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## Environmental Microbiology

# Bacterial ecology of abattoir wastewater treated by an anaerobic digester



Linda Jabari<sup>a,b</sup>, Hana Gannoun<sup>a,c</sup>, Eltaief Khelifi<sup>a</sup>, Jean-Luc Cayol<sup>b</sup>,  
Jean-Jacques Godon<sup>d</sup>, Moktar Hamdi<sup>a</sup>, Marie-Laure Fardeau<sup>b,\*</sup>

<sup>a</sup> Université de Carthage, Laboratoire d'Ecologie et de Technologie Microbienne, Institut National des Sciences Appliquées et de Technologie (INSAT), 2 Boulevard de la terre, B.P. 676, 1080 Tunis, Tunisia

<sup>b</sup> Aix-Marseille Université, Université du Sud Toulon-Var, CNRS/INSU, IRD, MOI, UM 110, 13288 Marseille cedex 9, France

<sup>c</sup> Université Tunis El Manar, Institut Supérieur des Sciences Biologiques Appliquées de Tunis (ISSBAT) 9, avenue Zouhaïer Essafi, 1006 Tunis, Tunisia

<sup>d</sup> INRA U050, Laboratoire de Biotechnologie de l'Environnement, Avenue des Étangs, F-11100 Narbonne, France

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## ABSTRACT

Wastewater from an anaerobic treatment plant at a slaughterhouse was analysed to determine the bacterial biodiversity present. Molecular analysis of the anaerobic sludge obtained from the treatment plant showed significant diversity, as 27 different phyla were identified. Firmicutes, Proteobacteria, Bacteroidetes, Thermotogae, Euryarchaeota (methanogens), and msbl6 (candidate division) were the dominant phyla of the anaerobic treatment plant and represented 21.7%, 18.5%, 11.5%, 9.4%, 8.9%, and 8.8% of the total bacteria identified, respectively. The dominant bacteria isolated were *Clostridium*, *Bacteroides*, *Desulfobulbus*, *Desulfomicrobium*, *Desulfovibrio* and *Desulfotomaculum*. Our results revealed the presence of new species, genera and families of microorganisms. The most interesting strains were characterised. Three new bacteria involved in anaerobic digestion of abattoir wastewater were published.

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## Introduction

For hygienic reasons, abattoirs use copious amounts of water in their processing operations (slaughtering and cleaning), which creates significant wastewater. In addition, the increased use of automated machines to process carcasses, along with the incorporation of washing at every stage, has increased water consumption in slaughterhouse facilities. The

high fat and protein content of slaughterhouse waste makes wastewater a good substrate for anaerobic digestion, due to its expected high methane yield.<sup>1</sup> Numerous microorganisms are involved in the anaerobic degradation of slaughterhouse waste, any step of which may be rate-limiting depending on the waste being treated as well as the process involved.

The microorganisms involved in anaerobic digestion have not been fully identified; however, at least four groups of microorganisms are involved in this process.<sup>2</sup> The first group

\* Corresponding author.

E-mail: [marie-laure.fardeau@univ-amu.fr](mailto:marie-laure.fardeau@univ-amu.fr) (M.-L. Fardeau).

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are the hydrolytic bacteria that degrade complex compounds (protein, carbohydrates, and fat) into simpler compounds, such as organic acids, alcohols, carbon dioxide (CO<sub>2</sub>) and hydrogen. The second group are the hydrogen producing acetogenic bacteria that use organic acids and alcohols to produce acetate and hydrogen. The third group contains homoacetogenic bacteria that can only form acetate from hydrogen, CO<sub>2</sub>, organic acids, alcohols, and carbohydrates. The fourth group comprises methanogens that form methane from acetate, CO<sub>2</sub>, and hydrogen. Hydrolytic, acetogenic, and methanogenic microorganisms play an equally important role in anaerobic digestion and methane production. Optimal methane production is only achieved via the interaction of multiple microorganisms,<sup>3</sup> and therefore, biodegradation of molecules in wastewater depends on the activity of all microbial groups involved.

Common fermentative bacteria include *Lactobacillus*, *Eubacterium*, *Clostridium*, *Escherichia coli*, *Fusobacterium*, *Bacteroides*, *Leuconostoc*, and *Klebsiella*. Acetogenic bacteria include *Acetobacterium*, *Clostridium*, and *Desulfovibrio*.<sup>2</sup> Methane producing organisms are classified under domain Archaea and phylum Euryarchaeota.<sup>4</sup>

In order to better understand the function of a bacterial population, a detailed description of the microbial ecosystem is necessary. One method is via molecular biology techniques.<sup>5</sup> Recent advances in the molecular analysis of bacterial ecosystems allow a better understanding of the specific microorganisms involved in wastewater treatment. There are only a few studies focused on microbial populations, diversity and evolution in reactors fed with complex organic wastes.<sup>1</sup> Therefore, little is known about the composition of these reactors. The development of advanced molecular biology techniques has contributed to the detection, quantification, and identification of the bacterial populations involved in the treatment of abattoir wastewater. For example, cloning and sequencing of 16S rRNA gene fragments provide information about the phylogeny of the microorganisms. Additionally, single stranded conformation polymorphism (SSCP) offers a simple, inexpensive and sensitive method for detecting whether or not DNA fragments are identical in sequence, and can greatly reduce the amount of sequencing necessary.<sup>6</sup>

This work aimed to study the bacterial ecology of an anaerobic digester through both bacterial culture and molecular biological techniques. The bacteria involved in the anaerobic digestion of abattoir wastewater were identified using classic microbiology techniques and molecular tools (sequencing of 16S rRNA and SSCP). Additionally, our results were compared with those of Gannoun et al.<sup>6</sup> to evaluate the effect of storage at 4 °C on the bacterial diversity of the sludge.

## Material and methods

### Origin of the sludge

Anaerobic sludge samples were collected from an upflow anaerobic filter that treats abattoir wastewater in Tunisia.<sup>7</sup> The digester operated under both mesophilic (37 °C) and thermophilic (55 °C) conditions. Samples were taken at the end of thermophilic phase and stored at 4 °C. The sludge was then analysed to determine the bacterial diversity present, first via bacterial culture.

### DNA extraction, PCR and SSCP analysis of the digester sludge

Four milliliters of the anaerobic sludge sample were centrifuged at 6000 rpm for 10 min. Pellets were re-suspended in 4 mL of 4 M guanidine thiocyanate–0.1 M Tris pH 7.5 and 600 μL of N-lauroyl sarcosine 10%. Two hundred and fifty microlitres of treated samples were transferred in 2 mL tubes and stored at –20 °C.

