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8 Selection of a pig manure contamination marker based on the influence of biological

9 treatment on the dominant faecal microbial groups

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13 Running title: microbial marker of pig manure

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20

21 **Abstract**
22

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

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42 **INTRODUCTION**

43 Brittany only represents 7% of France, but is the main pig production area and hosts
44 approximately 14 million fatteners per year. This high concentration of confined pig feeding
45 has led to over-application of manures to soil which contributes to water pollution. Physical
46 and biological manure treatment processes have been developed to limit nitrogen and
47 phosphorus pollution (5). As these treatments were not designed to eliminate microbial
48 pollution, even treated manure can contain pathogenic microorganisms (27) and agricultural
49 soils and water systems can thus potentially still be contaminated through surface runoff and
50 seepage. As manure application can increase the number of pathogens in the soil (18), pig
51 faeces may represent a significant risk to human health in Brittany. Currently, the bacteria
52 monitored to assess faecal contamination (*E. coli*, faecal coliforms and enterococci) do not
53 differentiate contamination from pig slurry from other animals or from pollution by humans.
54 It is thus important to develop analytic tools to specifically detect this source of pollution.
55 Many studies have already proposed potential markers for the detection of host-specific faecal
56 pollution (2, 3, 8, 12-15, 20, 37, 38, 48, 49). Much of this research has concentrated on
57 distinguishing human and animal sources of contamination (3, 8, 20, 30, 38). Some studies
58 have focused on identifying individual sources of animal pollution, and have described
59 molecular markers for faeces from duck (13), chicken (37), bovine (2, 3, 49) or cervids (6).
60 Concerning pigs, biomarkers have been proposed for faecal contamination but rarely for
61 manure, the bacterial composition of which differs from that of the faeces (9). Molecular
62 markers have been developed to target the 16S rDNA gene sequences of dominant *Eubacteria*
63 (2, 14, 43, 48) or methanogenic *Archeabacteria* (54) of the intestinal tract of pigs, whereas
64 Khatib *et al*. (29) targeted the STII toxin gene from enterotoxigenic *E. coli*. Among the
65 dominant groups of pig faecal *Eubacteria*, which include *Bacteroides-Prevotella*,
66 *Eubacterium-Clostridiacea*, *Lactobacillus-Streptococcus* (34, 45, 51, 58) and to a lesser

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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-Streptoccocus-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

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91 profiles. The specificity of this pig marker was then tested by assessing the host distribution in
92 a selection of faecal, manure and wastewater samples.
93

94 MATERIALS AND METHODS

95 Sample collection

96 Manure samples: Manures were collected from 17 piggeries located across Brittany. In these
97 farms, raw manure was stored for between two and eight weeks in a primary anaerobic tank,
98 followed by aeration treatment for a period of three to four weeks before final anaerobic
99 storage for between three and nine months. The chemical characteristics of the manures were
100 similar on all farms. The mean pH of the raw and treated manures was 7.5 and 7.8
101 respectively. The corresponding dry matter contents were 4.3% and 5.1% (wt/wt); total
102 Kjeldahl nitrogen contents 4.3 and 2.0 g litre^{-1} and soluble Chemical Organic Demand 9.7 and
103 2.4 g O_2 litre^{-1}, respectively. All manures stored in tanks were homogenised by mixing with a
104 propeller agitator for at least 30 minutes before sampling. A volume of 30 litres of manure
105 was removed and transferred to the laboratory. The samples were then re-mixed with a
106 propeller homogenizer. One litre of homogenised manure was transferred to a flask. Manure
107 was then centrifuged at 16,000 g to form a pellet of approximately 250 mg (wet weight). The
108 pellets were stored at -20°C.
109 Feacal samples: A total of 90 samples of animal faeces (30 bovine, 30 pig, 30 poultry faeces)
110 were collected from 62 farms across Brittany. Twenty-eight samples of human faeces from
111 healthy people were obtained from two French research institutes (IFREMER, Brest and
112 INRA, Jouy-en-Josas). Approximately 250 mg (wet weight) of each faeces were transferred
113 into a microtube and stored at -20°C.
114 Water samples: Fifteen urban wastewater (5 raw and 10 treated effluents) were collected from
115 locations across Brittany. Six independent samples of field runoff water (R1 to R6) were

