Assessing the occurrence of pharmaceuticals and antibiotic resistance genes during the anaerobic treatment of slaughterhouse wastewater at different temperatures

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Abstract

This study investigates the effect of psychrophilic, mesophilic and thermophilic temperatures on the anaerobic treatment of slaughterhouse wastewater, in terms of biogas production, occurrence of 30 pharmaceutical compounds of veterinary use, 4 antibiotic resistance genes (ARGs) which provide resistance to tetracyclines (*tetW*), fluoroquinolones (*qnrS*), , macrolide-lincosamide-streptogramin (*ermB*) and sulfonamides (*sul1*) antibiotics, as well as class I integron-integrase gene (*intI1*), related to horizontal gene transfer. The highest methane yield was obtained at a mesophilic temperature (35ºC) (323 mL CH_4/ g TCOD) followed by the yield obtained at thermophilic temperature (53ºC) (242 mL CH_4/ g TCOD). Regarding pharmaceuticals, chlortetracycline, oxytetracycline, tilmicosin, and lincomycin were the most abundant in the slaughterhouse wastewater, being detected predominantly in the solid phase (with median concentrations >200 μg/kg dry weight). On the other hand, ciprofloxacin,

ofloxacin, norfloxacin, lincomycin and ibuprofen were the most predominant in the anaerobic digestate regardless of the treatment temperature. Psychrophilic temperatures (21 ºC) exhibited moderate to low pharmaceuticals removal, while a large fraction of them were removed at a thermophilic temperature reaching 70-90 % removals for tetracycline, macrolides and one sulfonamide (sulfapyridine).

The highest relative abundance of the quantified ARGs was found at 53 ºC, suggesting that thermophilic temperatures normally associated with better removals of pathogens do not necessarily show better removals of antibiotic resistance genes.

Keywords: Anaerobic treatment; slaughterhouse wastewater; pharmaceutical compounds; antibiotic resistance genes; temperature.

1. Introduction

The meat processing industry has been recognized as one of the industrial sectors with the largest freshwater utilization, being equivalent to 29 % of the water consumption by agriculture (Gerbens-Leenes et al., 2013; Mekonnen and Hoekstra, 2012). This is expected to increase in the following decades, as predictions for the global meat production are also rising with current estimations assuming a consumption of 465 million tonnes by 2050 (Aziz et al., 2019). Slaughterhouses produce large volumes of wastewater and solid waste as a result of their production and cleaning activities. (Cuetos et al., 2008). Slaughterhouse wastewater (SWW) contains high amounts of carbohydrates, proteins and lipids and is considered an important source of pathogens and antibiotic resistant microbes, thus potentially contributing to the spread of these pollutants into the natural water bodies (Gros et al., 2019; Savin et al., 2020). In this sense, SWW is considered one of the most detrimental industrial wastewaters because

of its inadequate treatment in many countries, causing severe river and groundwater pollution (Aziz et al., 2019; Bustillo-Lecompte et al., 2016; Odekanle et al., 2020).

Different technologies are being used for the treatment of SWW, including physical–chemical methods and biological aerobic processes (Diez et al., 2020; Gannoun et al., 2009). However, anaerobic treatment processes are attracting attention due to its associated advantages: excellent organic matter removal, able to withstand higher organic loading rates as compared with other biological treatments, low generation of sludge, reduced costs, higher removal of pathogens and the conversion of the organic fraction into valuable by-products, such as biogas. This last aspect is especially interesting when taking into account the global energy crisis and continuous long-term increase in the demand and price of fossil fuels (Lin et al., 2018; Shilpi et al., 2019; Wu et al., 2016).

Within anaerobic digestion (AD), one of the most important factors to consider is temperature (T), having a direct effect on its performance and the stabilization of the waste. Three different operating temperature intervals are described in the literature: i) psychrophilic range (<25 ºC, PAD), ii) mesophilic range (30–40 °C, MAD) and iii) thermophilic range (50–60 °C, TAD) (Rodríguez-Valderrama et al., 2019). The optimum digestion temperature must be selected considering the potential biogas yield, quality of the digestate and heat requirement to achieve the desired temperature.

In general, it is assumed that increasing temperature results in an increase in biogas production. However, this is not always the case especially in waste with high content of proteins such as SWW, since the high levels of ammonia generated during protein degradation can inhibit the digestion process (Chae et al., 2008). Most of the studies dealing with SWW have been conducted in mesophilic and thermophilic temperatures.

