalis* (DSM 20104^T), *B. animalis* subsp. *lactis* (DSM 10140), *B. asteroides* (DSM 20089^T), *B. bifidum* (DSM 20456^T), *B. boum* (DSM 20432^T), *B. breve* (DSM 20213^T), *B. catenulatum* (DSM 16992^T), *B. choerinum* (DSM 20434^T), *B. coryneforme* (DSM 20216^T), *B. cuniculi* (DSM 20435^T), *B. dentium* (DSM 20084), *B. gallicum* (DSM 20093^T), *B. gallinarum* (DSM 20670^T), *B. globosum* (DSM 20092^T), *B. indicum* (DSM 20214^T), *B. infantis* (DSM 20088^T), *B. longum* (DSM 20219^T), *B. magnum* (DSM 20222^T), *B. merycicum* (DSM 6492^T), *B. minimum* (DSM 20102^T), *B. pseudocatenulatum* (DSM 20438^T), *B. pseudolongum* (DSM 20099^T), *B. pullorum* (DSM 20433^T), *B. ruminantium* (DSM 6489^T), *B. saeculare* (DSM 6531^T), *B. scardovii* (DSM 13734^T), *B. subtile* (DSM 20096^T), and *B. thermophilum* (DSM 20210^T).

Type strains were grown using the reinforced *Clostridium* medium (RCM) broth (Oxoid, Hampshire, England) and Columbia blood agar (CBA) (Difco, Le Pont de Claix, France) with addition of L-cysteine hydrochloride at 0.5 g/liter (Merck, Darmstadt, Germany) and glucose at 5 g/liter, incubated anaerobically.

Environmental samples. A total of 77 wastewater samples from various sources were obtained from April 2005 to May 2007. Twenty-one sewage samples were obtained from eight urban wastewater treatment plants (WTP) in the area of Catalonia. Two WTP were serving a population equivalent (PE) of less than 20,000, two between 20,000 and 100,000, three between 100,000 and 200,000, and one higher than 2,000,000. Nineteen wastewater samples were collected from two poultry slaughterhouses receiving weekly 60,000 animals each. A total of 24 samples were collected from pig sources (18 of them were slurries from storage tanks on two farms with 4,000 and 9,000 animals, and six samples were collected from wastewater in two pig slaughterhouses receiving weekly 12,500 and 15,000 pigs. Thirteen samples were obtained from bovine sources (six samples collected from bovine slurries from two different farms with around 200 calves each and seven from wastewater of five bovine slaughterhouses receiving between 900 and 7,750 animals weekly). Samples were maintained at 4°C until analysis (less than 4 h).

Nucleic acid isolation. Genomic DNA from bacterial strains was obtained using a protocol described elsewhere (24). In addition, genomic DNA from the environmental samples was extracted using the QIAamp DNA blood minikit (Qiagen GmbH, Hilden, Germany), by following the manufacturer's instructions, to facilitate the removal of PCR inhibitors.

Primers and PCK amplification. Specific amplification of the 16S rRNA genes of the *Bifidobacterium* genus was performed using a nested PCR to prevent a low amplicon yield. The first PCR was carried out with the primers Im26 and Im3 (Table 1) (23), which gave an amplicon of approximately 1,420 bp. This PCR product was later amplified using Bif164 and Bif662 (Table 1) (30, 51), giving a final fragment of approximately 520 bp. For DGGE analysis of the PCR product, a 40-bp GC clamp was attached to the 5' end of Bif662, and Bif164 was used as the forward primer (51). The reaction mixtures and PCR programs used were as

TABLE 1. Primers and host-specific probes for colony hybridization (CH) used in this study and their corresponding identities

Name	Sequence (5' to 3') ^a	Use	Target ^b	Reference(s)
Im26	GATTCTGGCTCAGGATGAACG	PCR and sequencing	<i>Bifidobacterium</i> genus specific	21
Im3	CGGTGCTCCCACTTTCATG	PCR and sequencing	<i>Bifidobacterium</i> genus specific	21
Bifi64	GGGTGGTAATGCCCGATG	DGE-PCR and sequencing	<i>Bifidobacterium</i> genus specific	28, 48
Bifi62	CCACCGTTACACCGGAA	PCR and sequencing	<i>Bifidobacterium</i> genus specific	28
Bifi62-GC	CGCCCCGCCGC GCCGGCGGGCGGGCGGGCACGGG GGG-CCACCGTTACACCGGAA	DGE-PCR	<i>Bifidobacterium</i> genus specific	48
27f	AGAGTTTGATCCTGGCTCAG	PCR and sequencing	Most eubacteria	55
1492r	TACGGTTACCTTGTACGAC	PCR and sequencing	Enterics and most eubacteria	55
HM-B	[DIG]GACTGGGAGCAAGCCCTTCGGGGTGAGTGTACCTT	CH	<i>B. adolescentis</i> , <i>B. ruminantium</i>	This study
PL	[DIG]CTTTATCGGGGAGCAGCGAGAGTAGTGTACC	CH	<i>B. gallinarum</i> , <i>B. pulloorum</i> , <i>B. saeculare</i> , <i>B. subtilis</i>	This study
PG-B	[DIG]GGGTGTAAACCGCTTTGTATTGGGAGCAAGCG	CH	<i>B. sinensis</i> , <i>B. minimum</i> , <i>B. indicum</i> , <i>B. asteroides</i> , <i>B. cornetiforme</i> , <i>B. mongoliense</i>	This study
CW-E	[DIG][CGCTTTTGTTC AAGGGCAAGGCACGGTTTCGGCCGT	CH	<i>B. animalis</i> , <i>B. pseudolongum</i>	This study

described elsewhere (23, 51). All reactions were performed in a 2400 GeneAmp PCR system (Perkin-Elmer, Foster City, CA).

