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ORIGINAL ARTICLE

Dynamics of antibiotic resistance genes and presence of putative pathogens during ambient temperature anaerobic digestion

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Keywords
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Abstract

Aims: This study was focused on evaluating the persistency of antimicrobial resistance (AR) genes and putative pathogenic bacteria in an anaerobic digesters operating at mesophilic ambient temperature, in two different year seasons: summer and winter.

Methods and Results: Abundance and dynamic of AR genes encoding resistance to macrolides (*erm*B), aminoglycosides (*aph*A2) and beta-lactams ($bla_{\text{TEM-1}}$) and persistency of potentially pathogenic bacteria in pilot-scale anaerobic digesters were investigated. AR genes were determined in the influent and effluent in both conditions. Overall, after 60 days, reduction was observed for all evaluated genes. However, during the summer, anaerobic digestion was more related to the gene reduction as compared to winter. Persistency of potentially pathogenic bacteria was also evaluated by metagenomic analyses compared to an in-house created database. *Clostridium*, *Acinetobacter* and *Stenotrophomonas* were the most identified.

Conclusions: Overall, considering the mesophilic ambient temperature during anaerobic digestion (summer and winter), a decrease in pathogenic bacteria detection through metagenomic analysis and AR genes is reported. Although the mesophilic anaerobic digestion has been efficient, the results may suggest medically important bacteria and AR genes persistency during the process.

Significance and Impact of the Study: This is the first report to show AR gene dynamics and persistency of potentially pathogenic bacteria through metagenomic approach in cattle manure ambient temperature anaerobic digestion.

Introduction

Untreated animal manure may comprise several disease-causing micro-organisms and constitutes reservoir of antimicrobial resistance (AR) genes. Large amounts of antibiotic compounds are applied in animal farming for growth promotion, prophylaxis and, at therapeutic levels, for treatment of infections. This practice may contribute to the increased percentages of resistant bacteria world-wide isolated from farm animals (Sahlström *et al.* 2004). In addition, the occurrence of enteropathogenic bacteria, such as enterococci, and Gram-negative rods (*Escherichia coli* and *Salmonella* spp.) has already been reported in cattle manure, and it is accepted that these animals play an important role in the spread of these potentially pathogenic bacteria to the surrounding environment (Sahlström *et al.* 2004; Venglovsky *et al.* 2009).

In an attempt to avoid the manure direct discharge into soil and aquatic ecosystems, anaerobic digestion technology is pointed out as an alternative to the sanitary

problems associated with the livestock areas (Holm-Nielsen *et al.* 2009). This technology is widely used in several countries resulting in biogas, and residues rich in nutrients, which can be used as fertilizers (Abubaker *et al.* 2012). Moreover, without any risk assessment, according to the literature, these fertilizers would be associated with the soil contamination by multiresistant potentially pathogenic bacteria (Eikmeyer *et al.* 2013). In this regard, temperature is pointed out as the dominant inactivating factor, as observed during mesophilic or thermophilic anaerobic digestion (Beneragama *et al.* 2013). Horizontal transfer of antibiotic resistance determinants is considered an important safety issue, and the input of resistance genes with effluent digesters could well contribute to the resistance problem in human antibiotic therapy (Heuer *et al.* 2011; Pruden *et al.* 2013).

Although the Brazilian climatic conditions are in the mesophilic range and most of the anaerobic digesters are operated at ambient temperature followed by land application (Kunz *et al.* 2009), there are no literature data available regarding risk assessments concerning the stability of potentially pathogenic bacteria or even inactivation of AR genes.

A recent report describes the isolation of multidrug-resistant bacteria from anaerobic digestion effluents and influent in ambient temperature pilot-scale digesters (Resende *et al.* 2014). From that study, samples were collected equally during two different periods (summer and winter) to provide new insights into further investigations related to the sanitary risks of the process concerning human and environmental health. At this point, this study provides original information on the persistency of the AR genes (macrolides, aminoglycosides and beta-lactams) and 16S rRNA gene sequencing for identification of potentially pathogenic bacteria. The AR genes evaluated in this study are representative of major genetic markers associated with the antimicrobial resistance considering drugs commonly used in dairy cattle management in Brazil, also relevant considering human medicine (Nero *et al.* 2007; Kemper 2008 Fonseca *et al.* 2009; Pribul *et al.* 2011).

Material and methods

Pilot-scale reactor conditions

Two reactors operating ambient temperatures in different seasons, with a 60-day retention time and 60-l working volume, were used. Fresh dairy cattle manure was collected weekly, in the same location, at the experimental Embrapa dairy cattle field located in Coronel Pacheco city, Minas Gerais state, Brazil.

The samples were transported immediately to the laboratory within 30 min, under refrigeration in ice box, and stored at 4°C, up to 96 h, according to the established procedures (Wang *et al.* 2012). As long as the dairy cows have been treated with beta-lactam (penicillin) in clinical or subclinical mastitis infections in the experimental Embrapa dairy cattle field, at the time of sample collection, the animals did not receive any antimicrobial drugs in the past 30 days.

Physical–chemical parameters were estimated as per standard methods (APHA 2005). Before being used as substrate in the pilot-scale digesters, the manure was diluted with cattle wastewater to a total solids (TS) concentration of 4–5%, and after for daily addition at an amount of 2 l.

The samples were collected equally from during two different periods: between January to March (2012) (summer) and June to July (2012) (winter). Ambient temperature was in the mesophilic range, between 14 and 25°C (average 19·5°C) in winter and 24–34°C (average 29·5°C) in summer.