Mesophilic temperatures are the most desired for AD as they imply lower electricity requirements than thermophilic treatments for heating. Moreover, at mesophilic more microbial groups can be active and present lower risk of ammonia inhibition and process instability (Schmidt et al., 2019). However, thermophilic temperatures have been shown to display better pathogen removal (i.e. fecal coliforms and salmonellae) which is an important aspect to consider when dealing with SWW (Loganath and Senophiyah-Mary, 2020).

SWW is also characterized by having a significant presence of pharmaceutical compounds (PhACs). This is not surprising since the use of veterinary PhACs is a common practice in the animal production industry. The consumption of these compounds depends on the level of industrialization in the farming sector and have a high variability among countries (Gonzalez and Angeles, 2017). For example, in China the total use of veterinary antibiotics in 2013 was approximately 80,000 tons as compared to 11,000 tons used in the US (Chollom et al., 2020). Within the European Union, Germany and Spain are the countries with the highest antibiotic consumption (Gonzalez and Angeles, 2017). The occurrence of PhACs during anaerobic treatment is widely reported in literature (Oliver et al., 2020; Varel et al., 2012), but no clear general trend has been found between the temperature applied and their effect on their removal. In fact, most of the studies concluded that a temperature increase favors the biodegradation of some PhACs and reduce the removal of others (Carballa et al., 2007; Davidsson et al., 2014; Gros et al., 2020; Zhou et al., 2015). Related to antibiotic resistance genes (ARGs), literature is even more contradictory, since some studies recommend thermophilic digestion for ARGs removal (Oliver et al., 2020; Zou et al., 2020) and others do not (Huang et al., 2019).

Overall, experimental evidence on the combined removal of PhACs and ARGs in complex wastewaters such as SWW is still limited (Chollom et al., 2020) and a direct comparison of their occurrence at different temperature ranges is still missing.

In this study the methane production potential of SWW coming from the inlet of an industrial slaughterhouse wastewater treatment plant (SWWTP) was investigated at different temperatures (21ºC, 35 ºC and 53 ºC). A characterization of the SWW and the inoculum was also conducted considering macro and micropollutants. The presence of 30 multiple-class PhACs was analyzed at before and after the anaerobic treatments to assess their possible removal, considering both solid and liquid phases. Finally, class I integron-integrase gene (*intI1*) related to horizontal gene transfer together with 4 different ARGs was quantified before and after the different anaerobic treatments tested.

2. Materials and methods

2.1 Anaerobic inoculum and slaughterhouse wastewater

The sludge used as inoculum was collected from a mesophilic (35º C) anaerobic digester from Girona WWTP (Catalonia, Spain). SWW was sampled from a pig slaughterhouse plant which handles approximately 2,000 m^3/d of SWW located in Catalonia-Spain.

Inoculum and SWW characteristics are shown in Table 1.

Table 1. Inoculum and SWW characteristics.

Parameter	Inoculum	SWW
pH	7.2±0.2	6.7±0.1
TCOD (mg/L)	13,520 ± 184	6,786 ± 147
SCOD (mg/L)	167 ± 6	2,254 ± 3

TS (g/L)	15.0 ± 0.2	n.m
VS (g/L)	9.8 ± 0.1	n.m
TSS (g/L)	n.m	1.7 ± 0.2
VSS (g/L)	n.m	1.5 ± 0.1
PO_4^{3-}-P (mg /L)	40.6±1.2	31.1±0.1
Cl^- (mg /L)	255±1	2,523±2
Na^+ (mg /L)	119±1	1,638±1
NH_4^+-N (mg/L)	511±5	173±1
Acetic acid (mg/L)	3.6±0.2	458±14
Propionic acid (mg/L)	n.d	355±28
Isobutyric Acid (mg/L)	n.d	81±2
N-Butyric acid (mg/L)	n.d	104±8
TKN (mg/L)	1,162±5	350±1

n.m: not measured; n.d: not detected.

2.2 Biochemical Methane Potential (BMP) tests

The maximum specific methane production from SWW was quantified using BMP tests. They were conducted under three different temperatures (psychrophilic "PAD=21ºC", mesophilic "MAD=35 ºC" and thermophilic "TAD=53 ºC"). 250 mL bottles (150 mL working volume) were used for the BMP tests with a ratio Inoculum/ Substrate (I/S) of 2 (Zahedi et al., 2017). After sealing the BMP bottles, they were placed in three different incubators controlled at 21 ºC, 35 ºC and 53 ºC. To ensure mixing, the bottles were placed in shakers at 150 rpm. All tests were conducted in triplicates. The biogas produced in the blanks (only with inoculum and without SWW) was subtracted from the biogas obtained in the other tests conducted with SWW. The BMP tests lasted for 28 days. Specific methane production (SMP) was expressed in milliliters of methane produced per gram of TCOD added (at normal condition, P=1 atm and T^a= 0ºC).

