Anaerobic treatment of swine manure under mesophilic and thermophilic temperatures: fate of veterinary drugs and resistance genes

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Abstract:

The effect of anaerobic treatment of swine manure at 35ºC (mesophilic) and 55ºC (thermophilic) on methane production, microbial community and contaminants of emerging concern was investigated. Pasteurization pretreatment and post treatment was also investigated in combination with anaerobic treatment at 35ºC. Specific methane production (SMP), 26 pharmaceutical compounds (PhACs) and five antibiotic resistance genes (ARGs) (*qnrS*, *tetW*, *ermB*, *sul1 and* bla_{TEM}) were evaluated. Mesophilic treatment resulted in the highest SMP regardless of whether pasteurization was applied. Marbofloxacin was the most abundant antibiotic in swine manure. In general, all groups of PhACs showed higher removals under thermophilic temperatures as compared to mesophilic. In general, pasteurization pretreatment followed by mesophilic anaerobic digestion provided the highest removals of ARGs. Finally, the genera *Streptococcus*, *Clostridium* and *Pseudomonas* which contain pathogenic species, were present in the swine manure. *Streptococcus,* which was the

most abundant, was decreased during all the treatments, while the others only decreased under certain treatments.

Keywords: Anaerobic treatment; swine manure; veterinary drugs; antibiotic resistance genes; temperature, microbial community composition

1. Introduction

Pork is one of the most consumed meats worldwide, with a global production of around 117 metric kilotons per year (Lassaletta et al., 2019). Its consumption levels have increased significantly during the last decade, especially in fast growing countries such as China (OECD/FAO, 2019). As a result of this increasing demand, pig farming has become more intensive, with larger farms and more pigs per area of land which leads to larger volumes of manure being produced that, if not properly managed, increases air, water, and soil pollution. The most common management practice for swine manure (SM), especially in Spain (the second producer of swine in Europe), is storing it in mostly uncovered tanks for three to six months prior to land spreading (Riaño and García-González, 2015) at rates that often exceed crop needs causing significant environmental impacts, especially in the regional aquifers due to the excessive nitrogen loading (Lassaletta et al., 2019; Penuelas et al., 2009). Another rising concern is the presence of contaminants of emerging concern, such as veterinary pharmaceuticals (PhACs) and antibiotic resistance genes which end up in the soil and the aquifers when this manure is spread into the land. Veterinary PhACs are commonly used in industrial farming for therapeutic purposes or for preventative treatment. The amount of veterinary PhACs and antibiotics used is highly variable depending on the country. Some northern European countries have minimized substantially their use while others, such the US, report high usage, with around 81% of their total antibiotic consumption being from farmed animals (FDA, 2012). The most important veterinary antibiotics include tetracyclines, fluoroquinolones, lincosamides and sulfonamides (Gros et al., 2019; Yin et al., 2020a), with 30 to 90% not absorbed by the animals and evacuated via faeces or urine (Zhang et al., 2018).. The excessive use of antibiotics might promote the growth of antibiotic resistant bacteria, resulting in the increase of antibiotic resistance genes (ARGs) (Zou et al., 2020) which has become a great environmental and public health concern (Oliver et al., 2020).

Biotechnologies such as anaerobic digestion (AD) are employed as remedial measures to reduce the environmental impacts of swine manure prior to its application to land. In fact, intensive farming might incentivize its application because the cost can be spread over a larger volume of production and farmers can also benefit by the production of biogas promoting circular economy. Temperature is considered the most important parameter in AD, with reactors normally operating at the mesophilic (30–40 °C, MAD) or thermophilic (50–60 °C, TAD) ranges (Rodríguez et al., 2019). Temperature has a direct effect on biogas production, microbial community succession and effluent characteristics (PhACs, ARGs and pathogens). Concerning to biogas, thermophilic temperatures can enhance digestibility and increase the growth of thermophilic microbes resulting in higher energy generation (Vergote et al., 2020). However, a temperature increase also results in an increase of free ammonia concentration (Anthonisen et al., 1976) that can be inhibitory for microorganisms specially in wastes with high content of nitrogen-ammonium, such as swine manure (Cao et al., 2020; Chae et al., 2008). On the other hand, the effect of temperature on PhACs or ARGs is not consistent, with some studies concluding that TAD could favor the biodegradation of PhACs and ARGs and others not (Carballa et al., 2007; Davidsson et al., 2014; Gros et al., 2020; Huang et al., 2019; Oliver et al., 2020; Sun et al., 2016; Zhou et al., 2015).

The removal of pathogens from manure is also considered when assessing any possible treatment. It is known that MAD is not sufficient to reduce the content of pathogens (i.e., fecal coliforms and *Salmonellae*), while TAD present a higher removal efficiency (Gannoun et al., 2009), but it is more unstable and expensive for this type of waste. To overcome this limitation, interest in thermal pre-treatment before mesophilic digestion has increased (Zhao and Liu, 2019). Sterilization of pathogenic microorganisms from wastewater can be achieved via a thermal pre-treatment (70 ºC for one hour) before AD (H. Li et al., 2017; Skiadas et al., 2005; Yin et al., 2020b). However, the effect of this pre-treatment on PhACs, ARGs and microbial community is not clear yet. A recent study evaluated the additional functioning of pasteurization pretreatment with mesophilic anaerobic treatment on the removal of chlortetracycline and oxytetracycline (Yin et al., 2020b). However, its effect on other veterinary PhACs, ARGs, and microbial community has not been reported so far and its efficiency as a post-treatment has not been studied yet.

The present paper investigates the removal and fate of PhACs, ARGs, microbial community composition, including pathogens and biogas production under different anaerobic treatment conditions including the sanitation effect of pasteurization as pre or post treatments from swine manure.

2. Material and methods

2.1. Inoculum and substrate

Anaerobic digester sludge coming from an anaerobic mesophilic digester located in a municipal wastewater treatment plant (WWTP) was used as inoculum. Swine manure was obtained from a pig farm located in Lleida (Catalonia-Spain), with a capacity of 600 pigs and a slurry storage tank of 500 m^3 which was emptied every 5-6 months. Inoculum and swine manure characteristics are shown in Table 1.