The pH value of the cattle manure (influent) was between 6·55 and 6·86, and the loading rates were around 13·3 g ml^{-1} of volatile solids (VS) in the summer and 12·4 g ml^{-1} of VS in the winter months. In all digesters, the percentage reduction in total solids of fermenting cattle manure was about 65% and VS was 68%. The average daily biogas produced in summer and winter months was 18·7 and 16 l per day, respectively. There was no significant difference in the methane content of the biogas produced in summer (59·2%) and winter (53·7%) months (ANOVA, $P = 0{\cdot}73$). There was no significant difference in the average methane yield between the reactors operated in the summer (0·11–1·18 l.g VS_{added}^{-1}) and winter (0·25–0·77 l.g VS_{added}^{-1}) months (ANOVA, $P = 0{\cdot}60$).

DNA extraction

Samples were identified according to season, day of collection and source (between the two digesters): samples collected during the summer and winter were denoted with the letters S (Summer) and W (Winter), respectively; the numbers (0–60) were added according to the sampling days during anaerobic digestion after initial load. The letters (a and b) corresponded to each of the digesters where the fermentation was carried out. In total, 58 samples from the digesters were collected at different time points, referred as 2 anaerobic digesters influent (S0 and W0) and 56 anaerobic digesters effluent samples starting from the 4th day after initial load (S4 to S60 and W4 to W60).

Influent (S0 and W0) and effluent (S4 to S60 and W4 to W60) samples (20 ml) were collected using sterile bottles. Upon receipt, aliquots of 10–15 ml of each sample were ultrasonic-treated by sonication on ice for 1 min

(cycle, 1·0; amplitude, 100%) with a UP100H Sonicator (Hielscher Ultrasound Technology, Teltow, Germany). Further, 5-ml aliquots of each sample were centrifuged 15 min at 14 000 ***g***, at 4°C, and the pellet was kept for genomic DNA extraction using the QIAamp DNA Stool Mini Kit, according to the manufacturer's instruction (Qiagen, Heidelberg, Germany). The DNA concentrations were quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at −20°C until use.

Quantitative PCR (qPCR)

Quantitative PCRs were used to quantify the abundance of three selected AR genes (*erm*B, *aph*A2 and $bla_{\text{TEM-1}}$, associated with macrolide, aminoglycoside and beta-lactams resistance, respectively), in the effluent or influent DNA samples (Table 1). Plasmids containing the target AR genes were used as standard DNA templates for each of the qPCRs. The plasmids were decimally diluted to range from 10^9 to 100 copy numbers. All of the qPCR amplifications and quantifications were performed using a CFX96 Real Time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Reaction mixtures (25 μl) contained 12·5 μl Sso-Advanced SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 1 μl forward and reverse primers (0·2 mmol l^{-1}), 5 μl of template DNA with two appropriate dilutions (0·1 and 1 ng μl^{-1}) and 5·5 μl ultra-pure PCR grade water. The following thermal program was used: initial incubation of 2 min at 95°C and 40 cycles of denaturation at 95°C for 15 s, annealing at the given temperatures (Table 1) for 1 min and a final melt curve stage with temperature ramping from 65 to 95°C. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products. Then, results were compared with a standard curve to obtain the number of target copies in the sample. PCR efficiency of each gene ranged from 94·7 to 102·3% with R^2 value more than 0·99 for the calibration curves.

Quantification of the bacterial 16S rRNA gene was also performed using the universal bacterial primer set W208 (250 nmol l^{-1}), W209 (250 nmol l^{-1}) and probe W210 (50 nmol l^{-1}) as previously described (Yu *et al.* 2005). For total bacteria, the standard curves were generated by amplification of serial 10-fold dilutions of a reference clone (Snell-Castro *et al.* 2005).

Baseline and threshold calculations were performed with the CFX® Manager software 3.0 (Bio-Rad Laboratories, Inc). The presence of PCR inhibitors was checked by serially diluting select samples and comparing PCR efficiencies with standards. All qPCRs were done in triplicate for both the standards and the microbial community DNA samples. The total bacteria abundance, *erm*B, *aph*A2 and $bla_{\text{TEM-1}}$ were finally expressed as the number of target copies per gram of effluent or influent. The relative abundance was then calculated by dividing the absolute abundance of each AR gene by total bacteria absolute abundance in each sample.

Sequencing of 16S rRNA gene and pathogen identification

DNA extracts from the final samples (S60a, S60b, W60a and W60b) were selected for high-throughput sequencing analysis. A mixture of amplicons generated from F515 and R928 primers (Table 1) were sequenced on a 454 GS-FLX Titanium sequencer (Roche Life Sciences, Branford, CT, USA) at the Molecular Research Laboratory (Shallowater, TX). After sequencing, all failed sequence reads, low-quality sequence ends and tags were removed. Mothur software package (Schloss *et al.* 2009) was used to remove the redundancy of the tag sequences and select