2.3 Analytical methods

Classical parameters in AD

Total solid (TS), volatile solid (VS), total suspended solid (TSS), volatile suspended solid (VSS), total Kjeldahl nitrogen (TKN), total chemical oxygen demand (TCOD) and soluble COD (SCOD) were analysed following standard methods (APHA, 1995). Ions and volatile fatty acids (VFA) were analyzed via ion chromatography (ICS5000, DIONEX) and gas chromatography (Trace GC Ultra ThermoFisher Scientific) respectively. pH and conductivity were measured using a pH meter and conductivity meter (Crison).

The biogas volume was measured using the same methodology reported in literature (Zahedi et al., 2018). At the beginning of each sampling the pressure of the headspace of each BMP bottle (volume of 100 mL) was recorded using a pressure sensor (PM7097, IFM). An infrared CH_4 sensor (GasTech S-Guard) was used to monitor the CH_4 content in the biogas.

Analysis of PhACs

PhACs concentrations were analyzed in the inoculum, SWW and outlet samples of the BMP tests. The analysis of 30 PhACs (Table S1) was carried out in the solid and aqueous phases, separately, using a method adapted from the one described in Gros et al. (2019). Methodology, chemicals and reagents are described in supplementary material..

The total concentration (liquid+solid) was calculated according to Gros et al. (2019) employing the following equation:

$$C = [C_{liquid} + C_{solid} * gr_p/gr_s * \%\ TS_{\ in\ the\ solid\ fraction}] \quad \text{(Eq. 1)}$$

where C_{liquid} *and* C_{solid} are the concentrations of PhACs quantified in the liquid and solid (lyophilizate) samples respectively, gr_p and gr_s are the weight of the solid fraction and the weight of the sample before centrifugation, respectively and % TS in the solid fraction was calculated as the weight after lyophilization/weight before lyophilization*100.

Eq. 2 was used to calculate the initial concentration of PhACs in the inlet:

$$Cin = [(CI * VI) + (CSWW * VSWW)/(VI + VSWW)] \quad \text{(Eq. 2)}$$

where CI and CSWW are the concentrations of PhACs measured in inoculum and SWW respectively, while VI and VSWW are the volumes of inoculum and SWW added in each BMP test, being 75 and 75 mL respectively.

DNA extraction and quantification of antibiotic resistance genes

Inoculum, SWW and outlet samples of the BMP tests were collected in triplicate and centrifuged at 4,000 *g* for 10 min at 4 ºC. The obtained pellet was suspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA and 1.2% Triton X-100) and treated with lysozyme (20 mg/mL) and proteinase K (10 mg/mL). The standard phenol-chloroform method was used for the extraction of genomic DNA (Sambrook and Russell, 2001), and Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA, USA) was used to determine its concentration.

Copy numbers of the four ARGs analysed (giving resistance to sulfonamides (*sul1*) antibiotics, tetracyclines (*tetW*), macrolide-lincosamide-streptogramin (*ermB*) and fluoroquinolones (*qnrS*)) were quantified by Real-time PCR (qPCR) assays (Marti et al., 2013; Subirats et al., 2017), These ARGs were chosen for being representatives of the most important antibiotic groups used in veterinary, and have been already identified in slaughterhouse samples in previous (Gros et al., 2019). Class I integrons (*intI1*), related

to horizontal gene transfer and the 16S rRNA gene, indicative for bacterial abundance were also quantified. The conditions detailed in Maeda et al., (2003) were used to quantify the selected genes. Briefly, a MX3005P system (Agilent Technologies; Santa Clara, CA, USA) was used for all qPCR assays, and dissociation curves were generated (from 60 to 95 °C). ANOVA or Student's t-test (p<0.05) were used to compare the data.

3. Results and discussion

3.1 Biogas production

The effect of the different temperatures tested (21 ºC, 35 ºC and 53 ºC) on the inoculum is presented in Figure S1. The fact that methane production was obtained under all temperatures indicates the presence of thermophilic, mesophilic and psychrophilic microorganisms in the inoculum. Fig. 1 shows the cumulative specific methane production (SMP). Results indicate that temperature had a significant influence on the anaerobic process. BMP tests conducted at 35ºC presented the highest production of methane and the shortest start-up. This is might be due to the fact that the biomass used as inoculum was withdrawn from an anaerobic digester working at 35 ºC and more time for adaptation to the PAD (21 ºC) and TAD (53 ºC) temperatures was required. A very low CH_4 production was observed during the first 10 days at 53 ºC but increased exponentially afterwards till reaching its maximum around day 15, according to the thermophilic blanks (Figure S1). The lowest CH_4 production rate was obtained under psychrophilic conditions (21 ºC) which presented a constant and linear increase till the end of the tests.

