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Prevalence and persistence of potentially pathogenic and antibiotic resistant bacteria during anaerobic digestion treatment of cattle manure

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HIGHLIGHTS

- Sustainable handling of anaerobic digestion and their effluents are needed.
- Effluents from biodigesters are widely spread into land used for the agriculture.
- Putative pathogenic bacteria persist after anaerobic digestion of cattle manure.
- Antimicrobial-resistant bacteria are prevalent in the effluents from biodigesters.
- Medically important bacteria imposes sanitary risks to the anaerobic digestion.

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ABSTRACT

Anaerobic digestion figures as a sustainable alternative to avoid discharge of cattle manure in the environment, which results in biogas and biofertilizer. Persistence of potentially pathogenic and drug-resistant bacteria during anaerobic digestion of cattle manure was evaluated. Selective cultures were performed for enterobacteria (ENT), non-fermenting Gram-negative rods (NFR) and Gram-positive cocci (GPC). Antimicrobial susceptibility patterns were determined and a decay of all bacterial groups was observed after 60 days. Multidrug-resistant bacteria were detected both the influent and effluent. GPC, the most prevalent group was highly resistant against penicillin and levofloxacin, whereas resistance to ampicillin, ampicillin-sulbactam and chloramphenicol was frequently observed in the ENT and NFR groups. The data point out the need of discussions to better address management of biodigesters and the implementation of sanitary and microbiological safe treatments of animal manures to avoid consequences to human, animal and environmental health.

1. Introduction

As long as the need for increased food production leads to the dairy industry growth, environmental concerns related to cattle manure management, which includes residues discharge into soil and aquatic ecosystems favoring the spread of putative pathogenic microorganisms are pointed out (Sahlström, 2003; Venglovsky et al., 2009).

In this regard, prospective studies towards new strategies for safe disposal of large quantities of cattle manure should take into account the sanitary and microbiological risks (Karim et al., 2005). To avoid direct discharge of manure into soil and aquatic ecosystems anaerobic digestion is pointed out as a sustainable alternative resulting in production of biogas and biofertilizer, whilst reducing the microbial load of the surrounding environments (Bagge et al., 2005; Saunders et al., 2012).

However, cattle farming is frequently referred as a reservoir for potentially pathogenic and antimicrobial resistant bacteria or also, reservoir of antibiotic resistance genes (Munir and Xagoraraki, 2011; Thames et al., 2012). To increase production related to prophylaxis, infectious diseases treatment and/or growth promoters, antimicrobial drugs are widely applied in animal husbandry (Heuer et al., 2011). As an ecological consequence, the presence of zoonotic pathogens in the environments and unintentional selection of bacteria that are resistant to antibiotics could have important human and animal health consequences, mainly when

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end up in agricultural lands (Walczak and Xu, 2011; Costa et al., 2013).

As long as anaerobic digestion has been considered an attractive method to promote a clean fuel from renewable feed stocks, such as animal manure, to develop a well-established technology, the optimization of anaerobic digestion processes requires effective operative control and possible correlation with reduction of pathogens (Holm-Nielsen et al., 2009).

In Brazil, as the most part of the country is located in the tropical region, the climatic conditions are mostly in the mesophilic range, and the majority of the anaerobic digesters are operated at ambient temperature followed by land application of the effluent (Kunz et al., 2009). In this regard, recycling of cattle manure at ambient temperature, i.e. production of energy and fertilizer from anaerobic digestion, would be of commercial and environmental interests, and no literature is available considering sanitary and ecological safety. Indeed, it is already reported concerns on the persistency of potentially pathogenic and antimicrobial-resistant bacteria during biogas and biofertilizers production, considering other anaerobic digestion models, but at constant temperatures (Beneragama et al., 2012).

In this regard, this study was focused on the evaluation of persistence of clinically relevant bacteria and their susceptibility patterns to antimicrobial drugs during anaerobic digestion effluents in continuous pilot-scale biodigesters, to accesses the sanitary risks of the process concerning human, animal and environmental health.

2. Methods

2.1. Pilot-scale biogas reactor and sample collection

Four experimental continuous biodigesters operating at ambient temperatures, with a 60 day retention time, and 60 L working volume were used. Fresh dairy cattle manure was collected from the experimental Embrapa dairy cattle field located in Coronel Pacheco city, Minas Gerais state, Brazil. The biodigesters was fed daily with influent of dairy cattle manure mixed with cattle wastewater (final total solids concentration 3–4%).

Temperature was measured by using an ordinary mercury thermometer during the sampling. Total solids (TS), volatile solids (VS), and pH of influent and effluent samples were measured according to standard methods (APHA, 2005). Biogas produced was measured every week by gas chromatography (Agilent Technologies, 7820A). All measurements were done in triplicate and the averages were taken as representative values.

In total, 58 samples from the biodigesters were collected at different times, referred to as the influent ($n = 2$, before feeding the biodigesters), and effluent ($n = 56$, samples were taken every 3–5 days during the digestion). Influent and effluent samples (20 mL) were collected using sterile bottles between January–March (2012) and May–June (2012). All samples were brought to the laboratory and processed within 1 h after collection.