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

Method	Target gene	Primer	Sequence 5′–3′	Annealing temp. (°C)	Reference
qPCR	*β*-Lactam resistance	$bla_{\text{TEM-1}}$	F: CATTTTCGTGTCGCCCTTAT R: GGGCGAAAACTCTCAAGGAT	58	Shi *et al.* (2013)
qPCR	Macrolide resistance	*erm*B	F: AGCCATGCGTCTGACATCTA R: CTGTGGTATGGCGGGTAAGT	55	Shi *et al.* (2013)
qPCR	Aminoglycoside resistance	*aph*A2	F: GATTGAACAAGATGGATTGC R: CCATGATGGATACTTTCTCG	55	Shi *et al.* (2013)
qPCR	16S rRNA—*Bacteria*	W208 W209 W210	F: ACTCCTACGGGAGGCAG R: GACTACCAGGGTATCTAATCC TaqMan: Yakima Yellow-TGCCAGCAGCCGCGGTAATAC-Tamra	60	Yu *et al.* (2005)
ePCR – pyrosequencing	16S rRNA—*Bacteria* and *Archaea*	515F 928R	F: GTG CCA GCM GCC GCG GTAA R: CCC CGY CAA TTC MTT TRA GT	–	Wang and Qian (2009)

unique tag sequences (each unique tag therefore represented different numbers of tag sequences). Briefly, flowgrams were quality-controlled and de-noised with the trim.flows, shhh.flows, trim.seqs and unique.seqs commands; aligned against the SILVA reference database (Pruesse *et al.* 2007) using align.seqs; and chimera-checked with the chimera.uchime command. Using the classify.seqs command, final sequence data set was classified considering the SILVA taxonomy distributed with Mothur. The number of sequences obtained was 17 531 from all samples. Operational taxonomic units (OTUs) were defined as group of sequences with a *P*-distance of 3% (or 97% similarity) succeeding construction of a distance matrix by commands dist.seqs and cluster.

OTUs were then taxonomically classified using BLASTn against our in-house created database of pathogenic 16S rRNA sequences. The 30 16S rRNA gene sequences analysed in this study included the sequences from known pathogenic bacteria. The 16S rRNA gene sequences corresponding to each species were retrieved from GenBank database. The most representative 16S rRNA gene was chosen for analysis according to the following standards: (i) strains with good phenotypic characterization; (ii) strains isolated as a source of contamination or infection in humans; and (iii) sequences with fewer undetermined bases.

For final classification, a bacterium species is defined as 'confidently identified by 16S rRNA gene sequencing' if there is >95% identify over an alignment and at least 249 bp assigned to the respective 16S rRNA gene sequence of the medically important bacteria species.

Nucleotide sequence accession numbers

Sequence data associated with this study are deposited in GenBank's Short Read Archive (SRA) under the accession number SRP041213.

Data analysis

To report an average performance of anaerobic digestion processes in both seasons, multiple sampling events were treated as replicates. Averages and standard errors of all data were determined. The treatment data of *erm*B, *aph*A2 and $bla_{\text{TEM-1}}$ gene abundance were first $\log_{10}$-transformed to improve normality and then analysed by one-way analysis of variance (ANOVA) procedure for independent samples to test for statistically significant differences. A *P*-value of <0·05 was considered to indicate significance. Co-occurrence of different AR genes was examined based on the association between the relative abundance of two gene classes by Pearson product-moment correlation coefficient.

Results

The dynamics of AR genes in influent and effluent samples from duplicate anaerobic digesters were investigated over a 60 days of fermentation in two different seasons (summer and winter), and in the final samples, pathogenic bacteria were searched using 16S rRNA sequencing data. For each samples during anaerobic process, the number of *erm*B, *aph*A2 and $bla_{\text{TEM-1}}$ gene copies per gram of influent or effluent was determined from the average of the two replicas operated during each season. The antibiotics removal efficiency was assessed by considering influent (day 0) and effluent (day 60) values. When all AR genes were normalized to 16S rRNA genes, relative abundance, the reductions in the different anaerobic processes on AR genes, maintained a remarkably similar trend.

Dynamics of the total bacteria

To compare absolute reductions of AR genes, gene quantities were compared with the absolute abundance of total bacterial 16S rRNA gene. All results were similar from each replica, in both digesters operated in the summer (ANOVA, $P = 0{\cdot}999$) as in the two operated in winter (ANOVA, $P = 0{\cdot}809$). For each samples during anaerobic process, the concentration of bacterial copies per gram of effluent or influent was carried out from the average of the two replicas operated during each season. The mean numbers of 16S rRNA gene copies were $2{\cdot}8 \times 10^9$ and $6{\cdot}4 \times 10^9$ in the summer and winter samples, respectively. No significant differences in abundances were found (ANOVA, $P = 0{\cdot}700$) in different seasons, suggesting that, independent of the season, a similar dynamic and abundance were obtained during the 60 days of fermentation.

Dynamic of macrolides resistance genes

The presence of *erm*B genes throughout the anaerobic digestion process in different seasons was evaluated, and the patterns observed in different time points are shown in Fig. 1. The influent contained substantial quantities of *erm*B genes, concentration of approx. 10^6 gene copies per gram, and they were similar in both seasons. The dynamics of *erm*B in both seasons exhibited a similar pattern, decreasing in a time-dependent manner. During the summer and winter months, the abundance of *erm*B was nearly 1-log-cycle lower in the effluent after 60 days of fermentation than the influent (samples of the first day). 93·9% of *erm*B were reduced in the anaerobic process in the summer and 84·0% in the winter months.