Microorganisms grow best at temperature ranges of mesophilic and thermophilic than in psychrophilic (Hagos et al., 2016). It has been shown that temperature increase the maximum specific growth rate of microbes (Bouskova et al., 2005) and the hydrolysis

step (Petropoulos et al., 2017). Generally, an increased temperature has a positive effect on the metabolic rate of microorganisms and accelerates the digestion processes (Hagos et al., 2016), but it is not always true, especially when the substrate has high amount of proteins or ammonia, as is the case of slaughterhouse wastewaters, increasing risk of free ammonia inhibition and process instability (Schmidt et al., 2019). In the present paper some free ammonia (it will be commented in the next section) inhibition on the methanogenic population under thermophilic conditions could have happened and it might explain the higher values of propionic acid obtained and the lower SMP in these tests. The lower SMP in PAD (71±17 mL CH_4/ g TCOD), as compared to the tests conducted at MAD is in agreement with Agler et al. who observed an approximate four times decrease in activity when the operating temperature decreased from 37 ºC to 22 ºC (Agler et al., 2010). Also the increase of methane solubility at lower temperatures results in less methane being released to the gas phase, decreasing the SMP (Noyola et al., 2006; Skouteris et al., 2012).

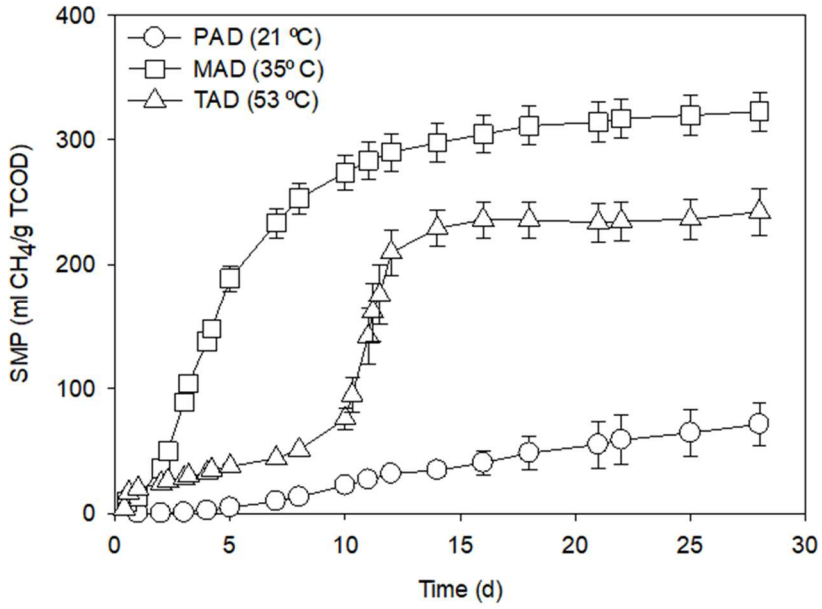

Figure 1 Cumulative specific methane production with standard deviation from the SWW at PAD (21 ºC), MAD (35 ºC) and TAD (53 ºC).

3.2 Effluent characteristics

Table 2 presents the results obtained from the characterization of the three different digestates at the end of the BMP tests.

Table 2. Parameters analyzed at the end of the BMP tests with standard errors.

Parameter	PAD (21 ºC)	MAD (35 ºC)	TAD (53 ºC)
pH	7.7±0.0	7.7±0.0	8.2±0.1
Conductivity (Sm/cm)	7.1±0.1	7.2±0.1	7.4±0.1
TCOD (mg/L)	7,980±10	7,770±70	7,560±85
SCOD (mg/L)	256±12	175±2	800±20
TS (g/L)	10.16±0.45	10.03±0.26	9.32±0.19
VS (g/L)	5.51±0.02	5.27±0.02	4.84±0.03
Cl^- (mg /L)	1,433±2	1,426±1	1,420±1
Na^+	890±1	883±1	895±1
$N\text{-}NH_4^+$ (mg/L)	482±0	522±2	627±8
Acetic acid (mg/L)	5.8±0.6	3.6±0.3	44.0±3.0
Propionic acid (mg/L)	n.d	n.d	274±10
Isobutyric Acid (mg/L)	n.d	n.d	88±1
N-Butyric acid (mg/L)	n.d	n.d	n.d
TKN (mg/L)	816±11	810±6	829±12

n.d: not detected

For all conditions, an average TCOD removal percentage of 23 % ± 3 % was obtained. The high amount of non-biodegradable COD present in the BMP tests and mostly coming from the inoculum explains this relatively low removal value observed. The inoculum comes from a full-scale anaerobic sludge digester operating with 20-30 days of hydraulic residence time (HRT). Under these HRTs, almost all the biodegradable material has already been eliminated, practically leaving organic matter that is difficult to biodegrade. In the BMP to maintain an I/S ratio of 2, half the volume was provided by the inoculum with a TCOD of 13,520 mg/L while the other half was provided by the

