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Evaluation of *Lactobacillus sobrius*/*L. amylovorus* as a New Microbial Marker of Pig Manure▽

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Based on a comparison of the dominant microbial populations in 17 pig manure samples and using a molecular typing method, we identified a species, *Lactobacillus sobrius* and *Lactobacillus amylovorus* (which now are considered a single species and are designated *L. sobrius*/*amylovorus* here), that was consistently found in manure. The aim of the present study was to confirm by real-time PCR the relevance of this species as a marker of pig fecal contamination. The specificity of *L. sobrius*/*amylovorus* was evaluated in human and animal DNA extracted from feces. The real-time PCR assay then was applied to water samples, including effluents from urban wastewater treatment plants, runoff water, and rivers. *L. sobrius*/*amylovorus* was consistently present in all samples of swine origin: 48 fecal samples, 18 from raw manure and 10 from biologically treated manure at mean concentrations of 7.2, 5.9, and 5.0 $\log_{10}$ cells/g, respectively. The species was not detected in any of the other livestock feces (38 samples from cattle and 16 from sheep), in the 27 human fecal samples, or in the 13 effluent samples from urban wastewater treatment plants. Finally, *L. sobrius*/*amylovorus* was not detected in runoff water contaminated by cattle slurry, but it was quantified at concentrations ranging from 3.7 to 6.5 $\log_{10}$ cells/100 ml in runoff water collected after pig manure was spread on soil. Among the stream water samples in which cultured *Escherichia coli* was detected, 23% tested positive for *L. sobrius*/*amylovorus*. The results of this study indicate that the quantification of *L. sobrius*/*amylovorus* using real-time PCR will be useful for identifying pig fecal contamination in surface waters.

Pig manure may contain pathogenic microorganisms that are harmful to humans and animals (11). These pathogens, which include bacteria, viruses, and protozoans, can survive for several weeks during the storage of manure and in the soil after manure is spread on the land (30). As a consequence, the large amount of manure that is produced and applied on land in many agricultural areas may impact water quality. It contributes to non-point source pollution, which is due partially to runoff from manured soil, especially when manure is spread just before rainfall. It is difficult to determine the origin of diffuse pollution, as it cannot be traced to a specific spot. Fecal indicators (*Escherichia coli*, fecal coliforms, and enterococci), which are commonly used to quantify fecal pollution, are present in the intestinal tracts of both humans and warm-blooded animals and thus cannot be used to distinguish contamination by pig manure from other sources of pollution. For this reason, alternative microbial indicators have been proposed for the identification of specific pollution sources.

During the past 10 years, a few library-independent methods have been developed for the identification of pig fecal contamination. They are based mostly on the PCR amplification of specific genes or sequences, such as the STII toxin gene from enterotoxigenic *E. coli* (16), the internal transcribed spacer (ITS) sequence from *Bifidobacterium thermacidophilum* subsp. *porcinum* (26), the 16S rRNA gene of *Bacteroides-Prevotella* (5, 27, 31), and the methyl coenzyme M reductase gene from a methanogenic *Archaea* member (41). However, some of these methods are only qualitative, like the detection of *B. thermacidophilum* subsp. *porcinum* or of the *mcrA* and STII toxin genes, and do not allow the level of contamination to be quantified. Furthermore, it is noteworthy that the archaeal *mcrA* gene was not detected in 16% of the pig feces analyzed (41), and that the presence of the STII toxin gene depends on the level of *E. coli* in the sample, which needs to be greater than 100 cells to avoid false positives (16). Okabe et al. (31) quantified a *Bacteroides-Prevotella* pig-specific marker (Pig-Bac2) in water samples using real-time PCR. However, this marker lacks specificity, as the Pig-Bac2 marker also was present in human and cow feces at a concentration of 7 and 8 $\log_{10}$ copies per g, respectively (31). Only one pig-specific *Bacteroidales* 16S rRNA gene marker (Pig-2-Bac), which was developed recently by Mieszkin et al. (27) using real-time PCR, appears to be suitable to quantify pig fecal contamination. However, one limit of targeting the *Bacteroidales* group could be their strictly anaerobic metabolism, which may influence their persistence in well-oxygenated water. Savichtcheva et al. (36) thus have reported that oxygen has a negative effect on the survival rate of *Bacteroides fragilis*. We thus consider it important to study biomarkers that are less sensitive to oxygen in order to extend the choice of tools for tracking sources of pollution by manure. Moreover, in the case of the downgrading of bathing or shellfish areas, when health and economic risks are involved, it could be useful to combine multiple markers to identify the source of pollution with certainty.