Extraction and purification of total genomic DNA was implemented according to the protocol developed by Godon et al.<sup>8</sup>

Highly variable V3 regions of bacterial 16S rRNA genes were amplified by PCR using bacterial (w49–w34) primers (Table 1). Samples were treated according to the protocol previously described by Delbès et al.<sup>9</sup>

For electrophoresis, PCR–SSCP products were diluted in water before mixing with 18.75 μL formamide (Genescan–Applied Biosystems) and 0.25 μL internal standards (ROX, Genescan–Applied Biosystems) according to the protocol of SSCP described by Delbès et al.<sup>9</sup>

SSCP analyses were performed on an automatic sequencer abi310 (Applied Biosystems). RNA fragment detection was done on the fluorescent w34 primer. The results obtained were analysed by GeneScan Analysis 2.0.2 Software (Applied Biosystems) as specified by Gannoun et al.<sup>6</sup> For bacterial identification, pyrosequencing of the DNA samples using a 454 protocol was performed (Research and Testing Laboratory, Lubbock, USA).

Methods of analysis for pyrosequencing data used herein have been described previously.<sup>10–14</sup> Sequences are first depleted of barcodes and primers. Then, short sequences under 200 bp are removed, sequences with ambiguous base calls are removed, and sequences with homopolymer runs exceeding 6 bp are removed. Sequences are then denoised and chimeras removed. Operational taxonomic units were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity).<sup>15–21</sup> Operational taxonomic units

**Table 1 – Sequences and target positions of primers used in this study.**

Primer	Sequence	Target	Reference
w34 <sup>a</sup>	TET-TTACCGGCTGCTGGCAC	16S rRNA universal	Gannoun et al. <sup>6</sup>
w49	ACGGTCCAGACTCCTACGGG	16S rRNA bacteria	Gannoun et al. <sup>6</sup>

<sup>a</sup> The primer w34 is marked at 5' end with fluorescent phosphoramidite-TET (Applied Biosystems).

were then taxonomically classified using BLASTn against a curated GreenGenes database<sup>22</sup> and compiled into each taxonomic level into both “counts” and “percentage” files. Counts files contain the actual number of sequences, while the percentage files contain the percentage of sequences within each sample that map to the designated taxonomic classification.

### Enrichment and isolation procedures of fermentative and SRB bacteria

The Hungate technique<sup>23</sup> was used throughout this study. Inoculations were done with 10% of culture. Samples were collected from the anaerobic filter at the end of thermophilic phase. A 0.5 mL aliquot of sample was inoculated into Hungate tubes containing 5 mL of basal medium.

For both SRB and fermentative bacteria, enrichment and isolation was done according to the protocol described previously by Hungate<sup>23</sup> and Khelifi et al.<sup>5</sup>

### Purification of the DNA, PCR amplification and sequencing of the 16S rRNA gene of isolated bacteria

Purification of the DNA, PCR amplification and sequencing of the 16S rRNA gene were performed as described previously.<sup>24</sup> The partial sequences generated were assembled using BioEdit version 5.0.9<sup>25</sup> and the consensus sequence of 1495 nucleotides was corrected manually for errors. The most closely related sequences in GenBank (version 178), the Ribosomal Database Project (release 10) identified using BLAST,<sup>26</sup> and the Sequence Match program,<sup>27</sup> were extracted and aligned. The consensus sequence was then adjusted manually to conform to the 16S rRNA secondary structure model.<sup>28</sup> Nucleotide ambiguities were omitted and evolutionary distances were calculated using the Jukes and Cantor option.<sup>29</sup> Dendrograms were constructed with the TREECON program using the neighbor-joining method.<sup>30</sup> Tree topology was re-examined by the bootstrap method (1000 replications) of re-sampling.<sup>31</sup> Its topology was also supported using the maximum-parsimony and maximum-likelihood algorithms.

### Data analyses

The two main factors taken into account when measuring diversity are richness and evenness. Richness is a measure of the number of different kinds of organisms present in a particular sample. Evenness (*E<sub>s</sub>*) compares the similarity of population sizes between each of the species present. The reciprocal of Simpson's index (diversity richness) (*1/D*), which is widely used for ecological studies, was also used as a measure of diversity. Richness and evenness were calculated and interpreted as described previously by Simpson<sup>32</sup> and Gannon et al.<sup>6</sup>

**Table 2 – The different phyla found in the digester.**

Phyla	Percentage of total (%)
Firmicutes <sup>a</sup>	21.7
Proteobacteria <sup>a</sup>	18.5
Bacteroidetes <sup>a</sup>	11.5
Thermotogae <sup>a</sup>	9.4
Euryarchaeota <sup>a</sup>	8.9
msbl6 (candidate division) <sup>a</sup>	8.8
Op8 (candidate division)	3.7
Chloroflexi	3.2
Ws3 (candidate division)	2.1
Op3 (candidate division)	1.9
Planctomycetes	1.9
Synergistetes	1.7
Crenarchaeota	1.4
spam (Candidate division)	1.0
op1 (Candidate division)	1.0
op9 (Candidate division)	0.8
gn02 (Candidate division)	0.4
ksb1 (Candidate division)	0.3
nkb19 (Candidate division)	0.3
tm6 (Candidate division)	0.3
Cyanobacteria	0.3
Spirochaetes	0.2
Actinobacteria	0.1
Dictyoglomi	0.1
Acidobacteria	0.1
Verrucomicrobia	0.1
Nitrospirae	0.1

<sup>a</sup> The most common phyla.

## Results and discussion

### Diversity and abundance of the bacterial communities in the bioreactor sludge using SSCP and DNA sequencing

There was significant microbial diversity of the upflow anaerobic filter, which operated under both mesophilic (37 °C) and thermophilic (55 °C) conditions. Twenty-seven different phyla were identified, and the six most common phyla (Firmicutes, Proteobacteria, Bacteroidetes, Thermotogae, Euryarchaeota (the methanogenic bacteria), and the msbl6 (candidate division)) represented 78.8% of the total (Table 2). The 21 less common phyla represented 21% of the total.