2.2. Microbiological quantitative methods

For bacterial counts, influent (1st day) and effluent samples (15th, 30th and 60th days) were collected and 10-fold serial diluted up to 10^{-8} in sterile saline solution (0.9% NaCl). Aliquots of 0.1 mL of each dilution were submitted to selective culture in different culture medium. The lowest dilution that produced microbial counts between 20 and 200 colonies was used to estimate the number of bacteria in the samples. The Gram positive cocci *Enterococcus* spp. were evaluated on Bile Esculin Agar (Himedia Laboratories, India) and *Staphylococcus* spp. on Hypertonic Manitol Agar (Himedia Laboratories, India) after incubation at 35.5 °C for 24 h. The Gram-negative bacteria were evaluated on Eosin-Methylene Blue Agar (Himedia Laboratories, India) after incubation at 37 °C for 24 h, and lactose fermenting (pink, purple or green metallic) and non-fermenting (colorless) colonies were counted. The experiments were performed in duplicates and results were expressed as mean bacterial counts.

2.3. Isolation and identification of bacterial samples

From the selective cultures for enterococci, staphylococci and Gram negative rods, three to five representative colonies were selected and sub-cultivated in Brain–Heart Infusion Agar (Himedia Laboratories) for stock by freezing and further experiments. For *Streptococcus* spp. isolation, the collected influent and effluent samples 10-fold serial diluted were streaked on sheep blood agar plates (Brain Heart Infusion supplemented with 5% of sheep blood) and incubated in a capnophilic atmosphere (5% CO_2). After incubation (18–48 h, 37 °C), pin point white colonies were selected.

The Gram positive cocci (staphylococci, enterococci and streptococci) were presumptively identified by morphotinctorial characteristics after Gram staining, as well as the ability to hydrolyze esculin, produce catalase and presence of zone of hemolysis. Species identification was performed using the commercial system BBL Crystal Rapid Gram-Positive ID Kit (Becton & Dickinson, USA), according to the manufacturer's instructions.

The Gram-negative bacteria were presumptively identified by morphotinctorial characteristics after Gram staining, as well as the ability of glucose, sucrose and lactose fermentation, oxidase and motility tests. Species identification was performed using API 20E (Bio Mérieux AB, Marcyl L'Etoile, France), according the manufacturer's instructions.

2.4. Antimicrobial susceptibility assays

The minimum inhibitory concentrations (MIC) for antimicrobial drugs were determined by the agar dilution method, according to the Clinical and Laboratory Standard Institute guideline (CLSI, 2012). Antibiotic stock solutions were added to melted Mueller–Hinton (Himedia) agar to obtain final concentrations ranging from 0.06 to 1024 μg mL^{-1}. The antimicrobial drugs were selected on the basis of microbial characteristics and clinical relevance as follows: (i) for Gram positive cocci catalase-positive (GPC/C+), penicillin (MedQuimica, Brazil), oxacillin (MedQuimica), vancomycin (MedQuimica), ampicillin-sulbactam (Cellofarm, Brazil), rifampin (Sigma Aldrich, USA), levofloxacin (Sigma Aldrich), trimethoprim-sulfamethoxazole (MedQuimica) and erythromycin (Sigma Aldrich); (ii) for Gram positive cocci catalase-negative (GPC/C−), penicillin, vancomycin, rifampin, levofloxacin and erythromycin; (iii) for Gram negative rods *Enterobacteriaceae* (ENT), ampicillin (Cellofarm), ampicillin-sulbactam, piperacillin-tazobactam (Novafarma, Brazil), cefepime (Biochimico, Brazil), meropenem (Biochimico), gentamicin (Novafarma, Brazil), amikacin (Teuto-Brasileiro Laboratorio, Brazil), levofloxacin, trimethoprim-sulfamethoxazole and chloramphenicol; and (iv) for non-fermenter Gram negative rods (NFR), piperacillin-tazobactam, cefepime, gentamicin, amikacin, trimethoprim-sulfamethoxazole, meropenem, levofloxacin and chloramphenicol.

The reference strains *Enterococcus faecalis* ATCC 51299, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were included as controls in the antimicrobial susceptibility assays for Gram-positive or Gram-negative bacteria and all tests were performed in duplicate. Using CLSI guidelines, the isolates were classified as sensitive, intermediate, or resistant to the tested antimicrobial agents (CLSI, 2012).

To determine the level of antibiotic resistance of the individual isolated bacteria, the multiple antibiotic resistance (MAR) index

was calculated by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics to which the isolates were exposed as previously described (Krumperman, 1983). A MAR value >0.2 was indicative of multiple antibiotic-resistant bacteria.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test were applied to compare production of gas and the distribution of different bacterial groups in different biodigesters. Student's *t*-test was used for comparison of the bacterial reduction rates. The significance level was set as $p < 0.05$. The microbial counts were converted to the logarithm of the number of colony forming units per mL of biowaste samples (log CFU mL^{-1}).