In the search for potential pig manure markers, we recently analyzed the dominant bacterial groups of 17 raw pig manure samples using 16S rRNA-targeted PCR and the CE-SSCP (capillary electrophoresis–single-strand conformation polymorphism) molecular typing method (26). Among the domi-

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nant bacterial groups (*Bacteroidales*, *Bifidobacterium*, *Eubacterium-Clostridiaceae*, and *Bacillus-Streptococcus-Lactobacillus*), we highlighted the presence of a microaerophilic species, *Lactobacillus sobrius*, which was isolated from piglet feces previously (19). Lactobacilli are known to establish a stable population in the intestinal tract of piglets soon after birth (28, 39) and to rapidly become a dominant population of their intestinal flora, at least in the first days after weaning (2, 14, 34). Their concentration in pig feces has been estimated at about 3×10^8 bacteria/g (9). Because of their protective effect against diarrhea, some species of *Lactobacillus*, including *L. sobrius*, particularly have been studied (20, 35). Konstantinov et al. (21) therefore designed a primer pair that specifically amplifies a fragment of the *L. sobrius* genome using real-time PCR. Finally, Jakava-Viljanen et al. (13) recently demonstrated very high similarity between the *L. sobrius* and *L. amylovorus* type and reference strains and representative porcine isolates based on their 16S rRNA gene sequence analysis. According to these results, *L. sobrius* and *L. amylovorus* constitute a single species and consequently are referred to as *L. sobrius*/*amylovorus* in this paper.

Given the abundance of *L. sobrius*/*amylovorus* in piglet feces (19, 37) and its systematic presence in raw manure (26), we tested this species as a new marker of pig fecal contamination. The aims of our study were (i) to confirm the specificity of *L. sobrius*/*amylovorus* to pig feces by analyzing five host groups (human, pig, cattle, poultry, and sheep), manure and by-products of manure treatment, runoff water, and urban wastewaters, and (ii) to estimate the suitability of this marker to identify pig fecal contamination found in surface waters. The concentrations of *L. sobrius*/*amylovorus* were estimated by real-time PCR using the primers designed by Konstantinov et al. (21). They were compared to the levels of *E. coli*, total lactobacilli, and, for river water samples, to the concentrations of the pig-specific *Bacteroidales* 16S rRNA genetic marker (Pig-2-Bac) developed by Mieszkin et al. (27).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study were *Lactobacillus acetotolerans* DSM20749^T, *L. acidophilus* DSM9126^T, *L. amylolyticus* DSM11664^T, *L. amylovorus* DSM20531^T, *L. crispatus* DSM20584^T, *L. delbrueckii* subsp. *delbrueckii* DSM20074^T, *L. hamsteri* DSM5661^T, *L. helveticus* DSM 20075^T, *L. johnsonii* DSM10533^T, *L. kalixensis* DSM16043^T, *L. kefiranofaciens* subsp. *kefiranofaciens* DSM5016^T, *L. kitasatonis* DSM16761^T, *L. reuteri* DSM8533^T, *L. sobrius* DSM16698^T, and *Escherichia coli* DSM30083^T. Strains were cultured under anaerobic conditions at 37°C on Man Rogosa Sharpe (MRS) agar (Biokar, France), except for *L. acetotolerans* DSM20749^T and *L. hamsteri* DSM5661^T, which were cultured on modified MRS medium (with 0.05% cysteine-hydrochloride and pH adjusted to 5.2) as recommended by DSMZ. *E. coli* was grown at 37°C on TBX medium (Biokar, France).