Gram-positive bacteria, including Firmicutes (low G+C), were the most common type of bacteria in the anaerobic digester, comprising 21.7% of the total. Both Gram-positive low G+C bacteria and the Bacteroidetes phylum are known for their fermentative properties. Furthermore Bacteroidetes play an important role in the degradation of complex polymers. Firmicutes and Bacteroidetes are also the two main groups encountered in the study by Godon et al.<sup>8</sup> of a fluidised bed anaerobic digester fed with vinasse. These two groups of bacteria hydrolyse the polymer substrates which are not degraded during the previous stages of anaerobic digestion (such as polysaccharides, proteins and lipids) into acetate, long chain fatty acids, CO<sub>2</sub>, formate and hydrogen.

Bacteria within the Proteobacteria phylum were also commonly found in the digester. These Gram-negative bacteria are considered to be some of the most cultivable microorganisms.<sup>33,34</sup> The Proteobacteria have an important

**Table 3 – The main genus and species of the digester.**

Strain	Percentage (%)	Strain	Percentage (%)
<i>Kosmotoga</i> spp. <sup>a</sup>	9.37	<i>Clostridium acetireducens</i>	0.97
<i>msbl6</i> (candidate division) <sup>a</sup>	8.75	<i>Segetibacter</i> spp.	0.97
<i>Desulfotomaculum thermocisternum</i> <sup>a</sup>	6.25	<i>Clostridium limosum</i>	0.90
<i>Rriemerella anatipestifer</i> <sup>a</sup>	5.48	<i>Sulfurovum lithotrophicum</i>	0.90
<i>Pelotomaculum thermopropionicum</i> <sup>a</sup>	4.44	<i>op9</i> (candidate division)	0.83
<i>op8</i> (candidate division) <sup>a</sup>	3.68	<i>Achromatium oxaliferum</i>	0.83
<i>Tthiohalomonas denitrificans</i> <sup>a</sup>	3.12	<i>Ureibacillus suwonensis</i>	0.76
<i>Thermobaculum terrenum</i> <sup>a</sup>	3.12	<i>Desulfobacterium catecholicum</i>	0.76
<i>Desulfobulbus elongatus</i> <sup>a</sup>	2.63	<i>Synergistes</i> spp.	0.76
<i>Methanobacterium</i> spp. <sup>a</sup>	2.43	<i>Desulfomicrobium</i> spp.	0.76
<i>Dysgonomonas mossii</i> <sup>a</sup>	2.22	<i>Desulfobulbus</i> spp.	0.69
<i>Caldanaerobacter uzonensis</i> <sup>a</sup>	2.15	<i>Methanobacterium beijingense</i>	0.62
<i>ws3</i> (candidate division) <sup>a</sup>	2.08	<i>Pseudomonas resinovorans</i>	0.62
<i>op3</i> (candidate division) <sup>a</sup>	1.94	<i>Rhodovibrio salinarum</i>	0.55
<i>Pirellula</i> spp. <sup>a</sup>	1.66	<i>Legionella birninghamiensis</i>	0.55
<i>Methanobacterium aarhusense</i> <sup>a</sup>	1.45	<i>Methanosaepta thermophila</i>	0.55
<i>Nitrosococcus oceani</i> <sup>a</sup>	1.45	<i>Acetobacterium wieringae</i>	0.55
<i>Coprococcus clostridium</i> sp. ss2/1 <sup>a</sup>	1.38	<i>Thermanaerovibrio acidaminovorans</i>	0.55
<i>Methanosphaerula palustris</i> <sup>a</sup>	1.11	<i>Thermosinus carboxydivorans</i>	0.55
<i>Parabacteroides goldsteinii</i> <sup>a</sup>	1.04	<i>Ruminococcus flavefaciens</i>	0.48
<i>Candidatus nitrososphaera gargensis</i> <sup>a</sup>	1.04	<i>Methanosaepta</i> spp.	0.48
<i>spam</i> (candidate division) <sup>a</sup>	1.04	<i>Rhodovulum euryhalinum</i>	0.48
<i>op1</i> (candidate division) <sup>a</sup>	1.04	<i>Thermomonas fusca</i>	0.48
<i>Methanobrevibacter curvatus</i>	0.97	<i>Mechercharimyces mesophilus</i>	0.41

The 48 most abundant species in the sample.

<sup>a</sup> The first 23 species are the prominent ones based on the PCR-SSCP and microbiological methods.

role in the hydrolysis and acetogenesis steps of anaerobic digestion, and include delta, gamma and beta varieties. *Deltaproteobacteria* contains many syntrophic anaerobic bacteria, which participate in sulphate reduction. Among the *Gammaproteobacteria*, there are many denitrifying bacteria or bacteria that accumulate phosphates.<sup>35</sup> The *Betaproteobacteria* are involved in nitrification, and are potentially also involved in denitrification.

Phylogenetic analysis of the domain Bacteria also helped to highlight the existence of a poorly known order, *Thermotogales*, which was relatively abundant within the digester at 9.4%. *Thermotogales* contains anaerobic bacteria that are heterotrophic with a fermentative metabolism.<sup>36</sup> These bacteria are also found in other anaerobic digestors.<sup>8</sup>

The *Planctomycetales* group represented 1.9% of the bacteria within the digester. Bacteria within *Planctomycetales* are limited to five kinds and only eight species are described. Aerobic heterotrophic *Planctomycetes* have been successfully isolated from brackish marine sediments, freshwater sediments, soil, hot springs, salt pits and tissues from giant tiger prawn postlarvae.<sup>37,38</sup> In addition, a special group of *Planctomycetes* were implicated in the oxidation of ammonia under anaerobic conditions in wastewater plants, coastal marine sediments, and oceanic and freshwater oxygen minimum zones.<sup>39</sup> Furthermore, a wide variety of *Planctomycetales* were found during analysis of samples from aquatic anaerobic environments, a sulphide- and sulphur-rich spring, activated sludge wastewater treatment plants and in anaerobic digestors.<sup>8,38,39</sup>

The *Chloroflexi* represented 3.2% of the digester's bacteria. *Chloroflexi* have been identified from many environments through 16S rRNA gene profiling, including marine and freshwater sediments. Despite this, the *Chloroflexi* remain a

relatively understudied bacterial lineage. At present, there are 19 complete genomes available for the *Chloroflexi*.<sup>40</sup>