To amplify 16S rRNA genes of total bacteria, the universal primers 27f and 1492r were used (Table 1) (57). The reaction mixture consisted of 1× *Taq* polymerase buffer (Eppendorf, Hamburg, Germany), 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate (dNTP), a 0.4 mM concentration of each primer, 2.5 U of *Taq* polymerase (Eppendorf, Hamburg, Germany), and 2 µl of the DNA extraction in a final volume of 50 µl. The reaction was performed under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s; and 72°C for 7 min.

Later, 5 µl of the PCR product was analyzed with 0.8 or 2% agarose gel to confirm successful amplification.

DGGE analysis of PCR products. DGGE analysis of PCR amplicons was performed using a DCode system apparatus (Bio-Rad, Hercules, CA). The PCR product was analyzed in 8% (wt/vol) polyacrylamide gels (30% acrylamide-bisacrylamide [37.5:1]). Polyacrylamide gels were run in 1× Tris-acetate acid-EDTA (TAE) (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA; pH 7.4) with an increasing concentration gradient ranging from 45% to 55% (at 100% denaturing conditions, the gel contains 7 M urea and 40% [vol/vol] formamide). PCR amplicons were separated by electrophoresis at 85 V at 60°C for 16 h.

The gels were stained for 45 min in 1× sodium chloride-Tris-EDTA buffer (STE) (100 mM sodium chloride, 10 mM Tris, 1 mM EDTA; pH 7.4) with SybrGold nucleic acid stain (Molecular Probes Inc., Eugene, OR) and viewed under UV by Image Master (Amersham Pharmacia Biotech). Gels were scanned, the PCR profiles were analyzed using the unweighted-pair group method analysis (UPGMA), and three distance measures were used (Pearson, Dice, and Jaccard). Jackknife analysis was done to assess the relative support for the tree topology. Clustering analyses were performed using Fingerprinting II software (Bio-Rad, Hercules, CA).

Sequence analysis of 16S rRNA gene amplicons. Bands obtained with DGGE analysis were excised with a sterile razorblade. DNA was extracted using a QIAEX II gel extraction kit (Qiagen GmbH, Hilden, Germany). The product was reamplified with the primers Bif164 and Bif662, mentioned above. Finally, amplicons were purified via QIAquick PCR purification (Qiagen GmbH, Hilden, Germany) and visualized with agarose gel. The sequencing reaction was achieved using a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and adding between 1 µl and 5 µl of DNA, depending on the DNA concentration. The product was purified and analyzed with an automated DNA sequencer (ABI PRISM 3700, Perkin Elmer; Serveis Científicotècnics, University of Barcelona).

All the 16S rRNA gene sequences were edited and assembled using version 7.0.1 of the BioEdit program (22). Sequences were subjected to similarity search analysis using the BLASTN software (Wisconsin Package version 10.2) and the Ribosomal Database Project II for species identification.

Bifidobacterium isolation and detection by colony hybridization. We then attempted to isolate the host-specific strains related with the specific DGGE bands from environmental samples. The main host-specific DGGE bands were used to design DNA probes for use in the colony hybridization technique. Two different approaches were followed to produce the DNA probes: (i) use of the whole DGGE band labeled with digoxigenin and (ii) design of specific short probes from the DGGE band sequences.

The DNA from DGGE bands was extracted from the gel, purified, and labeled with digoxigenin according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Labeling was performed by the incorporation of digoxigenin-11-deoxyuridine-triphosphate during the PCR with the Bif164 and Bif662 primers (18). The PCR conditions used were as mentioned above and described elsewhere. However, instead of the previous dNTP mix, 3.5 mM UTP-digoxigenin, 7 mM dTTP, and 10 mM dATP, dGTP, and dCTP were added (51).

To design short DNA probes, the software ClustalW 1.81 included in the BioEdit sequence alignment editor was used to detect specific regions of the DNA fragments. Detection of possible loops and internal hybridization was checked using Primer3 (version 0.4.0), an online program for primer design (<http://primer3.sourceforge.net/>). The designed probes labeled with digoxigenin were synthesized by Sigma (St. Louis, MO), and sequences are detailed in Table 1. Digoxigenin-labeled probes can be detected by using antibodies against this molecule.

