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Clostridium cluster I and their pathogenic members in a full-scale operating biogas plant

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Abstract A biogas production plant operating with main and secondary digesters (MD, SD) was analysed for the diversity of bacteria from *Clostridium* cluster I and its pathogenic members. The plant was run in two parallel lines, both receiving silages, and one, in addition, cattle manure (CM). Quantitative PCR of 16S rRNA genes from directly extracted DNA indicated that cluster I represented 0.2 to 5.6 % of the total bacterial communities. Its prevalence was particularly low in CM and also in SD compared to MD, indicating its decline during fermentation. In contrast, another highly abundant clostridial group, i.e. the "faecal" cluster XIVa, remained quantitatively unaffected during fermentation. A total of 85.1 % of 581,934 rRNA gene sequences gathered by group-specific PCR from the silages, CM and digesters could be assigned to cluster I. All remaining sequences fell into other clostridial groups. The three most dominant operational taxonomic units (OTUs) introduced with CM were from cluster I, and they declined during fermentation. Fermentation with CM significantly increased OTUs of clostridia outside of cluster I but not within. The only OTUs related to pathogens were detected for *Clostridium botulinum* with 0.18 % of all cluster I sequences in maize silage and less than 0.01 % in the other substrates and digester materials. These OTUs could be assigned to all four established *C. botulinum* groups, thus, potentially covering all seven neurotoxins. Mouse lethality tests of samples with suspected presence of *C. botulinum*, however, indicated no toxigenic potential and, thus, no risk associated with the rare occurrence of these OTUs.

Keywords Biogas plants · *Clostridium botulinum* · *Clostridium* cluster I · *Clostridium* cluster XIVa · 16S rRNA amplicon analyses · Pyrosequencing · Bacterial community analysis · Cattle manure · Silages

Introduction

Anaerobic digestion of organic material leads to the formation of methane-rich biogas. In recent years, the interest in the technical production of biogas in agriculture has increased since the formation of methane from manure or plant material poses an important renewable energy source (Weiland 2010). While the methane molecule itself is exclusively formed by archaea, the preceding activities, including hydrolysis of organic polymeric substances, acidogenesis, acetogenesis and hydrogen production are mainly provided by bacterial consortia, which typically include clostridia among their quantitatively dominant members (Kröber et al. 2009; Li et al. 2013; Sundberg et al. 2013; Ziganshin et al. 2013).

Clostridia represent a highly diverse group of gram-positive, obligatory anaerobic spore-forming bacteria within the phylum *Firmicutes*. The genus *Clostridium* itself is a phylogenetically highly heterogeneous group which encompasses bacteria from other spore-forming and non-spore-forming genera and families (Yutin and Galperin 2013). Therefore, *Clostridium* has been divided into several clusters, including *Clostridium* cluster I, which represents the genus *Clostridium* sensu stricto (Collins et al. 1994). Within this cluster I, there are some medically relevant species like *Clostridium perfringens*, the causative agent of gas gangrene, *Clostridium*

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tetani causing the tetanus disease, *Clostridium chauvoei* which causes blackleg, a highly mortal disease of ruminants, and *Clostridium botulinum*, the producer of the strong botulinum neurotoxin (BoNT) leading to botulism in humans and animals (Lindstrom et al. 2010; Peck et al. 2011). However, cluster I also contains many nonpathogenic members which can contribute with their metabolic diversity to the degradation of polymeric carbohydrates (Schellenberger et al. 2010), production of solvents (Keis et al. 1995) and organic acids (Schwarz et al. 2012) or of hydrogen (Bowman et al. 2010; Chang et al. 2008).

Bacteria from *Clostridium* cluster I have been detected in anaerobic sediments (Uz et al. 2007) and bioreactors-producing hydrogen (Park et al. 2014) or methane (Dohrmann et al. 2011) or in the gastrointestinal tract. In the latter, however, Clostridia from cluster XIVa often appear to be more prevalent (Becker et al. 2014; Rinttila et al. 2004; Ritchie et al. 2008; Wise and Siragusa 2007). In fact, clostridia from other clusters, e.g. cluster III, cluster IV or cluster XIVa, have similar metabolic potentials and they have been detected in similar environments like those from cluster I (Nakamura et al. 2009; Shrestha et al. 2011).

Currently, the contribution of specific *Clostridium* clusters to anaerobic metabolic transformations in the environment and bioreactors is still in an exploratory state and can at present not well be predicted. Considering the potential prevalence of *Clostridium* cluster I in methane-producing consortia, it is of utmost importance to understand whether pathogenic members of this cluster may also find suitable conditions for growth and, thus, cause unintended risks for humans and animals, especially in farm environments. Indeed, *C. chauvoei* was detected in biogas plants from farms with proven cases of blackleg (Bagge et al. 2009). For *C. botulinum*, e.g., pathogen multiplication in silage and the gastrointestinal tract of cattle with botulism has been reported, and potential risks for the contamination of dairy products have been discussed (Lindstrom et al. 2010). Different *C. botulinum* BoNT types were detected on farms, where bovine and human faeces as well as animal feed and domestic dusts were analysed (Krüger et al. 2012).

For methanogenic bioreactors, cultivation independent analyses of 16S rRNA genes by PCR amplification and DNA sequencing from directly extracted DNA revealed in previous studies the presence of 20 different operational taxonomic units (OTUs) from *Clostridium* cluster I, but phylogenetic analyses demonstrated that none of them was a close relative of a clostridial pathogen (Dohrmann et al. 2011). The phylogenetic analyses of several metagenomic libraries generated by next-generation high-throughput sequencing from directly extracted DNA, which comprised more than two million gene tags from various biogas reactors, indicated a high abundance of clostridia, but also, a very low presence of genes originating from *C. botulinum* or its close relatives (Eikmeyer et al. 2013).

The objective of this study was to apply the new potentials of high-throughput amplicon DNA sequencing of 16S rRNA genes from directly extracted DNA to characterize the diversity of bacteria from *Clostridium* cluster I at an unprecedented level of sensitivity and search for potential pathogens in a full-scale operating biogas plant. Bacterial community analyses were conducted on three different substrates, i.e. maize silage (MS), whole plant silage (WPS) and cattle manure (CM), entering the plant and on fermenting material from main (MD) and secondary digesters (SD). CM was considered as a potential source of *C. botulinum* and *C. chauvoei* because of recent reports on incidences of bovine botulism and blackleg in European farm environments (Bagge et al. 2009; Lindstrom et al. 2010). The architecture of the chosen biogas plant, which consisted of two parallel lines with main and secondary digesters allowed the comparison of bacterial communities in the presence and in the absence of CM. Samples indicating the presence of potential clostridial pathogens by phylogenetic analyses of 16S rRNA genes were further analysed by a reference laboratory for pathogen detection.

Materials and methods

Biogas plant and sampling

The biogas plant, built in 2008, is located in Germany and provides an amount equal to 2.4 MW_e to the natural gas grid. The plant consisted of two separate digestion lines each with one main digester (MD) followed by one secondary digester (SD). Each digester had a volume of 4250 m^3 and was operated at 40 °C. Each day, both lines received 39 t maize silage (MS) and 15 t of whole plant silage (WPS) from rye. Line I received in addition 43 m^3 cattle manure (CM). All substrates (MS, WPS, CM) and slurry samples from main digesters (MD I and MD II) and secondary digesters (SD I and SD II) were collected in June 2012. The material from each sample was split for analyses of the physicochemical process parameters and for molecular analyses of the microbial community composition. Furthermore, additional material of each sample was stored at −20 °C to be available for targeted testing of *C. botulinum* neurotoxin (BoNT) and other potential pathogens.

