



# Pig Manure Contamination Marker Selection Based on the Influence of Biological Treatment on the Dominant Fecal Microbial Groups

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Selection of a pig manure contamination marker based on the influence of biological  
treatment on the dominant faecal microbial groups

Running title: microbial marker of pig manure

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## Abstract

The objective of this study was to identify a microbial marker for pig manure contamination. We quantified the persistence of four dominant bacterial groups from the pig intestinal tract throughout manure handling in ten livestock operations (including aerobic digestion) using molecular typing. The partial 16S rRNA genes of *Bacteroides-Prevotella*, *Eubacterium-Clostridiaceae*, *Bacillus-Streptococcus-Lactobacillus* (BSL) and *Bifidobacterium* were amplified and analysed by capillary electrophoresis single strand conformation polymorphism (CE-SSCP). The most dominant bacterial populations were identified by cloning and sequencing their 16S rRNA genes. The results showed that *Bifidobacterium* and, to a lesser extent, members of the BSL group, were less affected by the aerobic treatment than either *Eubacterium-Clostridiaceae* or *Bacteroides-Prevotella*. Two *Bifidobacterium* species found in raw manure were still present in manure during land application, suggesting that they can survive outside the pig intestinal tract and also survive aerobic treatment. The 16S-23S rRNA ITS (Internal Transcribed Sequence) of one species, *Bifidobacterium thermacidophilum* subsp. *porcinum*, was sequenced and a specific pair of primers was designed for its detection in the environment. Using this nested PCR assay, this potential marker was not detected in samples from 30 bovine, 30 poultry, and in 28 human faeces or in 15 urban wastewater effluents. As it was detected in runoff waters after spreading of pig manure, we propose this marker as a suitable microbial indicator of pig manure contamination.

## INTRODUCTION

Brittany only represents 7% of France, but is the main pig production area and hosts approximately 14 million fatteners per year. This high concentration of confined pig feeding has led to over-application of manures to soil which contributes to water pollution. Physical and biological manure treatment processes have been developed to limit nitrogen and phosphorus pollution (5). As these treatments were not designed to eliminate microbial pollution, even treated manure can contain pathogenic microorganisms (27) and agricultural soils and water systems can thus potentially still be contaminated through surface runoff and seepage. As manure application can increase the number of pathogens in the soil (18), pig faeces may represent a significant risk to human health in Brittany. Currently, the bacteria monitored to assess faecal contamination (*E. coli*, faecal coliforms and enterococci) do not differentiate contamination from pig slurry from other animals or from pollution by humans. It is thus important to develop analytic tools to specifically detect this source of pollution. Many studies have already proposed potential markers for the detection of host-specific faecal pollution (2, 3, 8, 12-15, 20, 37, 38, 48, 49). Much of this research has concentrated on distinguishing human and animal sources of contamination (3, 8, 20, 30, 38). Some studies have focused on identifying individual sources of animal pollution, and have described molecular markers for faeces from duck (13), chicken (37), bovine (2, 3, 49) or cervids (6). Concerning pigs, biomarkers have been proposed for faecal contamination but rarely for manure, the bacterial composition of which differs from that of the faeces (9). Molecular markers have been developed to target the 16S rDNA gene sequences of dominant *Eubacteria* (2, 14, 43, 48) or methanogenic *Archeabacteria* (54) of the intestinal tract of pigs, whereas Khatib *et al.* (29) targeted the STII toxin gene from enterotoxigenic *E. coli*. Among the dominant groups of pig faecal *Eubacteria*, which include *Bacteroides-Prevotella*, *Eubacterium-Clostridiacea*, *Lactobacillus-Streptococcus* (34, 45, 51, 58) and to a lesser

67 extent, *Bifidobacterium* (40), the *Bacteroides-Prevotella* group has been particularly well  
68 studied (14, 22, 44). This marker of pig faeces was described by Okabe *et al.* (44) but the  
69 work was based on faeces sampled from only two farms and the number of clones analysed  
70 was low. Gourmelon *et al.* (22) also detected the presence of a specific marker of pig faeces  
71 belonging to the *Bacteroides-Prevotella* group in five stored manures. Although these studies  
72 revealed the presence of specific markers in faecal samples and in the subsequent pig  
73 manures, they did not address the possible disappearance of these anaerobic bacteria during  
74 storage or biological treatment of the manure.

75  
76 Due to the lack of data concerning the bacterial flora of manure, the aim of this study was (i)  
77 to compare the behaviour of the *Bacteroides-Prevotella* group with *Eubacterium-*  
78 *Clostridiaceae*, *Bacillus-Streptococcus-Lactobacillus* (BSL) and *Bifidobacterium* monitoring  
79 throughout the biological manure treatment and (ii) to search for a molecular marker amongst  
80 these groups of bacteria that was consistently present in the manure intended for land  
81 application. In the first part of the study, the persistence of the dominant bacteria throughout  
82 treatment was studied using molecular typing, Capillary Electrophoresis-Single Strand  
83 Conformation Polymorphism (CE-SSCP) (45) based on the analysis of the 16S rRNA genes.  
84 CE-SSCP is a fingerprinting technique in which single-stranded DNA fragments of the same  
85 length are separated based on the conformation of their secondary structure (23). The major  
86 advantages of this technique are its reproducibility between runs and its high resolution power  
87 with fewer false results than DGGE (25, 26).