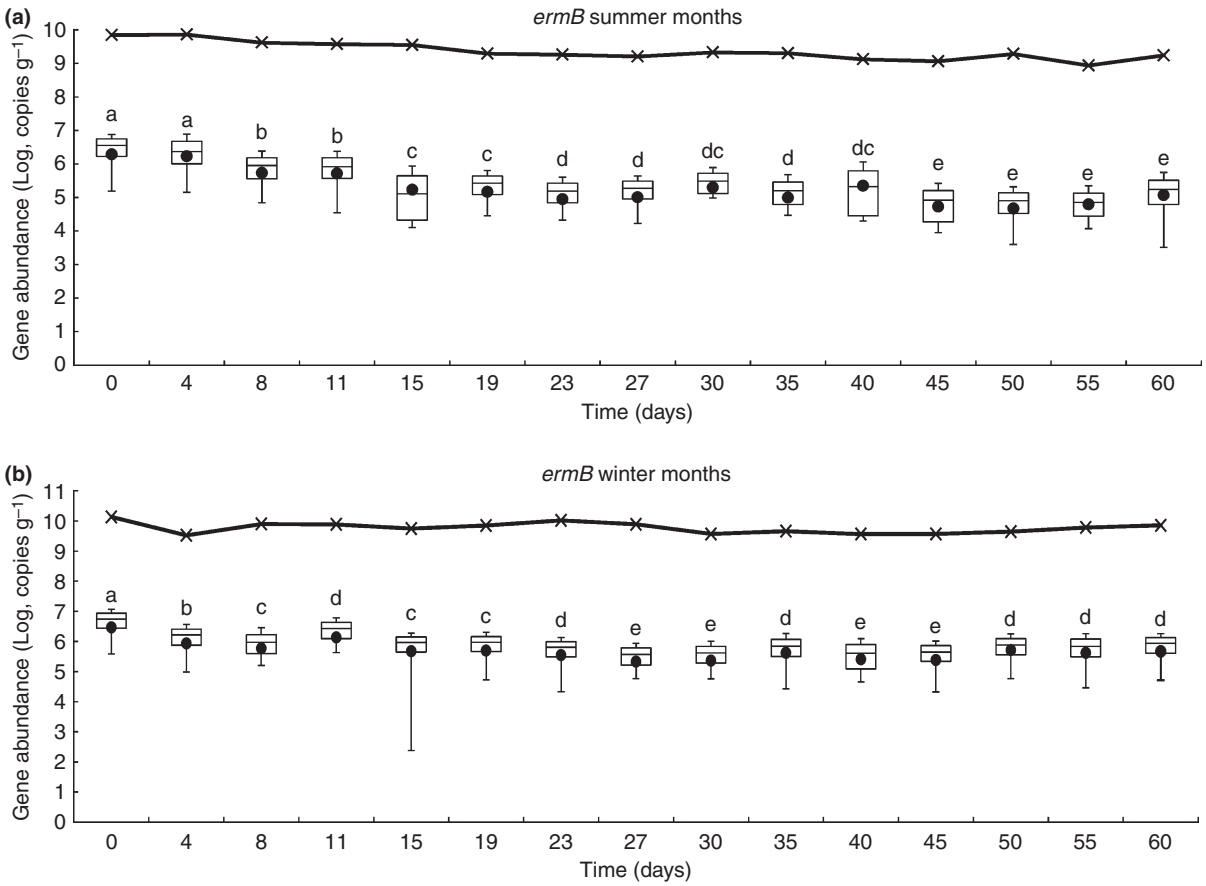

Figure 1 Box and whisker plot of absolute abundance of *erm*B gene during the anaerobic digestion in the summer (a) and winter months (b). All *erm*B data are expressed as $\log_{10}$ copies per gram of influent or effluents samples. Errors bars indicate maximum and minimum values; horizontal lines indicate median values; and boxes indicate values between the 25th and 75th percentiles. Different letters designate statistical significance ($P < 0{\cdot}05$). Values of symbols (•) and (×) indicate *erm*B mean and operation time variation of 16S rRNA mean, respectively.

Dynamic of aminoglycosides resistance genes

The quantities of *aph*A2 genes in different samples of anaerobic digestions process with varied temperatures are compared in Fig. 2. The abundance of *aph*A2 genes in influents not varied between the summer and winter anaerobic systems (nearly 10^4 gene copies). In winter months, the absolute abundance of *aph*A2 did not decrease significantly over the first 30 days of fermentation, and the decrease thereafter was smaller than observed for summer months. In the last samples (day 60), the concentrations were nearly 10^2 and 10^3 gene copies in the summer and winter, respectively. In the summer months, the removal rate was nearly 2·2-log-cycle, whereas in the winter months, it was 1-log-cycle reduction.

Dynamics of beta-lactams resistance genes

Quantitative changes of $bla_{\text{TEM-1}}$ in the different anaerobic reactors are shown in Fig. 3. In the initial samples, $bla_{\text{TEM-1}}$ was nearly 10^7copies per gram samples. In the summer conditions, the high quantities of $bla_{\text{TEM-1}}$ persisted well during 15 days and after that reduced in response to anaerobic digestion process, resulting in nearly 10^4 gene copies on day 60 (2·2-log-cycle reduction). In the winter months, there was also a slight decrease in the quantification of $bla_{\text{TEM-1}}$, resulting in nearly 10^4 gene copies on day 60 (0·84-log-cycle reduction).

Correlations and comparison between gene copy concentrations

In the initial samples, compared with the other genes evaluated, *aph*A2 genes were detected at the lowest abundance and $bla_{\text{TEM-1}}$ was the most abundant. Comparisons between winter and summer effluent samples showed that all results of quantification indicated an interaction between seasons' patterns affected the resistance determinant.