Physicochemical analyses

Slurry material from the biogas plant was analysed in accordance to the respective DIN EN protocols for its dry matter (DIN EN 12880 2001) and organic dry matter content (DIN EN 12879 2001), pH (DIN EN 15933 2012) and NH_4^+-N content (DIN EN 38406–5 1983). The ratio of volatile organic acids (VOA) and the total inorganic carbon (TIC) VOA/TIC was determined by titration with 0.5 M H_2SO_4.

Nucleic acid extraction

Sampled material was stored over night at 4 °C, and DNA was extracted on the following day. Each sample was split into two aliquots and both of them were separately analysed. Large particles present in the substrates and slurries from the digesters were separated during the first washing step with sterile phosphate buffered saline (PBS 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4; pH 7.4) by centrifugation at 500×*g* for 10 min. The supernatants were then centrifuged for 30 min at 11,800×*g*, and the pellets were washed once with PBS and, after another round of centrifugation, with sterile aqueous 0.85 % (*w*/*v*) KCl solution. Samples from the silages were pretreated to extract the microbial cell fraction as follows: 100-g portions were suspended in 700 ml PBS for 40 min at 4 °C in an orbital shaker (GFL, Burgwedel, Germany) at 20 rev min^{-1}. Large plant material was separated by a 2-mm sieve, and the filtrate entered the aforementioned protocol at 500×*g* centrifugation for 10 min. The nucleic acids were extracted using a phenol-chloroform extraction method described by Lueders et al. (Lueders et al. 2004), modified previously for the analyses of clostridial communities including spore lysis from biogas reactor materials (Method 5b) (Dohrmann et al. 2011). Genomic DNA was then quantified with Quant-iT PicoGreen dsDNA reagent (Life Technologies GmbH, Darmstadt, Germany) following the protocol of the manufacturer.

Quantification of microbial community (qPCR of 16S rRNA genes)

Bacterial and archaeal 16S rRNA gene copy numbers were quantified by separate real-time PCR, conducted in a StepOnePlus Real-Time PCR system (Life Technologies GmbH) using primer and probes described by Yu et.al. (Yu et al. 2005). PCRs were conducted in a volume of 20 µl, containing 2 µl of template DNA, 500 nM of each primer (custom synthesized by Eurofins Genomics, Ebersberg, Germany) and 200 nM of the respective probe (Eurofins) in the Maxima Probe qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA) including ROX. The probe to detect *Bacteria* was labelled with 5′-FAM and 3′-BHQ1 and that for *Archaea* with 5′-TAMRA and 3′-BHQ2. PCR started with 10 min at 95 °C followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s. Signals were detected at 60 °C. Standard curves were obtained from 10-fold dilutions of the pGEM-T vector (Promega, Mannheim, Germany) containing the 16S rRNA gene of *Bacillus subtilis* BD 466 (*Escherichia coli* positions 8–1513) or the 16S rRNA gene of *Methanobacterium oryzae* DSM 11106 (*E. coli* positions 109–1401), respectively. The PCR efficiency Eff was 95 % (R^2=0.999) of the standard curves for *Bacteria* and Eff=101 % (R^2=0.999) for *Archaea*. Ribosomal 16S rRNA genes of *Clostridium* cluster I (*C. perfringens* group) and of cluster XIVa (*Clostridium coccoides* - *Eubacterium rectale* group) were quantified by real-time PCR using primers described elsewhere (Rinttila et al. 2004). Each 25 µl PCR reaction contained 2 µl of template DNA, 500 nM of each primer (biomers.net GmbH, Ulm, Germany) in the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) including ROX. To quantify *Clostridium* cluster I, $MgCl_2$ was added to the reaction for a final concentration of 4 mM $MgCl_2$. PCR started with 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s, 55 °C for 20 s, 72 °C for 30 s and 30 s at 77 °C for signal detection of *Clostridium* cluster I or at 80 °C for cluster XIVa, respectively. Melting curves were recorded for the range from 70 to 95 °C. Standard curves were obtained from 10-fold dilutions of the pGEM-T vector (Promega) containing the 16S rRNA gene of *C. coccoides* DSM 935 (*E. coli* positions 8–1513) or a synthetic DNA (biomers.net GmbH, Ulm, Germany) corresponding to the 16S rRNA gene *rrsA* of *C. perfringens* ATCC 13124 (CP000246) (*E. coli* positions 55–181), respectively. The PCR efficiency Eff was 91 % (R^2=0.999) of the standard curves for *Clostridium* cluster I and 86 % (R^2=0.999) for cluster XIVa.

Group-specific rRNA gene amplicon pyrosequencing of members of *Clostridium* cluster I

To amplify *E. coli* positions 691 to 1359 of the 16S rRNA genes of *Clostridium* cluster I, the forward primer P930 (GTG AAA TGC GTA GAG ATT AGG AA) and the reverse primer P932 (GAT YYG CGA TTA CTA GYA ACT) (Le Bourhis et al. 2005) were selected and modified for 454 pyrosequencing applying the Lib-L emulsion PCR method on the GS FLX Titanium system (Roche, Mannheim, Germany). Forward primers were composed of the Lib-L primer A sequence, the key sequence, a sample specific multiplex identifier to trace the sequences back to the respective sample and P930. Fourteen different forward primers were used in combination with a single reverse primer consisting of Lib-L primer B sequence, the key sequence and P932. For primer sequences, see Supplemental material (Table S1). PCR conditions were 15 min at 95 °C, 30 cycles of 94 °C for 20 s, 58 °C for 30 s, 72 °C for 30 s and 5 min at 72 °C, respectively. The PCR master mix and product purification followed a previously described protocol (Dohrmann et al. 2012). Products from a minimum of three independent replicate amplifications (technical replicates) were pooled for pyrosequencing. Pyrosequencing was conducted by GATC (GATC Biotech AG, Konstanz, Germany). Two samples, namely single replicates of MS and WPS, respectively, failed in the pyrosequencing process.

Amplicon sequence quality evaluation and phylogenetic analyses

Sequences were processed with mothur v.1.32 and v.1.33 (Schloss et al. 2009) following the mothur 454 SOP version February 2014 including qfile, qwindowaverage=35, qwindowsize=50, reference = silva.bacteria.fasta, minlength=150 and average neighbour algorithm for clustering. Chimera sequences (6.6 %) were identified with chimera.uchime (http://drive5.com/uchime) and removed from the dataset. Sequences were grouped in OTUs at similarities of at least 97 % and classified in accordance to the RDP taxonomy with trainset9_032012. Summary.single was applied to calculate library coverage *C*. To get representative sequences for each OTU, get.oturep was applied with options column, list, name, label = 0.03, weighted = true and method = distance. For assignment of the representative sequences to the respective OTUs, the get.otulist command was used to create a list with all sequences in each OTU. For a detailed taxonomic analysis of the most abundant 20 OTUs, all sequences grouped in it were identified, picked from the primary fasta file and a consensus sequence was calculated for each OTU applying mothur commands align.seqs, filter.seqs and consensus.seqs with cutoff=50. Consensus sequences were thought to represent the OTU better than a single sequence. Additionally, the length of ca. 620 nt (without primer sequences), in contrast to the 148 nt of the representative sequences generated during the sequence processing, allowed for a more precise evaluation by NCBI megablast (www.ncbi.nlm.nih.gov/blast) and the RDP classifier (Wang et al. 2007).