Isolation of Bifidobacterium environmental strains. Sewage and wastewater from several animal slaughterhouses were used as the source of *Bifidobacterium* environmental strains. The agar media used to isolate *Bifidobacterium* species from the environmental samples were Beerens agar (4) and human bifidobacteria sorbitol-fermenting agar (HBSA) (32, 34). Then, samples were incubated at 37°C for 48 h under anaerobic conditions (GasPak; BBL, Hampshire, United

FIG. 1. Separation of the PCR product of the *Bifidobacterium* reference strains used in this study with genus-specific primers. Lanes: A, *B. adolescentis*; B, *B. ruminantium*; C, *B. catenulatum*; D, *B. animalis* subsp. *lactis*; E, *B. minimum*; F, *B. merycicum*; G, *B. subtilis*; H, *B. longum*; I, *B. longum* subsp. *infantis*; J, *B. saeculare*; K, *B. pullorum*; L, *B. pseudolongum* subsp. *pseudolongum*; M, *B. scardovii*; N, *B. breve*; O, *B. bifidum*; P, *B. pseudocatenulatum*; Q, *B. coryneforme*; R, *B. dentium*; S, *B. globosum*; T, *B. choerinum*; U, *B. angulatum*; V, *B. indicum*; W, *B. boum*; X, *B. thermophilum*; Y, *B. asteroides*. The denaturant gradient range was 45% (urea and formamide) in the upper part of the gel and 55% at the bottom.

Kingdom) with CO₂ atmosphere generators (Anaerocult A; Merck, Darmstadt, Germany). Colonies growing in these media were membrane transferred. Positively charged Hybond N⁺ nylon membranes (Amersham, Pharmacia Biotech, Buckinghamshire, United Kingdom) were used to perform the colony hybridization protocol as described elsewhere (43).

Confirmation of the identity of Bifidobacterium isolates. Some of the positive colonies for the different host-specific hybridization probes were cultivated using CBA and transferred to RCM broth. One milliliter of the growth cells was used to extract the DNA by following the protocol described elsewhere (24). The strains were identified by 16S rRNA gene sequence analysis using the *Bifidobacterium*-specific primers Im26 and Im3. Nevertheless, the universal primers 27f and 1492r were used when no amplification was observed with the *Bifidobacterium*-specific primers. The conditions used were as described above.

Sequence identity was checked using the BLASTN software (Wisconsin Package version 10.2) and the Ribosomal Database Project II for species identification.

Nucleotide sequence accession numbers. All sequences were submitted to GenBank under the following accession numbers: DGGE bands, JF519638 to JF519674; isolated *Bifidobacterium* strains, JF519675 to JF519695.

RESULTS

DGGE profiles of Bifidobacterium type strains. The PCR and DGGE conditions used in this study are suitable for the discrimination of almost all the reference strains of *Bifidobacterium* analyzed. However, five of the species could not be amplified with the primers used. These species were *B. animalis* subsp. *animalis* DSM 20104^T, *B. cuniculi* DSM 20435^T, *B. gallicum* DSM 20093^T, *B. gallinarum* DSM 20670^T, and *B. magnum* DSM 20222^T.

Each individual *Bifidobacterium* species displayed a unique DGGE band located at a single position on the gel (Fig. 1). However, *B. adolescentis* and *B. merycicum* displayed two bands located in different positions. A weak second band was also observed for *B. pseudolongum*, *B. pullorum*, and *B. saeculare*. Although the sequence of 16S rRNA genes was shown to be different by *in silico* analysis, the gradient conditions can give the same melting domains. Thus, more than one band may appear at or near any given position, hampering their identification. With the conditions used in the current study, most of the *Bifidobacterium* species were differentiated (Fig. 1). Con-

FIG. 2. DGGE profiles and dendrogram of the *Bifidobacterium* population of wastewater of different sources. □, human; ■, poultry; ▨, porcine; ▩, bovine samples. Jaccard and UPGMA clustering.

sequently, DGGE bands obtained on the analysis of environmental samples, which were identified as melting domains of more than one species by sequence analyses, were not considered for further selection of specific related bands to fecal origins.

Analysis of the DGGE profiles of environmental samples. The DGGE profiles varied with the source of the environmental samples. The profiles of the human and poultry samples were similar, but the pig and bovine samples were more heterogeneous (Fig. 2). Similar dendrograms were obtained by the different clustering algorithms used. Jackknife analysis was done to assess the relative support for the tree topology. The high stability for poultry and human clusters was assessed by Jackknife analysis (100% for each cluster). However, porcine and bovine clusters showed lower stability (76% and 56%, respectively), probably because of the observed heterogeneity of profiles.

Samples of human sources. Similar DGGE fingerprints were observed for all the human samples. Although up to nine separate bands were obtained in human profiles, only four of them were shared by all the samples (Fig. 3). Five of them were identified by sequence analysis. The sequences of all the DGGE bands were identical to, or closely related to, distinct species of *Bifidobacterium*, mainly *B. adolescentis* (Table 2).

Samples of animal sources. In case of poultry wastewater, a single band was detected for all the 19 samples analyzed (Fig. 2 and 3). This band showed 100% similarity with *B. saeculare*, *B. gallinarum*, and *B. pullorum*.

Higher heterogeneity was detected among the profiles obtained from the 24 samples from sources associated with pigs (Fig. 2 and 3). The number of bands detected ranged from two to nine. More bands were detected in slurries than in slaugh-

terhouse wastewater. The sequences of seven bands were analyzed, and their putative identities were assigned (Table 2). Three DGGE bands did not match (with more than 95% similarity) any of the sequences reported in the online databases.

The profiles of the bovine samples were highly diverse (Fig. 2 and 3). There was no relation between the diversity and the kind of the sample (feces or wastewater). Five of the bands were analyzed and identified with the most similar sequences in the online databases (Table 2).