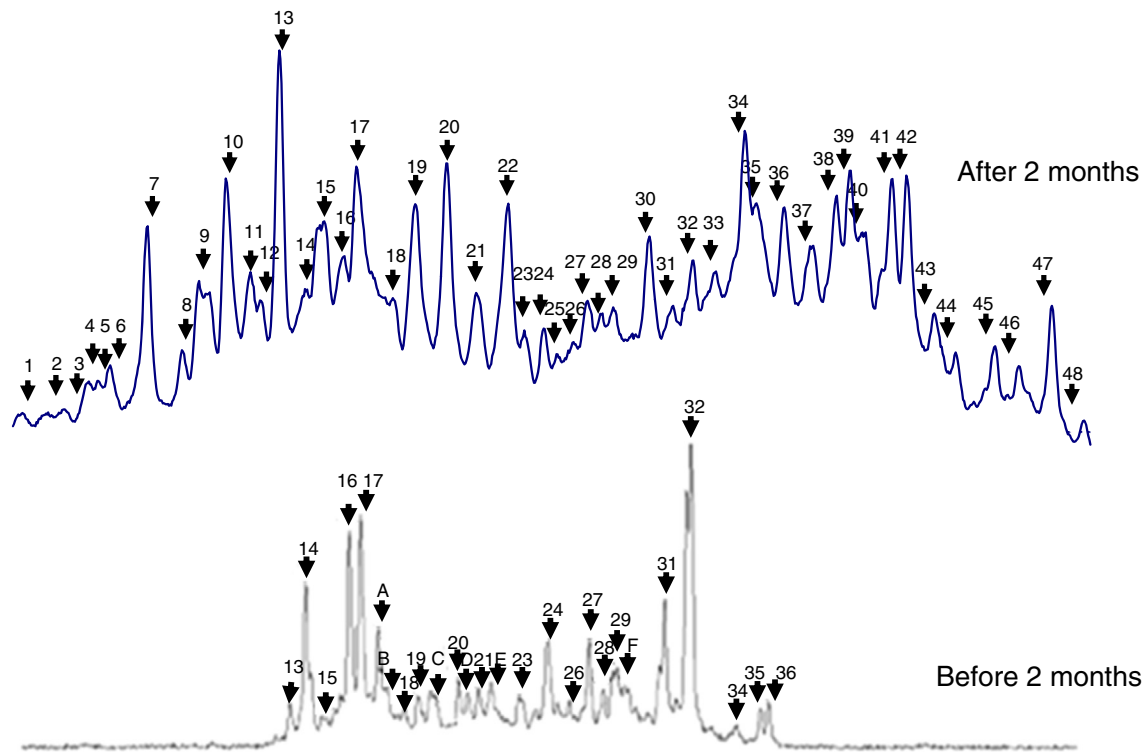
Tang et al.<sup>41</sup> showed that three phyla were involved in the mesophilic anaerobic treatment of synthetic rejection containing bovine serum albumin: *Bacteroidetes*, followed by *Firmicutes* and *Proteobacteria*. In another study by Fang et al.<sup>42</sup> that evaluated the anaerobic degradation of phenol rich rejection in an upflow anaerobic sludge blanket (UASB) reactor, eight phylogenetic groups were detected, namely *Thermotogales* (38.9% of clones), *Firmicutes* (27.8%), *Chloroflexi* (11.1%), candidate division OP8 (9.3%), candidate division OP 5 (5.5%), *Proteobacteria* (3.7%), *Bacteroidetes* (1.9%) and *Nitrospirae* (1.9%). These results are comparable with the results of our study.

The *Euryarchaeota*, which consist mainly of methanogenic bacteria, represented 8.9% of bacteria within the anaerobic digester. The *Crenarchaeota*, extreme thermoacidophiles, were also detected in the digester with an abundance of 1.4%. The digester seems to have limited archaeal diversity. These results are similar to the results of another study on the bacterial diversity of an anaerobic digester fed with vinasse, which contained only 4% of Archaea Bacteria sequences.<sup>8</sup>

Overall, there was significant bacterial diversity within the digester. There were 23 species each consisting of greater than 1%, and the most abundant species was *Kosmotoga* spp. with a percentage of 9.37% (Table 3).

#### SSCP analysis of the effect of storage on the diversity and abundance of bacterial communities within the bioreactor sludge

SSCP analyses (Fig. 1) show the results of two samples of sludge collected from the same digester at the end of the



**Fig. 1 – Effect of storage on the dynamics of single strand conformation polymorphism patterns of bacterial 16S rRNA gene amplification products of the anaerobic digester.**

thermophilic phase.<sup>6</sup> The second sample was stored at 4 °C for two months and showed different SSCP patterns. The analysis of the two SSCP patterns showed significant change in the bacterial community over time, which can be explained by the fact the sludge is not stable over time.

The dynamics of bacterial communities were monitored by PCR-SSCP methods. The profile obtained for the domain Bacteria is shown in Fig. 1. The SSCP pattern revealed the high diversity of bacteria, with at least 48 distinguishable peaks and about 23 prominent peaks. The bacterial diversity richness (1/D) and species evenness (Es) were used as a measure of diversity and abundance. The obtained values were 38.97 (which offers toward the number of species  $S=48$ ) (indicated maximum diversity) and 0.811 (which offers toward to 1: species in the sample are quite evenly distributed), respectively. This result is in agreement with other molecular studies based on the PCR-SSCP methods.

Several research groups confirmed our results and demonstrated that diversity in bacterial digestors varies depending on several factors. However, Zumstein et al.,<sup>43</sup> who studied the community dynamics in an anaerobic bioreactor using fluorescence PCR single-strand conformation polymorphism analysis, indicated that throughout the period of the study, rapid significant shifts in the species composition of the bacterial community occurred. In fact, the bacterial community was followed for two years through the analysis of 13 SSCP patterns, with one SSCP pattern every two months. The analysis of the SSCP patterns showed a continuous change in the bacterial community during that time. Typically, a microorganism initially present at low levels in the community grew, peaked

and decreased. Moreover, some microorganisms seemed to fluctuate simultaneously.

Keskes et al.<sup>44</sup> studied the effect of the prolonged sludge retention time on bacterial communities involved in the aerobic treatment of abattoir wastewater by a submerged membrane bioreactor. Their results showed that the biodiversity varied significantly in relation with the environmental conditions, particularly TSS.

The sludge microorganisms and associations of microorganisms may occupy the same ecological niche successively. They could correspond to the ecological unit called an ecotype.<sup>45</sup>

#### Isolation and identification of bacteria

Fermentative bacteria and SRB involved in the anaerobic digestion of organic matter in abattoir effluents were investigated by two approaches: classical microbiology and molecular taxonomy.