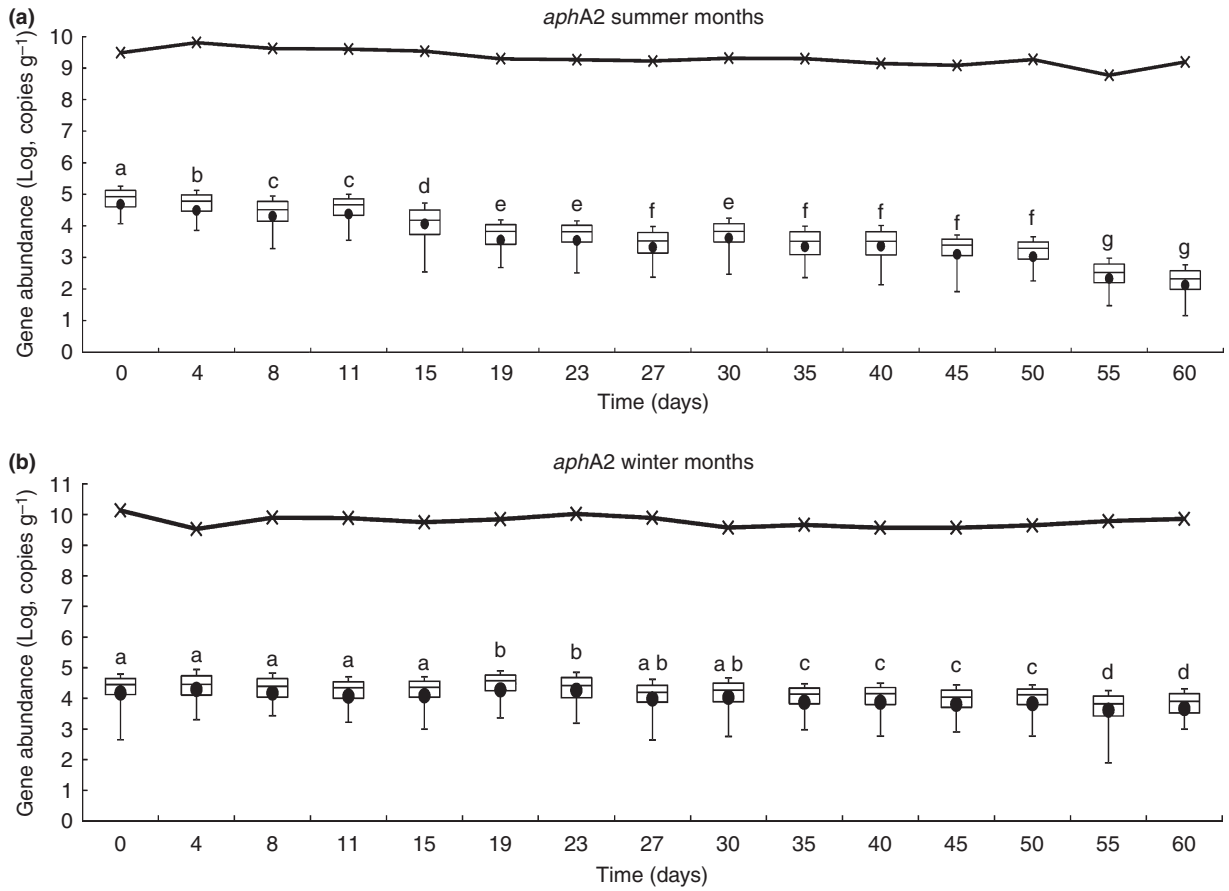

Figure 2 Box and whisker plot of absolute abundance of *aph*A2 gene during the anaerobic digestion in the summer (a) and winter months (b). All *aph*A2 data are expressed as $\log_{10}$ copies per gram of influent or effluents samples. Errors bars indicate maximum and minimum values; horizontal lines indicate median values; and boxes indicate values between the 25th and 75th percentiles. Different letters designate statistical significance ($P < 0{\cdot}05$). Values of symbols (●) and (×) indicate *aph*A2 mean and operation time variation of 16S rRNA mean, respectively.

Correlations with the different AR genes were examined by calculating the Pearson correlation coefficient between the *erm*B, *aph*A2 and $bla_{\text{TEM-1}}$ genes among all the influent and effluent samples evaluated (Table 2). The number of copies of all resistance genes was normalized to the basal level of 16S rRNA genes to minimize the variance caused by differences in background bacterial abundances. Positive correlation coefficient between AR genes tends to changes in abundance in the same direction, while a negative correlation coefficient indicates otherwise (Wang *et al.* 2012).

Among the relative abundance of all AR genes evaluated, all were positively correlated in both conditions. The strongest correlations observed were between *aph*A2 and $bla_{\text{TEM-1}}$ in summer months. Only a weak positive correlation between *aph*A2 and *erm*B in the winter month process was also observed (0·495).

Putative pathogens sequences

To identify possible pathogens sequences within each library in both seasonal conditions, BLAST analyses against our in-house created database of 30 pathogenic 16S rRNA sequences were accomplished. With the purpose of comparing the microbial structure among these samples, operational taxonomic units (OTUs) were determined for each sample at distance levels of 3%. A total of 17 531 sequences were classified into 1710 OTUs (1676 OTUs for the domain *Bacteria*; 34 OTUs for the *Archaea*) based on BLASTn results.