FIG. 3. DGGE profiles of the *Bifidobacterium* population of wastewater from different sources. Lanes: 1 and 2, ruminant; 3 to 6, pig; 7 to 10, human; 11 and 12, poultry.

TABLE 2. Frequency of detection and identity with the closest relatives of the DGGE bands from the sources analyzed: human, poultry, pig, and bovine

Sample source	Band name	Frequency ^a	Closest relative(s) (% sequence similarity) ^b
Human	HM-A	15/21	<i>B. adolescentis</i> (99)
	HM-B	21/21	<i>B. adolescentis</i> (100)
	HM-C	21/21	<i>B. adolescentis</i> (98)
	HM-D	18/21	<i>B. longum</i> (100)
	HM-E	21/21	<i>B. adolescentis</i> (98)
Poultry	PL-A	19/19	<i>B. saeculare</i> , <i>B. gallinarum</i> , <i>B. pullorum</i> (100)
Pig	PG-A	9/24	<i>B. minimum</i> (100)
	PG-B	18/24	No close relative identified
	PG-C	NI	<i>B. longum</i> (98)
	PG-D	NI	No close relative identified
	PG-E	NI	No close relative identified
	PG-F	NI	<i>B. pseudocatenulatum</i> (99)
	PG-H	22/24	<i>B. thermophilum</i> (100), <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (100)
Bovine	CW-A	8/13	<i>B. ruminantium</i> (99)
	CW-B	5/13	<i>B. adolescentis</i> (100)
	CW-C	5/13	<i>B. merycicum</i> (100)
	CW-E	10/13	<i>B. pseudolongum</i> (100)
	CW-F	9/13	<i>B. thermophilum</i> (100), <i>B. thermacidophilum</i> subsp. <i>thermacidophilum</i> (100)

^a Number of samples in which the band was detected/total number of samples analyzed. NI, not identified; we were unable to assign a band frequency due to the large number of bands in the profiles, their close proximity, and the differences between the DGGE gels.

^b According to the sequence match performed in the RDP. "No close relative identified" indicates that no species with a similarity higher than 95% was observed.

***Bifidobacterium* isolation from environmental samples.** Two different kinds of probes were designed to isolate the host-specific *Bifidobacterium* species from environmental samples. First we used the digoxigenin-labeled DGGE band as a probe. To this end, five of the most abundant DGGE bands (around 520 bp) for each host were selected and then labeled with digoxigenin. The bands chosen were HM-B (100% similarity with *B. adolescentis*), PL (100% similarity with *B. saeculare*), PG-B (unidentified *Bifidobacterium*), CW-E (100% similarity with *B. pseudolongum* subsp. *globosum*), and CW-F (100% similarity with *B. thermophilum* and *B. thermacidophilum*). Only the probes HM-B and CW-F detected the target species (Table 3). However, HM-B was nonspecific, since it detected other human-related species. *B. saeculare*, the unidentified *Bifidobacterium* species associated with pigs, and *B. pseudolongum* subsp. *globosum* could not be isolated using these probes. Therefore, in order to avoid possible lack of specificity associated with the length of the probe, new probes were designed (Table 1). The specific DGGE bands HM-B, PL, PG-B, and CW-E were aligned with ClustalW 1.81, and specific regions were identified. The lengths of the new specific probes were between 33 and 36 bp. A total of 1,357 colonies were screened by colony hybridization with the host-specific probes (Table 4). The human probe HM-B-p and the pig probe PG-B-p detected the corresponding targeted species: *B. adolescentis*

TABLE 3. Identification of the strains isolated from different wastewaters using the DGGE band as a probe for colony hybridization

DGGE band used as DNA probe (no. of positive colonies for CH ^a)	Isolated strain (% sequence identity ^b)
HM-B (5/16)	<i>B. adolescentis</i> (100) <i>B. longum</i> (100) <i>B. longum</i> (99) <i>B. pseudocatenulatum</i> (99) <i>B. pseudolongum</i> subsp. <i>pseudolongum</i> (100)
PL (23/60)	<i>B. longum</i> subsp. <i>suis</i> (97) <i>B. longum</i> subsp. <i>suis</i> (97) <i>B. longum</i> subsp. <i>suis</i> (97) <i>Lactobacillus agilis</i> (99) <i>L. agilis</i> (98) <i>Morganella morganii</i> (98) <i>M. morganii</i> (94)
PG-B (11/26)	<i>B. thermophilum</i> / <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (99) <i>B. thermophilum</i> / <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (93)
CW-E (17/44)	<i>B. thermophilum</i> / <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (99) <i>B. thermophilum</i> / <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (99)
CW-F (20/44)	<i>B. thermophilum</i> / <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (99) <i>B. thermophilum</i> / <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (99)

^a Number of positive colonies for colony hybridization/total number of colonies analyzed.

^b According to the sequence match performed in the RDP.

and an unidentified pig *Bifidobacterium* species. Although 594 and 312 colonies were screened by colony hybridization with PL-p and CW-E-p, respectively, neither of them isolated the targeted *Bifidobacterium* species. However, lack of specificity was found for CW-E-p, which detected *B. thermophilum* instead of *B. pseudolongum* subsp. *globosum* (Table 4).