To compare differences in abundance the number of sequences was rarefied to 34,027 sequences. A *t* test (Microsoft Excel, Microsoft Corporation, Redmond, WA) was applied to test for significant differences in abundance due to the different digester lines in MDs or SDs, respectively. Differences between MD and SD of each digester line were also evaluated. For all significantly affected OTUs, response ratios were calculated (Hedges et al. 1999). For each OTU identified as sensitive, a consensus sequence was calculated as mentioned before.

For comparisons of the phylogenetic diversity an approximately maximum-likelihood phylogenetic tree was calculated with FastTree Version 2.1.3 SSE3 (Price et al. 2010) and served for the calculation of a weighted UniFrac distance matrix (Lozupone and Knight 2005) with mothur. This matrix was used to calculate nonmetric multidimensional scaling (NMDS) with mothur and for analysis of similarities (ANOSIM) in *R* using the vegan package (R Core Team 2010).

Search for pathogen-linked 16S rRNA gene sequences within *Clostridium* cluster I

To identify rRNA gene sequence of *C. botulinum*, a set of reference sequences was generated from ARB-SILVA SSU 115 (Quast et al. 2013) with the requirements: name = *C. botulinum*, sequence length >1000, sequence quality >90 and alignment quality >90. These requirements were met by 268 sequences. Reference sequences and OTU representative sequences were aligned and clustered with the respective tools of the RDP pyrosequencing pipeline (Cole et al. 2009). Clusters which contained reference and OTU representative sequences were further analysed for their phylogenetic relation to *C. botulinum*. For each identified OTU, a consensus sequence was calculated as mentioned above and then analysed by ARB (Ludwig et al. 2004) including the SINA alignment (Pruesse et al. 2007). A maximum-likelihood tree was constructed based on 90 *Clostridium* cluster I sequences of at least 1300 nucleotides length applying the corresponding 50 % conservation filter and the AxML algorithm. Sequences retrieved in this study from the different substrates and digesters were added to the tree applying the same filter. Similarities between sequences were calculated with the respective tools implemented in ARB. In the same way as described above for *C. botulinum*, the amplicon dataset was also analysed for the presence of *C. tetani*, *C. chauvoei* and *C. perfringens*, respectively.

Bioassay of *C. botulinum* neurotoxins

Selected samples were analysed by the mouse lethality test for the presence of active BoNT. This bioassay was performed by an authorized company (Ripac-Labor GmBH, Potsdam, Germany) following the recommendations of the Friedrich-Loeffler-Institut (Dlabola et al. 2013). Retained material as well as the crude material, including large particles, remaining from DNA extraction was analysed directly for the presence of BoNT and after enrichment cultivation of *C. botulinum*. Thus, the results for each analysed sample were based on four independent analyses.

Deposition of DNA sequences

All DNA sequences retrieved from the different substrates and digester slurries were deposited at the European Nucleotide Archive and can be found under the Accession number PRJEB7129. In addition, consensus sequences of selected OTU are accessible under numbers LN558658 to LN558702 and LN613270.

Results

Quantification of microbial groups

Bacterial 16S rRNA genes detected in total DNA, directly extracted from the different materials, were in a range between 3×10^5 and 8×10^6 copies per ng DNA and generally higher in

the materials from digesters than in the substrates entering the biogas plant (Fig. 1a). The low bacterial copy numbers in MS were probably affected by the higher background of total DNA due to the presence of plant residual and fungal DNA (Rossi and Dellaglio 2007). Compared to *Bacteria*, the copy numbers of *Archaea*, which included the methanogens, were one to two orders of magnitude lower. The 16S rRNA genes from *Clostridium* cluster XIVa, i.e. the "faecal clostridia" (see "Materials and methods"; (Song et al. 2004)) represented 1.4 to 10.1 % of the total bacterial 16S rRNA genes in the different materials (Fig. 1b), with highest values for CM. In contrast, the contribution of *Clostridium* cluster I, potentially including *C. botulinum* and other clostridial pathogens, was particularly low in CM and, on average, also lower in the two silages. While there was a steep decline of cluster I from the main (MD) to the secondary digesters (SD), the prevalence of cluster XIVa did not significantly change. The contrasting prevalence of both clostridial clusters I and XIVa in the different materials indicated that these groups were not linked to each other.

Specificity of *Clostridium* cluster I 16S rRNA gene amplification

A total of 581,934 amplicon sequences, obtained from the substrates fed into the biogas plant and the two stages of the biogas production processes, passed the sequence quality analyses (Table 1). In total, 85.1 % of these sequences fell into *Clostridium* cluster I, the group targeted with the selected primers. The lowest primer specificities were detected for samples from the two SDs. Overall, a total of 653 different