The isolation of bacteria is preceded by an enrichment phase which favors the growth of a given microorganism (fermentative bacteria or SRB), selected according to the physical and nutritional conditions of the medium category. For this purpose, two different culture media were used, as described in the Materials and Methods section. The first culture medium was specially designed for the detection of fermentative bacteria and the second for SRB. Both culture media were tested at 37 °C and 55 °C. Glucose was used as an energy source to isolate fermentative bacteria, whereas lactate, acetate and  $H_2CO_2$  were reserved for the isolation of SRB.

**Table 4 – Phylogenetic affiliation of the 16S rRNA bacteria sequences of isolated strains.**

Name	Closest neighbor	Accession number	Origin	References	Similarity (%)
<i>Mesophilic fermentative bacteria (37 °C)</i>					
LIND8L2	<i>Clostridium novyi</i>	AB041865	Soil and feces	Sasaki et al. <sup>49</sup>	96%
LIND7H <sup>a</sup>	<i>Parabacteroides merdae</i>	AB238928	Human feces	Johnson et al. <sup>51</sup>	91%
LIND8A	<i>Clostridium sp 13A1</i>	AY554421	Anaerobic bioreactor treating cellulose waste	Burrell et al. <sup>50</sup>	99%
LINBA	<i>Clostridium sp D3RC-2</i>	DQ852338	Rumen yak china	Zhang et al. <sup>48</sup>	99%
LINBL1	<i>Clostridium sp D3RC-2</i>	DQ852338	Rumen yak china	Zhang et al. <sup>48</sup>	99%
LINBA2	<i>Clostridium sp D3RC-2</i>	DQ852338	Rumen yak china	Zhang et al. <sup>48</sup>	99%
LIND8A	<i>Clostridium sp D3RC-2</i>	DQ852338	Rumen yak china	Zhang et al. <sup>48</sup>	96%
<i>Thermophilic fermentative bacteria (55 °C)</i>					
LIND6LT2 <sup>a</sup>	<i>Parasporobacterium paucivorans</i>	AJ272036	Anaerobic digester treating solid waste	Lomans et al. <sup>57</sup>	87%
LIND8AT	<i>Uncultured bacterium</i>	DQ125705	Soils contaminated with uranium waste	Brodie et al. <sup>59</sup>	97%
LINBAT1	<i>Uncultured bacterium</i>	AF280825	Anaerobic digester treating pharmaceutical wastes	Lapara et al. <sup>60</sup>	99%
LINBLT2	<i>Uncultured bacterium</i>	AF280825	Anaerobic digester treating pharmaceutical wastes	Lapara et al. <sup>60</sup>	98%
LIND4FT1	<i>Caloramator coolhaasii</i>	AF104215	Anaerobic thermophilic granular sludge	Plugge et al. <sup>61</sup>	96%
LIND8HT	<i>Lutispora thermophila</i>	AB186360	Thermophilic bioreactor digesting municipal solid wastes	Shiratori et al. <sup>62</sup>	99%
LINBLT	<i>Clostridium thermosuccinogenes</i>	Y18180	Cattle manure, beet pulp, soil, sediment pond	Stackebrandt et al. <sup>72</sup>	98%
LINBHT2	<i>Clostridium tertium</i>	Y18174	Open war wounds	Stackebrandt et al. <sup>72</sup>	98%
<i>Mesophilic sulphate-reducing bacteria (37 °C)</i>					
LINBL	<i>Desulfohalobus propionicus</i>	AY548789	Fluidized-bed reactors treating acidic, metal-containing wastewater	Kaksonen <sup>67</sup>	99%
LINBH	<i>Desulfovibrio vulgaris</i>	AE017285	Soil, animal intestines and feces, fresh and salt water	Heidelberg et al. <sup>68</sup>	99%
LINBH2	<i>Desulfovibrio vulgaris</i>	AE017285	Soil, animal intestines and feces, fresh and salt water	Heidelberg et al. <sup>68</sup>	99%
LINBA1	<i>Desulfomicrobium baculatum</i>	AJ277895	Water-saturated manganese carbonate ore	Hippe <sup>73</sup>	99%
LINBH1	<i>Desulfomicrobium baculatum</i>	AJ277895	Water-saturated manganese carbonate ore	Hippe <sup>73</sup>	99%
<i>Thermophilic sulphate-reducing bacteria (55 °C)</i>					
LINBHT1 <sup>a</sup>	<i>Desulfotomaculum halophilum</i>	U88891	Oil production facilities	Tardy-Jacquenod et al. <sup>69</sup>	89%

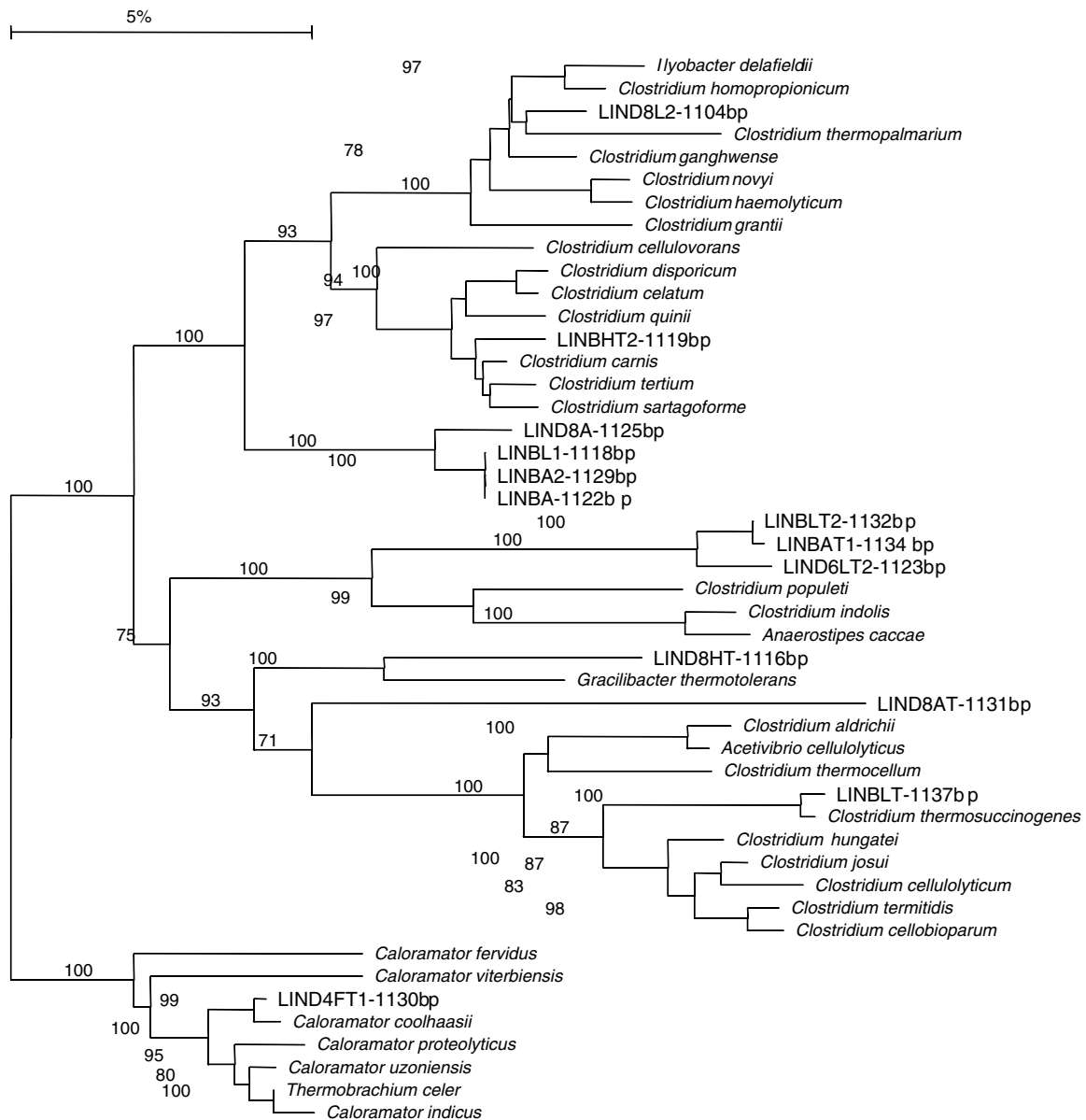
<sup>a</sup> These strains were characterized and published LIND7H,<sup>55</sup> LIND6LT2<sup>58</sup> and LINDBHT1.<sup>71</sup>