3. Results and discussion

3.1. Pilot-scale biogas reactor performance

Over the 60 days of hydraulic retention time in which influent of dairy cattle manure was fed, the pH value of the cattle manure (influent) was between 6.55 and 6.86 and ambient temperature was in the mesophilic range (between 25 and 35 °C). The pH was between 6.95 and 7.41 during the study. The percentage reduction in TS of fermenting cattle manure was about 65% and VS was 68%. The average daily biogas produced was 17 L/day, with a methane composition above 44.06–70.27%. There was no significant difference in methane composition among the pilot-scale digesters ($p = 0.499$).

Overall, there are few reports available to compare these results, considering anaerobic digestion of animal manure under ambient temperature at mesophilic range. It had already been shown that methane production by anaerobic digestion using animal manure as substrate, followed the same pattern in both mesophilic (35 °C) or ambient (16.8–29.5 °C) temperatures (Alvarez et al., 2006). Add to that, these data shows a similar methane production rate if compared to other studies with mesophilic bioreactors using, also, animal manure (Rico et al., 2007; Chae et al., 2008).

3.2. Bacterial prevalence during anaerobic digestion

The persistence of putative pathogenic bacteria such as Gram positive cocci or Gram negative rods justifies themselves the sanitary risks and microbiological relevance of the sustainable handling of anaerobic digestion and their effluents. From an ecological perspective, the occurrence of antimicrobial resistant bacteria calls to the spread and impacts of antimicrobial resistance both in human, animal and environmental health (Holm-Nielsen et al., 2009; Venglovsky et al., 2009; Costa et al., 2013).

The quantitative analysis for the viable microbial counts is presented in Fig. 1. During the experimental period, total viable count of GPC/C+ on Manitol Agar ranged from 2.65×10^5 CFU mL^{-1} in the influent (1st day) to 1.57×10^3 CFU mL^{-1} in effluent after 60 days of fermentation (99.40% reduction, $p = 7.9 \times 10^{-8}$). Throughout the evaluated time, decrease in GPC/C+ counts were observed between the initial load and 30 days ($p = 0.0001$), and between 30 and 60 days of anaerobic digestion ($p = 0.001$). The bacterial counts of GPC/C− on Bile Esculin Agar varied between 3.71×10^5 and 1.57×10^4 CFU mL^{-1} (95.76% reduction, $p = 0.0001$) and CFU reduction were observed between initial load and 15 days ($p = 0.0008$), and between 30 and 60 days of anaerobic digestion ($p = 0.0495$). The average ENT count on Eosin-Methylene Blue Agar ranged from 4.42×10^8 to 1.45×10^5 CFU mL^{-1}, while NFR varied between 5.43×10^8 and 9.44×10^5 CFU mL^{-1}, with average reduction of 99.96% ($p = 1.1 \times 10^{-11}$) and 99.82% ($p = 1.8 \times 10^{-8}$), respectively. From the initial load, bacterial counts considering these microbial groups (ENT and NFR) were observed only if compared to 15 days of anaerobic digestion ($p = 2.5 \times 10^{-12}$ and $p = 8.8 \times 10^{-9}$, respectively). Up to 60 days no significant bacterial counts were observed (ENT: $p = 0.166$, and NFR: $p = 0.152$, respectively). Overall, the reduction rates considering all the microbial groups from effluent samples were significantly different from influent samples ($p = 0.029$).

The observed GNR and enterococci densities in cattle manure (influent) were comparable to previously reported values which suggested 10^5–10^8 fecal organisms per gram (Walczak and Xu, 2011; Dungan et al., 2012).

The decay rate of viable bacteria after the period of anaerobic digestion of cattle manure is dependent on several factors, mainly,