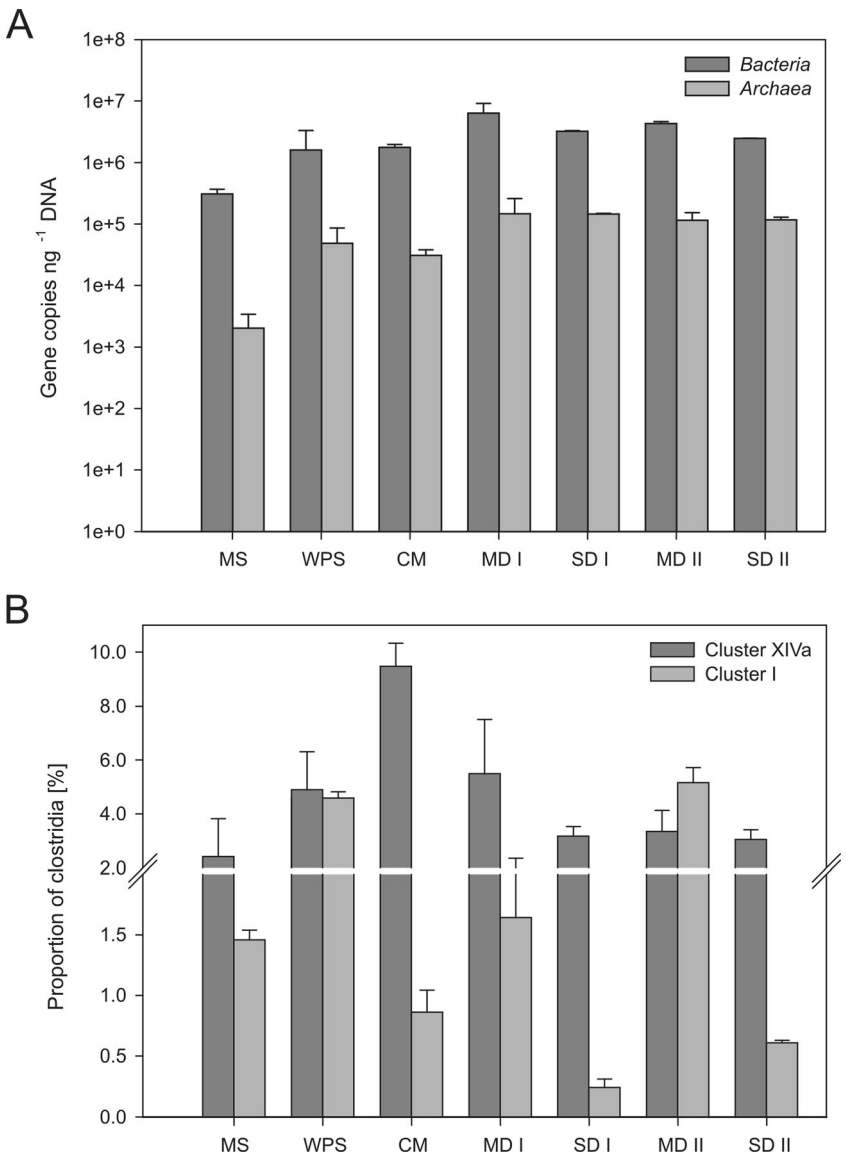


Fig. 1 Copy numbers of qPCR-amplified 16S rRNA genes of *Bacteria* and *Archaea* (**a**) and *Clostridia* clusters I and XIVa (**b**), in substrates entering the biogas plant of this study, i.e. maize silage (*MS*), whole plant silage (*WPS*) and cattle manure (*CM*), as well as materials collected from main digesters (*MD*) and secondary digesters (*SD*). Digesters indicated with *I* received *MS*, *WPS* and *CM*, while *CM* was not included into the substrates fed into digesters indicated with *II*

Table 1 Characterization and composition of the 16S rRNA gene amplicon libraries retrieved in this study

	Total	Substrates			Digester I		Digester II	
		MS	WPS	CM	MD	SD	MD	SD
Total number of amplicons	581,934	86,373	45,034	101,377	113,965	71,279	81,494	82,412
Amplicons assigned to *Clostridium* cluster I	495,487	85,020	39,497	87,643	107,519	27,265	80,045	68,498
Specificity for *Clostridium* cluster I (%)	85.1	98.4	87.7	86.5	94.3	38.3	98.2	83.1
Number of OTUs in *Clostridium* cluster I	653	148	60	92	241	247	140	268

MS maize silage, *WPS* whole plant silage, *CM* cattle manure, *MD* main digester, *SD* secondary digester

OTUs of *Clostridium* cluster I were detected. Phylogenetic analyses revealed that all sequences which fell outside of this cluster belonged, at the family rank, to other members of clostridia (Fig. 2).

Diversity and prevalence of 16S rRNA gene amplicons

The 581,934 amplicon sequences could be grouped into 2544 OTUs. For a balanced comparison, the dataset was rarefied to 34,027 sequences for each sample, which resulted in 2541 OTUs remaining. Of these, 318 OTUs contained ten or more sequences, which accounted for 98.5 % of all sequences.

The 20 most abundant OTUs, representing 91.3 % of all sequences, showed at least 97 % DNA sequence identity to previously identified rRNA genes, mainly originating from uncultured bacteria which had been retrieved from various habitats, including the gastrointestinal tract or faeces of humans and animals, composts and biogas plants (Table 2). The most abundant OTU (OTU 1), a member of *Clostridium* cluster I, represented almost 50 % of all sequences in CM and at least 11 % in the other materials, including those from the digesters. The prevalence of OTU 1 in MD of the line receiving CM declined during anaerobic digestion. In fact, all OTUs with more than 1 % abundance in CM, of which the first three were from cluster I, declined drastically during the digestion process of CM.

Bacteria from OTU 2, closely related to *Clostridium saccharoperbutylacetonicum*, were highly prevalent in the substrates especially MS, entering the biogas plant, but diminished, as indicated by their lower numbers in

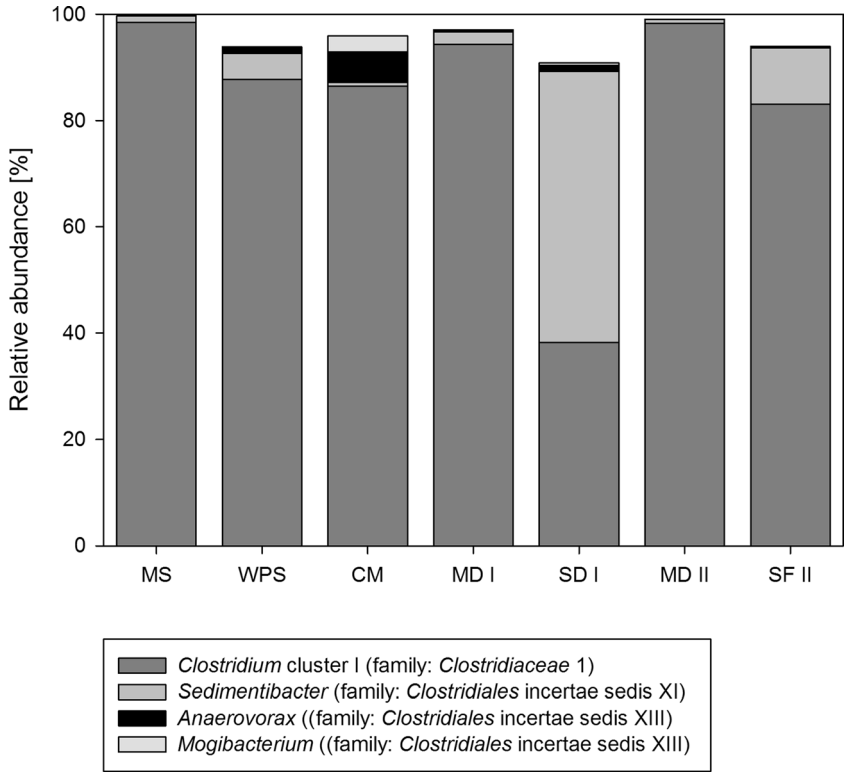


Fig. 2 Taxonomic affiliation of all 16S rRNA gene amplicon sequences detected with the *Clostridium*-specific PCR. For abbreviations of substrates, see legend of Fig. 1