SWW, with a TCOD content of 6,786 mg/L, resulting in an initial TCOD concentration in each BMP of around 10,150 mg/L. Therefore, even if 100 % of the organic matter present in the SWW was degraded (6,786 mg/L), the elimination percentages of TCOD would have been less than 40 %. In addition, taking into account that more than 30 % of the organic matter of the SWW can be non-biodegradable (Aziz et al., 2019; Ortner et al., 2020), a 23 % removal of TCOD indicates a good performance of the AD process.

In terms of effluent characteristics, very similar values were found in the BMPs conducted at 35 ºC and 21 ºC, despite the last ones only resulted in a 22 % of the methane detected at 35ºC. This was also observed by Connaughton et al. (2006), that found no similar COD removal efficiencies between mesophilic and psychrophilic conditions for AD of brewery wastewater while detecting a 50 % reduction in the SMP at the psychrophilic temperatures. As previously mentioned, the low SMP obtained under 21 ºC might be attributed to a reduction in the anaerobic activity of the biomass and also to an increase of the methane solubility (Agler et al., 2010; Noyola et al., 2006).

TKN values at the end of the BMP tests were more or less constant (805-837 mg/L) and lower than the values of the TKN that the inoculum is exposed to in the full scale anaerobic digester where it was withdrawn from (around 1,000 mg/L see Table 1). Ammonium values were influenced by the operational temperature due to higher hydrolysis of proteins at higher temperatures (Kim et al., 2003; Mehari et al., 2018). The concentration of ammonium at the end of the BMPs was 482±1 mg/L, 522±2 mg/L and 627±8 mg/L at 21 ºC, 35 ºC and 53 ºC, respectively. These values were lower than those reported (684-1239 mg/L) by Ortner (2020) after anaerobic mesophilic AD of SWW in a batch reactor (Hansen et al., 1998; Mahdy et al., 2020; Yenigün and Demirel, 2013). The concentration of free ammonia (FA), which has been reported to inhibit the

digestion process (Yenigün and Demirel, 2013) was calculated using the equation from Anthonisen et al., (1976) $((1.214\times NH_{4+N}\times 10^{pH})/(e^{6344/(273+T(^{o}C))}+10^{pH}))$. According to this formula our FA values were around 10, 28 and 225 mg $NH_{3\text{-}}N/L$ in PAD, MAD and TAD, respectively. It has been reported that inhibition of the methanogenic activity and VFA accumulation could occur at an FA level of 40 mg $NH_{3\text{-}N}/L$, indicating that the FA present in the thermophilic tests could be partially inhibit the production of biogas. However, it is not clear to which extend this concentration is inhibitory as other researchers have only found inhibition at FA concentrations of 400 mg $NH_{3\text{-}}N/L$ or even higher. (Hansen et al., 1998; Liu et al., 2019; Palatsi et al., 2011; Sutaryo et al., 2014; Yenigün and Demirel, 2013). Some inhibition on the methanogenic population under thermophilic conditions could explain the higher propionic values and the lower SMP in these tests. Higher values of VFA at thermophilic temperature has been also reported in the literature with other substrates (Hao and Wang, 2015).

3.3 Pharmaceutical compounds

16 out of the 30 targeted compounds were detected at the beginning or at the end of the BMP tests being predominant in the solid fraction (Table 3), as has been found in other studies (Boix et al., 2016; Gros et al., 2019; Yang et al., 2016). High differences were observed between the presence of these PhACs in the inoculum and the SWW. These differences are attributed to the fact that the inoculum came from a municipal WWTP and the SWW came from a meat processing industry.

275 **Table 3.** PhACs measured in the liquid and solid fractions from the inoculum, the SWW and the effluent of the BMP tests.