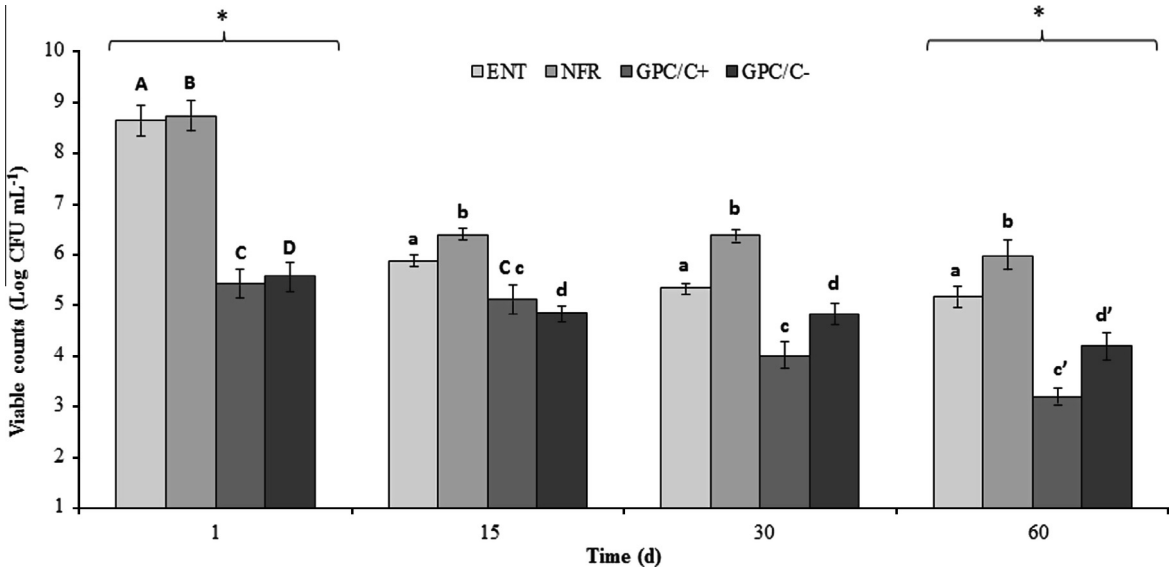

Fig. 1. Mean value of viable microbial counts (log CFU mL^{-1}) of initial load (1st day), and effluent samples (15th, 30th and 60th days), times 15–60, from pilot-scale anaerobic digesters. ENT: Gram-negative rods from the *Enterobacteriaceae* family; NFR: non-fermenting Gram-negative rods; GPC/C+: Gram-positive cocci/ Catalase-positive; GPC/C-: Gram-positive cocci/ Catalase-negative. Different letters including lowercase or uppercase letters and "*" indicate statistically significant differences ($p < 0.05$).

bacterial biology, and characteristics of anaerobic digestion including temperature and retention time (Smith et al., 2005; Pandey and Soupir, 2011). These data, together with good production rates of methane, indicate that a stable digestion process was occurring and that the digesters were achieving a performance typical of that achieved by full-scale ones. Add to that, these data are in agreement to Saunders et al. (2012) who reported increased rates of inactivation of indicator bacteria, *E. coli* and fecal coliform, in anaerobic digesters fed with dairy manure.

Six hundred and sixty ($n = 660$) bacterial strains were isolated from influent ($n = 67$ isolates) and effluent ($n = 593$ isolates) samples. Representative strains of GPC (staphylococci, enterococci and streptococci) were the most frequent ($n = 33$, 49.25%; $n = 353$, 59.53%), followed by ENT ($n = 28$, 41.79%; $n = 176$, 29.68%) and NFR ($n = 6$, 8.96%; $n = 57$, 9.61%) in influent and effluent, respectively. According to ANOVA, no significant difference was observed among the sampled sites (influent and effluent) considering the variations in the same bacterial group ($p = 0.093$). Considering the frequency of microbial recovery no significant difference was found between strains (GPC, ENT and NFR) in different samples, isolates were regularly distributed ($p = 0.209$).

Microbial identification of influent samples is shown in Table 1. Out of the 33 GPC, 12 different species were identified, 36.36% GPC/C+ and 63.64% GPC/C−. The most prevalent were *Enterococcus hirae*

Table 1
Species distribution of bacterial strains isolated from influent samples showing the frequency of identification between Gram positive cocci (GPC) and Gram negative, as enterobacteria (ENT) and non-fermenting Gram-negative rods (NFR).

Bacterial group (n) and frequency of species identification (%)		
Gram positive	Gram negative	
GPC (n = 33)	ENT (n = 28)	NFR (n = 6)
Enterococcus hirae (15.15)	*Escherichia coli* (82.14)	*Ralstonia pickettii* (50.00)
Enterococcus faecium (12.12)	*Enterobacter aerogenes* (7.14)	*Alcaligenes faecalis* (33.33)
Enterococcus casseliflavus/gallinarum (12.12)	*Enterobacter sakazakii* (7.14)	*Burkholderia cepacia* (16.67)
Kytococcus sedentarius (12.12)	*Salmonella choleraesuis ssp* (3.57)	
Staphylococcus capitis (9.09)		
Staphylococcus xylosus (9.09)		
Aerococcus viridans (6.06)		
Enterococcus avium (6.06)		
Enterococcus faecalis (6.06)		
Leuconostoc citreum (3.03)		
Leuconostoc pseudomesenteroides (3.03)		
Micrococcus species (3.03)		

GPC: Gram-positive cocci; ENT: Gram-negative rods from the *Enterobacteriaceae* family; NFR: non-fermenting Gram-negative rods.

Table 2
Species distribution of bacterial strains isolated from effluent samples showing the frequency of identification between Gram positive cocci (GPC) and Gram negative, as enterobacteria (ENT), non-fermenting Gram-negative rods (NFR) and others Gram negatives (GNR).