116 collected 40 to 50 min after six rainfall simulations on an experimental agricultural plot,
117 previously spread with either pig (samples R1 to R3) or bovine manure (R4 to R6). The
118 samples were collected and poured into 2 litre-flasks. Two samples were taken from two
119 lagoons which receive treated liquid manures from piggeries. The retention time for the
120 storage lagoons was between 5 days (L1) and 9 months (L2).
121 Volumes of approximately 200 mL of water were centrifuged at 4,000 g for 30 min and
122 pellets were transferred into microtubes for storage at -20°C.
123
124 **Enumeration of *E. coli***
125 *E. coli* were enumerated in all water samples using 3M^{TM} Petrifilm *E. coli* to estimate the
126 level of faecal contamination. Ten-fold serial dilutions were performed in peptone water up to
127 10^{-4}. The gel of the Petrifilm was rehydrated with 1 mL of water (diluted or not) and
128 incubated at 44°C for 24 h. Blue colonies (glucuronidase positive) were counted to determine
129 the concentration of *E. coli,* which was expressed in CFU/100 mL. All enumerations were
130 performed in triplicate.
131
132 **Collection Strains of *Bifidobacterium***
133 The strains used in this study were *B. animalis* subsp. *animalis*^{T} DSM 20104, *B. boum*^{T} DSM
134 20432, *B. longum* subsp. *suis*^{T} DSM 20211, *B. merycicum*^{T} DSM 6492, *B. pseudolongum*
135 subsp. *globosum*^{T} DSM 20092, *B. ruminantium*^{T} DSM 6489, *B. thermacidophilum* subsp.
136 *porcinum*^{T} DSM 17755 and *B. thermophilum*^{T} DSM 20210. All strains were cultured on the
137 medium described by Beerens (1) and incubated at 37°C in a jar under anaerobic conditions.
138 One mL of overnight culture of each strain was centrifuged at 17,000 g for 10 min. The
139 pellets were stored at -20°C.
140

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141 **Extraction of DNA**

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

145

146 **Bacterial group PCRs**

147 PCRs for each bacterial group were performed with a forward general bacteria primer W18
148 and a reverse group specific primer targeting BSL, *Eubacterium-Clostridiaceae*, *Bacteroides-*
149 *Prevotella* (GE08, GE09 and rBacPre respectively) and with a group specific primer pair for
150 the *Bifidobacterium* group (g-BIFID-F and g-BIFID-R) (Table 1). The reaction mix
151 comprised dNTP 0.2 mM, primers 350 nM each, 1x AccuPrime *Taq* DNA polymerase buffer
152 II, AccuPrime *Taq* DNA polymerase (Invitrogen) 2.5 U and 1 µL of manure DNA diluted five
153 times in water. The final reaction volume was 20 µL. The annealing temperature was 61, 55,
154 55, and 53°C for the BSL, *Eubacterium-Clostridiaceae, Bacteroides-Prevotella* and
155 *Bifidobacterium* groups, respectively. After a denaturation step at 94°C for 2 min, the
156 reactions were carried out by 30 cycles at 94°C for 30 s, at the annealing temperature for 90 s,
157 and at 68°C for 90 s. No final elongation was performed, as recommended by the supplier
158 (Invitrogen). The reaction was stopped by cooling the mixture to 10°C.
159 The size of the amplification products was confirmed by agarose gel electrophoresis (TBE 1X
160 and 0.7 or 1.5% agarose (wt/vol) for total bacteria and bacterial groups, respectively). The
161 PCR products were visualised under UV light after gel staining with ethidium bromide.
162 A volume of 1µL of each PCR product was used as a template for further PCR and CE-SSCP
163 analyses.