The sequences that were within a 95% similarity of a list of 30 known pathogen sequences were counted as potential pathogens and are presented in Table 3. Microbial identification through 16S rRNA genes showed persistence of these bacteria in final samples in both seasons. Among the total number of sequences obtained for the

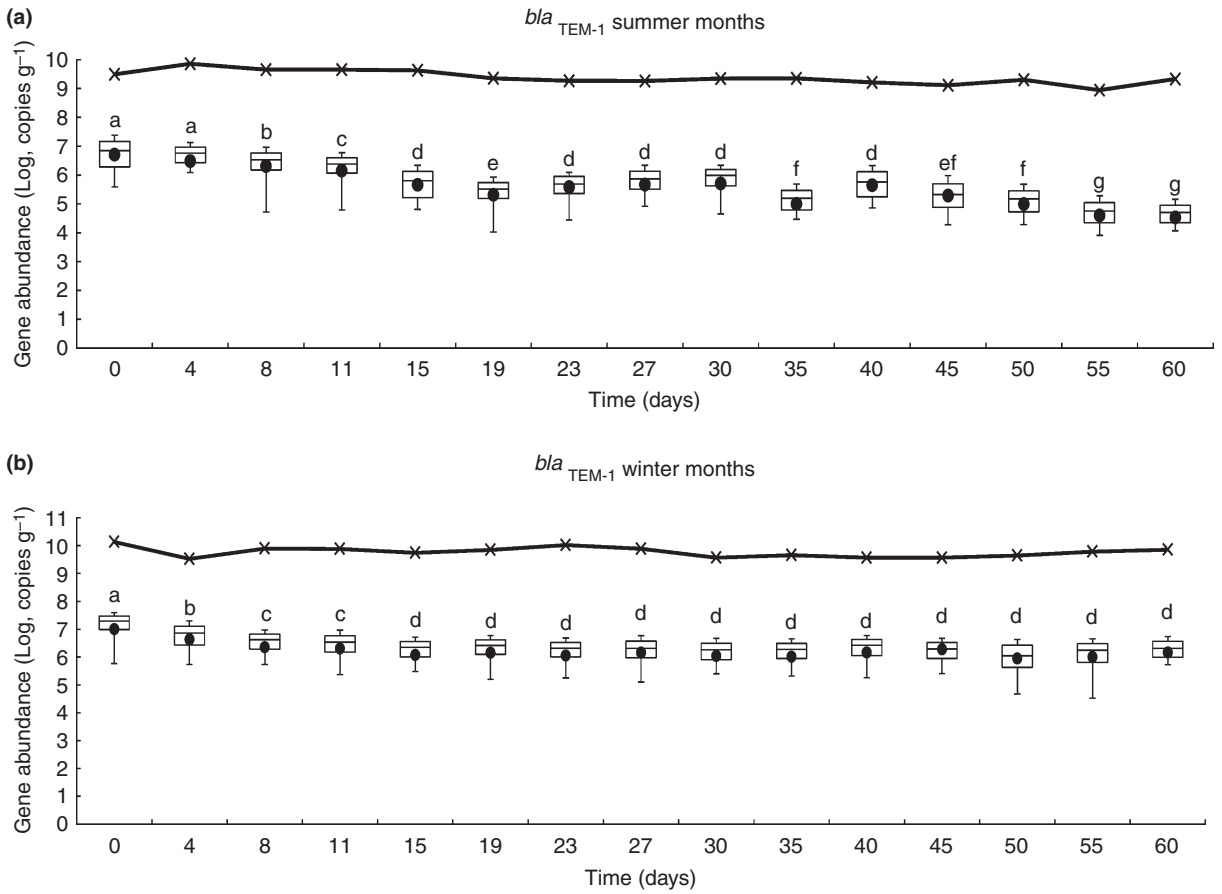

Figure 3 Box and whisker plot of absolute abundance of bla_{TEM-1} gene during the anaerobic digestion in the summer (a) and winter months (b). All bla_{TEM-1} data are expressed as $\log_{10}$ copies per gram of influent or effluents samples. Errors bars indicate maximum and minimum values; horizontal lines indicate median values; and boxes indicate values between the 25th and 75th percentiles. Different letters designate statistical significance ($P < 0{\cdot}05$). Values of symbols (●) and (×) indicate bla_{TEM-1} mean and operation time variation of 16S rRNA mean, respectively.

Table 2 Pearson correlation coefficient between relative abundance of different resistance (AR/16S-rRNA genes) in all anaerobic digestion samples

	*erm*B	*aph*A2	bla_{TEM-1}
*erm*B	1	0·495*	0·803*
*aph*A2	0·803†	1	0·720*
bla_{TEM-1}	0·812†	0·960†	1

*The top right triangular matrix is based on the relative abundance of winter months' anaerobic digestion samples.
†The bottom left triangular matrix is based on the relative abundance of summer months' anaerobic digestion samples.

different reactors, only about 0·73–1·38% in the summer and 2·59–2·60% in the winter samples represent sequence of putative pathogens.

Of the 30 pathogen sequence considered, only 16 different pathogens were identified within the databases by sequence similarity. Gram-positive, such as *Clostridium* and *Bacillus*, and Gram-negative, such as *Acinetobacter baumannii* and *Stenotrophomonas*, were the most identified bacteria in the final samples.

All results were similar from each replica, in both digesters operated in the summer (ANOVA, $P = 0{\cdot}191$) as in the two operated in winter (ANOVA, $P = 0{\cdot}993$). No significant differences in abundances of pathogens sequences were found between summer and winter samples (ANOVA, $P = 0{\cdot}463$), suggesting that independent of the season, all reactors obtained a similar abundance of pathogens after 60 days of fermentation.