88 The second part of the article describes the relevance of the potential marker of pig manure  
89 (*Bifidobacterium thermacidophilum* subsp. *porcinum*), selected according to the results of the  
90 CE-SSCP profiles and the subsequent identification of dominant peaks of the CE-SSCP

profiles. The specificity of this pig marker was then tested by assessing the host distribution in a selection of faecal, manure and wastewater samples.

## MATERIALS AND METHODS

### Sample collection

Manure samples: Manures were collected from 17 piggeries located across Brittany. In these farms, raw manure was stored for between two and eight weeks in a primary anaerobic tank, followed by aeration treatment for a period of three to four weeks before final anaerobic storage for between three and nine months. The chemical characteristics of the manures were similar on all farms. The mean pH of the raw and treated manures was 7.5 and 7.8 respectively. The corresponding dry matter contents were 4.3% and 5.1% (wt/wt); total Kjeldahl nitrogen contents 4.3 and 2.0 g litre<sup>-1</sup> and soluble Chemical Organic Demand 9.7 and 2.4 g O<sub>2</sub> litre<sup>-1</sup>, respectively. All manures stored in tanks were homogenised by mixing with a propeller agitator for at least 30 minutes before sampling. A volume of 30 litres of manure was removed and transferred to the laboratory. The samples were then re-mixed with a propeller homogenizer. One litre of homogenised manure was transferred to a flask. Manure was then centrifuged at 16,000 g to form a pellet of approximately 250 mg (wet weight). The pellets were stored at -20°C.

Faecal samples: A total of 90 samples of animal faeces (30 bovine, 30 pig, 30 poultry faeces) were collected from 62 farms across Brittany. Twenty-eight samples of human faeces from healthy people were obtained from two French research institutes (IFREMER, Brest and INRA, Jouy-en-Josas). Approximately 250 mg (wet weight) of each faeces were transferred into a microtube and stored at -20°C.

Water samples: Fifteen urban wastewater (5 raw and 10 treated effluents) were collected from locations across Brittany. Six independent samples of field runoff water (R1 to R6) were

collected 40 to 50 min after six rainfall simulations on an experimental agricultural plot, previously spread with either pig (samples R1 to R3) or bovine manure (R4 to R6). The samples were collected and poured into 2 litre-flasks. Two samples were taken from two lagoons which receive treated liquid manures from piggeries. The retention time for the storage lagoons was between 5 days (L1) and 9 months (L2). Volumes of approximately 200 mL of water were centrifuged at 4,000 g for 30 min and pellets were transferred into microtubes for storage at -20°C.

#### **Enumeration of *E. coli***

*E. coli* were enumerated in all water samples using 3M™ Petrifilm *E. coli* to estimate the level of faecal contamination. Ten-fold serial dilutions were performed in peptone water up to  $10^{-4}$ . The gel of the Petrifilm was rehydrated with 1 mL of water (diluted or not) and incubated at 44°C for 24 h. Blue colonies (glucuronidase positive) were counted to determine the concentration of *E. coli*, which was expressed in CFU/100 mL. All enumerations were performed in triplicate.

#### **Collection Strains of *Bifidobacterium***

The strains used in this study were *B. animalis* subsp. *animalis*<sup>T</sup> DSM 20104, *B. boum*<sup>T</sup> DSM 20432, *B. longum* subsp. *suis*<sup>T</sup> DSM 20211, *B. merycicum*<sup>T</sup> DSM 6492, *B. pseudolongum* subsp. *globosum*<sup>T</sup> DSM 20092, *B. ruminantium*<sup>T</sup> DSM 6489, *B. thermacidophilum* subsp. *porcinum*<sup>T</sup> DSM 17755 and *B. thermophilum*<sup>T</sup> DSM 20210. All strains were cultured on the medium described by Beerens (1) and incubated at 37°C in a jar under anaerobic conditions. One mL of overnight culture of each strain was centrifuged at 17,000 g for 10 min. The pellets were stored at -20°C.

## Extraction of DNA

DNA was extracted from the pellets stored at -20°C, using the QIAamp DNA stool kit (QIAGEN) in accordance with the manufacturer's instructions. The elution volume was 50 µL.