Bacterial group (n) and frequency of species identification (%)			
Gram positive	Gram negative		
GPC ($n = 353$)	ENT ($n = 176$)	NFR ($n = 57$)	Others GNR ($n = 7$)
Enterococcus faecium (15.01)	*Escherichia coli* (82.95)	*Alcaligenes faecalis* (17.58)	*Aeromonas hydrophila* (100.00)
Enterococcus hirae (12.18)	*Morganella morganii* (6.81)	*Pseudomonas alcaligenes* (17.58)	
Streptococcus bovis (7.65)	*Citrobacter freundii* (2.84)	*Ralstonia pickettii* (10.52)	
Aerococcus viridans (7.08)	*Enterobacter asburiae* (1.13)	*Burkholderia cepacia* (8.87)	
Enterococcus casseliflavus/gallinarum (6.52)	*Enterobacter cloacae* (1.13)	*Pseudomonas aeruginosa* (7.01)	
Enterococcus avium (6.52)	*Providencia stuartii* (1.13)	*Stenotrophomonas maltophilia* (7.01)	
Staphylococcus capitis (6.52)	*Raoultella terrigena* (1.13)	*Acinetobacter baumannii* (3.50)	
Staphylococcus xylosus (4.53)	*Serratia marcescens* (1.13)	*Acinetobacter junii* (3.50)	
Kytococcus sedentarius (4.25)	*Pantoe spp.* (0.56)	*Brevundimonas vesicularis* (3.50)	
Staphylococcus epidermidis (3.68)	*Providencia alcalifaciens* (0.56)	*Pseudomonas putida* (3.50)	
Enterococcus faecalis (3.12)	*Salmonella choleraesuis* (0.56)	*Pseudomonas stutzeri* (3.50)	
Streptococcus equinus (2.55)		*Achromobacter xylosoxidans* (1.75)	
Streptococcus salivarius (2.55)		*Acinetobacter haemolyticus* (1.75)	
Micrococcus luteus (2.27)		*Alcaligenes piechaudii* (1.75)	
Enterococcus durans (1.98)		*Moraxella osloensis* (1.75)	
Staphylococcus lentus (1.70)		*Ochrobactrum anthropi* (1.75)	
Enterococcus raffinosus (1.42)		*Pseudomonas fluorescens* (1.75)	
Pediococcus pentosaceus (1.42)		*Pseudomonas luteola* (1.75)	
Staphylococcus auricularis (1.42)		*Wautersia paucula* (1.75)	
Streptococcus criceti (1.42)			
Streptococcus vestibularis (1.42)			
Aerococcus urinae (1.13)			
Staphylococcus hominis (1.13)			
Streptococcus uberis (1.13)			
Leuconostoc citreum (0.85)			
Leuconostoc pseudomesenteroides (0.85)			
Lactococcus lactis (0.56)			
Kocuria varians/rosea (0.28)			

GPC: Gram-positive cocci; ENT: Gram-negative rods from the *Enterobacteriaceae* family; NFR: non-fermenting Gram-negative rods; GNR: Gram-negatives rods.

(15.15%), *Enterococcus faecium* (12.12%), *Enterococcus casseliflavus/gallinarum* (12.12%) and *Kytococcus sedentarius* (12.12%). Out of the 34 Gram-negatives rods, 82.35% were ENT, while 17.65% were NFR. For ENT, the most prevalent species were *Escherichia coli* (82.14%) and among the NFR, the most prevalent species were *Ralstonia pickettii* (50.00%) and *Alcaligenes faecalis* (33.33%).

The microbial identification of effluents samples is summarized in Table 2. CGP were the most observed with 28 different species identified, 25.21% CGP/C+ and 74.78% GPC/C−. The most prevalent were *Enterococcus faecium* (15.01%), *Enterococcus hirae* (12.18%) and *Streptococcus bovis* (7.65%). Similar to the influent samples, *Escherichia coli* (82.95%) was the most prevalent among the ENT, followed by *Morganella morganii* (6.81%) and *Citrobacter freundii* (2.84%). For NFR, 19 different species were observed, *A. faecalis* (17.58%) and *Pseudomonas alcaligenes* (17.58%) were most identified.

Enterococci and *Enterobacteriaceae* were the most identified microorganisms in all samples. It is known from previous studies that they are ubiquitous and potentially opportunistic pathogens. Add to that, they naturally occur in human and animal intestines and are recognized as being able to survive and to multiply under a wide range of stress conditions and hostile environments due to their high tolerance to variation of temperature and pH (Fisher and Philips, 2009; Costa et al., 2013). According to other previous studies, enterococci and *Enterobacteriaceae* populations seem to be predominant in mesophilic digesters of cattle manure (Bagge et al., 2005; Sawant et al., 2007), although the composition of microbial communities in different systems may differ due to manure handling practices and content, and environmental conditions (Sahlström, 2003).

3.3. Antimicrobial susceptibility

Out of 660 isolates, 239 strains (36.21%) were resistant to at least one of the tested antimicrobials. The drug susceptibility patterns are reported in Tables 3 and 4. Considering the GPC/C+ in influent samples, the highest frequency of resistance was to penicillin (83.33%). Antimicrobial resistance levels higher than 8.00% were observed against rifampin, erythromycin and levofloxacin. In contrast, vancomycin, ampicillin-sulbactam, oxacillin and trimethoprim-sulfamethoxazole were the most effective antimicrobials, with sensitivity rates of 100.00%. For the GPC/C−, 9.52% of resistance to penicillin was observed, especially considering the enterococci strains whereas intermediate resistance was observed against rifampin (9.52%) and erythromycin (14.28%). Considering ENT bacteria, the highest resistance rate was observed against to ampicillin (21.42% of resistance and 14.28% intermediate resistance). Resistance was also observed against to ampicillin-sulbactam, gentamicin and trimethoprim-sulfamethoxazole (>10%). No antimicrobial resistance was observed against meropenem, levofloxacin and cefepime. Antimicrobial resistance was not observed among NFR strains recovered from influents samples.