Table 1. Characteristics of the inoculum and substrate used in this study.

Parameter	Inoculum	Swine manure
pH	7.3±0.2	7.2±0.1
TCOD (mg/L)	18,100 ± 350	101,600 ± 135
SCOD (mg/L)	250 ± 2	19,060 ± 255
TS (g/L)	18.0 ± 0.1	73.0 ± 1.5
VS (g/L)	11.8 ± 0.1	54.73 ± 1.06
$P\text{-}PO_4^{3-}$ (mg /L)	28.6±1.0	22.2±0.1
Cl^- (mg /L)	170.1±1.1	1,973±2
Na^+ (mg /L)	76.6±0.2	1,180±26
$N\text{-}NH_4^+$ (mg/L)	638±8	3,218±112
P-TP (mg/L)	215 ± 1	1,594±42
TKN (mg/L)	1,438 ± 17	5,519±103

2.2. Biochemical Methane Potential (BMP) tests

BMP tests with an inoculum to substrate ratio of 2 were carried out in a 250 mL sealed bottles (150 mL working volume) following the procedure described in Zahedi et al., 2018.

BMP tests were evaluated under four different conditions (TAD (55 ºC), MAD (35 ºC), pre-pasteurization treatment + MAD (Past+MAD, swine manure was heated at 70 ºC for one hour before MAD) and MAD+post-pasteurization treatment (MAD+Past, after MAD, BMP was heated 70 ºC for one hour). Pasteurization or sanitation treatment were performed at 70 ºC for one hour, without shaking. Pasteurization as pre or post treatment of MAD digestion was assessed for pathogens removal. All bottles were flushed with nitrogen for 5 min and immediately sealed and placed in two incubators with a controlled temperature of 35ºC and 55ºC respectively and with 150 rpm of shaking to ensure enough mixing. Triplicates were done for each condition. Blank tests with only inoculum sludge were also included to assess the biogas production from the inoculum, which was subtracted from the biogas produced in the tests with manure. Specific methane production (SMP) was reported under normal conditions (Temperature = 0ºC and Pressure = 1 atm). More details about the calculation of the methane produced can be found in Supplementary information. The duration of the BMP tests was 20 days because the laboratories had to shut down due to covid-19 mobility restrictions which did not allow the authors to travel to the institute.

2.3. Chemical and microbial analysis

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total phosphate (TP), ammonium, total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) were determined according to standard methods (APHA, 2017). pH was measured using a Crison GLP pH meter. Volatile fatty acids (VFA) were determined by gas chromatography (Trace GC Ultra ThermoFisher Scientific). The volume of biogas produced and quantity of methane present were analyzed with a pressure and an infrared CH_4 sensor respectively, described in previous studies (Zahedi et al., 2018).

The analysis of 26 PhACs was conducted in swine manure, in the inoculum and in the AD effluents as stand-alone treatments and in the combinations with the pasteurization. In all samples, PhACs analysis were conducted in the solid and liquid phases, separated by centrifugation. The analysis of PhACs in the liquid phase was done by solid phase extraction (SPE). For swine manure, 10 mL were pre-concentrated, while for the other samples 25mL were used. For the analysis of PhACs in solids, samples were freeze-dried and 0.5g were measured, extracted using vortex in combination with ultrasonic extraction, and extracts were purified by SPE. In both cases, the protocol used is explained in Gros et al (2019). An Acquity ultra-high-performance-liquid chromatograph (Waters Corporation, MA, USA) coupled to a 5500 QTRAP hybrid quadrupole-linear ion trap tandem mass spectrometer (AB Sciex, Foster City, USA) was used for PhACs quantification according the procedure reported in Gros et al. (2019). Extraction recoveries, method detection and quantification limits for the PhACs analyzed in solid and liquid fractions are reported in tables S1 and S2 in the supplementary material. The equations used for the calculations of the total concentration of PhACs (including solid and liquid phases) are reported in Supplementary information.

To determine the abundance of ARGs as well as microbial community composition, the collected biomass was suspended in lysis buffer (2 mM EDTA, 20 mM Tris-HCl [pH 8.0] and 1.2% Triton X-100) and treated with proteinase K (10 mg/mL) and lysozyme (20 mg/mL). To extract genomic DNA, the method described in Sambrook and Russell (2001) was used. The copy numbers of five ARGs (*qnrS*, *tetW*, *ermB*, *sul1* and bla_{TEM} conferring resistance to fluoroquinolones, tetracyclines, macrolide-lincosamide-streptogramin (MLS), sulfonamides and β-lactam antibiotics, respectively) and the gene *intI1* (a proxy for gene mobilization) and the 16S rRNA gene (a proxy for bacterial abundance and used for data normalization) were quantified by real-time PCR (qPCR) assays following the previously described conditions (Marti et al., 2013; Lekunberri et al., 2017). Data were then compared using one-way analysis of variance (ANOVA) or Student's t-test, when necessary. A significance level was set at $p<0.05$.

High-throughput sequencing of the 16S rRNA gene was used to characterize microbial communities, which was conducted on Illumina MiSeq platform at the Sequencing and Genotyping Unit of the University of the Basque Country (UPV/EHU). More details about this analysis and the bioinformatics applied can be found in Supplementary Information.

2.4. Calculations

To calculate the free ammonia (FA) concentration the formula from Anthonisen et al., (1976) was used :

$$\text{FA (mg N-NH}_3\text{/L)} = ((1.214 \times \text{NH}_4^{+}\text{-N} \times 10\text{pH})/(\text{e}6344/(273+\text{T(ºC)})+10\text{pH})) \qquad \text{(Eq.1)}$$

To calculate the distribution coefficient (K_d) between the solid phase and the corresponding supernatant of the different PhACs analysed equation 2 was used:

$$K_{d\ \text{solid-liquid}} = (C_{i,\text{sorbed}} / C_{i\ \text{dissolved}}) \times 10^3 \qquad \text{(Eq.2)}$$

Where K_d is expressed in L/Kg, $C_{i,\text{sorbed}}$ is a concentration of pharmaceutical measured in the solid phase (ng/g dry weight) and $C_{i\ \text{dissolved}}$ is the one measured in the aqueous phase (ng/L).

