EFFECT OF ANAEROBIC STORAGE AND AEROBIC DIGESTION ON MICRO-ORGANISMS IN PIG MANURE: CULTURAL AND MOLECULAR APPROACHES

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SUMMARY

The impact of manure treatment was studied on bacterial populations using cultural methods and 16S rRNA targeted PCR Single-Strand-Conformation-Polymorphism analysis (SSCP). Aerobic treatment followed by anaerobic storage resulted in a reduction of between one to two logarithmic units of the numbers of *E coli* and enterococci, but this was not sufficient to eliminate *Salmonella* and *Listeria monocytogenes*. The dominant microbial community of the raw manures remained very stable with the persistence of four species whereas SSCP profiles of treated manures showed a greater diversity of bacterial population. Two species found in raw manure, *Bifidobacterium thermacidophilum subsp. porcinum* and *Lactobacillus sobrius*, could be proposed as manure indicators.

Keywords: manure, treatment indicators, pathogen, survival, SSCP, 16S rRNA

INTRODUCTION

Effluents from piggeries, that may contain viruses, parasites and pathogenic bacteria, can present a sanitary risk during its subsequent spreading on agricultural land. It has been observed that spreading has resulted in an increase in number of pathogenic microorganisms in soil (Gessel *et al.*, 2004). The health risk increases when manure is spread on soil where certain crops (e.g. salads, fruit and some vegetables) that are not intended to be cooked are grown (Nicholson *et al.*, 2005). In response to the recent strengthening of European regulation concerning the recycling of animal by-products (regulation n° 1774/2002), it is important to study the effectiveness of manure treatments which include aerobic processes developed primarily for biological nitrogen removal.

The behaviour of the microorganisms in manure is generally studied using either cultural or molecular methods. The cultural approach is adapted to the detection of indicator bacteria (*E. coli*, enterococci and *Clostridium perfringens*) and specific pathogenic bacteria. It enables numeration of low levels of pathogens. Nevertheless, the absence of specific media and the existence of viable but non cultivable forms introduce a bias on the number detected. Thus, among the 10^9 to 10^{10} bacteria /mL enumerated in manure using direct microscopic counting, only 10 to 20% can be cultured (Cotta *et al.*, 2003). Furthermore, the inventory of the faecal flora of 24 pigs revealed the presence of 375 phylotypes (molecular equivalent of a species) for which only 17% were close to

known species (Leser *et al.*, 2002). The molecular approach may thus appear more relevant for the detection of faecal microorganisms even if, it cannot yet target subdominant microbial groups. As expected, no pathogenic bacteria or classical bacterial indicator have been detected during the molecular inventories of faecal matter and manure (Leser *et al*., 2002, Snell-Castro *et al.*, 2005).

The aim of this study was to compare the impact of the simple anaerobic storage of manure with a more complex aerobic/anoxic treatment on the bacteria of sanitary interest and on the manure dominant microbial community. The originality of the study consisted in the use of both cultural and molecular methods.

MATERIAL AND METHODS

Sampling was carried out between March and July 2006 at 17 piggeries located in Brittany (France). Samples were taken from 27 anaerobic storage tanks : 17 from the raw manure storage tanks (primary tank) and 10 from the treated manure storage tanks after aerobic digestion (secondary tank). Before sampling, manure that had been stored in the tanks between 3 weeks and 6 months were homogenised by mixing. Microbial analysis was performed by (i) cultural methods and (ii) PCR amplification of the 16S ribosomal RNA bacterial genes followed by Single-Strand Conformation Polymorphism analysis (SSCP) of the PCR products.

Cultural methods

E. coli was enumerated using 3M Petrifilm *E. coli* (incubated 24 hours at 44°C). Detection of *E. coli* was based on enumeration of blue colonies according to the manufacturers' directions. Enterococci were enumerated as black colonies on Bile Esculine Azide agar (4 h, 44°C) resulting from a transfer of the red colonies obtained on a first plating onto Slanetz-Barkley agar (48 h, 37°C). Spores of *Clostridium perfringens* were enumerated according to the Most Probable Number (MPN) method previously described by Pourcher *et al.* (2006). *Salmonella* was enumerated according to the MPN method described by AFNOR (Anonymous, 2004). The presence of *Listeria monocytogenes* was detected in ten grams of manure using an Oxoid Novel Enrichment Broth-Listeria (ONE Broth) (24h, 30°C) followed by plating onto a chromogenic Agar Listeria according to Ottaviani and Agosti (ALOA). Typical colonies on ALOA agar were subjected to PCR for lysteriolysin O genes (*hlyA*) detection using the primers previously described by Bohnert *et al.* (1992).