With regards to bacteria isolated from effluent samples, among the 8 antibiotics tested for GPC/C+, resistance was not observed

Table 3
Drug susceptibility patterns of bacteria recovered in influent samples from pilot-scale anaerobic digesters.

Microbial group and tested antimicrobial drugs		Minimum inhibitory concentrations (μg mL^{-1})			Susceptibility patterns[a] (%)		
		$MIC_{50\%}$	$MIC_{90\%}$	Range	S	IR	R
GPC/C+[b]	Rifampin	0.06	0.24	0.06–8.00	83.34	8.33	8.33
	Vancomycin	0.24	1.00	0.12–2.00	100.00	–	–
	Ampicillin-sulbactam	0.24	0.50	0.06–4.00	100.00	–	–
	Penicillin	0.50	2.00	0.06–16.00	16.67	–	83.33
	Erythromycin	0.24	0.50	0.06–16.00	75.01	16.66	8.33
	Oxacillin	0.24	0.24	0.06–0.24	100.00	–	–
	Levofloxacin	0.50	8.00	0.50–16.00	50.01	16.66	33.33
	Trimethoprim-sulfamethoxazole	0.30	2.40	0.30–19.00	100.00	–	–
GPC/C−[c]	Rifampin	1.00	1.00	0.06–2.00	90.48	9.52	–
	Vancomycin	2.00	2.00	0.06–4.00	100.00	–	–
	Penicillin	0.25	1.00	0.06–32.00	90.48	–	9.52
	Erythromycin	0.06	1.00	0.06–16.00	80.96	14.28	4.76
	Levofloxacin	1.00	2.00	0.24–2.00	100.00	–	–
ENT[d]	Meropenem	0.06	0.06	0.06–1.00	100.00	–	–
	Gentamicin	1.00	64.00	0.25–128.00	82.15	–	17.85
	Amikacin	2.00	4.00	1.00–32.00	96.43	3.57	–
	Levofloxacin	0.06	0.24	0.06–1.00	100.00	–	–
	Trimethoprim-sulfamethoxazole	0.06	76.00	0.03–76.00	82.15	–	17.85
	Cefepime	0.06	0.12	0.06–1.00	100.00	–	–
	Piperacillin-tazobactam	2.00	4.00	1.00–32.00	96.43	3.57	–
	Chloramphenicol	4.00	8.00	4.00–32.00	96.43	–	3.57
	Ampicillin-sulbactam	4.00	64.00	1.00–128.00	78.58	7.14	14.28
	Ampicillin	8.00	32.00	2.00–512.00	64.30	14.28	21.42
NFR[e]	Meropenem	0.25	0.25	0.06–0.25	100.00	–	–
	Gentamicin	0.25	0.25	0.12–0.25	100.00	–	–
	Amikacin	0.50	1.00	0.25–2.00	100.00	–	–
	Levofloxacin	0.06	0.12	0.06–0.12	100.00	–	–
	Trimethoprim-sulfamethoxazole	2.40	4.80	0.60–38.00	100.00	–	–
	Cefepime	0.50	0.50	0.12–4.00	100.00	–	–
	Piperacillin-tazobactam	1.00	1.00	1.00–8.00	100.00	–	–
	Chloramphenicol	4.00	8.00	4.00–8.00	100.00	–	–

[a] S: sensitivity; IR: intermediate resistance; R: resistance.
[b] GPC/C+: Gram-positive cocci/Catalase-positive (n = 12).
[c] GPC/C−: Gram-positive cocci/Catalase-negative (n = 21).
[d] ENT: Gram-negative rods from the *Enterobacteriaceae* family (n = 28).
[e] NFR: non-fermenting Gram-negative rods (n = 6).

Table 4
Drug susceptibility patterns of bacteria recovered in effluent samples from pilot-scale anaerobic digesters.