Fecal samples. A total of 132 samples of fresh animal feces (38 cowpats of cattle, 48 feces of pig, 34 droppings of poultry, and 16 feces of sheep) were collected from 64 farms across Brittany. Twenty-seven samples of human feces from healthy people were obtained from two French research institutes (IFREMER [Brest] and INRA [Jouy-en-Josas]). From each well-mixed sample of fresh feces, subsamples of approximately 250 mg (wet weight) were taken and transferred into a microtube and stored at −20°C.

Manure and lagoon water samples. Manure and lagoon water samples were collected from 18 piggeries located across Brittany as previously described (26). Samples were centrifuged at 16,000 × *g* to form a pellet of approximately 250 mg (wet weight). The pellets were stored at −20°C.

Water samples. Six independent samples of field runoff water were collected 40 to 50 min after six rainfall simulations on an experimental agricultural plot previously spread with either cattle or pig manure. A rainfall simulator was placed under a tent to prevent wind and natural rain perturbation. To represent an extreme storm event in spring, simulated rainfall was applied at an intensity of 67 mm · h^{-1}. All samples were poured into 2-liter flasks. Volumes of approximately 200 ml of water were centrifuged at 4,000 × *g* for 30 min, and pellets were transferred into microtubes and stored at −20°C.

Thirteen samples of treated urban wastewater were collected from locations across Brittany and Pays de la Loire (France). Samples of river surface water (1 liter) were collected from 30 sites located in rural areas in Brittany. For these samples, 100 ml of river water was filtered through a 0.2-μm-pore-size polycarbonate filter. When it was not possible to filter due to clogging, the water samples were centrifuged as described above. Filtrates or pellets were transferred into microtubes and stored at −20°C.

Enumeration of *E. coli*. *E. coli* cells were enumerated in all water samples using 3M *E. coli* petri film to estimate the level of fecal contamination as described previously (26). The concentration of *E. coli* was expressed in CFU/ml.

Extraction of genomic DNA of strains. Genomic DNA was isolated from a 1-ml exponential-phase culture of each strain collected using the Promega Wizard purification kit (Promega). DNA pellets were suspended in 100 μl of rehydration solution (10 mM Tris, 1 mM EDTA). The integrity of genomic DNA was monitored by 1.5% agarose gel electrophoresis in 1× Tris-borate-EDTA and ethidium bromide staining and quantified using a Nanodrop ND1000 microspectrophotometer (Labtech, France).

Extraction of genomic DNA from samples. DNA was extracted from the pellets, and filters were stored at −20°C using the QIAamp DNA stool kit (Qiagen) in accordance with the manufacturer's instructions, except that the samples were homogenized in buffer ASL and heated at 95°C for 5 min to lyse bacterial cells. The elution volume was 50 μl.

Quantitative PCR. PCR amplification was performed using a Bio-Rad CFX96 real-time PCR instrument with Bio-Rad CFX Manager software, version 1.1 (Bio-Rad, Hercules, CA).