Isolation and culture of bacteria allowed us to study the biodiversity of these bioreactors as well as highlighted the dominant bacteria in the different conditions tested. The isolation of the different strains was carried out on an agar medium. This step helped to isolate strains that are identified based on their morphological differences (size, shape, and color).

Following this step, molecular analysis of the isolated strains was performed. After extraction of the bacterial DNA, the 16S rDNA was amplified by PCR. The quality of the amplified DNA was visualised with ultra-violet light after migration on gel agarose at 1%. The PCR product was subsequently sequenced to identify the different strains. The genes coding for 16S rRNA have been chosen as taxonomic markers, as they have a universal distribution and conserved function. These

genes possess both highly conserved areas, providing information on the evolution of distant species, and variable areas, to differentiate organisms belonging to the same genus, and eventually the same species.<sup>46</sup>

Each microorganism was then identified by comparing the 16S rDNA sequences with those of known microorganisms, and each was cataloged in computer databases. Given the large number of isolated bacteria in recent underwent restriction analysis by ARDRA technique to eliminate identical strains restriction profiles to keep only the amplifias of different strains priori which will be the object of phylogenetic studies. Phylogenetic affiliations of the 16S rRNA of bacteria sequences are presented in Table 4 with the closest relatives of isolated mesophilic and thermophilic fermentative bacteria and SRB.



**Fig. 2 – Phylogenetic tree based on the gene encoding the 16S RNA showing the positioning of fermentative mesophilic and thermophilic bacteria isolated from the anaerobic digester.**

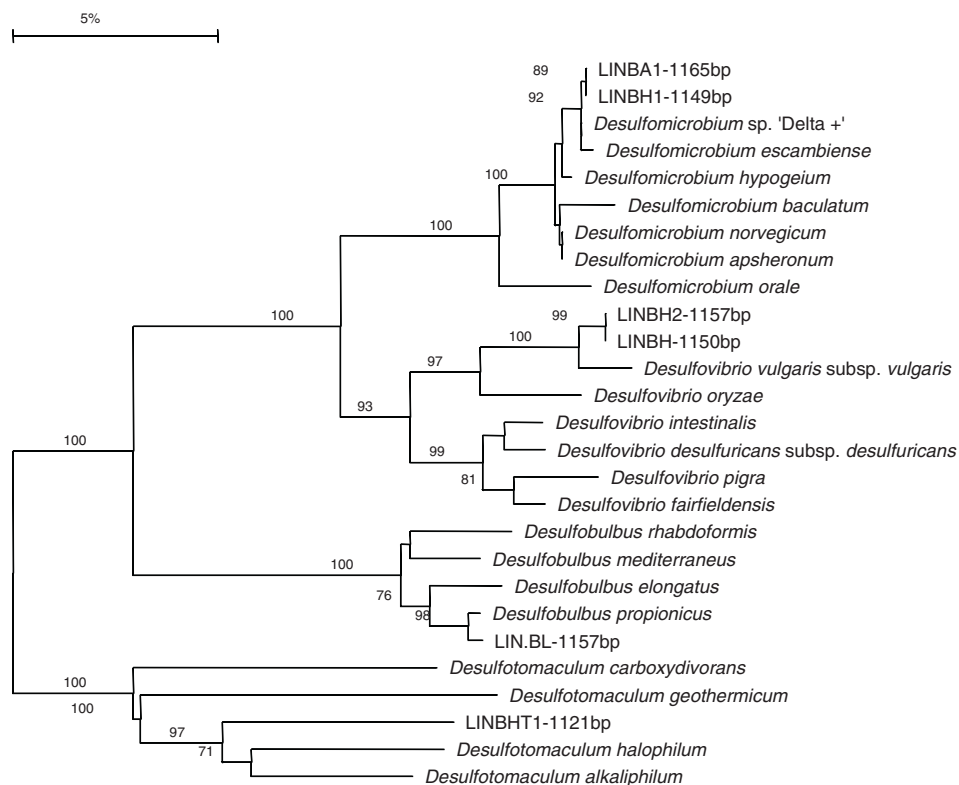
#### Fermentative bacterial communities in the bioreactor sludge

Strains LINBA, LINBL1, LINBA2 are closest phylogenetically to *Clostridium* sp. D3RC-2 with a percentage sequence similarity of 99%. This bacterium was firstly detected in the rumen of a yak in China<sup>47,48</sup> but it is not yet described. The strain LIND8A shares 96% of sequences with *Clostridium* sp. D3RC-2. This strain seems to be a new species and differs from the latter. The strain LIND8L2 has also been recently affiliated with *Clostridia* species. LIND8L2 is a strain similar to *Clostridium novyi* with 96% sequence similarity. *C. novyi* is a pathogenic bacterium phylogenetically close to *C. botulinum* and *C. haemolyticum*.<sup>49</sup> The nearest strain to LIND8A is *Clostridium* sp 13A1, previously isolated from an anaerobic bioreactor treating cellulose wastes,<sup>50</sup> with 99% sequence similarity.