Compounds	Inlet				Outlet					
	Inoculum		SWW		21 ºC		35 ºC		53 ºC	
	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	n.d	n.d	8.44±0.56	n.d	n.d	n.d	n.d	n.d	n.d
Tilmicosin	n.d	n.d	1.75±0.09	206.43±6.56	blq	110.44±4.00	blq	88.47±8.43	blq	28.28±16.42
Lincomycin	n.d	n.d	20.07±0.34	506.80±121.1	13.68±0.14	330.32±0.89	7.29±0.22	264.65±21.21	7.60±0.26	n.d
Tetracycline	n.d	455.08±30.08	n.d	n.d	n.d	356.13±5.51	n.d	361.51±30.08	n.d	238.90±27.89
Oxytetracycline	n.d	128.81±10.94	Blq	357.20±7.97	n.d	173.74±2.27	n.d	193.15±12.93	n.d	81.30±8.06
Chlortetracycline	n.d	30.96±4.65	0.50±0.02	10236±1363	n.d	1783±34	n.d	1646±120	n.d	550±87
Ciprofloxacin	0.66±0.08	13798±740	n.d	n.d	0.67±0.08	12720±512	0.88±0.07	14245±329	2.39±0.19	15275±4113
Ofloxacin	blq	2884±155	blq	47±24	blq	2583±122	0.31±0.05	2318±166	1.04±0.06	2569±534
Enrofloxacin	n.d	37.90±0.21	blq	90.11±4.98	n.d	58.64±0.55	n.d	58.64±0.93	n.d	64.55±4.80
Flubendazole	n.d	n.d	blq	38.06±0.43	blq	blq	blq	blq	blq	blq
Norfloxacin	n.d	2617±510	n.d	n.d	n.d	2476±189	n.d	2745±437	n.d	2289±232
Azithromycin	n.d	234±33	n.d	n.d	n.d	134±8	n.d	97±5	n.d	blq
Sulfadiazine	n.d	n.d	0.24±0.001	4.11±0.30	0.21±0.01	3.26±0.46	0.13±0.01	3.68±0.10	0.19±0.01	2.73±0.14
Sulfapyridine	n.d	20.87±0.85	n.d	n.d	n.d	8.47±0.05	n.d	10.55±0.34	n.d	blq
Diclofenac	1.03±0.05	76.25±10.79	n.d	n.d	0.76±0.01	34.83±1.08	0.58±0.06	33.66±1.19	0.80±0.10	22.09±4.21
Ibuprofen	15.71±0.30	236.41±24.27	0.42±0.04	n.d	7.45±0.06	83.53±8.58	8.03±0.39	114.69±1.61	11.29±0.30	78.10±0.73

276 n.d: non-detected; blq: below limit of quantification; * Refers to Kg of dry weight.

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278 Chlortetracycline, oxytetracycline (tetracyclines), tilmicosin (macrolide), and
279 lincomycin (linsosamide) were the most abundant PhACs in the SWW (with median
280 concentrations >200 μg/kg dry weight in the solid fractions) and are widely used
281 antibiotics in animal breeding operations. Other compounds, such as the
282 fluoroquinolone antibiotics ofloxacin or enrofloxacin, sulfadiazine (sulfonamide),
283 tiamulin (macrolide) or the anti-helminthic flubendazole, also used in animal
284 production, were detected in liquid and solid fractions of the SWW at concentrations
285 lower 1 μ g/L and 100 μ g/kg dry weight, respectively. All these compounds are
286 antibiotics commonly used as veterinary drugs to treat or prevent respiratory tract
287 infections, pneumonia, gastrointestinal disease and general bacterial infections in pigs,
288 cattle and sheep.