Molecular analysis

Manure samples were centrifuged for 10 min at 17,500 g. About 0.25 g of each pellet was transferred into a microtube and immediately stored at – 20°C. DNA extractions were performed on one pellet using the QIAamp DNA mini stool kit (QIAGEN). The manure microbial communities were analyzed by PCR amplification of the V3 region of microbial 16S rRNA genes using primers targeting the total bacteria or specific microbial groups as described by Peu *et al.* (2006) and Matsuki *et al*. (2004). Four microbial groups were targeted: *Bacillus-Streptococcus-Lactobacillus* (BSL), *Eubacterium-Clostridium* (EC), *Bacteroides-Prevotella* (BP) and *Bifidobacterium*. Amplification was performed with a MJ Mini thermocycler (Bio-rad). The resulting PCR products were then separated by SSCP capillary electrophoresis with an ABI 310 genetic analyzer (Applied Biosystems) as described by Delbes *et al.* (2000). The dominant populations observed after SSCP electrophoresis were identified by the cloning and sequencing of there corresponding PCR product according to the methodology previously described by Peu *et*

al. (2006). DNA sequences were identified by comparison with their closest relatives available in databases using BLAST from the National Centre for Biotechnology Information and the Ribosomal Database Project.

RESULTS

Faecal indicators dynamic in pig manure

Overall, for the 17 piggeries, indicator counts in raw manure varied from 6×10^3 to 1×10^8 for *E. coli*, from 6×10^4 to 7×10^6 for enterococci and from 2×10^3 to 1×10^6 for *C. perfringens* per g of wet weight. The variation observed (about 2 logarithmic units) for *E. coli* and enterococci are in the same order of magnitude than those reported by Hill and Sobsey (2003) and Vanotti *et al.* (2005) for liquid manures. *Salmonella* and *L. monocytogenes* were detected in 60 and 30% of the 17 raw manure samples respectively. The proportion of *Salmonella* is in agreement with those reported by Chinivasagam *et al.* (2004) and Watabe *et al.* (2003) who isolated *Salmonella* respectively in 31% and 71.4% of the analyzed manures. The difference of proportion and levels of *Salmonella* in manures are affected by numerous factors such as geographic location, size of the breeding, age of livestock and dietary changes. The presence of *L. monocytogenes* is probably due to its ubiquitous character as it was suggested by Garrec *et al.* (2003) who regularly detected this bacterium in sludge of waste water treatment plants. The 10 treatment systems studied showed a reduction of *E. coli*, enterococci and *Salmonella* (Table 1), with average reductions of 3.1 $\log_{10}$ for *E. coli*, 1.4 $\log_{10}$ for enterococci, 1.2 $\log_{10}$ for *Salmonella* but only 0.2 $\log_{10}$ for *C. perfringens*. The absence of reduction of *C. perfringens* spores confirms the large resistance of this bacterium to the treatment, as it was previously observed by Pourcher *et al.* (2005) during the composting of municipal sludge.

Table 1. Concentrations of bacteria (per gram of wet weight) and occurrence of *L. monocytogenes* in raw manures and the treatment by-products from 17 piggeries

manure treatment	type of manure		*E. coli*	*Enterococci*	*C. perfringens*	*Salmonella*	*L. monocytogenes*
anaerobic storage[a]	raw manure	mean	**$3.5\ 10^5$**	**$8.3\ 10^5$**	**$3.5\ 10^4$**	**41**	**0%[e]**
	(7)[c,]	(SD)[d]	($4.1\ 10^5$)	($1.4\ 10^6$)	($2.9\ 10^4$)	(99)	
anaerobic storage[b]	raw manure	mean	**$1.1\ 10^7$**	**$1.9\ 10^6$**	**$2.0\ 10^5$**	**3.8**	**50%**
	(10)	(SD)	($3.1\ 10^7$)	($2.2\ 10^6$)	($3.7\ 10^5$)	(7.4)	
aerobic digestion followed by anaerobic storage[b]	treated manure (10)	mean (SD)	**$9.7\ 10^3$** ($4.1\ 10^3$)	**$7.1\ 10^4$** ($5.9\ 10^4$)	**$1.4\ 10^5$** ($2.4\ 10^5$)	**0.2** (0.5)	**20%**

[a] piggeries without N removal treatment; [b] piggeries with N removal treatment; [c] number of samples; [d] standard deviation;
[e] frequency of detection of *Listeria monocytogenes* (%)

The succession of aerobic digestion and anaerobic sludge storage clearly affected the survival of vegetative bacterial forms. However, faecal indicators and pathogen bacteria were present in treated manure and persisted up to the time of spreading onto the fields.