3. Results and discussion

3.1. Methane production and classical parameters of anaerobic digestion

The effect of the different conditions tested on the specific methane production (SMP) is shown in Fig. 1.

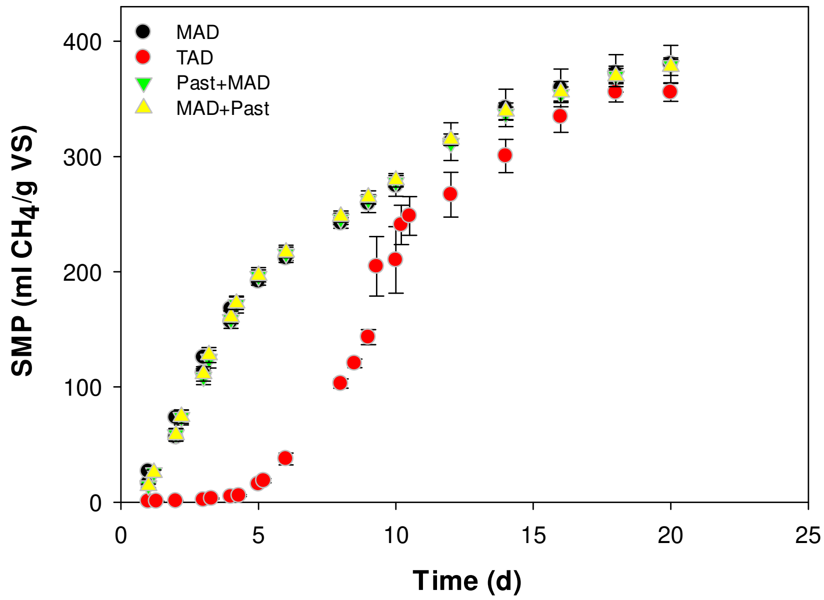

Figure 1. Specific methane production from the swine manure under the different conditions tested.

BMP tests conducted at MAD presented shortest start-up and it is probably because the inoculum was acclimated at 35ºC and required more time to be able to start performing at 55ºC (Gannoun et al., 2009). However, the fact that started an exponential methane production after 4 days indicates that the inoculum also contained thermophilic microbes.

The whole duration time of the BMP was 20 days. The profiles show that the exponential CH_4 production phase was completed as the methane increase detected in the last days was substantially decreased for all the treatments.

The average values of SMP obtained (Table 2) were around 350 and 380 mL CH_4/ g VS at thermophilic and mesophilic conditions, respectively and in the line with previous studies of AD of swine manure that ranged between 220-470 mL CH_4/ g VS (Cao et al., 2020; Kafle and Chen, 2016; Lu et al., 2020; Rodríguez et al., 2017). The lower values observed at thermophilic conditions could be explained by the higher free ammonia (FA) values calculated using equation 1. At MAD FA values were around 25 mg NH_3-N/L, while at TAD these were close to 200 mg NH_3-N/L. Concentrations of 40 mg NH_3-N/L have been reported as inhibitory for methanogens, with other authors suggesting an inhibitory FA range of 100-400 mg NH_3-N/L depending on the sludge (Hansen et al., 1998; Palatsi et al., 2011; Sutaryo et al., 2014; Gebreeyessus and Jenicek, 2016;). This inhibition can be causing the lower methane production detected in these BMPs and also the slight accumulation of propionic acid detected under the TAD conditions (Table 2). This is in agreement with other studies with swine manure as substrate (Cao et al., 2020) and with other substrates (Hao and Wang, 2015). In all the reactors, the VFA values were below the inhibitory values (3,000-6,000 mg /L) (Owusu-Agyeman et al., 2020; Pullammanappallil et al., 2001).

Regarding to the thermal pre-treatment or pasteurization effect on the mesophilic SMP, no differences were observed between MAD and Past+MAD (≈ 380 mL/g VS). Our results contradict previous findings (Yin et al., 2020b). Yin et al. observed that using pasteurisation pre-treatment of swine manure before MAD, SMP could increase from 244 to 254 mL/g VS. The reason why no increase in the SMP occurred in the MAD after using pasteurisation pre-treatment could be due to the fact that no significant solubilization of carbohydrates and proteins or change in the manure composition occurred after 1 h at 70 ºC (Appels et al., 2010; Vergote et al., 2020). Even, Appels et al. observed that the efficiency of the subsequent anaerobic digestion slightly decreased for sludge pre-treated at 70 º C, being only higher temperatures interesting for the increase of the energy recovery (Appels et al., 2010).
Other reason which can explain the differences observed with Yin et al. could be differences in the ammonia concentration of the pig manure (data non given in Yin et al. study). Bonmatí et al. examined the effect of thermal pre-treatment at 80 ºC for 3 h on mesophilic digestion of pig manure using slurries with different total ammonia concentration (TAN) and they observed that thermal pre-treatment had a different effect on the methane production depending on the type of slurry; it was only positive with slurries containing low TAN concentration (Bonmatí et al., 2001).
After completing the BMP tests, the concentration of TKN ranged between 1300-1700 mg/L.

Table 2. SMP and classical parameters analyzed at the end of the different tests.