The closest phylogenetic relatives of the strain LIND7H<sup>T</sup> are *Parabacteroides merdae*,<sup>51</sup> *P. goldsteinii*,<sup>52,53</sup> and *P. gordonii*,<sup>54</sup> with 91.4%, 91.3% and 91.2% sequence similarity, respectively. This novel strain was identified and characterised by Jabari et al.<sup>55</sup> On the basis of phylogenetic inference and phenotypic properties, LIND7H<sup>T</sup> is proposed as the type strain of a novel genus and species within the family *Porphyromonadaceae*, *Macellibacteroides fermentans* gen. nov., sp. nov.

Strains isolated in mesophilic conditions were determined to belong to Firmicutes and Bacteroidetes, which are known for their fermentative activity and which are the two groups mainly encountered in the study by Godon et al.<sup>8</sup> on an anaerobic digester. They hydrolyse the polymer substrates not degraded during the stages of remediation (such as polysaccharides, proteins and lipids) to acetate, long chain fatty acids, CO<sub>2</sub>, formate and hydrogen.





**Fig. 3 – Phylogenetic tree based on the gene encoding the 16S RNA showing the positioning of SRB isolated from the anaerobic digester.**

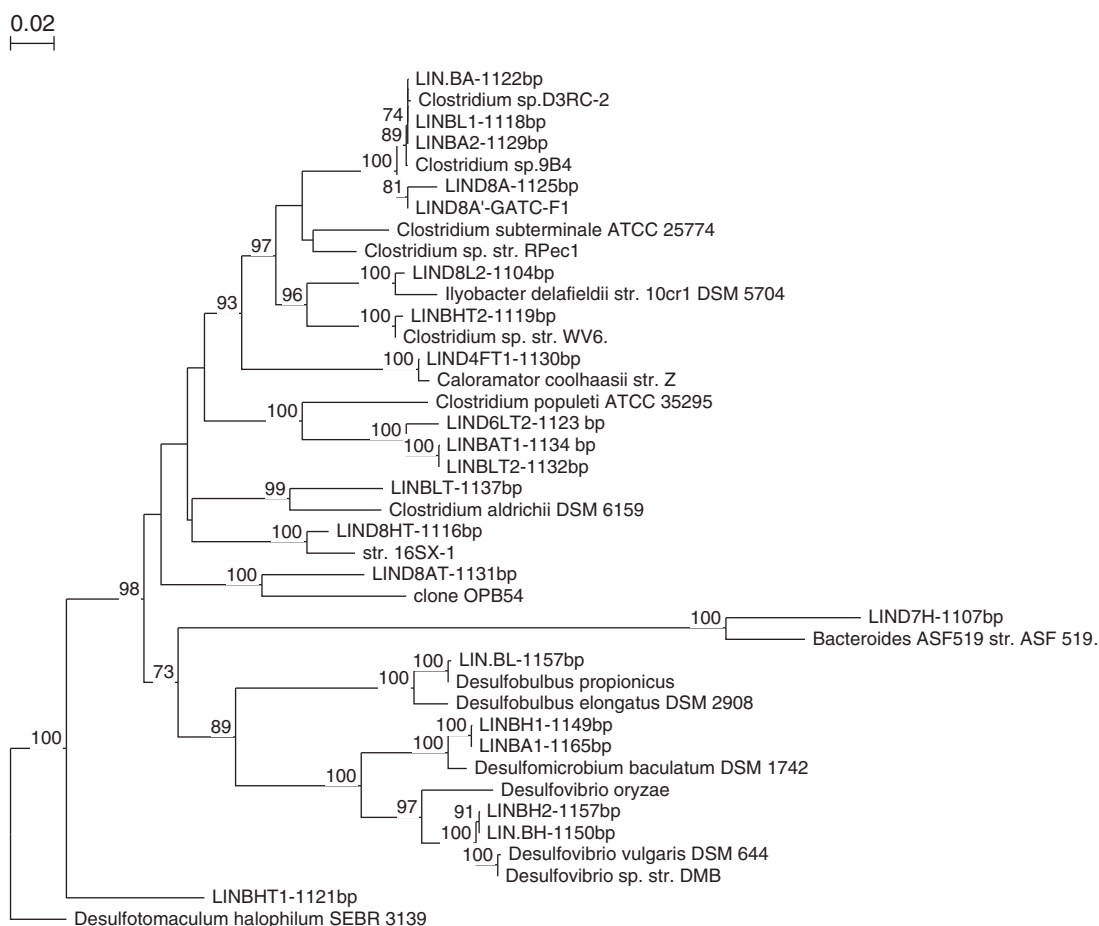
The isolation of fermentative, thermophilic bacteria obtained some strains classified as “uncultured bacterium” indicating they cannot be grown on synthetic material in a laboratory. These strains are LIND6LT2, LIND8AT, LINBAT1 and LINBLT2. The strain LIND6LT2 was detected in an anaerobic digester treating solid waste in thermophilic conditions.<sup>56</sup> The closest phylogenetic relative to this strain is *Parasporobacterium paucivorans* with 87.17% sequence similarity.<sup>57</sup> This novel strain was initially identified and characterised by Jabari et al.<sup>58</sup> On the basis of phylogenetical and physiological properties, the strain LIND6LT2<sup>T</sup> is proposed as the strain type of *Deftuviitalea saccharophila* gen. nov., sp. nov., placed in *Deftuviitaleaceae* fam. nov., within the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*. The strain LIND8AT has 97% similarity with a strain classified as uncultivable bacterium, probably involved in metal reduction.<sup>59</sup>

According to the phylogenetic tree (Fig. 2), the strains LINBAT1 and LINBLT2 are phylogenetically close. These sequences were also identified within an anaerobic digester treating pharmaceutical wastes.<sup>60</sup> The closest relative to strain LIND4FT1 is *Caloramator coolhaasii*, with 96% sequence similarity which suggests that this strain is a new species. *C. coolhaasii* has been isolated from an anaerobic granular sludge bioreactor that degrades glutamate. It is moderately thermophilic and strictly anaerobic.<sup>61</sup> LIND8HT strain is close to *Lutispora thermophile*,<sup>62</sup> with 99% sequence similarity which is firstly isolated from an enrichment culture derived from an anaerobic thermophilic bioreactor treating artificial solid wastes.