## Bacterial group PCRs

PCRs for each bacterial group were performed with a forward general bacteria primer W18 and a reverse group specific primer targeting BSL, *Eubacterium-Clostridiaceae*, *Bacteroides-Prevotella* (GE08, GE09 and rBacPre respectively) and with a group specific primer pair for the *Bifidobacterium* group (g-BIFID-F and g-BIFID-R) (Table 1). The reaction mix comprised dNTP 0.2 mM, primers 350 nM each, 1x AccuPrime *Taq* DNA polymerase buffer II, AccuPrime *Taq* DNA polymerase (Invitrogen) 2.5 U and 1 µL of manure DNA diluted five times in water. The final reaction volume was 20 µL. The annealing temperature was 61, 55, 55, and 53°C for the BSL, *Eubacterium-Clostridiaceae*, *Bacteroides-Prevotella* and *Bifidobacterium* groups, respectively. After a denaturation step at 94°C for 2 min, the reactions were carried out by 30 cycles at 94°C for 30 s, at the annealing temperature for 90 s, and at 68°C for 90 s. No final elongation was performed, as recommended by the supplier (Invitrogen). The reaction was stopped by cooling the mixture to 10°C.

The size of the amplification products was confirmed by agarose gel electrophoresis (TBE 1X and 0.7 or 1.5% agarose (wt/vol) for total bacteria and bacterial groups, respectively). The PCR products were visualised under UV light after gel staining with ethidium bromide.

A volume of 1 µL of each PCR product was used as a template for further PCR and CE-SSCP analyses.

## Analysis by CE-SSCP PCR



We used a nested PCR where the first PCR (described above) was done with the group specific primers to target the microbial groups of interest. As the amplified DNA fragments are larger than the V3 region, each group specific PCR product was amplified again in a second PCR using the bacterial W34-W49 primers to target the V3 region and label the DNA fragment with the fluorescent dye present on primer W49. These two primers were used specifically for SSCP since they target the 16S rDNA V3 region that is the right length (200 base pairs) and has the necessary diversity for SSCP analysis of microbial communities. This approach facilitates the PCR reactions and enables careful comparison of the different patterns which are generated with the same primers.

The reaction mix comprised dNTP 0.2 mM, primers 390 nM, 1x *Pfu* turbo buffer, *Pfu* turbo (Stratagene): 0.625 U, 1 µL of the PCR products amplified previously. The final reaction volume was 20 µL. The amplification conditions were one cycle at 94°C for 2 min followed by 25 cycles of 30 s at 94°C, 30 s at 61°C then 30 s at 72°C and a final elongation step of 10 min at 72°C. The resulting PCR products were then separated by SSCP capillary electrophoresis using an ABI 310 genetic analyser (Applied Biosystems) as described by Delbes *et al.* (10) but using a CAP 5.58% - Glycerol 10% polymer (Applied Biosystem).

### **Cloning and sequencing**

For each bacterial group, cloning was performed on a mixture of two PCR products selected according to their SSCP profiles (with the most numerous and highest peaks). The mixed PCR products were cloning and transformed in *E. coli* competent cells using the StrataClone PCR cloning kit (Stratagene, La Jolla, CA) following the manufacturer' instructions, except for the ligation time which was increased from 5 to 15 min.

A total of 275 clones were further analysed: 96 for the *Eubacterium-Clostridiaceae* group (48 from raw manures and 48 from treated manures), 35 for the BSL group (11 from raw manures

and 24 from treated manures), 72 for the *Bacteroides-Prevotella* group (48 from raw manures and 24 from treated manures) and 72 for the *Bifidobacterium* group (24 from raw manures and 48 from treated manures).

The clones were randomly picked and their inserts were screened by nested PCR and CE-SSCP as follows; in the first step, plasmid inserts were amplified by PCR with plasmid targeted primers T7 (5'-TAATACGACTCACTATAGGG-3') and P13 (5'-GACCATGATTACGCCA-3') (Stratagene, La Jolla, CA). The reaction mix was dNTP 0.2 mM, primers 700 nM each, 1x RedTaq Buffer, RedTaq polymerase 2.5 U and deionised water to bring the volume to 25 µL. The amplification conditions were 10 min at 94°C followed by 25 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final elongation step at 72°C for 10 min. One µL of these PCR products was used to perform a CE-SSCP PCR as described above. Inserts yielding a peak that co-migrated with distinguishable peaks from the manure CE-SSCP profiles were sequenced for peak identification.

A total of 139 clones were sequenced. Sequence reactions were performed with the Ouest Genopole Sequencing Facility (CNRS, Roscoff, France) using primer T7. DNA sequences were identified by comparison with their closest relative available in databases using Blast from the National Centre for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>) and the Ribosomal Database Project II (<http://rdp.cme.msu.edu/>).

## **Specific amplification of the *Bifidobacterium thermacidophilum* subsp. *porcinum* Intergenic Transcribed Spacer (ITS)**