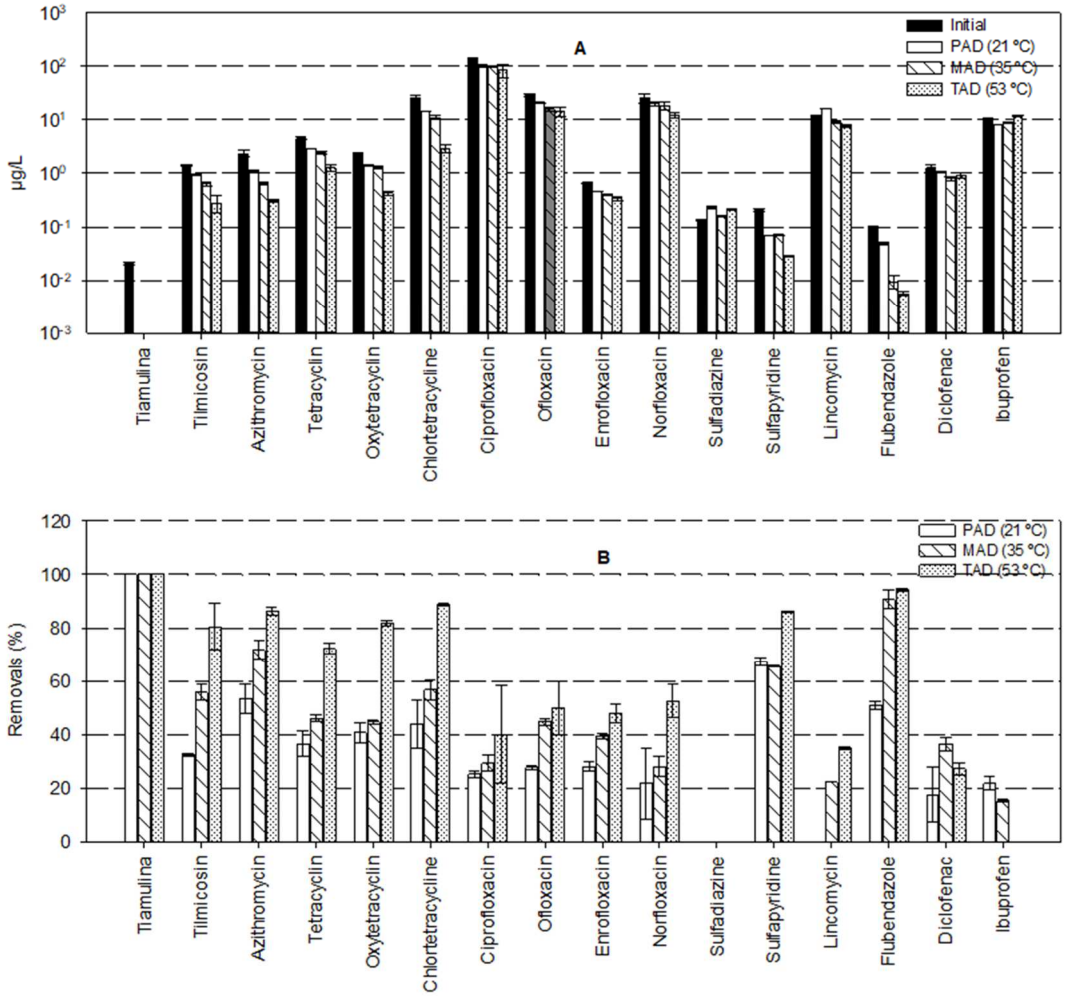
289 Regarding the inoculum, ciprofloxacin, ofloxacin, norfloxacin (fluoroquinolones),
290 azithromycin (macrolide), tetracycline and ibuprofen (analgesic/anti-inflammatory)
291 were the most predominant compounds (with median concentrations >200 μg/kg in the
292 solid fractions). Tetracycline, chlortetracycline and oxytetracycline, enrofloxacin
293 (fluoroquinolone antibiotic), sulfapyridine (sulfonamide antibiotic) and diclofenac (anti-
294 inflammatory), also used in human medicine, were detected in the liquid and solid
295 fractions of the inoculum at concentrations below 1 μ g/L and 200 μ g/kg dry weight.

296 Results indicate that SWW is a considerable source of PhACs when used as substrate
297 for anaerobic digestion; in fact, the occurrence of tilmicosin, lincomycin, flubenzole and
298 sulfadiazine in the inlet of the BMP was only attributed to SWW inputs (Table 3 and
299 Figure 2). On the contrary, ciprofloxacin, norfloxacin, azithromycin, tetracycline and
300 ibuprofen were introduced in the BMPs only through the inoculum. Chlortetracycline
301 and oxytetracycline were detected in both SWW and inoculum, but they were
302 predominant in the SWW.

303 Figure 2A depicts the average concentration of the pharmaceuticals in the inlet and in
304 the AD outlet at the different temperatures tested (logarithmic scale) considering both
305 liquid and solid phases (see equation 1 material and methods section). The initial
306 concentration in the BMP tests was calculated according the equation 2 (see material
307 and methods section). To facilitate the comparison among the different conditions
308 tested, the reduction of the individual PhACs is depicted in Figure 2B. Most PhACs
309 (except lincomycin (at 21 ºC), sulfadiazine (at any temperature) and ibuprofen (at 53
310 ºC)) were reduced during the AD process although their removal percentages differed
311 among the different treatments. Other literature studies have reported no removal or
312 even an increase in concentration for ibuprofen, sulfadiazine and lincomycin in
313 anaerobic conditions which is agrees with our findings (Feng et al., 2017a; Gonzalez-
314 Salgado et al., 2020; Gros et al., 2019). An increase in the levels of PhACs can be due
315 to the transformation of metabolites to the original compounds during the anaerobic
316 treatment as was reported in an anaerobic sewer system (Jelic et al., 2015), chemical
317 changes in the SWW and inoculum during their removal or analytical limitations, such
318 as matrix effects (Gros et al., 2020). In general, the removals of all groups of antibiotics
319 were positive and influenced by temperature. BMP tests conducted at psychrophilic
320 temperature (21 ºC) resulted in lower PhACs removal as compared to the tests
321 conducted at mesophilic (35 ºC) or thermophilic (53 ºC) temperatures, which displayed
322 the highest removals. These results are in line with other literature, with AD resulting in
323 higher PhACs removal when operating under thermophilic conditions (Feng et al.,
324 2017b; Samaras et al., 2014; Youngquist et al., 2016) and in disagreement with others
325 that suggested that temperature was not important for the reduction of PhACs (Boix et
326 al., 2016; Gonzalez-Gil et al., 2016; Malmborg and Magnér, 2015). The reduction
327 percentages for tetracyclines, macrolides and fluoroquinolones were doubled under