164

165 **Analysis by CE-SSCP PCR**

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166 We used a nested PCR where the first PCR (described above) was done with the group
167 specific primers to target the microbial groups of interest. As the amplified DNA fragments
168 are larger than the V3 region, each group specific PCR product was amplified again in a
169 second PCR using the bacterial W34-W49 primers to target the V3 region and label the DNA
170 fragment with the fluorescent dye present on primer W49. These two primers were used
171 specifically for SSCP since they target the 16S rDNA V3 region that is the right length (200
172 base pairs) and has the necessary diversity for SSCP analysis of microbial communities. This
173 approach facilitates the PCR reactions and enables careful comparison of the different
174 patterns which are generated with the same primers.
175 The reaction mix comprised dNTP 0.2 mM, primers 390 nM, 1x *Pfu* turbo buffer, *Pfu* turbo
176 (Stratagene): 0.625 U, 1 µL of the PCR products amplified previously. The final reaction
177 volume was 20 µL. The amplification conditions were one cycle at 94°C for 2 min followed
178 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
179 min at 72°C. The resulting PCR products were then separated by SSCP capillary
180 electrophoresis using an ABI 310 genetic analyser (Applied Biosystems) as described by
181 Delbes *et al*. (10) but using a CAP 5.58% - Glycerol 10% polymer (Applied Biosystem).
182
183 **Cloning and sequencing**
184 For each bacterial group, cloning was performed on a mixture of two PCR products selected
185 according to their SSCP profiles (with the most numerous and highest peaks). The mixed
186 PCR products were cloning and transformed in *E. coli* competent cells using the StrataClone
187 PCR cloning kit (Stratagene, La Jolla, CA) following the manufacturer' instructions, except
188 for the ligation time which was increased from 5 to 15 min.
189 A total of 275 clones were further analysed: 96 for the *Eubacerium-Clostridiaceae* group (48
190 from raw manures and 48 from treated manures), 35 for the BSL group (11 from raw manures

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191 and 24 from treated manures), 72 for the *Bacteroides-Prevotella* group (48 from raw manures
192 and 24 from treated manures) and 72 for the *Bifidobacterium* group (24 from raw manures
193 and 48 from treated manures).
194 The clones were randomly picked and their inserts were screened by nested PCR and CE-
195 SSCP as follows; in the first step, plasmid inserts were amplified by PCR with plasmid
196 targeted primers T7 (5'-TAATACGACTCACTATAGGG-3') and P13 (5'-
197 GACCATGATTACGCCA-3') (Stratagene, La Jolla, CA). The reaction mix was dNTP 0.2
198 mM, primers 700 nM each, 1x Red*Taq* Buffer, Red*Taq* polymerase 2.5 U and deionised water
199 to bring the volume to 25 µL. The amplification conditions were 10 min at 94°C followed by
200 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
201 for 10 min. One µL of these PCR products was used to perform a CE-SSCP PCR as described
202 above. Inserts yielding a peak that co-migrated with distinguishable peaks from the manure
203 CE-SSCP profiles were sequenced for peak identification.
204 A total of 139 clones were sequenced. Sequence reactions were performed with the Ouest
205 Genopole Sequencing Facility (CNRS, Roscoff, France) using primer T7. DNA sequences
206 were identified by comparison with their closest relative available in databases using Blast
207 from the National Centre for Biotechnology Information (NCBI,
208 http://www.ncbi.nlm.nih.gov/BLAST/) and the Ribosomal Database Project II
209 (http://rdp.cme.msu.edu/).
210

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

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

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216 to bring the volume to 20 µL. The amplification programme was as described by Cardinale *et*
217 *al*. (7) except for the elongation temperature (72°C) which was adapted to Red*Taq*
218 polymerase. The PCR product was sequenced by the Ouest Genopole Sequencing Facility
219 (CNRS, Roscoff, France). The sequence obtained was aligned, using the ClustalW2 software
220 (52), to the seven ITS sequences of *Bifidobacterium* strains present in Genbank (*B. breve,*
221 *B. adolescentis, B. longum, B. choerinum, B. animalis, B. thermophilum* and
222 *B. pseudolongum*) and to the ITS sequence of *B. longum* biotype *suis* that was obtained in this
223 study as described above. Based on the comparison of these sequences, a pair of primers
224 specific to *B. thermacidophilum* subsp. *porcinum* was designed (GE35 / GE36) (Table 1).
225 Specific detection of *B. thermacidophilum* subsp. *porcinum* was then performed using a
226 nested PCR. All *Bifidobacterium* ITSs were first amplified using the primer pair ITSF/ITSR
227 as described above. The resulting PCR products were diluted 10 times and 1 µL was used as
228 template for a second PCR using the primer pair GE35 / GE36. The GE35 / GE36 PCR
229 reaction mix comprised 1x AccuPrime *Taq* DNA polymerase buffer II, AccuPrime *Taq*
230 polymerase 2.5 U (Invitrogen), primers 350 nM each, and deionised water to bring the total
231 volume to 20 µL. The PCR was performed using the following conditions: one cycle at 94°C
232 for 2 min, 30 cycles at 94°C for 30 s, 59°C for 30 s, and 68°C for 1.5 min.
233
234 **Nucleotide sequences accession numbers**.
235 Sequences were deposited in the EMBL database under accession numbers AM991308 to
236 AM991325.
237
238 **RESULTS**
239 **Comparison of the dominant microbial groups of raw and treated manures**