Microbial group and tested antimicrobial drugs		Minimum inhibitory concentrations (μg mL^{-1})			Susceptibility patterns[a] (%)		
		$MIC_{50\%}$	$MIC_{90\%}$	Range	S	IR	R
GPC/C+[b]	Rifampin	0.06	1.00	0.06–8.00	91.02	6.74	2.24
	Vancomycin	0.24	2.00	0.06–2.00	100.00	–	–
	Ampicillin-sulbactam	0.24	2.00	0.06–16.00	98.88	1.12	–
	Penicillin	1.00	8.00	0.06–16.00	25.85	–	74.15
	Erythromycin	0.24	2.00	0.06–32.00	70.79	19.10	10.11
	Oxacillin	0.24	0.24	0.06–0.50	91.02	–	8.98
	Levofloxacin	2.00	64.00	0.06–64.00	49.45	16.85	33.70
	Trimethoprim-sulfamethoxazole	2.40	9.50	0.30–128.00	95.51	–	4.49
GPC/C−[c]	Rifampin	0.12	4.00	0.06–8.00	91.67	6.82	1.52
	Vancomycin	0.50	4.00	0.06–4.00	100.00	–	–
	Penicillin	0.25	2.00	0.06–32.00	97.35	–	2.65
	Erythromycin	0.06	1.00	0.06–16.00	85.23	12.50	2.27
	Levofloxacin	1.00	2.00	0.06–4.00	96.59	3.41	–
ENT[d] and others GNR	Meropenem	0.06	0.25	0.06–2.00	99.45	0.55	–
	Gentamicin	1.00	1.00	0.06–128.00	99.45	–	0.55
	Amikacin	4.00	4.00	0.25–32.00	99.45	0.55	–
	Levofloxacin	0.06	0.12	0.06–1.00	100.00	–	–
	Trimethoprim-sulfamethoxazole	0.06	4.80	0.06–38.00	100.00	–	–
	Cefepime	0.06	0.12	0.06–8.00	100.00	–	–
	Piperacillin-tazobactam	2.00	4.00	0.06–32.00	99.45	0.55	–
	Chloramphenicol	4.00	8.00	0.06–32.00	92.90	4.37	2.73
	Ampicillin-sulbactam	8.00	128.00	1.00–512.00	75.96	7.65	16.39
	Ampicillin	8.00	64.00	0.06–1024.00	63.95	18.57	17.48
NFR[e]	Meropenem	0.06	0.50	0.06–8.00	96.50	–	3.50
	Gentamicin	0.25	4.00	0.06–64.00	96.50	–	3.50
	Amikacin	1.00	8.00	0.12–128.00	96.50	–	3.50
	Levofloxacin	0.12	0.50	0.06–2.00	100.00	–	–
	Trimethoprim-sulfamethoxazole	9.50	38.00	0.6–304.00	91.33	–	8.77
	Cefepime	0.50	4.00	0.06–64.00	96.50	–	3.50
	Piperacillin-tazobactam	1.00	4.00	0.06–256.00	98.25	–	1.75
	Chloramphenicol	4.00	32	0.06–32.00	73.69	7.01	19.29

[a] S: sensitivity; IR: intermediate resistance; R: resistance.
[b] GPC/C+: Gram-positive cocci/Catalase-positive (n = 89).
[c] GPC/C−: Gram-positive cocci/Catalase-negative (n = 264).
[d] ENT: Gram-negative rods from the *Enterobacteriaceae* family (n = 176) and others GNR (n = 7).
[e] NFR: non-fermenting Gram-negative rods (n = 57).

against vancomycin. Penicillin was found to be the less effective drug (74.15% resistance), followed by levofloxacin (33.70% resistance) and erythromycin (10.11% resistance). In the GPC/C− group low resistance rates were observed against rifampin, penicillin and erythromycin. Considering the Gram negative bacteria, the ENT strains were resistant only against ampicillin and ampicillin-sulbactam (>10% resistance), whereas for the NFR strains, antimicrobial resistance was observed against almost all the tested drugs, especially chloramphenicol (19.29% resistance), with exception of levofloxacin, for which no resistance was observed.

The multiple antibiotic resistance (MAR) index is presented in Table 5. According to this parameter, 55.65% of the isolated bacteria is characterized as multi-resistant strains (MAR > 0.2). The index ranged from 0.12 to 0.50 and 0.12 to 0.62 for GPC from influent and effluent samples respectively. Of the GPC/C+ isolated from the influent samples, 33.33% showed MAR > 0.2, whereas considering the effluent samples, 62.92% showed MAR > 0.2. For GPC/C−, 4.76% showed MAR > 0.2 considering the isolates from influent, whereas 2.65% of the bacteria isolated from the effluent displayed MAR > 0.2.

Table 5
Frequency of drug-resistant bacteria and multiple antibiotic resistance (MAR) index among microbial groups isolated from pilot-scale anaerobic digesters samples.

Samples	Microbial group	Frequency of resistance (%)	MAR (frequency of determination %)		
			<0.2	>0.2	Range
Influent	GPC/C+[a]	83.33	50.00	33.33	0.12–0.50
	GPC/C−[b]	28.57	23.81	4.76	0.12–0.25
	ENT[c]	50.00	21.43	28.57	0.10–0.50
	NFR[d]	0	0	0	0
Effluent	GPC/C+	88.76	25.84	62.92	0.12–0.62
	GPC/C−	17.42	14.77	2.65	0.12–0.37
	ENT and others GNR[e]	37.15	10.92	26.23	0.10–0.30
	NFR	29.83	15.79	14.04	0.12–0.62

[a] GPC/C+: Gram-positive cocci/ Catalase-positive.
[b] GPC/C−: Gram-positive cocci/Catalase-negative.
[c] ENT: Gram-negative rods from the *Enterobacteriaceae* family.
[d] NFR: non-fermenting Gram-negative rods.
[e] GNR: Gram-negatives rods.

With regards to ENT strains isolated from the influent samples, 28.57% showed MAR > 0.2, whereas for ENT and others GNR isolated from the effluent samples, 26.23% showed MAR > 0.2. For NFR strains isolated from the effluent samples MAR > 0.2 was observed for 14.04% of the isolated bacteria.