Discussion

According to the literature, it is important to highlight the sanitary safety during the anaerobic digestion, especially if its final products, that is anaerobic digestion effluent (biofertilizer), are intended for environmental use (Walsh *et al.* 2012). As cattle manure is considered a reservoir for potentially pathogenic and antimicrobial resistant bacteria, a safe load reduction for these micro-organisms or its

Table 3 Percentage of sequences belonging to selected pathogenic bacterial 16S rRNA evaluated during the anaerobic digestion in final samples of the summer and winter months anaerobic digestion

Species (NCBI sequence identification)	% of total			
	S60a	S60b	W60a	W60b
Acinetobacter baumannii ATCC 17978 (NC_009085)	0·19	0·02	1·72	0·61
Burkholderia cepacia (GQ359110)	ND	ND	ND	ND
Bacillus anthracis str. H9401 (NC_017729)	0·15	0·02	0·22	0·41
Bacteroides fragilis 638R (FQ312004)	ND	ND	0·02	ND
Campylobacter jejuni subsp. *jejuni* (CP001900)	ND	ND	ND	ND
Citrobacter freundii ATCC 8090 (ANAV01000042)	ND	ND	ND	ND
Clostridium botulinum NCTC 2916 (NZ_ABDO02000001)	ND	ND	0·02	ND
Clostridium difficile CD196 (NC_013315)	0·17	0·30	0·13	0·64
Clostridium perfringens (NZ_ABDY01000045)	0·04	0·02	0·02	0·03
Clostridium tetani E88 (NC_004557)	ND	ND	ND	ND
Corynebacterium diphtheriae HC01 (NC_016786)	0·04	0·07	0·09	0·47
Enterococcus faecalis ATCC 4200 (NZ_GG670380)	ND	0·07	0·07	0·12
Enterococcus faecium (AY172570)	ND	0·07	0·07	0·12
Escherichia coli ATCC 8739 (NC_010468)	ND	ND	ND	ND
Fusobacterium nucleatum subsp. *nucleatum* ATCC 25586 (NC_003454)	ND	ND	ND	ND
Haemophilus influenzae 86-028NP (NC_007146.2)	ND	ND	ND	ND
Klebsiella pneumoniae pneumoniae HS11286 (CP003200)	ND	0·05	ND	ND
Listeria monocytogenes (S55472.1)	ND	ND	ND	ND
Mycobacterium tuberculosis F11 (NC_009565.1)	ND	ND	ND	ND
Pseudomonas aeruginosa NCGM2.S1 (AP012280)	0·04	ND	ND	ND
Neisseria meningitidis M01-240149 (CP002421)	ND	ND	ND	ND
Salmonella enterica subsp. *enterica* serovar *choleraesuis* str. SC-B67 (NC_006905.1)	ND	0·05	ND	ND
Staphylococcus aureus subsp. *aureus* (NZ_GG730226)	ND	ND	ND	ND
Staphylococcus epidermidis RP62A (NC_002976)	ND	ND	ND	ND
Stenotrophomonas maltophilia K279a (NC_010943)	0·10	0·02	0·24	0·20
Streptococcus agalactiae GD201008-001 (CP003810)	ND	0·23	ND	ND
Streptococcus pneumoniae ATCC 700669 (NC_011900)	ND	0·23	ND	ND
Streptococcus pyogenes MGAS5005 (NC_007297)	ND	0·23	ND	ND
Vibrio cholerae O1 *biovar eltor* str. N16961 (NC_002505)	ND	ND	ND	ND
Yersinia enterocolitica subsp. *enterocolitica* (NC_008800)	ND	ND	ND	ND
Total percentage	0·73	1·38	2·59	2·60
Total sequence	5183	4338	4594	3416

ND, not detected.

transferable genes figures as one of the sanitary concerns regarding the technological use of anaerobic digestion (Sahlström *et al.* 2004; Eikmeyer *et al.* 2013).

With regard to the antimicrobial resistance phenomenon, there are several calls on the impacts of antimicrobial use in both human and veterinary medicine (Heuer *et al.* 2011). In an attempt to access such sanitary risks, genetic markers representative of those associated with antimicrobial resistance commonly used in both human and veterinary medicine were chosen (Nero *et al.* 2007; Fonseca *et al.* 2009; Pribul *et al.* 2011). The gene *erm*B is related to macrolides, such as erythromycin, clarithromycin and azithromycin, besides lincosamide and streptogramin resistance; *aph*A2 is related to the resistance against aminoglycosides such as amikacin, gentamicin, kanamycin, neomycin, streptomycin and tobramycin; and $Bla_{\text{TEM-1}}$ is related to the resistance against penicillins such as benzylpenicillin and amoxicillin, and cephalosporins such as cephalothin, cefazolin and cefoxitin (Syrogiannopoulos *et al.* 2003; Ramirez and Tolmasky 2010; Salverda *et al.* 2010).

Thus, to contribute to the understanding on the persistence of AR genes and putative pathogenic bacteria in cattle manure anaerobic digestion operating at ambient temperature, this study investigated the influence of the seasonal patterns either in summer (average temperature 29·5°C) or in winter (average temperature 19·5°C) and operation time (during 60 days of fermentation) on AR genes load, and in the final samples, pathogenic bacteria were searched using 16S rRNA sequencing data.

Copies of 16S rRNA genes displayed a similar trend in both anaerobic processes (summer and winter) during the 60 days of fermentation. The mean concentrations of 16S rRNA in both seasons were on the same order as

values, and this result is in accordance with other studies which observed total bacterial load during anaerobic digestion (Alexander *et al.* 2011).