Parameter	MAD (35 ºC)	TAD (55 ºC)	Past+MAD	MAD + Past
SMP (ml CH_4/g VS)	380±17	356±8	379±5	377±8
pH	7.2±0.1	7.8±0.0	7.3±0.1	7.4±0.0
TCOD (mg/L)	9,526±1,275	10,076±747	8,976±855	8,008±150
TS (g/L)	18.1±5.4	19.4±1.7	20.0±1.7	21.5±3.0

VS (g/L)	11.4±3.4	12.3±1.0	11.3±2.7	12.2±2.2
TKN (mg /L)	1,379±72	1,700±6	1,553±39	1,700±30
NH_4 (mg/L)	1,040±6	1,100±18	917±3	889±8
Acetic acid (mg/L)	4.2±0.3	16.7±0.1	4.5±0.4	7.1±0.1
Propionic acid (mg/L)	n.d	2.8±0.7	n.d	1.3±0.0
Isobutyric Acid (mg/L)	n.d	10.0±0.8	n.d	n.d
N-Butyric acid (mg/L)	n.d	n.d	n.d	n.d

n.d: non detectable

3.2. Veterinary pharmaceuticals and antibiotics

PhACs were analyzed in the solid and liquid phases of the swine manure and 10 out of the 26 targeted compounds could be detected. From these 10 PhACs, 9 were antibiotics (2 macrolides, 2 tetracyclines, 3 fluoroquinolones, 1 sulfonamide and 1 lincosamide) and 1 anthelmintic drug. Later, these 10 PhACs were analysed in the inoculum and in the effluents from the BMP bottles. Results are depicted in Table 3 (and for a direct comparison, see Table S3 in Supplementary information). All the PhACs were predominant in the solid fractions in agreement with previous studies (Yang et al., 2016; Gros et al., 2019). Only 4 compounds (2 tetracyclines: chlortetracycline and oxytetracycline and 2 fluoroquinolones: ciprofloxacin and norfloxacin) out of the 10 PhACs were detected in both swine manure and inoculum, being chlortetracycline and oxytetracycline predominant in the manure and ciprofloxacin and norfloxacin predominant in the inoculum. These differences are because the inoculum comes from an anaerobic digester from a municipal WWTP and swine manure from a pig farm, thus it is expected to find differences in the compounds detected in these samples. The differences found in terms of PhACs composition in the swine manure and in the inoculum agree with what is reported in the literature (Auguet et al., 2017; Ben et al., 2008; Ghirardini et al., 2020; Gros et al., 2019; Pazda et al., 2019). Marbofloxacin, a bactericidal with a broad spectrum of activity mainly used for respiratory infections in pigs (Lei et al., 2017), was the most abundant PhACs in the swine manure (>1000 µg/kg in the solid fractions). Other compounds, such as ciprofloxacin or norfloxacin (fluoroquinolones), oxytetracycline (tetracycline) sulfadiazine (sulfonamide), lincomycin (lincosamide) or the anti-helminthic flubendazole, were detected in the swine manure at median concentrations between 100 and 1000 µg/kg in the solid phases, respectively and tiamulin and tilmicosin (macrolides) and chlortetracycline were detected in the swine manure at median concentrations below 100 µg/kg in the solid phases. Regarding the inoculum, ciprofloxacin and norfloxacin (fluoroquinolones) were the most predominant (>1000 µg/kg in the solid phase), followed by oxytetracycline and chlortetracycline (tetracyclines) (< 110 µg/kg in the solid phases). These compounds show a strong sorption behavior, and this would explain their detection at relevant concentrations in both manure and inoculum solid fractions. Ciprofloxacin is a fluoroquinolone antibiotic of major human consumption and has been widely detected in urban wastewater and sewage sludge (Giebułtowicz et al, 2020; Jia et al., 2012), thus explaining the high concentrations detected for this compound in the inoculum in comparison with the other substances.

Table 3. Median concentrations of the PhACs detected in swine manure, inoculum and in the AD outlets, in both liquid (µg/L) and solid fractions (µg/kg in d.w.)

Compounds	Inlet						Outlet					
	Swine manure		Inoculum		MAD		TAD		Past+MAD		MAD+Past	
	liquid fraction (µg/L)	solid fraction (µg/kg)	liquid fraction (µg/L)	solid fraction (µg/kg)	liquid fraction (µg/L)	solid fraction (µg/kg)	liquid fraction (µg/L)	solid fraction (µg/kg)	liquid fraction (µg/L)	solid fraction (µg/kg)	liquid fraction (µg/L)	solid fraction (µg/kg)
Tiamulin	n.d	53.3±5.2	n.d	n.d	n.d	15.7 ±1.2	n.d	16.8±0.8	n.d	15.7 ±0.3	n.d	16.9±0.5
Tilmicosin	0.67±0.16	24.4±0.0	n.d	n.d	blq	42.1±0.1	blq	41.0±2.79	blq	47.0±1.0	blq	40.1±0.4
Oxytetracycline	1.50±0.09	277.4±16.8	n.d	106.0+±0	n.d	155.9±0.7	n.d	138.3±19.3	n.d	145.7±15.2	n.d	172.9±23
Chlortetracyclin	n.d	81.1.±0.2	n.d	13.5±0.9	n.d	39.9±1.4	n.d	16.2±0.6	n.d	36.3±5.0	n.d	43.0±1.3
Ciprofloxacin	n.d	126.2±2.1	0.3±0.0	9,000±13	0.2±0.0	4,744±691	0.4±0.0	4,348±607	0.3±0.0	4,870±172	0.9±0.1	5,017±90
Marbofloxacin	6.5±0.0	1,310±19	n.d	n.d	0.2±0.0	1,025±36	0.4±0.0	1,060±5	0.2±0.0	1,035±69	0.5±0.0	1,188±50
Norfloxacin	26.3±1.0	30.4±11.7	11.1±0.2	1,335±7	11.3±0.8	640±25	11.7±0.4	176±21	11.8±1.8	633±74	12.1±1.7	600±15
Sulfadiazine	41.9±2.5	570±76	n.d	n.d	16.5±0.0	229±17	40.4±5.7	382±3	19.3±0.3	250±3	15.2±0.8	158±85
Lincomycin	7.7±0.0	195.3±2.7	n.d	n.d	0.5±0.0	34.1±0.0	2.2±0.0	46.1±4.1	0.49±0.0	31.1±7.2	0.9±0.0	17.7±0.7
Flubendazole	0.74±0.03	354±15	n.d	n.d	0.11±0.00	59.5±3.9	0.13±0.00	51.0±0.3	0.11±0.00	63.3±1.5	0.16±0.00	63.3±0.2

n.d.: non-detected; blq: below limit of quantification.