Table 2 Taxonomic assignment of the 20 most abundant OTUs

OTU		Closed relative to consensus sequence				Taxonomic assignment[b]	Number of sequences	Abundance of OTU (% of all OTUs)[c]						
								Substrates			Line I		Line II	
OTU no.	Accession no.[a]	Description	Accession no.	Sequence identity (%)	Habitat information			MS	WPS	CM	MD I	SD I	MD II	SD II
1	LN558658	Uncultured bacterium	JQ187120.1	99	Human faeces	*Clostridium* cluster I	10,8658	*15.1*	*37.3*	*49.7±0.9*	*29.5±3.5*	*19.6±2.1*	11.0±3.27	*23.7±1.7*
2	LN558659	*Clostridium saccharoperbutylacetonicum*	NR_102516.1	99	Soil	*Clostridium* cluster I	34,289	*64.1*	2.55	*10.7±2.3*	3.52±1.24	0.770±0.150	1.14±0.48	0.917±0.110
3	LN558660	Uncultured bacterium	EU250947.1	99	Compost	*Clostridium* cluster I	55,139	0.285	*14.6*	0.004±0.001	*9.96±0.53*	2.36±0.07	*42.1±0.4*	*19.1±4.6*
4	LN558661	Uncultured bacterium	FJ678669.1	98	Cattle intestine	*Clostridium* cluster I	41,875	1.71	*8.33*	*20.1±0.1*	*19.6±0.7*	5.20±0.82	5.66±1.51	5.93±0.53
5	LN558662	Uncultured bacterium	CU918096.1	98	Anaerobic digester	*Sedimentibacter*	37,503	1.71	2.41	0.150±0.032	1.75±0.17	*40.5±4.1*	0.567±0.510	*10.1±8.77*
6	LN558663	Uncultured bacterium	FJ205828.1	99	Biogas plant	*Clostridium* cluster I	27,246	*1.90*	6.75	0.001±0.001	9.24±2.72	1.29±0.07	*16.4±0.4*	8.76±2.82
7	LN558664	Uncultured bacterium	FJ205822.1	99	Biogas plant	*Clostridium* cluster I	24,736	1.75	6.46	0	9.00±3.76	1.10±0.09	*14.5±0.4*	7.62±3.07
8	LN558665	*Clostridium* sp. M2/40	HG917868.1	99	Biogas plant	*Clostridium* cluster I	11,417	0.629	4.91	0.240±0.010	2.37±1.91	3.21±0.20	0.504±0.210	7.68±2.95
9	LN558666	Uncultured bacterium	FN985641.1	99	Biogas plant	*Sedimentibacter*	6379	0.835	1.75	0	0.744±0.280	*6.13±0.32*	0.175±0.120	1.03±0.79
10	LN558667	Uncultured bacterium	HQ156043.1	96	Biogas plant	Unclassified *Bacteria*	5104	0.024	1.91	0.040±0	1.16±0.99	2.02±0.23	0.410±0.300	2.90±1.67
11	LN558668	Uncultured bacterium	AB507570.1	99	Sheep rumen	Unclassified *Clostridiales*	3950	0	0.370	4.82±0.29	0.212±0.140	0.442±0.100	0.012±0	0.134±0.040
12	LN558669	Uncultured bacterium	AY854283.1	99	Zebu intestine	*Clostridiales* incertae sedis XIII	2973	0.006	0.194	3.58±0.29	0.157±0.080	0.407±0.110	0.010±0	0.116±0.050
13	LN558670	Uncultured bacterium	HQ741662.1	97	Human intestine	*Clostridium* cluster I	1826	0.032	0.009	0.026±0.026	0.431±0.250	1.15±0.40	0.050±0.050	1.00±0.99
14	LN558671	Uncultured bacterium	FJ205822.1	99	Biogas plant	*Clostridium* cluster I	1761	0.106	0.317	0	0.677±0.080	0.144±0	0.936±0.220	0.619±0.070
15	LN558672	Uncultured bacterium	FJ205828.1	99	Biogas plant	*Clostridium* cluster I	1673	0.100	0.282	0	0.691±0.140	0.098±0.020	0.864±0.100	0.614±0.140
16	LN558673	Uncultured bacterium	FN995959.1	97	Biogas plant	*Clostridiales* incertae sedis XIII	1627	0	0.514	1.92±0.36	0.075±0.040	0.132±0	0	0.010±0.010
17	LN558674	Uncultured bacterium	EU250947.1	98	Compost	Unclassified *Clostridiaceae* 1	1613	0	0.094	0.003±0.003	0.870±0.590	0.253±0.020	0.588±0.540	0.610±0.590
18	LN558675	Uncultured bacterium	HQ741662.1	97	Human intestine	*Clostridium* cluster I	1614	0.247	0.003	0.003±0.003	1.16±0.88	0.175±0.010	0.492±0.440	0.419±0.420
19	LN558676	Uncultured bacterium	FN985641.1	99	Biogas plant	*Sedimentibacter*	1658	0.047	0.461	0	0.148±0.090	1.81±0.22	0.019±0.020	0.209±0.130
20	LN558677	Uncultured bacterium	EU250947.1	98	Compost	Unclassified *Clostridiaceae* 1	1586	0	0.015	0.001±0.001	1.08±0.78	0.115±0	0.816±0.760	0.316±0.310

The dataset for each sample was rarefied to 34,027 sequences. Italicised numbers indicate the three most dominant OTUs for the respective materials

[a] Maximum length consensus sequences

[b] RDP classifier result

[c] Arithmetic mean and standard deviation. Each material was analysed in duplicate except MS and WPS

both SDs compared to MDs and substrates. In contrast, OTU 5, a *Sedimentibacter* relative, was more prevalent in the SDs than in the substrates or MDs. Three relatives of *Clostridium* cluster I, i.e. OTU 3, 6 and 7, which together represented 26.2 % of all sequences, were more abundant in MDs than in SDs or substrates, suggesting an active role in the digestion process. These OTUs were mostly present in WPS, indicating this substrate as a potential source.