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240 For each bacterial group, the CE-SSCP profiles obtained from the 10 raw and treated manures
241 were aligned and compared (Fig. 1 to 4). The *Eubacterium-Clostridiaceae* profiles provided
242 the lowest resolution with a high background level below the peaks underlining the
243 complexity of this bacterial group (Fig. 1). The raw manure profiles shared 9 to 11 co-
244 migrating peaks and a similar number of distinct peaks before and after treatment. However,
245 in most cases, the peaks present in raw manures did not co-migrate with the peaks of treated
246 manures.
247 The BSL group profiles provided a lower background signal than that observed for the
248 *Eubacterium-Clostridiaceae* group (Fig. 2). The profiles of raw and treated manures consisted
249 of 10 and 12 peaks, respectively. After aerobic treatment, seven peaks from the treated
250 manures co-migrated with peaks from the raw manure profiles.
251 The CE-SSCP profiles of the *Bacteroides-Prevotella* and *Bifidobacterium* groups differed
252 from the BSL and *Eubacterium-Clostridiaceae* group profiles by the absence of background
253 and the small number of peaks detected (Fig. 3 and 4). These profiles yielded three and two
254 dominant peaks respectively, consistently preceded by smaller artifactual peaks which were
255 also visible with purified clones (data not shown). These artifactual peaks were probably
256 produced either during migration in capillary electrophoresis or during PCR amplification. In
257 the latter, they would represent a small proportion of PCR fragments that have ended
258 prematurely. The three peaks from the *Bacteroides-Prevotella* group detected in all raw
259 manures were not detected in treated manures, which contained two other distinguishable
260 peaks (Fig. 3B). The first peak (BA3) was common to all treated manures whereas the
261 position of the second peak (BA4) differed from one sample to another. The profiles of the
262 *Bifidobacterium* group were characterized by two peaks which were detected in all raw and
263 treated manures (Fig. 4).
264

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265 **Identification of the major peaks of each group**

266 The dominant peaks were identified by cloning and sequencing of the corresponding 16S
267 rRNA gene fragments. A total of 275 clones were screened by CE-SSCP and 139 were
268 sequenced. The phylogenetic affiliation of the clones corresponding to the major peaks of the
269 CE-SSCP profiles is presented in Table 2. Only 37.5% of the *Eubacterium-Clostridiaceae*
270 16S rDNA sequences found in raw manure demonstrated more than 97% similarity to
271 sequences in databases. Four of the dominant peaks in the *Eubacterium-Clostridiacea* raw
272 manure profiles were identified, but no identity could be assigned to peaks obtained from the
273 treated manure profiles. The closest relative of the four sequences identified were sequences
274 from uncultured bacteria from various sources, including the effluent treatment plant, the
275 solid waste digester and the pig manure storage pit.