All target sequences and primer sequences used in this study are presented in Table 1. Primer sequences and PCR programs used for the quantification of lactobacilli and *L. sobrius*/*amylovorus* are described in Su et al. (37) and Konstantinov et al. (21), respectively. The reaction mixture consisted of 12.5 μl of IQ SYBR green Supermix (Bio-Rad), a 200 nM concentration of each primer, 2 μl of 1/10 diluted DNA, and 9.5 μl of water to reach a final volume of 25 μl. *E. coli* was quantified by the real-time PCR protocol described by Khan et al. (15). The reaction mixture consisted of 12.5 μl of IQ SYBR green Supermix (Bio-Rad), a 350 nM concentration of each primer, 2 μl of 1/10 diluted DNA, and 9.5 μl of water to reach a final volume of 25 μl. *Bacteroidales* marker Pig-2-Bac was quantified using TaqMan chemistry according to the protocol described by Mieszkin et al. (27). The reaction mixture consisted of 12.5 μl of IQ supermix (Bio-Rad), 300 nM concentrations of each primer, a 200 nM concentration of Pig-2-Bac113MGB probe, 2 μl of 1/10 diluted DNA, and 8.5 μl of water to reach a final volume of 25 μl. Each assay was conducted in triplicate in 96-well plates (Bio-Rad).

Standard curves for DNA. The standard curves for genomic DNA were constructed using serially diluted DNA from *L. sobrius* DSM16698^T and *E. coli* DSM30083^T. In brief, the concentrations of *L. sobrius* and *E. coli* were estimated using the standard plate count method to be 3.5×10^8 and 1×10^9 CFU/ml, respectively. Cells were enumerated during the exponential growth phase. Total DNA was extracted from the two reference strains and 10-fold serially diluted in sterile deionized water to yield 7×10^0 to 7×10^5 cells per reaction mixture for *L. sobrius* and lactobacilli and 2.7×10^1 to 2.7×10^6 cells per reaction mix for *E. coli*. Results of real-time PCR thus were expressed as cells per ml of water or per g of manure.

For the quantification of the *Bacteroides* Pig-2-Bac marker, standard curves were generated from serial dilutions of a known concentration of linear plasmid DNA (kindly supplied by S. Mieszkin, IFREMER, Brest, France) linearized with NotI enzyme (Roche Diagnostics) (27).

RESULTS

Primer specificity. The set of primers developed by Konstantinov et al. (27) was tested on 14 reference strains of *Lactobacillus*. As expected, only *L. sobrius* DSM16698^T and *L. amylovorus* DSM20531^T, which were reclassified recently as the same species (13), gave a signal, whereas closely related strains such as *L. kitasatonis* DSM16761^T or *L. acidophilus* DSM9126^T did not give a signal (data not shown).

TABLE 1. Sequences of the primers and probes used in this study

Primers and probes	Primer and probe sequence (5′→3′)	Size of amplicon (bp)	Annealing temp (°C)	Final concn (nM)	Target	Reference
Pig-2-Bac						
Pig-2-Bac41F	GCATGAATTTAGCTTGCTAAATTTGAT	116	60	300	Pig-specific *Bacteroidales*	Mieszkin et al. (27)
Pig-2-Bac163Rm	ACCTCATACGGTATTAATCCGC			300		
Pig-2Bac113MGB	(VIC)TCCACGGGATAGCC(NFQ-MGB)[a]			200		
Lactobacilli						
LAC1	AGCAGTAGGGAATCTTCCA	320	60	200	Lactobacilli	Su et al. (37)
Lab0677	CACCGCTACACATGGAG			200		
L. sobrius/*amylovorus*						
OTU171_RDA_F	TTCTGCCTTTTTGGGATCAA	175	60	200	*L. sobrius*/*amylovorus*	Konstantinov et al. (21)
OTU171_RDA_R	CCTTGTTTATTCAAGTGGGTGA			200		
E. coli						
IEC-UP	CAATTTTCGTGTCCCCTTCG	450	58	350	*E. coli*	Khan et al. (15)
IEC-DN	GTTATTGATAGTGTGTCGAAA			350		

[a] NFQ-MGB, nonfluorescent quencher-minor groove binder.