DISCUSSION

PCR with specific primers followed by DGGE analysis enables us to differentiate between some of the *Bifidobacterium* species. The position of a PCR product in the gel depends on the melting domains formed as it advances across the gel. Different sequences have different melting domains, thus resulting in different positions in the gel. However, it is not always possible to discriminate between amplicons with different sequences. In some cases, different strains migrate to the same position, as do *B. thermophilum*, *B. boum*, and *B. indicum* or *B. pullorum*, *B. saeculare*, *B. pseudolongum*, and *B. scardovii*. Although 16S rRNA gene sequences from these strains proved to be different, they may have had the same melting domains. In other cases, some species can display more than one DGGE band. Two different bands were clearly described in *B. adolescentis* and *B. merycicum*. Species of the *Bifidobacterium* genus have between one and five operon copies of the 16S rRNA gene, which differ in their sequences (12). The detection of

TABLE 4. Identification of the strains isolated from different wastewaters with the designed probes for colony hybridization

Probe (no. of colonies positive for CH ^a)	Isolated strain (% sequence identity ^b)
HM-B (5/132)	<i>B. adolescentis</i> (99) <i>B. pseudocatenulatum</i> (100) <i>B. pseudocatenulatum</i> (99) <i>B. pseudocatenulatum</i> (98) <i>B. catenulatum</i> (98)
PL (0/594)	
PG-B (10/319)	<i>Lactobacillus reuteri</i> (100) <i>B. thermophilum</i> / <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (100) <i>B. thermophilum</i> / <i>B. thermacidophilum</i> subsp. <i>porcinum</i> (97) Unknown bacterium
CW-E (20/312)	<i>B. thermacidophilum</i> subsp. <i>thermacidophilum</i> (100) <i>B. thermophilum</i> (99)

^a Number of samples in which the band was detected/total number of samples analyzed.

^b According to the sequence match performed in the RDP.

more of one band using DGGE was already reported by Sakotari et al, who described three different bands for *B. adolescentis*: two different operons of the 16 rRNA gene differing by a few nucleotides and a third band containing heteroduplexes of both sequences (51). Other limitations of this technique already reported were also detected in this study and include (i) the presence of a commonly detected band in all the samples located in the upper part of the gel and (ii) the presence of a doublet with two adjacent bands. For the first limitation, the sequence analysis showed a mix of different sequences described as possible PCR heteroduplexes, which due to their instability remain in the upper part of the DGGE gel (51). The second effect had been related to an abortion of the elongation reaction during PCR caused by the GC clamp (hairpin formation) (51). This can result in DNA molecules with slightly different migration.

Despite these limitations, differentiation between *Bifidobacterium* species in environmental samples was possible with this PCR and DGGE analysis. In previous studies, the bacterial diversity of feces from individuals was compared. High bacterial variability among samples was described as a function of age, diet, or geographic area (32, 52, 57). In the present study, wastewater from different origins was analyzed. Wastewater showed similar profiles (diversity) for both human and poultry samples. Wastewater samples include a blend of a large number of individual feces and are more representative of fecal pollution sources. Individual fecal samples can be highly variable and, as such, are not representative of populations. Different *Bifidobacterium* species can show different survival, and an “average effect” occurs. The average effect consists of a decrease in the diversity of nondominant bacteria with a low occurrence in individuals, which emphasizes the most abundant species with higher occurrence (59). Because previous studies reported a sensitivity of the DGGE able to detect bacteria that constitute up to 1% of the total bacterial community (40), we have to consider that only the most dominant

bacteria will be detected with this technique. Therefore, the most abundant human-related *Bifidobacterium* species and those with the best survival are usually distributed among the different sewage samples analyzed, which enables the identification of a possible MST marker.

Human-related *B. adolescentis* and *B. longum* were detected in all the sewage samples together with two unidentified species. These two species shared 98% similarity with *B. adolescentis* for the 520-bp fragment analyzed with sequence analysis. The *B. adolescentis*, *B. longum*, and *B. catenulatum* group have been described as the main species detected in adult human gut and wastewater (33, 36, 51, 58). The results obtained in the present study confirm *B. adolescentis* as a possible MST marker of human fecal pollution (10, 32). In a study performed in France, *B. adolescentis* was detected in most posttreated water from wastewater treatment plants, suggesting its use in surface water polluted by sewage, indicating recent fecal pollution (58). Another study could detect *B. adolescentis* for 24 h in summer and 72 h in winter by PCR and for 120 h in summer and more than 240 h in winter with quantitative PCR once the bacterium was released in the environment (11).

Low diversity of the *Bifidobacterium* genus was observed in samples from poultry sources, and the same single band for all samples was described. The species was identified as *B. saeculare*. Analysis *in silico* reported a difference of just two nucleotides with *B. pullorum*. The same DGGE profile for both species was obtained when reference species were analyzed, hampering their discrimination with this technique. Previous studies also reported a lower presence of the *Bifidobacterium* genus in the gut of poultry, represented mainly by *B. pullorum* (20, 61).