Strains LINBLT and LINBHT2 were affiliated with *C. tertium* and *C. thermosuccinogenes*, respectively, each with 98% sequence similarity.

The mesophilic and thermophilic fermentative strains which were isolated were also designated to the group *Firmicutes*, which seems to be the most abundant group in our anaerobic digester. Almost all isolates were represented by *Clostridium* species. This is not surprising, because during the hydrolysis phase in bioreactors, macromolecules such as polysaccharides, lipids, proteins and nucleic acids are cleaved, typically by specific extracellular enzymes, producing monomers (monosaccharides, fatty acids, amino acids and nitrogen bases) which are transported into the cell where they are fermented. The bacteria involved in this stage have a strictly anaerobic or facultative metabolism. During the acidogenesis phase, these monomers are metabolised by fermentative microorganisms to primarily produce volatile fatty acids (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate), but also alcohols, sulphide (H<sub>2</sub>S), CO<sub>2</sub> and hydrogen. Acidogenesis leads to simplified products of fermentation, and the bacteria involved in this step may be facultative or strictly anaerobic. Strictly anaerobic bacteria of the genus *Clostridium* are often a large fraction of the population participating in the anaerobic step of acid formation.

Fermentative bacteria were abundant, particularly the proteolytic *Clostridium* species. These species hydrolyse proteins to polypeptides and amino acids, while lipids are hydrolysed via oxidation to long-chain fatty acids, and glycerol



**Fig. 4 – Phylogenetic tree based on the 16S RNA gene of bacteria isolated from digester treating abattoir wastewaters.**

and polycarbohydrates are hydrolysed to sugars and alcohols. After that, fermentative bacteria convert the intermediates to volatile fatty acids, hydrogen and CO<sub>2</sub>.<sup>6</sup>

#### **Sulphate-reducing bacterial communities in the sludge bioreactor**

Sulphate-reducing bacteria were isolated in mesophilic and thermophilic conditions on various substrates. In anaerobic digestion, the acidogenesis products are converted into acetate and hydrogen in the acetogenesis phase. The hydrogen is normally used by the microbial community's methanogenic hydrogenophiles to reduce CO<sub>2</sub> to methane (CH<sub>4</sub>) while acetate is converted by methanogenic acetoclasts to CH<sub>4</sub>.

The presence of sulphate in the medium may change the flow of the substrate available for methanogens. In fact, the SRB may oxidise a portion of the substrate (mainly via the hydrogen) using sulphate as an electron acceptor. In such situations, the substrate is converted to sulphur if the pH of the medium is acidic. These methanogenic bacteria can therefore compete with other bacterial groups such as sulphate-reducing bacteria.<sup>63</sup> The SRB may also be involved in the hydrolysis<sup>64</sup> and acetogenesis steps.<sup>65</sup> In addition, the SRB are known to play a key role in the biodegradation of a number of environmental pollutants.<sup>66</sup>

The strain LINDBL, isolated at 37 °C (Table 4) in the presence of 20 mM lactate, is most closely related to *Desulfobulbus propionicus* with a 99% sequence similarity. *D. propionicus* was first isolated from a fluidised bed bioreactor treating effluent containing acid and metals.<sup>67</sup> Strains LINDBH and LINDBH2 were affiliated phylogenetically to *Desulfovibrio vulgaris* with 99% sequence similarity. Both strains were isolated at 37 °C on basal medium supplemented with H<sub>2</sub>CO<sub>2</sub> (2 bars) as a substrate. *D. vulgaris* is used as a model for the study of SRB to analyse the mechanisms of metal corrosion and to treat toxic metal ions from the environment.<sup>68</sup>

LINDBA1 and LINDBHT1 are two mesophilic strains that were isolated on basal medium for SRB using two different substrates, the first one in the presence of 20mM acetate and the second one in the presence of H<sub>2</sub>CO<sub>2</sub> (at 2 bars). These strains have *Desulfomicrobium baculatum* as their closest relative, with 99% sequence similarity. Phylogenetic analysis demonstrated that the strain LINDBHT1<sup>T</sup> belonged within the genus *Desulfotomaculum*. This strain (LINDBHT1<sup>T</sup>) had *Desulfotomaculum halophilum*<sup>69</sup> and *Desulfotomaculum alkaliphilum*<sup>70</sup> as its closest phylogenetic relatives with approximately 89% sequence similarity. LINDBHT1<sup>T</sup> is a novel anaerobic thermophilic sulphate-reducing bacterium. This bacterium constitutes a new species of the genus *Desulfotomaculum*, *D. peckii*<sup>71</sup> (Fig. 3).

Various SRB were isolated from the anaerobic digester which shows that they are involved in the degradation process. In order to gain an overall idea of the cultivable bacterial diversity of the digester, we grouped all of the bacteria isolated on to the same phylogenetic tree (Fig. 4).

Analysis of the microbial populations obtained from the anaerobic sludge samples in both mesophilic and thermophilic conditions led to the isolation of many morphologically distinct bacteria. Molecular and microbial analyses showed that fermentative bacteria (primarily *Clostridium* spp. and *Parabacteroides* spp.), *Desulfobulbus* spp., *Desulfomicrobium* spp., *Desulfovibrio* spp. and *Desulfotomaculum* spp. were the prominent members of the bacterial community in the bioreactor (Fig. 4). The diversity of the microbial community within the digester may reflect the metabolic diversity of microorganisms involved in anaerobic digestion. The interactions of this complex microbial community allows for complete degradation of natural polymers such as polysaccharides, proteins, lipids and nucleic acids into methane and carbon dioxide.

## Conclusion

In conclusion, the use of both bacterial culture and molecular techniques enabled us to establish a picture of the existing microbial biodiversity in an anaerobic digester. The culture approach was essential, especially with regard to culture and/or isolation of microorganisms with no known cultivable representative. Further research in this area can only improve our knowledge of microbial anaerobic digestors, including the role of different microbial populations involved in anaerobic degradation of waste, which will improve control of these treatment processes. A comprehensive molecular inventory would also support and complement our study of the microbial diversity of anaerobic cultures as it would link information on the diversity and function of microbial communities in their environment.

## Conflicts of interest

The authors have no conflict of interest to declare.

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