Detection of *L. sobrius*/*amylovorus* in feces. To estimate the relative abundance of *L. sobrius*/*amylovorus* in feces of different hosts, we compared the concentrations of this species to those of lactobacilli and *E. coli* (Table 2). A total of 163 samples were analyzed, including 48 pig feces, 87 feces samples from other animals, and 28 human feces. *L. sobrius*/*amylovorus* was present in 100% of the pig feces samples at a mean concentration of 7.2 $\log_{10}$ cells/g. The primers did not amplify products in human, cattle, and sheep feces. They amplified 5 of the 34 poultry droppings, which represent closely the composition of the fecal matter excreted. In all the samples of pig feces, the concentrations of *L. sobrius*/*amylovorus* always were within the same order of magnitude as those observed for lactobacilli and *E. coli*, whereas this species represented less than 0.1‰ of lactobacilli and of *E. coli* in the five poultry feces.

Influence of manure treatment on the *L. sobrius*/*amylovorus* population. We then tested the persistence of *L. sobrius*/*amylovorus* during manure management and our ability to detect it in pig farm effluents intended for spreading or irrigation. Target bacterial genomes and cultured *E. coli* were quantified in raw manure and in manure stored in a tank for 6 to 9 months after a biological treatment to remove nitrogen. Lagoon water samples that consisted of supernatant from treated manure stored in tanks also were collected and analyzed (Table 3).

L. sobrius/*amylovorus* was consistently detected in raw manure at a mean concentration of 5.9 $\log_{10}$/g. It is interesting that despite the aerobic biological treatment and the ensuing anaerobic storage for several months, the decrease in the number of *L. sobrius*/*amylovorus* cells did not exceed 1 $\log_{10}$. These results underline the persistence of the biomarker throughout the aerobic biological treatment. In lagoon water samples that were slightly contaminated, as indicated by the very low level of *E. coli* (close to the detection limit of the cultural method), *L. sobrius*/*amylovorus* was quantified in 50% of the samples at a level of 1.7 $\log_{10}$ cells/ml. In the two lagoon water samples where *E. coli* was not found, we did not detect *L. sobrius*/*amylovorus*.

Quantification of *L. sobrius*/*amylovorus* in urban effluent and water samples. Real-time PCR assays using *L. sobrius*/*amylovorus*, lactobacilli, and *E. coli* primer sets and the enumeration of cultured *E. coli* were performed on water samples. Nineteen water samples were impacted by human and animal fecal waste. They included six runoff water samples from a soil surface after the spreading of cattle slurry or pig manure and 13 treated effluent samples from urban wastewater treatment plants. Thirty surface water sources were sampled in rural areas with no prior knowledge of the level or the origin of pollution, except for one sample that was collected in a stream close to a field where manure had been spread 3 days prior to a rainy period. The quantification and the prevalence of *L. sobrius*/*amylovorus* were also compared to those of the *Bacteroidales* Pig-2-Bac marker developed by Mieszkin et al. (27) (Tables 4 and 5).

TABLE 2. Concentrations of *L. sobrius*/*amylovorus*, lactobacilli, and *E. coli* ($\log_{10}$ cells/gram) in feces of different origins

Source of tested fecal sample	No. of samples tested	No. of positive samples[a]	Concn (min-max)		
			L. sobrius/*amylovorus*	Lactobacilli	*E. coli*
Pig	48	48	7.2 (4.8–8.3)	7.3 (5.8–8.4)	8.0 (4.9–9.4)
Poultry	34	5	3.1 (2.7–3.4)	7.6 (6.3–8.3)	8.8 (6.5–9.8)
Human	27	0	—[b]	5.7 (3.2–6.8)	7.9 (4.4–9.2)
Cattle	38	0	—	4.7 (3.4–5.4)	6.9 (5.5–7.2)
Sheep	16	0	—	5.3 (3.2–5.7)	5.7 (4.5–6.2)

[a] Number of samples in which *L. sobrius*/*amylovorus* was quantified.
[b] —, not detected

TABLE 3. Concentrations of *L. sobrius/amylovorus*, lactobacilli, and *E. coli* (log_{10} cells/g or ml) in pig farm effluent