276

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

283

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

289

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290 The sequences of the two peaks of the *Bifidobacterium* profiles, obtained either from the raw
291 or treated manures, were 99 to 100% similar to *B. thermacidophilum subsp. porcinum* isolated
292 from piglet faeces (peaks Bi1 and Bi1b) (60) and 98 to 100% similar to *Bifidobacterium*
293 *pseudolongum* subsp. *pseudolongum* isolated from porcine cecum (peaks Bi2 and Bi2b) (50).
294

295 **Specificity of GE35 / GE36 primers**

296 Among the four groups of bacteria analysed in this study, only two species,
297 *B. thermacidophilum* subsp. *porcinum* and *B. pseudolongum* subsp. *pseudolongum,* co-
298 migrated with a peak that was systematically detected in all raw and treated manure CE-SSCP
299 profiles. Given that *Bifidobacterium pseudolongum* subsp. *pseudolongum* has previously been
300 observed in various animal faeces (4), the *B. thermacidophilum* subsp. *porcinum* strain was
301 selected for further analyses. However, this species is genotypically too similar to
302 *B. thermophilum* (57) to be differentiated at the 16SrRNA gene sequence level. The design of
303 specific primers thus required targeting of the 16S-23S rRNA intergenic transcribed spacer
304 region. A specific pair of primers (GE35 / GE36) was designed and tested on *B.*
305 *thermacidophilum* subsp. *porcinum*^{T} and on seven other *Bifidobacterium* type strains
306 representative of taxa of animal origin as previously described by Ventura *et al.* (56). The test
307 showed that the primer set produced species-specific amplicons from *B. thermacidophilum*
308 subsp. *porcinum*^{T} and did not amplify any PCR products from the seven other strains (Table
309 3).
310

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

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314 *thermacidophilum* subsp. *porcinum* marker was only found in pig faeces when nested PCR
315 and the GE35 / GE36 primers were used.
316
317 *B. thermacidophilum* subsp. *porcinum* and the concentration of *E. coli* were observed in
318 manure and in water samples using our nested PCR assay (Table 5). Regardless of the level of
319 *E. coli*, *B. thermacidophilum* subsp. *porcinum* was not detected in urban effluents of human
320 origin, or in runoff water impacted by bovine manure contamination in spite of the presence
321 of *E. coli*. In the case of runoff waters obtained after application of pig manure, the three
322 samples showed positive amplification. *B. thermacidophilum* subsp. *porcinum* was also found
323 in raw and treated manure and in two types of lagoon supplied with treated pig liquid manure.
324 In lagoon L1 (with a retention time of five days) the concentration of *E. coli* was 4.5×10^{6}
325 CFU/ 100 mL and a positive amplification of the target bacteria was observed, whereas in
326 lagoon L2 (with a retention time of nine months) neither *E. coli* nor *B. thermacidophilum*
327 *subsp. porcinum* were detected.
328
329 **DISCUSSION**

330 Although pig-specific genetic markers have been proposed to trace faecal pollution in the
331 environment, their application has mainly focused on faecal samples (14, 22, 43, 44, 54) and
332 data concerning manure intended for spreading are scarce (22, 29). Cotta *et al*. (9) reported a
333 difference in composition between the bacterial communities of pig faeces and stored manure.
334 Furthermore, Peu *et al*. (45) observed differences in the bacterial community in fresh manure
335 located below the animals and manure stored in outdoor tanks. To be considered as suitable, a
336 microbial indicator of pig contamination must be abundant and found not only in faeces but
337 also in stored manure intended for land application.
338

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339 Whereas studies concerning faecal markers have usually focused on a particular group of
340 bacteria, we used a broader strategy (i.e. four groups instead of one) with the aim of
341 identifying a potential microbial marker of pig contamination present both in raw and treated
342 manures. The behaviour of four pig faecal bacterial groups (34, 45, 53, 58) was monitored
343 throughout pig manure biological treatment using molecular typing (CE-SSCP). These
344 bacterial groups were selected either because they are dominant in manure microbial
345 communities (*Eubacterium-Clostridiacea*, *Bacteroides-Prevotella*, BSL) or due to their
346 known host specificity. Thus phylogenetic groups of the *Bacteroides-Prevotella* have been
347 associated with pig faeces (14, 22, 43, 44) and the genus *Bifidobacterium* consists of species
348 from animal or human (17, 40) origin.
349
350 -SSCP profiles
351 The 17 raw manures analysed revealed the remarkable consistency of the SSCP profiles of the
352 four bacterial groups (Fig. 1 to 4) regardless of the geographical location of the piggeries
353 sampled and of the storage period of the manures. In practice, in Brittany piggeries, raw
354 manure stores are rarely aerated and slurry tanks are not operated as closed batch reactors but
355 are subject to regular additions of fresh manure. The major difference from one manure to
356 another is thus the length of storage, which ranges from weeks to months depending on the
357 storage capacity of the tank. This consistency of the bacteria profile could be explained by the
358 similarity of farm management practices (diet and the age of the animals) and manure storage
359 conditions. Leung and Topp (35) and Peu *et al*. (45) obtained similar results using molecular
360 techniques to monitor pig manure microbial community dynamics during storage in a
361 laboratory-scale reactor and a manure storage tank for a period of three months, respectively.
362 These data suggested that the dominant bacterial populations of manure stored under anoxic
363 conditions are not strongly influenced by the length of storage.