Concerning the different anaerobic treatments, ciprofloxacin and marbofloxacin (fluoroquinolones) were the most predominant in the solid phase (>1000 µg/kg in the solid phases) which is in line with the highest values of these compounds in the inoculum and swine manure (solid phase). However, in the liquid phase the most predominant antibiotics were norfloxacin and sulfadiazine (with median concentrations > 11 µg/L in the liquid fractions) which were also predominant in the liquid phase of swine manure.

Reduction of PhACs during the different anaerobic treatments is depicted in Figure 2. The removal differed between compounds, where all substances (except tilmicosin, marbofloxacin and sulfadiazine) were reduced during the 20 days of duration of the BMP tests. In general, all groups of PhACs showed higher removal in TAD as compared to MAD (except for lincomycin). These results agree with some studies that indicate an enhancement of PhACs removal when anaerobic digestion is operated around 55ºC (Samaras et al., 2014; Feng et al., 2017) but disagree with others that suggest that temperature does not affect the removal of PhACs (Malmborg and Magnér, 2015; Gonzalez-Gil et al., 2016;). Regarding the effect of pasteurization treatment in combination with AD on PhACs removal, results suggest that this treatment does not influence the removal of PhACs. When comparing the tests conducted under mesophilic conditions to the ones where a combination of pasteurization with mesophilic treatment was applied, some differences were observed. The application of a thermal pre-treatment such as pasteurisation has been reported in the literature to promote thermal hydrolysis of some pharmaceutical compounds. A recent study observed that when applying pasteurisation (70 ºC and 1 h) before MAD, oxytetracycline concentrations decreased from 180 mg/kg TS to 17 mg/kg TS, while with only MAD these final values were around 27 mg/kg TS (Yin et al., 2020a). This agrees with our findings where higher removals of oxytetracycline and chlortetracycline were detected in PAST+MAD vs MAD+PAST or MAD alone. Also, the fact that PAST+MAD showed better removals for these two compounds as compared with the MAD+PAST treatment indicates a thermal hydrolysis promotion of antibiotic biodegradability in AD but not only to the thermal hydrolysis. However, for the other 5 PhACs (tiamulin, ciprofloxacin, norfloxacin, sulfadiazine and flubendazole) 70 ºC for 1hour seem not to be enough to produce significant effects neither before nor after AD treatment. The differences observed between tetracyclines and the other groups of antibiotics could be due to the lower thermal stability of the tetracyclines as compared with others antibiotic groups, as ß-lactams or fluoroquinolones (Svahn and Björklund, 2015; Junza et al., 2014; Yi et al., 2016; Zhang and Li, 2018). Yin et al. showed that tetracyclines could be reduced by 90% within 5 min at 130 °C. However for fluoroquinolones, only 20% of decrease was observed when the samples were heated at 120 °C for 20 min (Junza et al., 2014), suggesting that fluoroquinolones have higher thermal stability than tetracyclines.

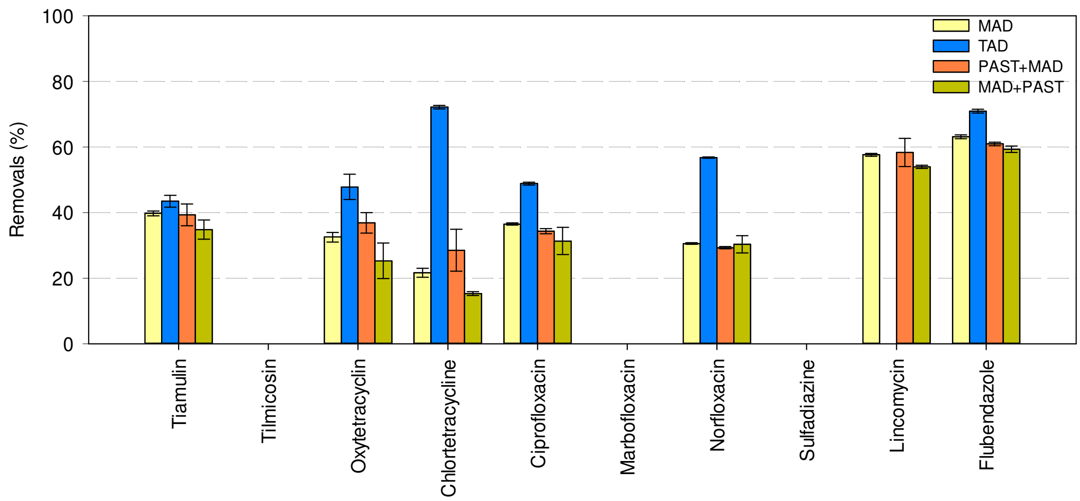

Figure 2. Reduction of PhACs under the different anaerobic treatments tested.

Another important point is that temperature not only favors biodegradation, but also the desorption of the pharmaceuticals from the solid to the aqueous phase at the end of the anaerobic treatment. This can be observed in Figure 3, where the distribution coefficient between the solid and the liquid phase of the PhACS detected in both phases is shown (data are presented in logarithmic scale). TAD compared to MAD, show lower solid liquid distribution coefficient, even in marbofloxacin and sulfadiazine which were not biodegraded (see Figure 2). This can be attributed to hydrolysis, which is considered the main chemical transformation route in PhACs degradation, and is improved at higher temperatures (Yi et al., 2016). The same authors observed an increase on tetracycline hydrolysis rate when temperature was increased 10ºC. Other studies observed that desorption of other antibiotics, as ofloxacin, norfloxacin, ciprofloxacin or lomefloxacin from the solid to the aqueous phase was improved by increasing the temperature (Li et al., 2017; Zhang and Li, 2018). When comparing among the different MAD treatments, solid-liquid distribution coefficient was very similar between MAD and PAST+MAD but lower than MAD+PAST. This could be explained because in the PAST+MAD treatment, the PhACs solubilized by the initial pasteurization could be biodegraded during the subsequent MAD treatment, while in the MAD+PAST treatment, the pasteurization was done at the end and therefore the solubilized PhACs due to the increased temperature of the pasteurization could not be consumed.