The total ITS sequence of *B. thermacidophilum* subsp. *porcinum*<sup>T</sup> was amplified by PCR using the primer set ITSF/ITSR designed by Cardinale *et al.* (7). The reaction mix was 1x RedTaq buffer, RedTaq polymerase 5U, dNTP 0.2 mM, primers 700 nM and deionised water

to bring the volume to 20  $\mu$ L. The amplification programme was as described by Cardinale *et al.* (7) except for the elongation temperature (72°C) which was adapted to RedTaq polymerase. The PCR product was sequenced by the Ouest Genopole Sequencing Facility (CNRS, Roscoff, France). The sequence obtained was aligned, using the ClustalW2 software (52), to the seven ITS sequences of *Bifidobacterium* strains present in Genbank (*B. breve*, *B. adolescentis*, *B. longum*, *B. choerinum*, *B. animalis*, *B. thermophilum* and *B. pseudolongum*) and to the ITS sequence of *B. longum* biotype *suis* that was obtained in this study as described above. Based on the comparison of these sequences, a pair of primers specific to *B. thermacidophilum* subsp. *porcinum* was designed (GE35 / GE36) (Table 1). Specific detection of *B. thermacidophilum* subsp. *porcinum* was then performed using a nested PCR. All *Bifidobacterium* ITSs were first amplified using the primer pair ITSF/ITSR as described above. The resulting PCR products were diluted 10 times and 1  $\mu$ L was used as template for a second PCR using the primer pair GE35 / GE36. The GE35 / GE36 PCR reaction mix comprised 1x AccuPrime Taq DNA polymerase buffer II, AccuPrime Taq polymerase 2.5 U (Invitrogen), primers 350 nM each, and deionised water to bring the total volume to 20  $\mu$ L. The PCR was performed using the following conditions: one cycle at 94°C for 2 min, 30 cycles at 94°C for 30 s, 59°C for 30 s, and 68°C for 1.5 min.

#### **Nucleotide sequences accession numbers.**

Sequences were deposited in the EMBL database under accession numbers AM991308 to AM991325.

## **RESULTS**

### **Comparison of the dominant microbial groups of raw and treated manures**

For each bacterial group, the CE-SSCP profiles obtained from the 10 raw and treated manures were aligned and compared (Fig. 1 to 4). The *Eubacterium-Clostridiaceae* profiles provided the lowest resolution with a high background level below the peaks underlining the complexity of this bacterial group (Fig. 1). The raw manure profiles shared 9 to 11 co-migrating peaks and a similar number of distinct peaks before and after treatment. However, in most cases, the peaks present in raw manures did not co-migrate with the peaks of treated manures.

The BSL group profiles provided a lower background signal than that observed for the *Eubacterium-Clostridiaceae* group (Fig. 2). The profiles of raw and treated manures consisted of 10 and 12 peaks, respectively. After aerobic treatment, seven peaks from the treated manures co-migrated with peaks from the raw manure profiles.

The CE-SSCP profiles of the *Bacteroides-Prevotella* and *Bifidobacterium* groups differed from the BSL and *Eubacterium-Clostridiaceae* group profiles by the absence of background and the small number of peaks detected (Fig. 3 and 4). These profiles yielded three and two dominant peaks respectively, consistently preceded by smaller artifactual peaks which were also visible with purified clones (data not shown). These artifactual peaks were probably produced either during migration in capillary electrophoresis or during PCR amplification. In the latter, they would represent a small proportion of PCR fragments that have ended prematurely. The three peaks from the *Bacteroides-Prevotella* group detected in all raw manures were not detected in treated manures, which contained two other distinguishable peaks (Fig. 3B). The first peak (BA3) was common to all treated manures whereas the position of the second peak (BA4) differed from one sample to another. The profiles of the *Bifidobacterium* group were characterized by two peaks which were detected in all raw and treated manures (Fig. 4).

## Identification of the major peaks of each group

The dominant peaks were identified by cloning and sequencing of the corresponding 16S rRNA gene fragments. A total of 275 clones were screened by CE-SSCP and 139 were sequenced. The phylogenetic affiliation of the clones corresponding to the major peaks of the CE-SSCP profiles is presented in Table 2. Only 37.5% of the *Eubacterium-Clostridiaceae* 16S rDNA sequences found in raw manure demonstrated more than 97% similarity to sequences in databases. Four of the dominant peaks in the *Eubacterium-Clostridiaceae* raw manure profiles were identified, but no identity could be assigned to peaks obtained from the treated manure profiles. The closest relative of the four sequences identified were sequences from uncultured bacteria from various sources, including the effluent treatment plant, the solid waste digester and the pig manure storage pit.

Two of the three dominant peaks of the *Bacteroides-Prevotella* raw manure profiles and peak BA3 of the treated manure profiles were identified. The closest relative of the *Bacteroides-Prevotella* sequences was found in various sources, but not in pig faeces or manure (Table 2). As mentioned above, a specific *Bacteroides-Prevotella* peak was found to be present in each treated manure profile. One of them (BA4) was cloned and sequenced. Its closest relative was a *Bacteroidetes* identified in microbial fuel cells fed with wastewater (46).

Two peaks of the BSL profiles of raw manure were assigned (BSL 3 and 7). BSL3 was 91% similar to its closest relative, a turkey intestinal tract microorganism. The sequence of peak BSL7 was 100% similar to *Lactobacillus sobrius* isolated from piglet faeces (31). The two BSL peaks identified in treated manure were only about 88% similar to cloned DNA from an estuarine sediment.