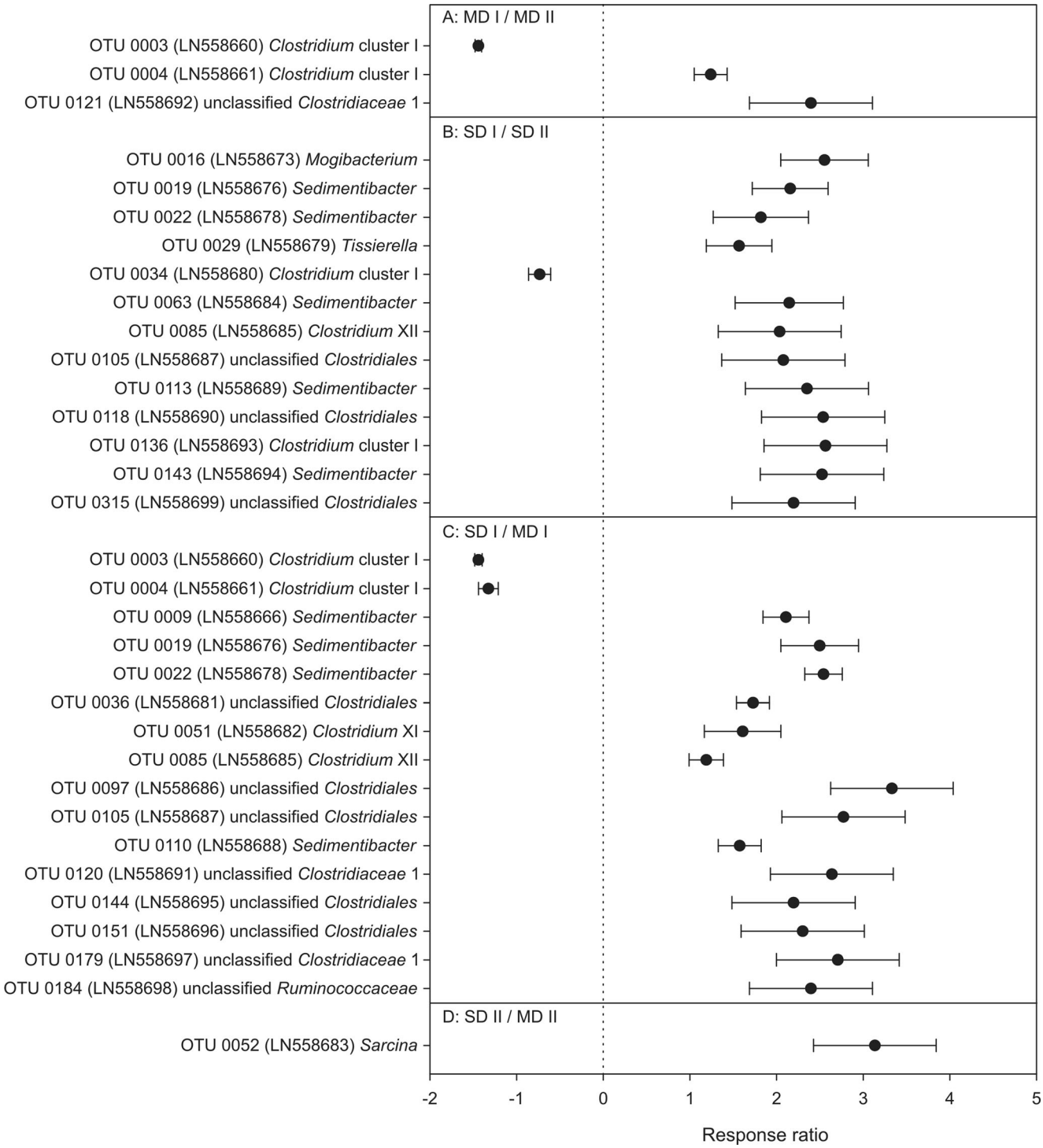


Fig. 3 Identification of OTUs which responded significantly different when digesters from line I and line II and/or main and secondary digesters were compared to each other. For each OTU, the most accurate taxonomic affiliation possible, based on RDP analyses, is indicated

Effect of the substrates and the digesters compartment on the clostridial community

Of all 2544 OTUs, only 27 responded significantly to the different conditions in the digesters, i.e. either the presence or absence of CM or the differences prevalent in SD compared to MD. Six OTUs, among them were two from cluster I, responded significantly to both CM and digester compartments (main vs. secondary) (Fig. 3). A comparison between the digesters receiving CM or not, revealed only three responsive OTU in the MDs, but 13 in the SDs. Interestingly, 14 of the 16 responsive genera were more abundant in digesters with CM, suggesting that they might have been involved in the degradation of substrates specific for CM. Only 4 of these 16 responsive OTU originated from *Clostridium* cluster I, while the other 12 were relatives of *Sedimentibacter*, *Mogibacterium* and other non-cultured isolates outside of this cluster. Two of the cluster I sequences declined in their abundance in response to CM. In contrast, all clostridia outside of cluster I increased in presence of CM.

NMDS was utilized to visualize the differences in the communities' phylogenetic structure including all rarefied sequences (n=34,027 for each sample) (Fig. 4). Each substrate and digester was characterized by distinct clostridial communities, and replicates were, with the exception of SD II, more similar to each other than those of different origin. The analyses indicated that distinct clostridial communities were present in MD and SD and those differences also existed between the digesters receiving CM or not.

In an additional step, all samples were characterized for their physicochemical properties (Supplemental material; Table S2), and ANOSIM revealed that significant effects (p<0.05) on the clostridial diversity were linked to the amount of organic dry matter (oDM)(R=0.96) and the concentrations of VOA (R=0.92) and NH^{+}_{4} (R=0.92) (Supplemental material, Table S3).

Presence of *C. botulinum* and other potentially pathogenic members of cluster I

Phylogenetic analyses of the 581,934 amplicon DNA sequences retrieved in this study did not indicate the presence of OTUs related to *C. perfringens*, *C. tetani or C. chauvoei*, respectively. In contrast, 4 OTUs were identified with sequence identity of above 97 % to *C. botulinum* (Table 3). The highest prevalence of sequences, i.e. 150, was found with MS, and phylogenetic analyses assigned this OTU 0066 to *C. botulinum* group III (BoNT types C or D) (Fig. 5). Sequences of OTU 0541 assigned to group II (BoNT types B, E or F) were detected in CM, and sequences of OTU 0208 assigned to group I (BoNT types A, B or F) were found in all digesters. The two sequences of OTU 1209, one detected in MS and the other in the SD of line II (no CM), belonged to group IV (*Clostridium argentinense*, BoNT type G). While the overall contribution of C. *botulinum* related 16S rRNA gene sequences in MS represented 0.18 % of *Clostridium* cluster I, the occurrence in other materials was below 0.01 %.

Three samples with different *C. botulinum* groups from different substrates, i.e. MS, CM and MD I, were selected for mouse lethality tests. None of these tests indicated the presence of BoNT-producing bacteria, either by direct analyses or after incubation for enrichment of *C. botulinum* in appropriate growth media (Table 3). Thus, the indication for the presence of toxigenic *C. botulinum* by 16S rRNA gene-based analyses was not confirmed.

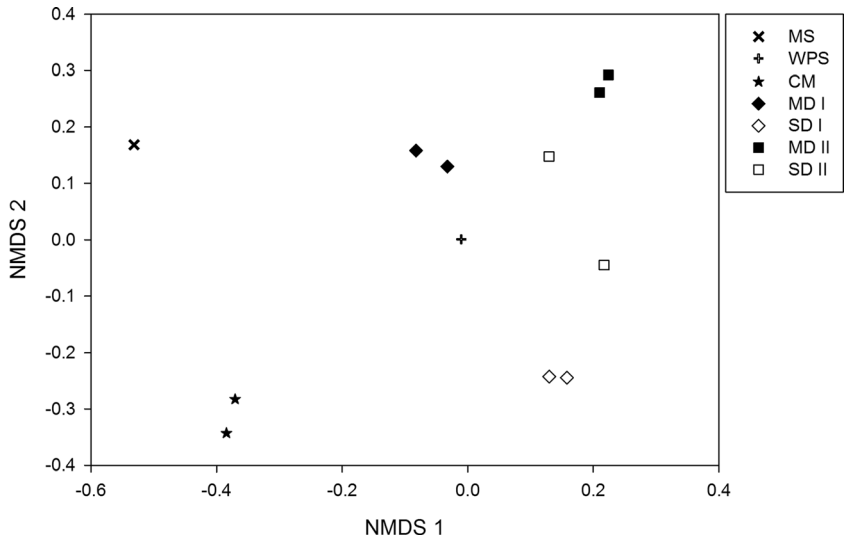


Fig. 4 NMDS ordination based on weighted UniFrac distances derived from FastTree phylogeny (stress=0.11; R^2=0.95). For abbreviations, see legend of Fig. 1

Table 3 Detection of OTUs (based on 16S rRNA gene sequences) closely related to *Clostridium botulinum* from different substrates and digester materials

	Substrates			Digester I		Digester II	
	MS	WPS	CM	MD	SD	MD	SD
Sequence numbers[a]							
OTU 0066	150	1	0	0	0	0	0
OTU 0208	0	0	0	9	2	3	3
OTU 0541	0	0	4	0	0	0	0
OTU 1209	1	0	0	0	0	0	1
Contribution to cluster I (%)	0.18	0.003	0.005	0.008	0.007	0.004	0.004
Result of mouse lethality tests							
Direct[b]	Negative	n.a.	Negative	Negative	n.a.	n.a.	n.a.
After enrichment	Negative	n.a.	Negative	Negative	n.a.	n.a.	n.a.