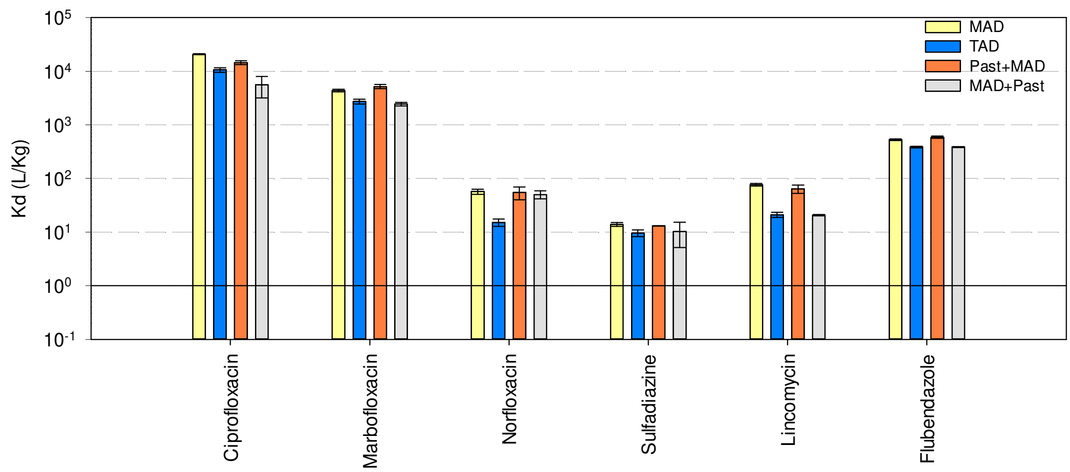

Figure 3. Solid-liquid distribution coefficient (K_d) of the PhACs detected in both phases under the different anaerobic treatments tested.

3.3. Antibiotic resistance genes

Five ARGs (*ermB*, *qnrS*, *sul*, bla_{TEM} and *tetW*), *intI1* and 16S rRNA genes were quantified in the inoculum, the swine manure and at the end of the anaerobic treatments. 16S rRNA gene copy numbers were used to normalize the relative abundances and the results are showed in Figure 4, where the presence of each gene within the overall microbial community is estimated.

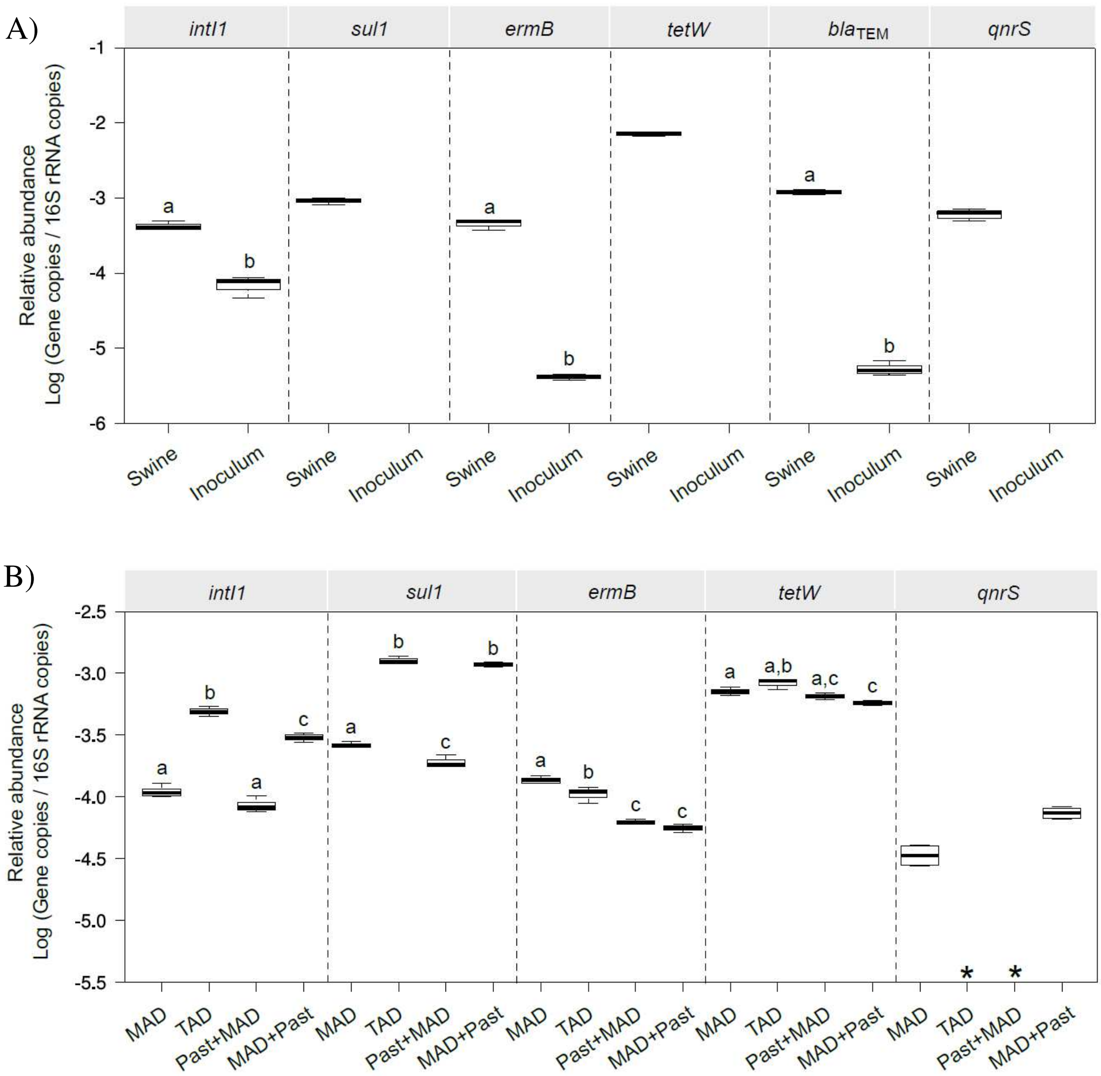

Figure 4. Amount of ARGs (relative abundance) in inoculum and swine manure (A) and at the end of the BMP tests from the different conditions tested (B). Different superscripts indicate significant difference (p<0.05). An asterisk (*) denotes values below the limit of quantification.