The sequences of the two peaks of the *Bifidobacterium* profiles, obtained either from the raw or treated manures, were 99 to 100% similar to *B. thermacidophilum* subsp. *porcinum* isolated from piglet faeces (peaks Bi1 and Bi1b) (60) and 98 to 100% similar to *Bifidobacterium pseudolongum* subsp. *pseudolongum* isolated from porcine cecum (peaks Bi2 and Bi2b) (50).

### **Specificity of GE35 / GE36 primers**

Among the four groups of bacteria analysed in this study, only two species, *B. thermacidophilum* subsp. *porcinum* and *B. pseudolongum* subsp. *pseudolongum*, co-migrated with a peak that was systematically detected in all raw and treated manure CE-SSCP profiles. Given that *Bifidobacterium pseudolongum* subsp. *pseudolongum* has previously been observed in various animal faeces (4), the *B. thermacidophilum* subsp. *porcinum* strain was selected for further analyses. However, this species is genotypically too similar to *B. thermophilum* (57) to be differentiated at the 16SrRNA gene sequence level. The design of specific primers thus required targeting of the 16S-23S rRNA intergenic transcribed spacer region. A specific pair of primers (GE35 / GE36) was designed and tested on *B. thermacidophilum* subsp. *porcinum*<sup>T</sup> and on seven other *Bifidobacterium* type strains representative of taxa of animal origin as previously described by Ventura *et al.* (56). The test showed that the primer set produced species-specific amplicons from *B. thermacidophilum* subsp. *porcinum*<sup>T</sup> and did not amplify any PCR products from the seven other strains (Table 3).

The host specificity of the species was then examined using the set of primers on DNA originating from human, pig, bovine, and poultry faeces (Table 4). All faecal samples gave a positive signal at the first universal ITS targeted PCR, but the presence of *B.*

*thermacidophilum* subsp. *porcinum* marker was only found in pig faeces when nested PCR and the GE35 / GE36 primers were used.

*B. thermacidophilum* subsp. *porcinum* and the concentration of *E. coli* were observed in manure and in water samples using our nested PCR assay (Table 5). Regardless of the level of *E. coli*, *B. thermacidophilum* subsp. *porcinum* was not detected in urban effluents of human origin, or in runoff water impacted by bovine manure contamination in spite of the presence of *E. coli*. In the case of runoff waters obtained after application of pig manure, the three samples showed positive amplification. *B. thermacidophilum* subsp. *porcinum* was also found in raw and treated manure and in two types of lagoon supplied with treated pig liquid manure. In lagoon L1 (with a retention time of five days) the concentration of *E. coli* was  $4.5 \times 10^6$  CFU/ 100 mL and a positive amplification of the target bacteria was observed, whereas in lagoon L2 (with a retention time of nine months) neither *E. coli* nor *B. thermacidophilum* subsp. *porcinum* were detected.

## DISCUSSION

Although pig-specific genetic markers have been proposed to trace faecal pollution in the environment, their application has mainly focused on faecal samples (14, 22, 43, 44, 54) and data concerning manure intended for spreading are scarce (22, 29). Cotta *et al.* (9) reported a difference in composition between the bacterial communities of pig faeces and stored manure. Furthermore, Peu *et al.* (45) observed differences in the bacterial community in fresh manure located below the animals and manure stored in outdoor tanks. To be considered as suitable, a microbial indicator of pig contamination must be abundant and found not only in faeces but also in stored manure intended for land application.

Whereas studies concerning faecal markers have usually focused on a particular group of bacteria, we used a broader strategy (i.e. four groups instead of one) with the aim of identifying a potential microbial marker of pig contamination present both in raw and treated manures. The behaviour of four pig faecal bacterial groups (34, 45, 53, 58) was monitored throughout pig manure biological treatment using molecular typing (CE-SSCP). These bacterial groups were selected either because they are dominant in manure microbial communities (*Eubacterium-Clostridiaceae*, *Bacteroides-Prevotella*, BSL) or due to their known host specificity. Thus phylogenetic groups of the *Bacteroides-Prevotella* have been associated with pig faeces (14, 22, 43, 44) and the genus *Bifidobacterium* consists of species from animal or human (17, 40) origin.

#### -SSCP profiles

The 17 raw manures analysed revealed the remarkable consistency of the SSCP profiles of the four bacterial groups (Fig. 1 to 4) regardless of the geographical location of the piggeries sampled and of the storage period of the manures. In practice, in Brittany piggeries, raw manure stores are rarely aerated and slurry tanks are not operated as closed batch reactors but are subject to regular additions of fresh manure. The major difference from one manure to another is thus the length of storage, which ranges from weeks to months depending on the storage capacity of the tank. This consistency of the bacteria profile could be explained by the similarity of farm management practices (diet and the age of the animals) and manure storage conditions. Leung and Topp (35) and Peu *et al.* (45) obtained similar results using molecular techniques to monitor pig manure microbial community dynamics during storage in a laboratory-scale reactor and a manure storage tank for a period of three months, respectively. These data suggested that the dominant bacterial populations of manure stored under anoxic conditions are not strongly influenced by the length of storage.