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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 *Bacteroide*s 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

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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

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414 highlighted the host specificity of *Bifidobacterium thermacidophilum* subsp. *porcinum,* which
415 was previously described in the pig intestinal tract (41, 60), as it was not detected in bovine,
416 poultry, human faeces nor in urban wastewaters containing domestic sewage. Our results
417 showed that using nested PCR, it was possible to detect *Bifidobacterium thermacidophilum*
418 subsp. *porcinum* in water samples contaminated by manure. This is in agreement with the
419 study of King *et al*. (30) who also used nested PCR to detect *B. adolescentis* in samples of
420 water impacted by human activities. As already reported by Lamendella *et al.* (33) and King
421 *et al.* (30), our results confirm that certain species of *Bifidobacterium* might represent a good
422 target population for assessing faecal contamination above a background level for example
423 associated with heavy rainfall events.
424
425 -Conclusions
426 The comparison of dominant pig manure microbial communities throughout manure treatment
427 using CE-SSCP allowed a large number of raw and treated manures to be screened. This
428 demonstrated that *Bifidobacterium* and, to a lesser extent, members of the BSL group were
429 less affected by the handling and treatment of manure than *Eubacterium-Clostridiaceae* and
430 *Bacteroides-Prevotella*. These data show that the *Bifidobacterium* species found in manure
431 can persist outside the pig intestinal tract and that *B. thermacidophilum* subsp. *porcinum* can
432 be used as an indicator of manure contamination in the environment.
433
434
435
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442

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

648

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

652

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

658

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

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

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

[a] M = A/C ; N = A/T/C/G ; Y = C/T ; [b] 6FAM = 6 carboxyfluorescein; [c] primer label

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TABLE 2: phylogenetic affiliation of 16S rDNA sequences

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

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

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

Bifidobacterium strain[a]	PCR Product formation
B. boum^T DSM 20432	-
B. thermophilum^T DSM 20210	-
Bifidobacterium thermacidophilum subsp. *porcinum*^{T} DSM 17755	+
B. merycicum^T DSM 6492	-
B. pseudolongum subsp. *globosum*^{T} DSM 20092	-
B. ruminantium^T DSM 6489	-
B. animalis subsp. *animalis*^{T} DSM 20104	-
B. longum subsp. *suis*^{T} DSM 20211	-

[a] Specificity was tested with chromosomal DNA from *Bifidobacterium* previously detected in animal faeces

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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)[a]	30
Bovine (n = 30)	0
Poultry (n=30)	0
Human (n=28)	0

[a] number of samples

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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	*E. coli* counts (CFUs/100 mL)[a]	Number of positive samples for the target bacteria/total
Raw manure (pig)	-	4.0×10^{6} (4.2×10^{6})	17/17
Treated manure (pig)	-	5.1×10^{4} (3.3×10^{4})	10/10
Lagoon with a retention time of 5 days (L1) (pig)	treated liquid manure	4.5×10^{6} (4.1×10^{5})	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^{3})	3/3
Runoff water (R4 to R6)	bovine manure spread on field	7.5×10^{3} (8×10^{2})	0/3
Raw waste water	Urban effluent (mainly human)	1.8×10^{6} (1.7×10^{6})	0/5
Treated waste water	Urban effluent (mainly human)	3.3×10^{3} (4.3×10^{3})	0/10

[a] mean values (standard deviation)