The quantity of the targeted ARGs presented significant differences between the inoculum and the swine manure, being more abundant in the manure and with some *sul1*, *tetW* and *qnrS* genes only detected in this stream. The genes conferring resistance to tetracyclines were the most abundant (≈-2.0±0.1 log [*tetW* copies/16S rRNA copies]) gene detected in swine manure while the relative abundance of the other four ARGs and *intI1* gene were ≈-3.2±0.2 log [gene copies/16S rRNA copies].

The most abundant ARGs detected after the anaerobic treatment (Figure 4B) were *tetW* and *sul1* which also agrees with other studies of AD with the same substrate (Gros et al., 2019; Huang et al., 2019; Zhang et al., 2020). In contrast, bla_{TEM} was not detected after any treatment, indicating that anaerobic digestion can remove this resistance gene. Past+MAD showed the highest removals ($p<0.05$) for *intI1* and *sul1* and was equally effective as MAD+Past for *ermB*.

The relative abundances of *sul1* and *intI1* gene at TAD and MAD+Past were much higher ($p<0.05$) than at MAD and Past+MAD. Higher values of horizontal transfer genes in TAD vs MAD could be explained due to increased levels of stress in the anaerobic biomass probably caused by the high concentrations of free ammonia (FA) at thermophilic temperatures (Zhang et al., 2020). At MAD, FA values were around 25 mg NH_3-N/L, while at TAD FA reached levels close to 200 mg NH_3-N/L. Other possible reason is the increase in the *Pseudomonas* genera (see Figure 5; TAD and MAD+Past) in these samples since these genera is associated to the increase in these genes (https://card.mcmaster.ca/ontology/36549).

When comparing the different treatments conducted at mesophilic temperature, MAD+Past show an increase on the abundance of ARGs as compared to MAD or Past+MAD. This could be due to temperature stress as suggested by Poole (2012) or also to the high FA levels achieved in this high temperature (for 1 hour the temperature was 70 ºC, reaching FA concentrations around 170 mg NH_3-N/L). This stress was not produced in the Past+MAD, because only the substrate was pasteurized and after it was anaerobically degraded for twenty days, while in MAD+Past, after the AD all the BMP was pasteurized.

3.4. Microbial diversity

Microbial community analyses were conducted in the swine manure, inoculum and after the anaerobic treatments. A total of 1,118,549 sequences were obtained from the swine manure, inoculum and after the anaerobic treatments. The library size of each sample was then normalized to the smallest number of sequences (28,706) in order to minimize any bias due to the difference in the total number of sequences. The number of OTUs observed at a 97% taxonomic cut-off ranged from 787 (in the swine samples) to 1,790 (in the inoculum samples). Shannon diversity index and Chao richness estimators were also determined (Table 4), indicating higher diversity and richness in the inoculum as compared with the other samples. Interestingly, TAD showed a significantly higher diversity and richness than MAD ($p<0.05$). Although direct comparisons with other studies cannot be established due to methodological differences, the results seem to be consistent with a previous study (Gao et al., 2018), in which TAD had higher diversity and richness than MAD during the treatment of municipal solid waste.

Table 4. Measures of α diversity for the different samples.

Sample	**No. of OTUs**	**Shannon diversity index**	**Chao1 richness estimator**
Swine	787 ± 81 ^{a}	5.32 ± 0.04 ^{a}	1,035 ± 97 ^{a}
Inoculum	1,790 ± 28 ^{b}	5.71 ± 0.02 ^{b}	2,741 ± 106 ^{b}
MAD	1,063 ± 17 ^{c}	4.55 ± 0.02 ^{c}	1,543 ± 25 ^{c}
TAD	1,431 ± 20 ^{d}	5.35 ± 0.02 ^{a}	2,376 ± 185 ^{d}

Past+MAD	955 ± 18 ^{e}	4.30 ± 0.04 ^{d}	1,369 ± 90 ^{c}
MAD+Past	1,379 ± 13 ^{d}	5.13 ± 0.01 ^{e}	2,101 ± 105 ^{d}

Values are means of three replicates ± standard deviation. Different superscript letters indicate significant differences (p<0.05) among the samples.

The microbial community structure present in the swine manure differed significantly from the one present in the inoculum (Supplementary material). As expected, the microbial community structure at the end of the anaerobic tests was similar to the inoculum, as this contributed to a higher percentage in solids in the BMP. However, the treatment at thermophilic conditions resulted in a significantly different microbial community as compared to the inoculum, probably due to the fact that only a fraction of the microorganisms present in the inoculum were able to survive under these conditions.

Although a relatively high degree of similarity among the bacterial communities was observed at the phylum level (Supplementary material), differences with respect to community structure and composition were significant at the genus level. In fact, the microbial community composition (at the genus level) in the AD samples showed high differences between the different anaerobic treatments; but in general it could be concluded that unclassified *Bacteroidales*, *Bellilinea, Sedimentibacter, Rectinema, Treponema* (except to Past+MAD) and unclassified *Thermotogae* were the six most abundant genera throughout the MAD, MAD+Past and Past+MAD. On the other hand, unclassified *Bacteroidales*, *Clostridium, Rectinema* and unclassified *Thermotogae* were the most abundant genera at TAD (Fig. 5). All of the above genera are typical in anaerobic reactors and are known to participate in the degradation of organic matter (Dong et al., 2018; Liczbiński and Borowski, 2021; Lu et al., 2019; Wang et al., 2017).