364

365 Biological treatment of manure, comprising nitrification-denitrification by alternating periods  
366 of aerobic and anoxic conditions, caused changes in the composition of *Eubacterium-*  
367 *Clostridium* and of the *Bacteroides-Prevotella* groups. These results are in agreement with  
368 those of Leung and Topp (35), who observed significant changes in bacterial manure  
369 populations during aeration. It is interesting to note that the four bacterial groups targeted in  
370 this study, which are classified as anaerobes, presented different behaviours throughout  
371 treatment suggesting different tolerance levels to oxygen. The composition of the  
372 *Eubacterium-Clostridium* and of the *Bacteroides-Prevotella* groups changed significantly,  
373 resulting in the disappearance of the dominant peaks found in raw manure, whereas new  
374 peaks appeared in treated manure. It has previously been reported that the presence of oxygen  
375 has significant effects on the survival ability of faecal *Bacteroides* spp and *Eubacterium-*  
376 *Clostridium* groups (16, 47). In contrast, *Bifidobacterium* and to a lesser extent BSL appeared  
377 to be less sensitive to biological treatment because most of their peaks were detected both in  
378 raw and treated manure. The different behaviour during treatment indicates that the BSL and  
379 *Bifidobacterium* groups are potentially more robust markers of manure contamination.

380

381 -Identification of peaks of SSCP profiles

382 From the 16 peaks identified (Table 2), only six were identical or closely related to other  
383 sequences obtained specifically from pig faeces or manure. The scarcity of data available on  
384 the bacterial populations of treated urban or animal effluents could explain the small number  
385 of sequence matches, particularly with *Eubacterium-Clostridium* groups. Peak C5 was closely  
386 related (98% similarity) to an uncultured *Clostridium* previously found in a manure storage  
387 pit (58) and peak BSL7 was identified as *Lactobacillus sobrius*, which has previously been  
388 described in piglet (32) and pig faeces (28). However, none of these peaks was found in

389 treated manure whereas the two *Bifidobacterium* peaks were found in both raw and treated  
390 manure. These peaks presented 100% similarity with *B. pseudolongum* subsp. *pseudolongum*,  
391 which has been isolated from various animal faeces (17), and with *B. thermacidophilum*  
392 subsp. *porcinum*, which has been recently described in pig and piglet faeces (41, 60).

393  
394 The absence of members of the *Bacteroides-Prevotella* group as a potential marker was  
395 surprising because several phylotypes of this group have previously been found in pig faeces  
396 (14, 22, 34, 43, 58) and manure (35, 45, 58). This absence could be explained by the use of  
397 the CE-SSCP technique which over-represents the dominant bacterial populations when these  
398 populations make up more than 1% of the total community (36). The presence of two very  
399 dominant peaks in the raw and treated manure may have masked the diversity of less  
400 dominant species. These two peaks were not closely related to bacteria isolated from pig  
401 faeces or manures and presented poor similarity (92%) with uncultured bacteria from rumen  
402 and rhizosphere.

#### 403 404 -*B. thermacidophilum* subsp. *porcinum* targeting

405 According to the results of the SSCP analyses, which highlighted the presence of  
406 *B. thermacidophilum* subsp. *porcinum* in manures, the host specificity of this genetic marker  
407 was then determined. As this species is closely related to *B. thermophilum* and *B. boum* (56)  
408 the 16S rDNA did not allow discrimination of the target bacteria. Nevertheless, the use of a  
409 nested PCR for the ITS region of 16S and 23S rDNA led to differentiation between  
410 *B. thermacidophilum* subsp. *porcinum* from *B. thermophilum* and *B. boum* (Table 3).  
411 Lamendella *et al.* (33) reported that certain species of the genus *Bifidobacterium* were present  
412 in various environments whereas other species had a preferential host such as *B. boum* and *B.*  
413 *thermophilum*; these authors only detected the latter in pig faeces (33). Our results also

highlighted the host specificity of *Bifidobacterium thermacidophilum* subsp. *porcinum*, which was previously described in the pig intestinal tract (41, 60), as it was not detected in bovine, poultry, human faeces nor in urban wastewaters containing domestic sewage. Our results showed that using nested PCR, it was possible to detect *Bifidobacterium thermacidophilum* subsp. *porcinum* in water samples contaminated by manure. This is in agreement with the study of King *et al.* (30) who also used nested PCR to detect *B. adolescentis* in samples of water impacted by human activities. As already reported by Lamendella *et al.* (33) and King *et al.* (30), our results confirm that certain species of *Bifidobacterium* might represent a good target population for assessing faecal contamination above a background level for example associated with heavy rainfall events.

#### -Conclusions

The comparison of dominant pig manure microbial communities throughout manure treatment using CE-SSCP allowed a large number of raw and treated manures to be screened. This demonstrated that *Bifidobacterium* and, to a lesser extent, members of the BSL group were less affected by the handling and treatment of manure than *Eubacterium-Clostridiaceae* and *Bacteroides-Prevotella*. These data show that the *Bifidobacterium* species found in manure can persist outside the pig intestinal tract and that *B. thermacidophilum* subsp. *porcinum* can be used as an indicator of manure contamination in the environment.