Source of samples (*n*)	Concn (min-max) (%)[a]			
	L. sobrius/amylovorus	Lactobacilli	*E. coli* qPCR	*E. coli* culture[b]
Raw manure stored in a pit (18)	5.9 (5.0–6.7)	6.1 (5.1–6.7)	6.9 (5.7–7.5)	5.4 (2.3–6.6)
Biologically treated manure (10)	5.0 (4.0–6.0)	5.1 (4.3–5.5)	5.7 (4.5–6.3)	2.7 (1.6–3.0)
Lagoon water (8)	1.7 (0.7–2.1) (50)	3.8 (3.4–3.9) (50)	—[c]	0.7 (0-1.1) (75)

[a] Percentage of positive samples when the number is below 100%.
[b] Enumerated by the culture method.
[c] —, no data.

Contrary to lactobacilli that were consistently found in waters regardless of their origin and the level of *E. coli*, *L. sobrius/amylovorus* was not detected in any effluents of urban wastewater treatment plants. *L. sobrius/amylovorus* was quantified at concentrations ranging from 3.7 to 6.5 log_{10} cells/100 ml in runoff water samples collected after the spreading of pig manure, whereas it was not detected in runoff water contaminated by cattle slurry. Furthermore, *L. sobrius/amylovorus* was quantified in three samples of surface water in which the Pig-2-Bac marker also was present (Table 5).

DISCUSSION

A high concentration of piggeries in a restricted area, as is the case in Brittany, leads to the fecal contamination of surface waters after manure is spread on the soil. The transfer of pathogenic microorganisms from manure can pose a risk to human health, especially in coastal areas where shellfish farming and nautical activities are widespread. It is thus important to possess tools to accurately identify and quantify pig fecal contamination in sensitive areas to be able to act directly on the source of pollution. However, few quantitative methods are available to differentiate pig and other animal or human sources of pollution. Only one specific biomarker, Pig-2-Bac, which was developed by Mieszkin et al. (27) and does not require culture, can be used to quantify pig fecal contaminations in environmental water samples. The present study tested the relevance of a new pig biomarker, *L. sobrius/amylovorus*, using the set of primers designed by Konstantinov et al. (21). This biomarker targets two species, *L. amylovorus* and *L. sobrius*, which were described by Nakamura (29) and by Konstantinov et al. (19), respectively, and now are considered a single species due their high level of genomic similarity (13). To our knowledge, this is the first report that targets lactobacilli as a biomarker of fecal contamination.

The concentrations of lactobacilli estimated by real-time PCR in the 48 pig feces analyzed in this study ranged between 5.8 and 8.4 log_{10} cells per g of feces and were slightly lower (ca. 0.7 log_{10} less) than those of *E. coli*, as previously observed by Furet et al. (9). These data also are comparable to the concentrations observed in the intestinal tract of piglets that range between 5.6 and 9.3 log_{10} cells per g (18, 34, 37, 40). The variability of these concentrations may be explained by the differences in age and diet of the animals (17, 18).

We also observed variability of the concentrations of lactobacilli and *E. coli* in feces of humans and other animals. However, mean concentrations of *E. coli* were in the same order of magnitude as those reported for human feces by Furet et al. (9) and Firmesse et al. (8) with SYBR green PCR. They also were similar to the concentrations of *E. coli* in cattle feces reported by Furet et al. (9) and of *Enterobacteriaceae* in ileum and cecum of poultry observed by Olsen et al. (32) using the FISH method.