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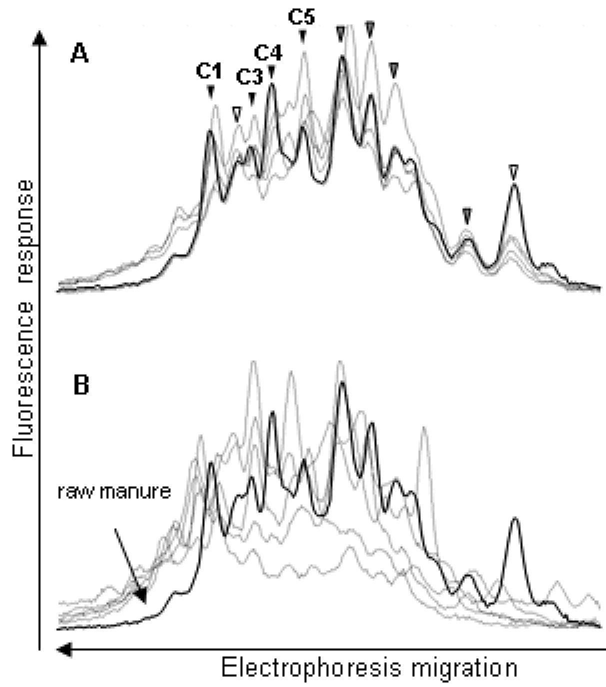

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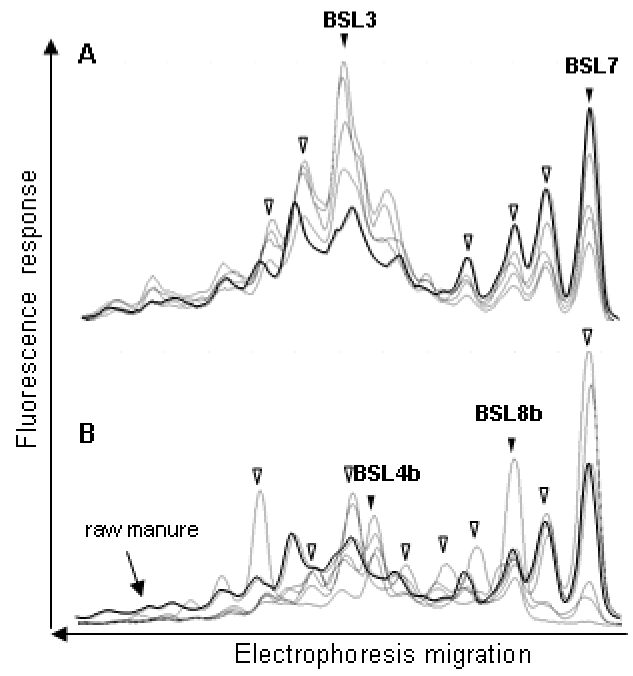

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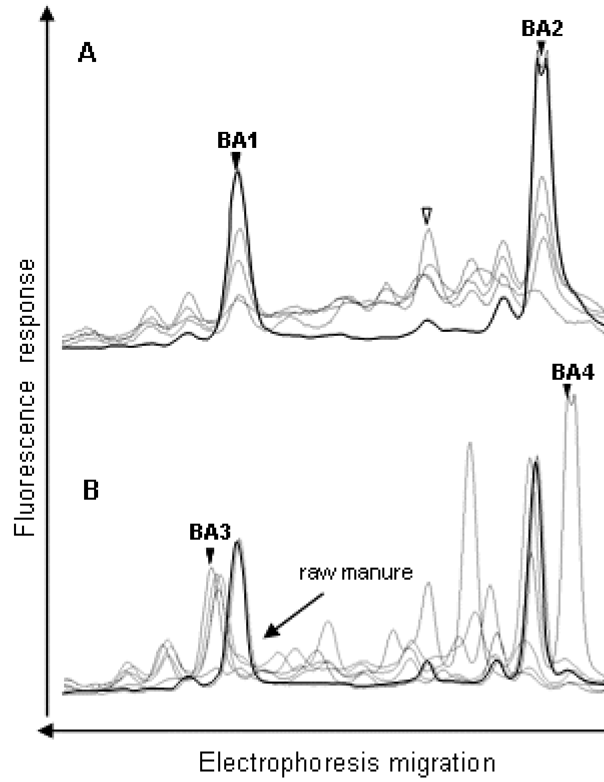

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