Considering the purpose of anaerobic digestion effluent usage as biofertilizer in agriculture, it is important to highlight that such effluents are suitable and accepted only if the sanitary safety is satisfactorily guaranteed. Regarding pathogens, the regulations for use of manure for agricultural purposes are based on three principles: (i) a requirement for treatment, to reduce the amount of pathogens, as anaerobic digestion; (ii) confirmation of treatment; and (iii) assurance of the microbiological quality of the manure (Venglovsky et al., 2006; Martensa and Böhm, 2009). The results obtained in this study, the mesophilic anaerobic digestion of dairy manure causes a reduction of various pathogens. Although, there is another aspect to the agricultural use of effluents biodigesters, the occurrence of resistant bacteria.

In this model, as a matter of concern, an important proportion of bacteria strains recovered from the influent samples exhibited resistant to different antibiotics, and practically the same pattern of resistance was detected in effluent samples. The data show several patterns of resistance from both commensal and opportunistic pathogens which may proliferate through the environment and allow the spread of resistance genes through bacterial genetic recombination with consequences to the human and animal antimicrobial chemotherapy (Walsh et al., 2012).

A few studies have reported the prevalence of antimicrobial resistance among bacteria isolated from effluents of anaerobic digesters. Among these, back in the 80's Abdul and Lloyd (1985) reported the occurrence of resistant *E. coli* strains in mesophilic digestion at 37 °C of pig waste. More recently, Beneragama et al. (2011) observed a persistence of drug-resistant bacteria in thermophilic co-digestion of dairy manure and waste milk at 55 °C until the end of the process. Beneragama et al. (2012) reported a survival of multidrug-resistant bacteria in mesophilic digestion at 37 °C of dairy manure and waste milk after 22 days of digestion, whereas no resistant-bacteria were observed after thermophilic digestion at 55 °C. These data may suggest that temperature is an important characteristic concerning drug-resistant bacteria persistence during anaerobic digestion, along with microbial completion and fermentation length.

The MAR index is widely used to refer multidrug resistant organisms in several environments. The index was introduced in 1983 and no suggestions are made regarding the minimum of antimicrobial agents in the test panel. More recently, new insights are being made regarding the definition of multidrug-resistant bacteria. For example, the definition of multidrug resistant bacteria in clinical field described by Magiorakos et al. (2012) consider oxacillin as a multiresistance marker among staphylococcal populations, when associated, especially to nosocomial environment. High frequency of MAR > 0.2 among the bacteria isolated in the effluent samples it is reasonable to state that as it is performed, the effluent of biodigesters may play an important role as environmental reservoir of antimicrobial resistance genes. The abundance of these multidrug-resistant bacteria may reflect a microbial adaptive response to the empirical use of antimicrobials as prophylactics or therapeutics in cattle farm (Costa et al., 2013). As an ecological consequence, the adaptive mechanisms to the selective pressure imposed by antimicrobials may co-select for bacterial strategies to survive under stress conditions (Diniz et al., 2004).

Parveen et al. (2006) demonstrates regional and seasonal differences in MAR profiles among livestock farms. However, sanitary risks may become higher when antibiotic-resistant microbes are persistent along potentially pathogenic bacteria, as some species isolated in this study. These bacteria are extremely efficient in horizontal gene transfer and may contribute to the spread and maintenance of resistance genes among different bacterial populations. Considering the biofertilizer as a final product after anaerobic digestion of cattle manure, it is accepted that persistent antimicrobial-resistant bacteria may survive in the soil for up to one month after land application (Nicholson et al., 2005). In this regard, with the increasing interest for biogas production and use of effluents on arable land, becomes it import to consider the persistence of clinically important bacterial populations to sanitary safety and public health management (Bagge et al., 2005; Abubaker et al., 2012). Considering the nature of the cattle manure, it may be suggested that improvement by performing additional treatment before anaerobic digestion would interfere with initial load of methanogenic bacteria, reflecting on the biogas output, but this hypothesis was not evaluated in this study. By the other hand, it has already been reported a decay in potentially pathogenic and drug-resistant bacteria counts during thermophilic digestion (Beneragama et al., 2012). Anyway, considering the digestion at mesophilic range presented, additional steps such as effluent heating would aggregate economical costs in the final process. Other choices of post-treatment system (e.g. stabilization lagoons, ozone oxidation and anaerobic ammonium oxidation) are also suggested to efficiently remove the multidrug resistance bacteria (Berneta and Béline, 2009; Di Iaconi, 2012). Further prospective studies are needed to better discuss the extent of the antimicrobial resistance phenomena in cattle management and its consequence to the cattle manure recycling strategies with implications for human, animal and environmental health.

4. Conclusion

The practice of spreading of effluents from biodigesters into land used for the agriculture production is widely used. However, the presence of antimicrobial-resistant bacteria or putative pathogenic microorganisms may lead to sanitary and ecological risks. Discussions are needed concerning the use of antimicrobial drugs in animal farms and surrounding environments. Environmental regulations should address the sanitary and microbiological safety concerning the use of effluents of ambient temperature biodigesters especially regarding the persistence of putative pathogens and antimicrobial resistant bacteria.

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