Our study showed that *L. sobrius/amylovorus*, which was detected consistently at concentrations ranging between 4.8 and 8.3 log_{10} cells per g in pig feces, represents at least 80% of the lactobacilli. These data are in agreement with those of Su et al. (37) and Pieper et al. (34), who observed between 5.3 and 8.6 log_{10} *L. sobrius* in the ileum and small intestine of weaning piglets. Our data thus highlight the fact that *L. sobrius/amylovorus*, which was previously quantified in piglets (18, 34, 37, 40), also is abundant in the feces of adult pigs. While the presence of *L. sobrius* has been described only in the intestinal tract of piglets (19, 34, 37), the natural habitat of *L. amylovorus* is less known. *L. amylovorus* was initially described in maize silage (29). However, recently Brusetti et al. (3), who used

TABLE 4. Concentrations of *L. sobrius/amylovorus*, lactobacilli, and *E. coli* (log_{10} bacteria/ml) in water samples and concentrations of host-specific Pig-2-Bac marker in surface water (log_{10} copies/ml)

Source (no. of samples)	Origin of pollution	Concn (min-max) (%)[a]				
		L. sobrius/amylovourus	Lactobacilli	*E. coli* qPCR	*E. coli* culture[b]	Pig-2-Bac
Urban treated wastewater (13)	Human	>LD[c] (0)	2.7 (2.1–3.4)	4.0 (2.5–4.9)	2.4 (0–3.1)	
Runoff water[d] (3)	Cattle	>LD (0)	4.7 (4.5–4.8)	4.7 (3.5–5.3)	3.3 (2.3–3.7)	
Runoff water (3)	Pig	3.3 (1.7–4.5)	4.7 (4.4–4.9)	3.7 (3.5–3.8)	2.0 (1.8–2.1)	
Surface water (1)	Pig	1.8	3.1	3.0	0	3.0
Surface water (29)	Unknown	2.0 (1.8–2.2) (7)	4.0 (2.1–4.5)	3.5 (2.5–4.4)	0.5 (0–1.7) (41)	2.8 (2.1–3.0) (7)

[a] Percentage of positive samples when the number is below 100%.
[b] Number of *E. coli* cells estimated by the culture method.
[c] LD, under the limit of detection.
[d] Runoff water collected after the application of slurry or manure.

TABLE 5. Prevalence of *E. coli* and the pig markers and among the 30 surface water samples

Condition (presence/absence[a])			No. of samples meeting the conditions
E. coli (culture method)	*L. sobrius*/*amylovorus*	Pig-2-Bac	
−	−	−	17
−	+	−	0
−	−	+	0
−	+	+	0
+	−	−	10
+	−	+	0
+	+	−	0
+	+	+	3

[a] −, absence; +, presence.

length heterogeneity-PCR to study the succession of the lactic bacteria during maize ensiling, did not report the presence of *L. amylovorus*.

In our study, *L. sobrius*/*amylovorus* was not found in the cattle, sheep, and human samples. These results are consistent with the fact that neither *L. amylovorus* nor *L. sobrius* has been found by molecular inventory methods in intestinal or fecal microflora of humans (6, 12, 38). More importantly, while we always found lactobacilli and *E. coli* in the urban wastewater effluents, the latter at concentrations similar to those reported by Wéry et al. (42), we never detected *L. sobrius*/*amylovorus* in such samples.

The presence of *L. sobrius*/*amylovorus* at low concentrations in 5 of the 34 poultry feces is in accordance with the study of Cauwerts et al. (4), who isolated *L. amylovorus* by culture in the intestine of chicken. However, it is important to underline that when detected in poultry, the concentration of *L. sobrius*/*amylovorus* was 4 $\log_{10}$ below the level observed in pig feces. Given its low prevalence and concentration, it is not surprising that *L. sobrius*/*amylovorus* has not been identified using molecular tools in the intestinal tracts of chicken and turkey, the dominant lactobacilli being *L. delbrueckii* and *L. acidophilus* or *L. aviarius* and *L. salivarius* for chicken (10, 24, 25) and *L. aviarius* for turkey (23). Because the natural habitat of *L. sobrius*/*amylovorus* is not well documented, it was important to test manure and surface water samples for the presence of this biomarker to show that it still was present in pig manure but not ubiquitous in the environment, and that we were able to detect it in contaminated samples.