Several known pathogenic bacteria belong to the *Streptococcus*, *Clostridium* and *Pseudomonas* genera which presented the highest abundance in the present study.

Despite not all the members of these genera are pathogenic, their increase or decrease might be used as an indication of the pathogenic potential of the sample. From these, *Streptococcus* was the only that was reduced in all the anaerobic treatments tested, especially in MAD and MAD+Past, while *Pseudomona*s and *Clostridium* were only reduced in MAD and Past+MAD, being *Clostridium* sharply increased after the TAD and slightly after MAD+Past.

Raw data have been deposited in the NCBI BioProject database under accession number PRJNA738863.

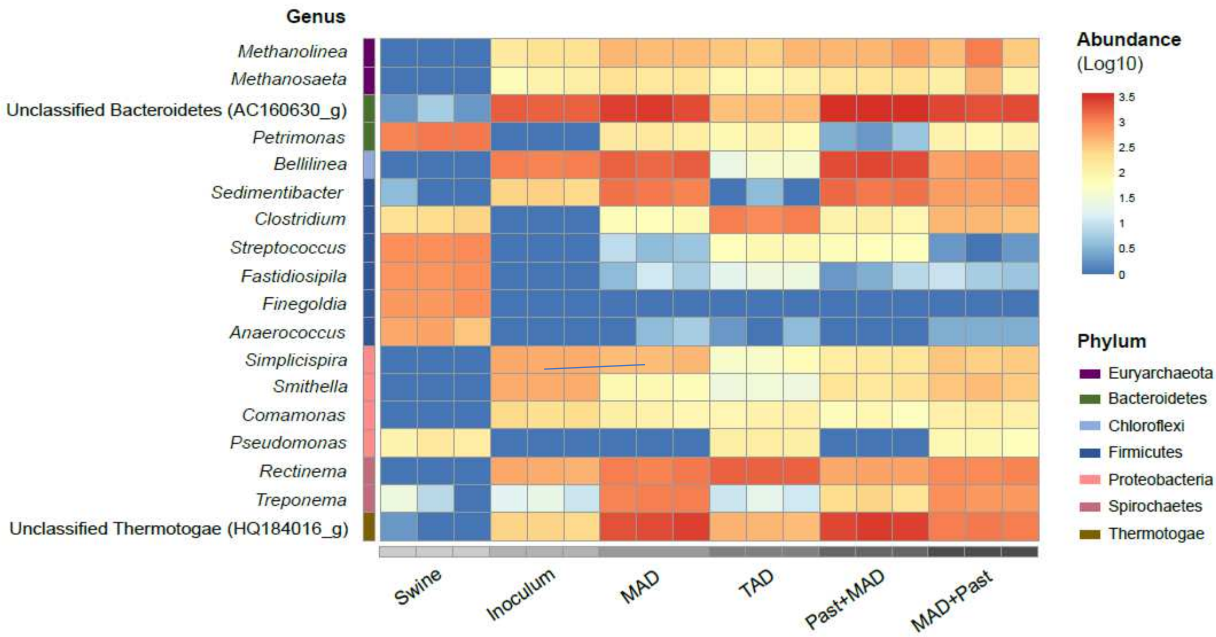

Figure 5. Heatmap of abundance of different genera present in the swine manure, inoculum and after the different AD treatments.

3.5. Overall understanding and implications

The main concern about the use of swine manure as fertilizer is the uncontrolled release of nitrogen into the soils that causes nitrate pollution in the groundwater from many regions with intensive pig farming. In this sense, European policies are focused on the regulation of this aspect, limiting the maximum quantities of organic nitrogen released into the land. However, there is an increasing concern about the presence and distribution of pharmaceutical compounds in the environment and the associated antibiotic resistance bacteria or antibiotic resistance genes. Therefore, controlling diffuse pollution caused by emerging pollutants present in all manures is becoming urgent.

This paper focuses on the anaerobic treatment of swine manure, with special attention on the fate of different PhACs used as veterinary drugs during anaerobic digestion processes. While removals differ among the compounds (from 0 to 70%), TAD treatment is the option providing the best results. Important to consider also is the solid-liquid distribution which shows that the biggest fraction of the assessed PhACs remains in the solid phase. Therefore, one possibility to reduce the release of these PhAcs into the environment could be the use of only the liquid fraction as fertilizer.

The anaerobic treatment is also used to reduce the solids content and their pathogenic potential. The reduction of solids causes a decrease in the overall genes content, including ARGs. However, to really compare among the different treatments, the evaluation of ARGs presented in this paper has been done in relative abundance. Results indicate that swine manure contains significant amounts of all ARGs tested but most of them are reduced during the different anaerobic treatments. It is important to stress that microbial analysis detected 7 genera of microbes in the swine manure, with 3 of them known to contain pathogenic microorganisms (*Streptococcus, Clostridium and Pseudomonas*). Although the link between the detected ARGs and the pathogenic microorganisms cannot be made, the high abundance of these genera in the swine manure and the relatively high numbers of ARGs increases the probability that some pathogens show resistance to some of the antibiotics present in the manure. And this highlights again the need of proper treatments for swine manure, to reduce the release of antibiotic resistance pathogens into the environment which is a major threat for our environment and health.

4. Conclusions

The conclusions obtained are:

- Mesophilic temperature provided the highest SMP in BMPs conducted during 20 days with swine manure.
- The highest PhACs removals were observed at TAD. After the anaerobic treatments, the desorption of PhACs from solid to aqueous phase was the highest in the TAD followed by MAD+PAST.
- Past+MAD are more effective in removing *sul1*and *intI1*genes than other tested conditions. For *ermB*, Past+MAD was equally effective than MAD+Past. *bla*$_{TEM}$ was the only ARG completely removed under all the conditions.
- Only *Streptococcus* decreased in all the treatments after AD, especially in MAD and MAD+Past, while *Pseudomona*s and *Clostridium* were only decreased in MAD and Past+MAD, being *Clostridium* sharply increased after the TAD and slightly after MAD+Past.

E-supplementary data of this word can be found in online version of the paper.

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