Greater *Bifidobacterium* diversity was detected in samples from pig and bovine sources, represented by a major number of species and high variability within the samples, hampering the identification of a common and host-specific species. The lack of homogeneity among samples could be explained through the different diets and/or prebiotics fed to the animals, depending on the farms and ages of the animals (37, 54). A band corresponding to *B. thermophilum*/*B. thermacidophilum* was detected in almost all the pig samples and in some of the bovine samples. Both species are closely related, and their discrimination is difficult by 16S rRNA gene analysis (35). The analysis of pig samples showed three bands not related with any previously identified species in the databases, which increases the number of unidentified species of this genus. The human-related species *B. longum* and *B. pseudocatenulatum* and one band with *B. minimum* related to sewage (56) were also detected in pig samples. Most of the previous studies analyzing pig microbiota coincided in detecting mainly *B. pseudolongum* subsp. *pseudolongum*, *B. thermophilum*, and *B. thermacidophilum* subsp. *porcinum*. The 16S-23S rRNA internal transcribed sequence (ITS) of the latter species had already been proposed as an MST indicator (35). This species was detected in runoff water after manure was spread in the fields, suggesting it as a good pig fecal indicator. In this work, it has also been detected in cattle samples, suggesting that it is not an indicator of specific farming. Although the *Bifidobacterium* genus is not among the main bacteria from the ruminant gut (27), some species, such as *B. thermophilum* and *B. pseudolongum* subsp. *globosum*, have been associated with bovine

samples (29, 56). These two species were the most frequently detected in the DGGE profiles of bovine samples, followed by *B. ruminantium* and *B. merycicum* (both related to bovine) and *B. adolescentis* (human-related species). *B. ruminantium* and *B. adolescentis* are very closely related species. The 520-bp fragment analyzed allowed the differentiation of some of these species but not others, for instance, distinguishing between *B. adolescentis* and *B. ruminantium*, which are both important species for host identification. The analysis of different fragments of DNA could be taken into consideration for further analysis to identify some of these species.

In the second part of this study, the environmental isolation of selected *Bifidobacterium* species was planned in order to select possible MST indicators for further studies. Different results can be obtained in the analysis of bacterial populations, depending on whether molecular or culture-dependent techniques are used. On the one hand, not all environmental bacteria can be cultivated with the media currently available. And on the other hand, lack of specificity was found when using the colony hybridization with species-specific probes in this study. The lack of specificity was greater when the whole DGGE fragment (~520 bp) was used as a probe. *B. adolescentis* and a pig-related *Bifidobacterium* species that has yet to be identified were recovered successfully with the small probes. The poultry (*B. saeculare*)- and bovine (*B. pseudolongum* subsp. *globosum*)-related species could not be isolated from the environment, perhaps because they could not grow on Beerens or HBSA medium or possibly due to the low abundance of these species in the samples, as a result of which they were masked by the most abundant microbiota. Species other than *Bifidobacterium* spp., such as *Morganella morganii* and *Lactobacillus* spp., which can grow in the selected media, were also isolated.

In summary, there is variability between the diversity of *Bifidobacterium* populations from wastewater from human and other animal sources, which allows us to select host-related species that may be used as MST markers. *B. adolescentis* has been confirmed as a human MST marker, although it was detected in a few bovine samples. *B. saeculare*, *B. pseudolongum* subsp. *globosum*, and a hitherto identified *Bifidobacterium* sp. could be used as MST markers for poultry, bovine, and pig sources, respectively. *B. thermophilum* and *B. thermacidophilum* were detected in almost all the bovine and pig samples. Colony hybridization in selective media by host-specific probes isolated *B. adolescentis* and the pig-related *Bifidobacterium* species. However, the other selected species could not be isolated from the environment, and unspecific bacteria were detected instead.

ACKNOWLEDGMENTS

This study was supported by the Spanish Government, research project CGL2007-65980-C02-01.

Analyses with Fingerprinting II software were done at Centre de Recerca en Seguretat Animal (Universitat Autònoma de Barcelona).