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FIGURE 1. Comparison of the *Eubacterium-Clostridiaceae* group CE-SSCP profiles from five raw manures (A) and five treated manures (B). One raw manure profile (in bold) is also shown in part B of the figure for comparison. The peaks corresponding to the dominant bacterial populations are indicated by arrowheads. The white arrows correspond to unidentified peaks, the grey arrows to peaks identified by one sequence only, and black arrows to peaks identified by at least two sequences. Peaks that could be identified are designated C1 to C5, as in Table 3.

FIGURE 2. Comparison of the BSL group CE-SSCP profiles from five raw manures (A) and five treated manures (B). The legend corresponds to the legend of Fig. 1. Peaks that could be identified are designated BSL3, BSL7, BSL4b and BSL8b as in Table 3.

FIGURE 3. Comparison of the *Bacteroides-Prevotella* group SSCP profiles from five raw manures (A) and five treated manures (B). The legend corresponds to the legend of Fig. 1. Peaks that could be identified are designated BA1, BA2, BA3 and BA4 (further characterized in Table 3). Because of their strong dominance over the profiles, peaks BA2 and BA4 saturated the fluorescence detector when other peaks were detectable.

FIGURE 4. Comparison of the *Bifidobacterium* SSCP profiles from five raw manures (A) and five treated manures (B). The legend corresponds to the legend of Fig. 1. Peaks that could be identified are designated Bi1, Bi2, Bi1b and Bi2b as shown in Table 3.

TABLE 1: Sequences and target positions of the primers used in this study

	Sequence (5'-3') <sup>a</sup>	<i>E. coli</i> position	16s rRNA target	Reference
W18	GAGTTTGATCMTGGCTCAG	9	Bacteria	Godon <i>et al.</i> (21)
W34	ACGGTCCAGACTCCTACGGG	330	V3 Bacteria	Delbès <i>et al.</i> (11)
W49	6FAM-TTACCGCGGCTGCTGGCAC <sup>b</sup>	500	V3 Universal	Delbès <i>et al.</i> (11)
GE08	ATTYACCGCTACACATG	679	<i>Bacillus</i> spp. <i>Lactobacillus</i> spp. <i>Pediococcus</i> spp. <i>Leuconostoc</i> spp. <i>Weissella</i> spp. <i>Streptococcus</i> spp.	Heilig <i>et al.</i> (24)
GE09	CCCTTTACACCCAGTAA	561	<i>Clostridiaceae</i>	Van Dyke <i>et al.</i> (55)
rBacPre	TCACCGTTGCCGGCGTACTC	887	<i>Prevotella</i> <i>Bacteroides</i>	Wood <i>et al.</i> (59)
g-BIFID-F	CTCCTGGAAACGGGTGG	153	<i>Bifidobacterium</i>	Matsuki <i>et al.</i> (39)
g-BIFID-R	GGTGTTCTTCCCGATATCTACA	699	<i>Bifidobacterium</i>	Matsuki <i>et al.</i> (39)
ITSF	GTCGTAACAAGGTAGCCGTA		Total ITS (universal primer)	Cardinale <i>et al.</i> (7)
ITSR	GCCAAGGCATCCACC		Total ITS (universal primer)	Cardinale <i>et al.</i> (7)
GE35	ATGGTATCGCGGGGGTTCGTC		ITS <i>B. thermacidophilum</i> subsp. <i>porcinum</i>	This study
GE36	GAACACCCGGGAAGGAA		ITS <i>B. thermacidophilum</i> subsp. <i>porcinum</i>	This study

<sup>a</sup> M = A/C ; N = A/T/C/G ; Y = C/T ; <sup>b</sup> 6FAM = 6 carboxyfluorescein; <sup>c</sup> primer label