Analysis of the occurrence and abundance of AR genes showed that $bla_{\text{TEM-1}}$, *aph*A2 and *erm*B were prevalent in the influent and effluent samples of anaerobic digestion. Plasmid-mediated *erm*B genes generally confer resistance to macrolide, lincosamide and streptogramin and have been commonly detected in cattle environments (Chen *et al.* 2007; Negreanu *et al.* 2012). Previous studies also observed the occurrence of $bla_{\text{TEM-1}}$ and *aph*A2 in intestinal bacteria and in the environment (Shi *et al.* 2013). All these genes have been reported as highly transferable among both Gram-negative and Gram-positive bacteria (Roberts 2008; Lachmayr *et al.* 2009).

Comparatively, the genes showed different initial abundance, and $bla_{\text{TEM-1}}$ and *erm*B were in higher amounts in the influent. These observations support previous research showing that resistance to these antibiotics is widespread and highlight persistent offering selective pressure in these wastes, resulting in drug-resistant bacteria recover, even if during animal management, antibiotics had never been used (Alexander *et al.* 2008; Heuer *et al.* 2011; Munir and Xagoraraki 2011). The spread of genes encoding for antimicrobial resistance among ruminant microbiota and manure bacteria is potentially relevant. As an adaptive consequence, the genetic mobility of such genes warrants the bacteria survivor, playing a role as a long-term reservoir of antibiotic resistance determinants (Ghosh and LaPara 2007).

The results revealed that ambient temperature anaerobic process was effective in reducing the initial load of AR genes. More detailed analysis of the AR gene dynamics showed that the genes were reduced to lower amounts and declined over time in the digesters. Thus, the abundance and removal of AR genes varied along fermentation process probably by seasons and operation time patterns. Such differences could outcome the differences in the survival ability of host bacteria (Wang *et al.* 2012). Only few studies have been conducted to quantitatively assess the effects of ambient temperature anaerobic digestion on AR genes in cattle manure. However, it has been stated that temperature and retention time would be critical parameters in bacterial survival process (Sahlström 2003). This probably would explain the slight efficacy of summer digestion in reducing AR genes. Furthermore, as shown by Ma *et al.* (2011), gene transfer might have been hindered by increase in temperature because microbial processes during anaerobic digestion may result in extracellular DNA damage, through hydrolysis and biodegradation. It is important to highlight that 16S rRNA levels were stable during the fermentation time. As such, the reduction in AR genes in both seasons is not referred to the overall decrease in total bacteria.

Further to support the above hypothesis, a correlation analysis was performed. The data show positive correlation on the interactions between relative abundance of each gene evaluated in different season. Thus, ambient temperature anaerobic digestion might have different implications for different AR genes in both conditions, and the specific nature of these relations needs to be better addressed in further studies (Nõlvak *et al.* 2013).

To access eventual interactions between detection of resistance genes in digester effluent samples and occurrence of medically important micro-organisms, potentially pathogenic bacteria were screened in the digester effluents by metagenomic analyses. Microbial identification through 16S rRNA genes showed persistence of these bacteria in final samples in both seasons. Slight increases in the relative abundance of pathogens (% of total) were identified in the winter than in summer samples analysed, but no significant difference was found. This result might indicate the discrete influence of temperature on the change in the community bacteria. However, several factors including system operation time may affect the amount of certain species. For further discussion of this hypothesis, studies should be conducted with higher number of digesters. Yet, Massé *et al.* (2011) indicated that temperature could diversify the abundance of bacteria pathogens in animal manure anaerobic digestion.

Gram-positive, such as *Clostridium* and *Bacillus*, and Gram-negative, such as *Acinetobacter* and *Stenotrophomonas*, were the most identified bacteria in the final samples. It is recognized from other studies that these bacteria are ubiquitous and opportunistic pathogens. Similar results previously reported showed that lowering these pathogens load is a major concern for any subsequent use of anaerobic digester effluents as biofertilizers (Watcharasukarn *et al.* 2009; Eikmeyer *et al.* 2013). Add to that, these results show that only small proportions were identified as pathogens. However, it has to be considered that the evaluated reactors were continuously fed with cattle manure. Hence, the pathogenic and AR genes load would not be reduced to the undetectable amounts.

Using real-time quantitative PCR to quantify AR genes, we may not evidence or discuss on the microbial viability, and whether the detected genetic markers are actually functional. On the other hand, the methodological approach used may have several advantages because it is based on genotype, and allows discussion on the presence of different genetic markers which might be available for vertical or horizontal transfer in the environment (Diehl and LaPara 2010; Burch *et al.* 2013).

Considering the digestion at mesophilic range presented, the AR removal and prevalence of bacteria pathogens did not follow a simple or stabilization trend with ambient temperature variation (summer and winter). Considering

the practice of spreading of effluents from digesters into land used for the agriculture production, the presence of AR genes or putative pathogenic micro-organisms may lead to sanitary and ecological risks (Resende *et al.* 2014).

The data presented in this study raise significant concerns and information highly applicable to support a safe technological handle of anaerobic digestion using cattle manure as substrate. Even with the narrow temperature range, AR gene copy numbers were reduced form initial load and the detection of medically important bacteria did not vary between summer and winter seasons. The functional viability and persistence in both bacteria and AR genes remains in the open environment still remains unclear. Further prospective and quantitative studies should address the extent of the antimicrobial resistance phenomena in effluents management with implications for human, animal and environmental health.

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Conflict of interest

No conflict of interest declared.

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