Like lactobacilli and *E. coli*, *L. sobrius*/*amylovorus* persisted in raw manure and was only slightly impacted by the biological treatment and storage of manure. These data are in agreement with the studies of Leung and Topp (22) and Peu et al. (33), who observed very few changes in the bacterial communities during the anaerobic storage of manure. It is important to note that *L. sobrius*/*amylovorus* was quantified in lagoon waters stored for 9 to 12 months only in the samples in which cultured *E. coli* was detected. These results suggest that, like *E. coli*, *L. sobrius*/*amylovorus* has the ability to persist in water for extended periods of time. Furthermore, after pig manure was spread on a field before a rainfall event, the transfer of *L. sobrius*/*amylovorus* was similar to that of *E. coli* measured by real-time PCR, as both bacteria were found in runoff water at similar concentrations.

To validate the specificity of our marker, we quantified the Pig-2-Bac marker described by Mieszkin et al. (27) in samples of natural river water using TaqMan PCR technology and compared its presence to that of lactobacilli and *E. coli*. In samples of surface water collected in rural areas, *E. coli* and lactobacilli measured by real-time PCR always were quantified at similar concentrations ranging between 5.5 and 6.9 $\log_{10}$ cells/100 ml, whereas cultured *E. coli* was detected in 13 of the 30 surface water samples. The difference in concentration between *E. coli* measured by molecular and cultural methods could be explained by the fact that this bacteria can enter a viable but nonculturable state (1) or by the amplification of DNA from dead cells. The systematic detection of lactobacilli in surface waters could be due to the existence of viable but nonculturable cells or to the presence of ubiquitous species, given the considerable diversity of this group of bacteria in ecological habitats (7). Interestingly, *L. sobrius*/*amylovorus* and Pig-2-Bac were detected simultaneously in three surface water samples, one of them being collected after a rainfall event less than 10 m from a field where pig manure had been spread. Our data not only highlight the fact that *L. sobrius*/*amylovorus* cells are not ubiquitous in the environment but also confirm the specificity of our marker and its ability to be transferred via runoff into surface water. Furthermore, our results confirm that the contamination of rivers by pig manure appears to be relatively frequent in Brittany, as *L. sobrius*/*amylovorus* was present in 23% of the rivers in which cultured *E. coli* was detected.

This study showed the potential for using *L. sobrius*/*amylovorus* to identify the contamination of water by pig manure. One concern might be a high concentration of poultry farms producing *L. sobrius*/*amylovorus* in the environment. However, the prevalence and the concentration of *L. sobrius*/*amylovorus* in poultry droppings were very low. In addition, poultry farms more often produce manure diluted with litter material and largely dried prior to land application, thus substantially reducing the concentration of *L. sobrius*/*amylovorus* if present. Even if such dilution did not apply, we have observed in the runoff experiment that, regardless of the bacteria, the concentration in the runoff water was ca. 1,000th of that in pig manure. This result implies that the concentration of *L. sobrius*/*amylovorus* originating from poultry manure is less than 1 cell/ml in the subsequent runoff water.

The quantification of this biomarker by real-time PCR makes it a promising tool for microbial source tracking. Its usefulness is enhanced by its systematic presence in manure, whether treated or not, collected in various farms located in different areas of Brittany. The persistence of *L. sobrius*/*amylovorus*, its transfer via runoff, and its quantification in river waters mean that this bacterium is relevant for the detection of contamination by pig manure. However, since it is not possible to exclude the relatively minor presence of *L. sobrius*/*amylovorus* in the feces of wild animals, this new marker should be used with other pig markers such as Pig-2-Bac to improve the detection accuracy of the method. Furthermore, future studies will concentrate on the behavior of *L. sobrius*/*amylovorus* under different environmental conditions.

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