Digester I received MS, WPS and CM, while digester II only received MS and WPS, respectively

MS maize silage, *WPS* whole plant silage, *CM* cattle manure, *MD* main digester, *SD* secondary digester, *n.a* not analysed

[a] Sum of duplicates for all materials except MS and WPS for which no duplicates were available (see "Materials and methods")

[b] Crude sample material

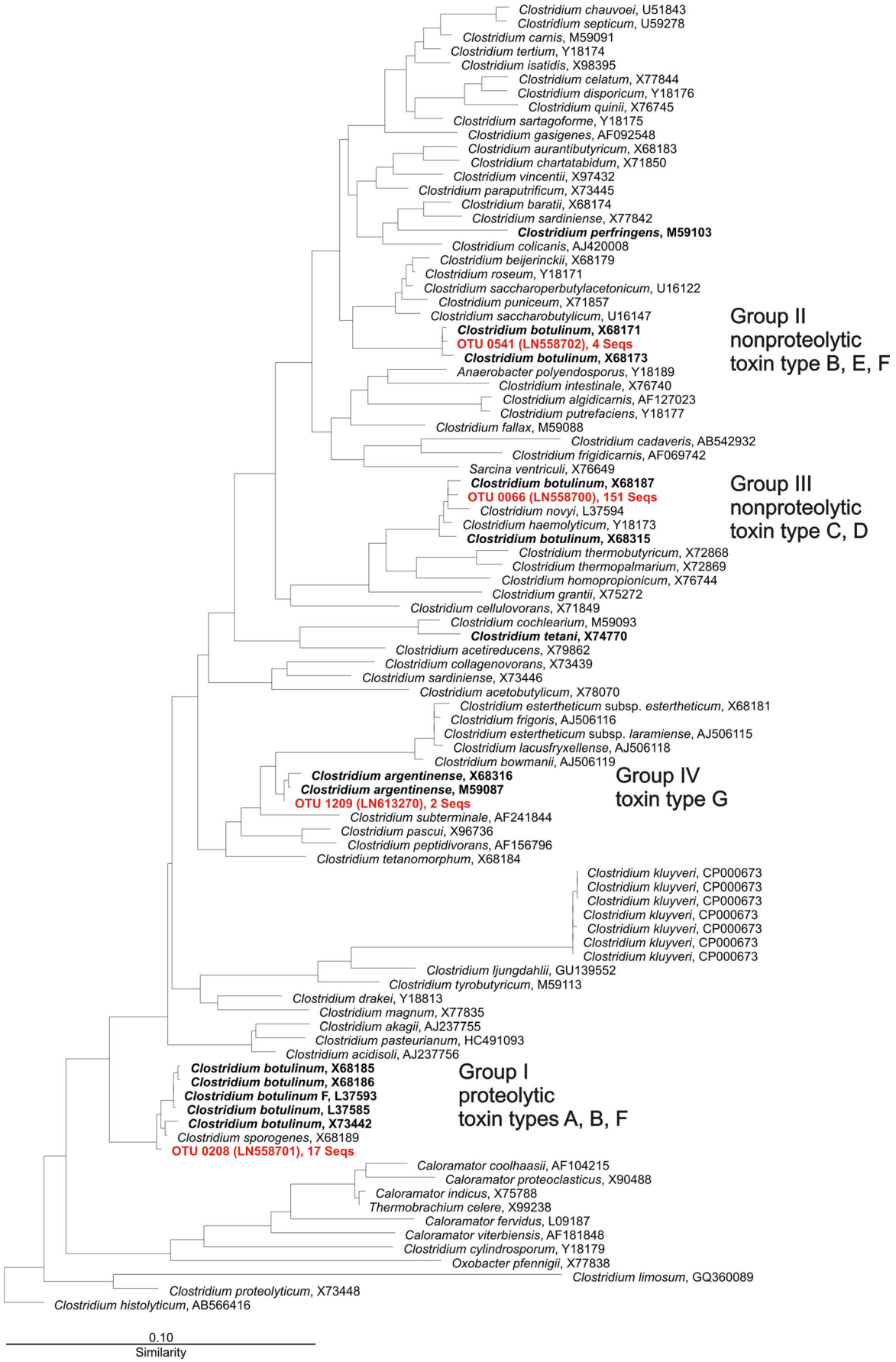
Discussion

The biogas plant selected for this study was in full operation during the time the samples were collected. The population sizes of *Bacteria* and *Archaea*, as indicated by domain specific qPCR of their 16S rRNA genes, were similar to those reported for other commercial biogas plants run with similar substrates, including manure and silages (Nettmann et al. 2008) (Sundberg et al. 2013). The analyses of the three substrates entering the biogas plant of this study revealed in CM lower overall abundance for *Clostridium* cluster I compared to both silages, while, as expected, the contribution of "faecal" cluster XIVa sequences was higher in the manure. The relatively high incidence of both clostridial clusters in silages, in the range of 1 to 6 % of all bacterial genes, was in fact surprising, considering that in contrast to manure (Lu et al. 2014), clostridia generally are not expected to be among the dominant bacterial community members in such materials (Duniere et al. 2013; McGarvey et al. 2013). Clostridia in silages may be introduced with soil (Duniere et al. 2013) or manure used crop fertilization.

Bacterial diversity in this study was analysed by sequencing 16S rRNA gene fragments, but in contrast to other studies in which universal bacterial primers were applied (Bengelsdorf et al. 2013; Kröber et al. 2009; Sundberg et al. 2013), this study applied, for higher sensitivity, group specific primers targeting *Clostridium* cluster I. The selected primers, however, also amplified rRNA genes from some phylogenetic neighbours of cluster I, originating from some *incerta sedis* families of the *Clostridiales*. This primer inaccuracy had already been observed in a preceding study based on the sequencing of 66 amplicons (Dohrmann et al. 2011); while in this study, almost 9000 times more sequences were analysed. This inaccuracy was, in fact, quite useful in this study when the response of single OTUs to the conditions in the digesters receiving CM and those without were compared to each other since they apparently revealed different patterns of responses between *Clostridium* cluster I and other clostridial groups.

Due to the architecture of the biogas plants selected for this study, with two parallel lines each consisting of one main and one secondary digester and both receiving the same basic substrates, it was possible to investigate the effect of CM addition on the clostridial diversity in a comparative approach. The CM impact on the community structure in fact became evident in NMDS analyses where the communities from digesters receiving CM were clearly distinct from those without CM. However, at the OTU level, the clostridia could rarely be assigned to a specific substrate. This was mainly linked to the fact that, generally, the OTUs from CM also occurred in one or both of the silages. Therefore, it was not surprising that the three dominant OTUs from cluster I of CM also occurred in digesters which did not receive CM (see Table 2). Only three OTUs, of which two, however, were the second and

Fig. 5 Maximum likelihood tree of *Clostridium* cluster I showing the affiliation of 16S rRNA genes obtained from OTU consensus sequences. *Groups I* to *IV* indicate the four distinct groups of *C. botulinum* strains. Reference sequences for identification of *C. botulinum* are indicated in *bold*; OTUs of this study identified as close *C. botulinum* relatives in *red*