REFERENCES

- Andersson, A. F., et al. 2008. Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS One* **3**:e2836.
- Bachoon, D. S., C. M. Miller, C. P. Green, and E. Otero. 2010. Comparison of four polymerase chain reaction methods for the rapid detection of human fecal pollution in marine and inland waters. *Int. J. Microbiol.* **2010**:595692.
- Ballesté, E., X. Bonjoch, L. A. Belanche, and A. R. Blanch. 2010. Molecular indicators used in the development of predictive models for microbial source tracking. *Appl. Environ. Microbiol.* **76**:1789–1795.
- Beerens, H. 1991. Detection of bifidobacteria by using propionic acid as a selective agent. *Appl. Environ. Microbiol.* **57**:2418–2419.
- Bernhard, A. E., and K. G. Field. 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **66**:4571–4574.
- Bernhard, A. E., and K. G. Field. 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Appl. Environ. Microbiol.* **66**:1587–1594.
- Biavati, B., and P. Mattarelli. 2006. The family Bifidobacteriaceae, p. 322–382. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), *The prokaryotes*, vol. 3. Springer, New York, NY.
- Blanch, A. R., et al. 2004. Tracking the origin of faecal pollution in surface water: an ongoing project within the European Union research programme. *J. Water Health* **2**:249–260.
- Blanch, A. R., et al. 2006. Integrated analysis of established and novel microbial and chemical methods for microbial source tracking. *Appl. Environ. Microbiol.* **72**:5915–5926.
- Bonjoch, X., E. Ballesté, and A. R. Blanch. 2004. Multiplex PCR with 16S rRNA gene-targeted primers of bifidobacterium spp. to identify sources of fecal pollution. *Appl. Environ. Microbiol.* **70**:3171–3175.
- Bonjoch, X., F. Lucena, and A. R. Blanch. 2009. The persistence of bifidobacteria populations in a river measured by molecular and culture techniques. *J. Appl. Microbiol.* **107**:1178–1185.
- Candela, M., B. Vitali, D. Matteuzzi, and P. Brigidi. 2004. Evaluation of the *rm* operon copy number in *Bifidobacterium* using real-time PCR. *Lett. Appl. Microbiol.* **38**:229–232.
- Dick, L. K., et al. 2005. Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. *Appl. Environ. Microbiol.* **71**:3184–3191.
- Dorai-Raj, S., J. O'Grady, and E. Colleran. 2009. Specificity and sensitivity evaluation of novel and existing *Bacteroidales* and *Bifidobacterium*-specific PCR assays on feces and sewage samples and their application for microbial source tracking in Ireland. *Water Res.* **43**:4980–4988.
- Eckburg, P. B., et al. 2005. Diversity of the human intestinal microbial flora. *Science* **308**:1635–1638.
- Fasoli, S., et al. 2003. Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *Int. J. Food Microbiol.* **82**:59–70.
- Fiksdal, L., J. S. Maki, S. J. LaCroix, and J. T. Staley. 1985. Survival and detection of *Bacteroides* spp., prospective indicator bacteria. *Appl. Environ. Microbiol.* **49**:148–150.
- García-Aljaro, C., M. Muniesa, J. Jofre, and A. R. Blanch. 2004. Prevalence of the *stx2* gene in coliform populations from aquatic environments. *Appl. Environ. Microbiol.* **70**:3535–3540.
- Gavini, F., et al. 2006. *Bifidobacterium* species isolated from animal feces and from beef and pork meat. *J. Food Prot.* **69**:871–877.
- Gong, J., et al. 2008. Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. *J. Appl. Microbiol.* **104**:1372–1382.
- Gourmelon, M., et al. 2010. Development of microbial and chemical MST tools to identify the origin of the faecal pollution in bathing and shellfish harvesting waters in France. *Water Res.* **44**:4812–4824.
- Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**:95–98.
- Kaufmann, P., A. Pfeifferkorn, M. Teuber, and L. Meile. 1997. Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. *Appl. Environ. Microbiol.* **63**:1268–1273.
- Kellogg, D. E., J. J. Sninsky, and S. Kwok. 1990. Quantitation of HIV-1 proviral DNA relative to cellular DNA by the polymerase chain reaction. *Anal. Biochem.* **189**:202–208.
- Kildare, B. J., et al. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. *Water Res.* **41**:3701–3715.
- King, E. L., D. S. Bachoon, and K. W. Gates. 2007. Rapid detection of human fecal contamination in estuarine environments by PCR targeting of *Bifidobacterium adolescentis*. *J. Microbiol. Methods* **68**:76–81.
- Kocherginskaya, S., R. I. Aminov, and B. A. White. 2001. Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches. *Anaerobe* **7**:119–134.
- Kreider, C. A. 1998. Persistence of PCR-detectable *Bacteroides distasonis* from human feces in river water. *Appl. Environ. Microbiol.* **64**:4103–4105.
- Lamendella, R., J. W. Santo Domingo, C. Kely, and D. B. Oerther. 2008. Bifidobacteria in feces and environmental waters. *Appl. Environ. Microbiol.* **74**:575–584.
- Langendijk, P. S., et al. 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl. Environ. Microbiol.* **61**:3069–3075.
- Layton, A., et al. 2006. Development of *Bacteroides* 16S rRNA gene Taq-