Bacterial 16S rRNA gene dynamic in manure

The composition of the raw manures microbial communities was determined by 16S rRNA gene-targeted PCR amplification and subsequent SSCP electrophoresis of the PCR products (Figure 1). This technique allows the representation of a microbial community as a profile of peaks where each dominant peak is representative of a PCR product and by implication, a bacterial species. The SSCP profiles obtained for the 17 raw manures were relatively homogeneous regardless of the time of manure storage (ranging from 3 weeks to 6 months), suggesting a weak impact of the anaerobic storage on the microbial community. The profiles contained about 32 distinguishable peaks that emerged from a background of subdominant bacterial diversity (Figure 1a). As observed previously by Peu *et al.* (2006) during anaerobic manure storage, the profiles could be subdivided in 3 groups of peaks corresponding to the *Clostridiaceae*, *Bacteroidetes* and BSL microbial groups. By contrast, the SSCP Profiles obtained for the 10 treated manures differed strongly from those of raw manures by a greater apparent diversity of peaks (Figure 1b). These changes of profiles reveal an important evolution of the corresponding microbial communities and thus a strong impact of the aerobic treatment on the raw manure as previously observed by Leung and Topp (2001). The harsh conditions of aerobic treatment probably inhibited some of the strict anaerobic microbial groups of raw manure and supported the growth of other environmental species. These important changes in the dominant microbial groups correspond also to the decrease of the subdominant groups to which belong indicator bacteria.

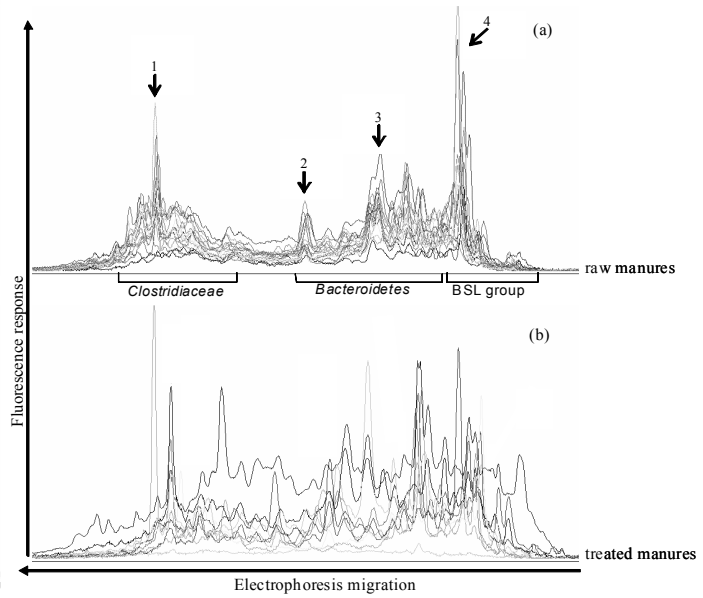

Figure 1. Comparison of bacterial 16S rRNA gene-targeted PCR – SSCP profiles for 17 raw manures sampled in primary storage tanks (a) and for 10 treated manures sampled in secondary storage tanks (b). SSCP electrophoresis was performed from right to left. The horizontal and vertical axes indicate time (number of scans) and the detection of fluorescently labelled PCR products, respectively. Arrows indicate dominant peaks present in a majority of raw manures.

The search for new bacterial indicators of pig manure

Interestingly, four of the dominant peaks of raw manures (noted as 1 to 4 on figure 1a) were present in the majority of the samples analysed. They were investigated to see if they could be used as microbiological markers of pig manure. They were all related to uncultured bacteria. Peak 1 was closely related to *Clostridium* (accession number DQ309375) with a 93% sequence similarity. Peaks 2, 3 and 4 were related to *Bacteroidales* with 92–93% sequence similarity (accession numbers AB240481, AB240481 and AB175368, respectively). As these 4 species were not specific to pig manure, the same strategy was carried out again but targeting more specific bacterial subgroups (EC, BP, BSL groups and *Bifidobacterium*). Two SSCP peaks were remarkably present in several raw manures. They were assigned to *Bifidobacterium thermacidophilum subsp. porcinum* (accession number AY148470, sequence similarity of 98%) and *Lactobacillus sobrius* (accession number AY700063, sequence similarity of 99%), two species previously identified in intestinal tract of both pig and piglet (Zhu and Dong, 2003; Konstantinov *et al.*, 2006). However, comparison with other livestock effluents (poultry and cattle) is in progress to confirm their specificity.

CONCLUSION

The value of this study lies in the large number of piggeries included which enables the determination of the general characteristics of the bacterial community of raw and treated manures. The results underline the existence of a potential risk of spreading *Salmonella* which were detected in 60% of the 17 raw manures and in 20% of the 10 treated manures analysed. The N removal treatment results in a decrease in *E coli* and enterococci concentrations, but is not however sufficient to completely eliminate the pathogenic bacteria and it has no effect on the spores of *C perfringens*. The molecular analyses highlighted (i) the strong impact of aerobic treatment on the raw manure microbial community and (ii) the presence of specific populations in the raw manures belonging to *Bifidobacterium* and *Lactobacillus* groups that could be proposed as new indicators of the effectiveness of treatment.

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