TABLE 2: phylogenetic affiliation of 16S rDNA sequences

Peak designation*	Sequence length (bp)	Closest relative			Source	Reference
		Name (accession no.) for closest match	Affiliation group	% similarity		
C1	525	Clone B-87 (AY676487)	<i>Clostridiaceae</i>	97	Bovine teat canal	Gill <i>et al.</i> 2006 (19)
C3	530	Clone M75 (DQ640962)	<i>Clostridiaceae</i>	88	Effluent treatment plant	Kalia <i>et al.</i> 2007 (unpublished)
C4	524	Clone A35 D28 L B B12 (EF559222)	<i>Clostridiaceae</i>	99	Mesophilic solid waste digester	Li <i>et al.</i> 2007 (unpublished)
C5	525	Clone P316 (AF261803)	<i>Clostridiaceae</i>	98	Manure storage pit	Whitehead and Cotta. 2004 (unpublished)
BA1	707	Clone BRC82 (EF436368)	<i>Bacteroidetes</i>	92	Rumen water buffalo	Mao <i>et al.</i> 2007 (unpublished)
BA2	844	Clone SRRT42 (AB240481)	<i>Bacteroidetes</i>	92	Rhizosphere biofilm of phragmites	Nakamuera <i>et al.</i> 2005 (unpublished)
BA3	662	Clone Z144 (EU029356)	<i>Bacteroidetes</i>	94	Raw milk	Raats and Halpern 2007 (unpublished)
BA4	405	Clone oca46 (AY491639)	<i>Bacteroidetes</i>	94	Waste water	Phung <i>et al.</i> 2006 (46)
BSL3	674	clone WTB_Y48 (EU009859)	<i>Mollicutes</i>	91	Turkey intestinal tract	Bent <i>et al.</i> 2007 (unpublished)
BSL 7	674	<i>L. sobrius</i> (AY700063)	<i>Lactobacillus</i>	100	Piglet intestinal tract	Konstantinov <i>et al.</i> 2006 (32)
BSL4 b	645	Clone R8C-A3 (AY678482)	<i>Mollicutes</i>	88	Estuarine sediment	Nielsen <i>et al.</i> 2004 (42)
BSL8 b	647	Clone R8C-A3 (AY678482)	<i>Firmicutes</i>	86	Estuarine sediment	Nielsen <i>et al.</i> 2004 (42)
Bi1	513	<i>B. thermacidophilum</i> subsp. <i>porcinum</i> (AY148470)	<i>Bifidobacterium</i>	99	Piglet intestinal tract	Zhu <i>et al.</i> 2003 (60)
Bi2	522	<i>B. pseudolongum</i> subsp. <i>pseudolongum</i> (AY174109)	<i>Bifidobacterium</i>	100	Porcine cecum	Simpson <i>et al.</i> 2003 (50)
Bi1 b	513	<i>B. thermacidophilum</i> subsp. <i>porcinum</i> (AY148470)	<i>Bifidobacterium</i>	100	Piglet intestinal tract	Zhu <i>et al.</i> 2003 (60)
Bi2 b	514	<i>B. pseudolongum</i> subsp. <i>pseudolongum</i> (AY174109)	<i>Bifidobacterium</i>	98	Porcine cecum	Simpson <i>et al.</i> 2003 (50)

\* Sequences from this study have been deposited in EMBL under accession numbers AM991308 to AM991325

TABLE 3: Specificity of PCR product formation with primer set GE035/GE036 tested on collection strains of *Bifidobacterium*

<i>Bifidobacterium</i> strain <sup>a</sup>	PCR Product formation
<i>B. boum</i> <sup>T</sup> DSM 20432	-
<i>B. thermophilum</i> <sup>T</sup> DSM 20210	-
<i>Bifidobacterium thermacidophilum</i> subsp. <i>porcinum</i> <sup>T</sup> DSM 17755	+
<i>B. merycicum</i> <sup>T</sup> DSM 6492	-
<i>B. pseudolongum</i> subsp. <i>globosum</i> <sup>T</sup> DSM 20092	-
<i>B. ruminantium</i> <sup>T</sup> DSM 6489	-
<i>B. animalis</i> subsp. <i>animalis</i> <sup>T</sup> DSM 20104	-
<i>B. longum</i> subsp. <i>suis</i> <sup>T</sup> DSM 20211	-

<sup>a</sup>Specificity was tested with chromosomal DNA from *Bifidobacterium* previously detected in animal faeces



TABLE 4: Results of *Bifidobacterium thermacidophilum* subsp. *porcinum* PCR tested on DNA from human and animal faeces

Origin of faeces	Number of positive samples
Pig (n = 30) <sup>a</sup>	30
Bovine (n = 30)	0
Poultry (n=30)	0
Human (n=28)	0

<sup>a</sup> number of samples

TABLE 5: *E. coli* counts and detection of *Bifidobacterium thermacidophilum* subsp. *porcinum* in manure and in waters impacted by human activity and contaminated by manure

Type of sample	Origin of the contamination	<i>E. coli</i> counts (CFUs/100 mL) <sup>a</sup>	Number of positive samples for the target bacteria/total
Raw manure (pig)	-	4.0×10 <sup>6</sup> (4.2×10 <sup>6</sup> )	17/17
Treated manure (pig)	-	5.1×10 <sup>4</sup> (3.3×10 <sup>4</sup> )	10/10
Lagoon with a retention time of 5 days (L1) (pig)	treated liquid manure	4.5×10 <sup>6</sup> (4.1×10 <sup>5</sup> )	1/1
Lagoon with a retention time of 9 months (L2) (pig)	treated liquid manure	not detected	0/1
Runoff water (R1 to R3)	pig manure spread on field	9.7×10 (3.3×10 <sup>3</sup> )	3/3
Runoff water (R4 to R6)	bovine manure spread on field	7.5×10 <sup>3</sup> (8×10 <sup>2</sup> )	0/3
Raw waste water	Urban effluent (mainly human)	1.8×10 <sup>6</sup> (1.7×10 <sup>6</sup> )	0/5
Treated waste water	Urban effluent (mainly human)	3.3×10 <sup>3</sup> (4.3×10 <sup>3</sup> )	0/10

<sup>a</sup> mean values (standard deviation)