- Man-based real-time PCR assays for estimation of total, human, and bovine faecal pollution in water. *Appl. Environ. Microbiol.* **72**:4214–4224.
32. Lynch, P. A., B. J. Gilpin, L. W. Sinton, and M. G. Savill. 2002. The detection of *Bifidobacterium adolescentis* by colony hybridization as an indicator of human faecal pollution. *J. Appl. Microbiol.* **92**:526–533.
 33. Mangin, I., et al. 2006. Characterization of human intestinal bifidobacteria using competitive PCR and PCR-TTGE. *FEMS Microbiol. Ecol.* **55**:28–37.
 34. Mara, D. D., and J. I. Oragui. 1983. Sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution. *J. Appl. Bacteriol.* **55**:349–357.
 35. Marti, R., P. Dabert, and A. M. Pourcher. 2009. Pig manure contamination marker selection based on the influence of biological treatment on the dominant fecal microbial groups. *Appl. Environ. Microbiol.* **75**:4967–4974.
 36. Matsuki, T., K. Watanabe, R. Tanaka, M. Fukuda, and H. Oyaizu. 1999. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. *Appl. Environ. Microbiol.* **65**:4506–4512.
 37. Molbak, L., L. E. Thomsen, T. K. Jensen, K. E. Bach Knudsen, and M. Boye. 2007. Increased amount of *Bifidobacterium thermacidophilum* and *Megasphaera elsdenii* in the colonic microbiota of pigs fed a swine dysentery preventive diet containing chicory roots and sweet lupine. *J. Appl. Microbiol.* **103**:1853–1867.
 38. Morrison, C. R., D. S. Bachoon, and K. W. Gates. 2008. Quantification of enterococci and bifidobacteria in Georgia estuaries using conventional and molecular methods. *Water Res.* **42**:4001–4009.
 39. Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695–700.
 40. Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* **73**:127–141.
 41. Myers, R. M., T. Maniatis, and L. S. Lerman. 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods Enzymol.* **155**:501–527.
 42. Nebra, Y., X. Bonjoch, and A. R. Blanch. 2003. Use of *Bifidobacterium dentium* as an indicator of the origin of fecal water pollution. *Appl. Environ. Microbiol.* **69**:2651–2656.
 43. Nebra, Y., J. Jofre, and A. R. Blanch. 2002. The effect of reducing agents on the recovery of injured *Bifidobacterium* cells. *J. Microbiol. Methods* **49**:247–254.
 44. Nielsen, D. S., et al. 2003. Case study of the distribution of mucosa-associated *Bifidobacterium* species, *Lactobacillus* species, and other lactic acid bacteria in the human colon. *Appl. Environ. Microbiol.* **69**:7545–7548.
 45. Okabe, S., N. Okayama, O. Savichtcheva, and T. Ito. 2007. Quantification of host-specific *Bacteroides-Prevotella* 16S rRNA genetic markers for assessment of fecal pollution in freshwater. *Appl. Microbiol. Biotechnol.* **74**:890–901.
 46. Petersson, A., et al. 2009. Denaturing gradient gel electrophoresis (DGGE)-based monitoring of intestinal lactobacilli and bifidobacteria of pigs during a feeding trial. *Arch. Anim. Nutr.* **63**:112–126.
 47. Rajilic-Stojanovic, M., H. Smidt, and W. M. de Vos. 2007. Diversity of the human gastrointestinal tract microbiota revisited. *Environ. Microbiol.* **9**:2125–2136.
 48. Reischer, G. H., D. C. Kasper, R. Steinborn, A. H. Farnleitner, and R. L. Mach. 2007. A quantitative real-time PCR assay for the highly sensitive and specific detection of human faecal influence in spring water from a large alpine catchment area. *Lett. Appl. Microbiol.* **44**:351–356.
 49. Requena, T., et al. 2002. Identification, detection, and enumeration of human bifidobacterium species by PCR targeting the transaldolase gene. *Appl. Environ. Microbiol.* **68**:2420–2427.
 50. Resnick, I. G., and M. A. Levin. 1981. Assessment of bifidobacteria as indicators of human fecal pollution. *Appl. Environ. Microbiol.* **42**:433–438.
 51. Satokari, R. M., E. E. Vaughan, A. D. Akkermans, M. Saarela, and W. M. de Vos. 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **67**:504–513.
 52. Simpson, J. M., V. J. McCracken, H. R. Gaskins, and R. I. Mackie. 2000. Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. *Appl. Environ. Microbiol.* **66**:4705–4714.
 53. Simpson, J. M., V. J. McCracken, B. A. White, H. R. Gaskins, and R. I. Mackie. 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J. Microbiol. Methods* **36**:167–179.
 54. Tzortzis, G., A. K. Goulas, J. M. Gee, and G. R. Gibson. 2005. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. *J. Nutr.* **135**:1726–1731.
 55. van der Wielen, P. W., D. A. Keuzenkamp, L. J. Lipman, F. van Knapen, and S. Biesterveld. 2002. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microb. Ecol.* **44**:286–293.
 56. Ventura, M., D. van Sinderen, G. F. Fitzgerald, and R. Zink. 2004. Insights into the taxonomy, genetics and physiology of bifidobacteria. *Antonie Van Leeuwenhoek* **86**:205–223.
 57. Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697–703.
 58. Wery, N., C. Monteil, A. M. Pourcher, and J. J. Godon. 2010. Human-specific fecal bacteria in wastewater treatment plant effluents. *Water Res.* **44**:1873–1883.
 59. Whitford, M. F., R. J. Forster, C. E. Beard, J. Gong, and R. M. Teather. 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. *Anaerobe* **4**:153–163.
 60. Yampara-Iquise, H., G. Zheng, J. E. Jones, and C. A. Carson. 2008. Use of a *Bacteroides thetaiotaomicron*-specific alpha-1-6, mannanase quantitative PCR to detect human faecal pollution in water. *J. Appl. Microbiol.* **105**:1686–1693.
 61. Zhu, X. Y., and R. D. Joerger. 2003. Composition of microbiota in content and mucus from cecae of broiler chickens as measured by fluorescent in situ hybridization with group-specific, 16S rRNA-targeted oligonucleotide probes. *Poult. Sci.* **82**:1242–1249.
 62. Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie, and H. R. Gaskins. 2004. Molecular ecological analysis of the gastrointestinal microbiota: a review. *J. Nutr.* **134**:465–472.
 63. Zoetendal, E. G., A. von Wright, T. Vilpponen-Salmela, K. Ben-Amor, A. D. Akkermans, and W. M. de Vos. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl. Environ. Microbiol.* **68**:3401–3407.