Clostridium chauvoei, U51843
Clostridium septicum, U59278
Clostridium carnis, M59091
Clostridium tertium, Y18174
Clostridium isatidis, X98395
Clostridium celatum, X77844
Clostridium disporicum, Y18176
Clostridium quinii, X76745
Clostridium sartagoforme, Y18175
Clostridium gasigenes, AF092548
Clostridium aurantibutyricum, X68183
Clostridium chartatabidum, X71850
Clostridium vincentii, X97432
Clostridium paraputrificum, X73445
Clostridium baratii, X68174
Clostridium sardiniense, X77842
Clostridium perfringens, M59103
Clostridium colicanis, AJ420008
Clostridium beijerinckii, X68179
Clostridium roseum, Y18171
Clostridium saccharoperbutylacetonicum, U16122
Clostridium puniceum, X71857
Clostridium saccharobutylicum, U16147
Clostridium botulinum, X68171
OTU 0541 (LN558702), 4 Seqs
Clostridium botulinum, X68173
Anaerobacter polyendosporus, Y18189
Clostridium intestinale, X76740
Clostridium algidicarnis, AF127023
Clostridium putrefaciens, Y18177
Clostridium fallax, M59088
Clostridium cadaveris, AB542932
Clostridium frigidicarnis, AF069742
Sarcina ventriculi, X76649
Clostridium botulinum, X68187
OTU 0066 (LN558700), 151 Seqs
Clostridium novyi, L37594
Clostridium haemolyticum, Y18173
Clostridium botulinum, X68315
Clostridium thermobutyricum, X72868
Clostridium thermopalmarium, X72869
Clostridium homopropionicum, X76744
Clostridium grantii, X75272
Clostridium cellulovorans, X71849
Clostridium cochlearium, M59093
Clostridium tetani, X74770
Clostridium acetireducens, X79862
Clostridium collagenovorans, X73439
Clostridium sardiniense, X73446
Clostridium acetobutylicum, X78070
Clostridium estertheticum subsp. estertheticum, X68181
Clostridium frigoris, AJ506116
Clostridium estertheticum subsp. laramiense, AJ506115
Clostridium lacusfryxellense, AJ506118
Clostridium bowmanii, AJ506119
Clostridium argentinense, X68316
Clostridium argentinense, M59087
OTU 1209 (LN613270), 2 Seqs
Clostridium subterminale, AF241844
Clostridium pascui, X96736
Clostridium peptidivorans, AF156796
Clostridium tetanomorphum, X68184
Clostridium kluyveri, CP000673
Clostridium kluyveri, CP000673
Clostridium kluyveri, CP000673
Clostridium kluyveri, CP000673
Clostridium kluyveri, CP000673
Clostridium kluyveri, CP000673
Clostridium kluyveri, CP000673
Clostridium ljungdahlii, GU139552
Clostridium tyrobutyricum, M59113
Clostridium drakei, Y18813
Clostridium magnum, X77835
Clostridium akagii, AJ237755
Clostridium pasteurianum, HC491093
Clostridium acidisoli, AJ237756
Clostridium botulinum, X68185
Clostridium botulinum, X68186
Clostridium botulinum F, L37593
Clostridium botulinum, L37585
Clostridium botulinum, X73442
Clostridium sporogenes, X68189
OTU 0208 (LN558701), 17 Seqs
Caloramator coolhaasii, AF104215
Caloramator proteoclasticus, X90488
Caloramator indicus, X75788
Thermobrachium celere, X99238
Caloramator fervidus, L09187
Caloramator viterbiensis, AF181848
Clostridium cylindrosporum, Y18179
Oxobacter pfennigii, X77838
Clostridium limosum, GQ360089
Clostridium proteolyticum, X73448
Clostridium histolyticum, AB566416
Group II
nonproteolytic
toxin type B, E, F
Group III
nonproteolytic
toxin type C, D
Group IV
toxin type G
Group I
proteolytic
toxin types A, B, F
0.10
Similarity

third most dominant OTU from cluster I, responded significantly to the presence of CM in the main digesters. These two exhibited opposing responses and their responses could be linked to their abundance in CM: OTU 4 represented 20 % of the clostridial population in CM and was more abundant in MD I, while OTU 3 was nearly absent in CM (0.004 %), but well represented in WPS (14.6 %); it was more abundant in MD II. The distinction of clostridial clusters as well as OTU in this study was based on phylogenetic analyses; but for clostridia, these do not correlate with differences in carbon source utilization (Gupta and Gao 2009; Rainey and Stackebrandt 1993), which can be considered as a main factor structuring the communities in the digesters.

Indications for the presence of *C. botulinum*, but not of other pathogens from *Clostridium* cluster I were obtained from all samples analysed in this study. However, its content of the community of *Clostridium* cluster I was very small, ranging from 0.18 % in MS to 0.003 % in WPS. Considering the contribution of the cluster I sequences to the total bacterial community, as established by qPCR of their 16S rRNA gene, the detection of a single *C. botulinum* 16S rRNA gene would require the analysis of a background of 270,000 to 370,000 bacterial genes in MDs, and, a background of 2 to 8 million sequences from SDs. It should be noted that the species name "*C. botulinum*" is defined on the ability of these bacteria to form the botulinum neurotoxin (BoNT) rather than on phylogenetic grounds (Peck et al. 2011). Therefore, *C. botulinum* groups I to IV are distinguished by distinct phylogenetic lineages (Hutson et al. 1996), and each of these groups is characterized by a potential to produce different BoNT types, e.g. group I produces A, B or F, and group III, C or D ((Collins and East 1998), see also Fig. 5). Interestingly, the suspected *C. botulinum* OTUs detected in this study encompassed representatives of all four groups, thus indicating a potential that all seven BoNT types could theoretically be produced. In a farm environment, including silages, other studies revealed the presence of *C. botulinum* BoNT types A, B, C, D and E (Krüger et al. 2012; Lindstrom et al. 2010; Notermans et al. 1981). Even though the rare occurrence of sequences indicating the presence of the four *C. botulinum* groups and the fact that the two fermentation lines in the biogas plant were not represented by any replicate digesters do not allow any detailed statistical analysis of the environmental conditions which would affect the prevalence of these groups, it appears clear that none of them multiplied on its path through the biogas plants, independent of whether CM was supplied or not.

While the limitation of the 16S rRNA gene-based approach lies in its inability to ultimately confirm the presence of *C. botulinum* BoNT, the beauty of this approach is that it allows the testing of a large number of environmental samples without sacrificing laboratory animals. Due to the chosen high-throughput DNA sequencing approach of the 16S rRNA gene amplicons from cluster I, unprecedented detection thresholds of up to one gene copy in a background of two million bacterial genes could be achieved. All three samples, which included together the presence of all four *C. botulinum* groups, were tested negative in mouse lethality tests. Since the toxicity analyses also included previous enrichment steps, it can be concluded that no vegetative cells or spores of *C. botulinum*, as defined by their capacity to produce BoNT, were present in the materials analysed. Thus, OTUs assigned to the four *C. botulinum* groups originated from species in which the BoNT encoding genes must have been absent, dysfunctional or not expressed. The presence of silent BoNT genes in *C. botulinum* strains is not unusual (Dineen et al. 2003; Hutson et al. 1996). Non-toxigenic *C. botulinum* derivates could be a result of the instability of BoNT encoding genomic regions, which are typically linked to insertion sequences, transposons or other mobile genetic elements (Brüggemann 2005; Hill et al. 2009).

In summary, this study demonstrates how environmental samples can be analysed independent of cultivation based on high-throughput 16S rRNA gene amplicon sequencing for the diversity of *Clostridium* cluster I sequences and search with high sensitivity for the presence of suspected bacterial pathogens without sacrificing laboratory animals. The abundance and diversity of 16S rRNA amplicons retrieved in this study suggest that bacteria from *Clostridium* cluster I, in contrast to other clostridia, played only a minor role in the fermentation of the organic material, including CM, in the selected full-scale operating biogas plant, and no risk was identified for an unintended presence or proliferation of pathogens from *Clostridium* cluster I.

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