thermophilic conditions as compared to psychrophilic temperatures. Macrolides (except tiamulin, which was totally degraded) showed average reduction values of 43±15 %, 64±12 % and 83±4 % at psychrophilic, mesophilic and thermophilic temperatures, respectively. In the case of tetracyclines the mean reduction percentages were 41±4 % (21 ºC), 49±7 % (35 ºC) and 81±8 % (53 ºC) and for fluoroquinolones a reduction of 26±2 % (21 ºC), 36±8 % (35ºC) and 48±6 % (53 ºC) was achieved.

A possible reason why higher PhACs removals were observed at higher temperatures could be that thermophiles have higher metabolic activity, and also that the solubility and bioavailability of some persistent organic pollutants are greatly increased, and thus, the degradation of the pollutants by thermophiles can be faster and complete (Jing-lan et al., 2012).



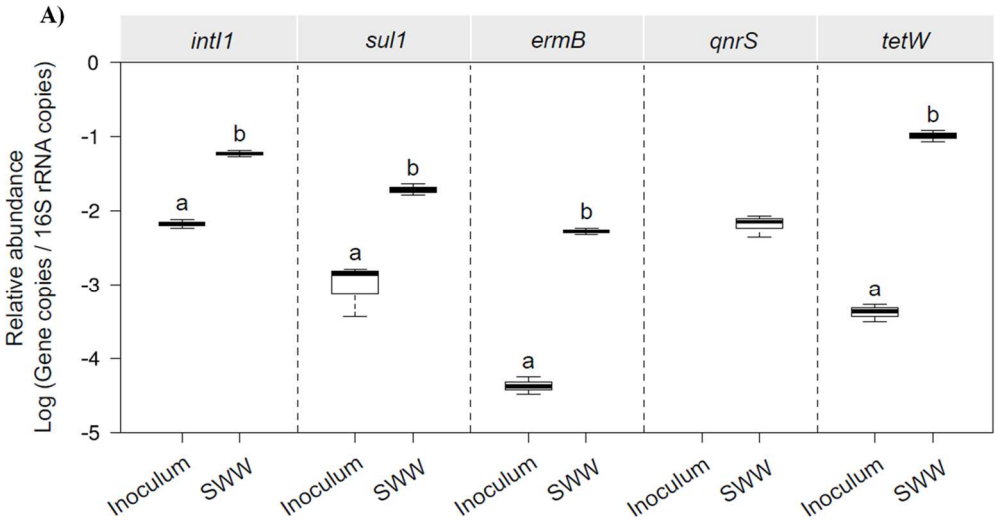
339

340 **Figure 2.** A) Concentration of PhACs at the beginning (initial) and at the end of the
341 BMP tests at different temperatures (21ºC, 35ºC and 53ºC); B) Removals of PhACs in
342 the BMP tests conducted at 21 ºC, 35 ºC and 53 ºC.

343 3.4 Antibiotic resistance genes

344 The four target ARGs, *qnrS*, *tetW*, *ermB* and *sul1* (conferring resistance to
345 fluoroquinolones, tetracyclines, macrolide-lincosamide-streptogramin (MLS) antibiotics
346 and sulfonamides, respectively), *intI1* (genetic marker of anthropogenic influence and
347 potential mobilization of ARGs) and 16S rRNA genes were measured in the inoculum,
348 the SWW and at the end of the BMP tests conducted at different temperatures. Their

relative abundances were normalized to the 16S rRNA gene copy number so an estimation of the presence of the targeted gene within the overall microbial community could be estimated (Figure 3). Results are expressed in logarithmic scale, where values of -1, -2, -3 and -4 indicate the presence of 1 resistance gene for every 10, 100, 1,000 and 10,000 copies of 16S rRNA gene, respectively.



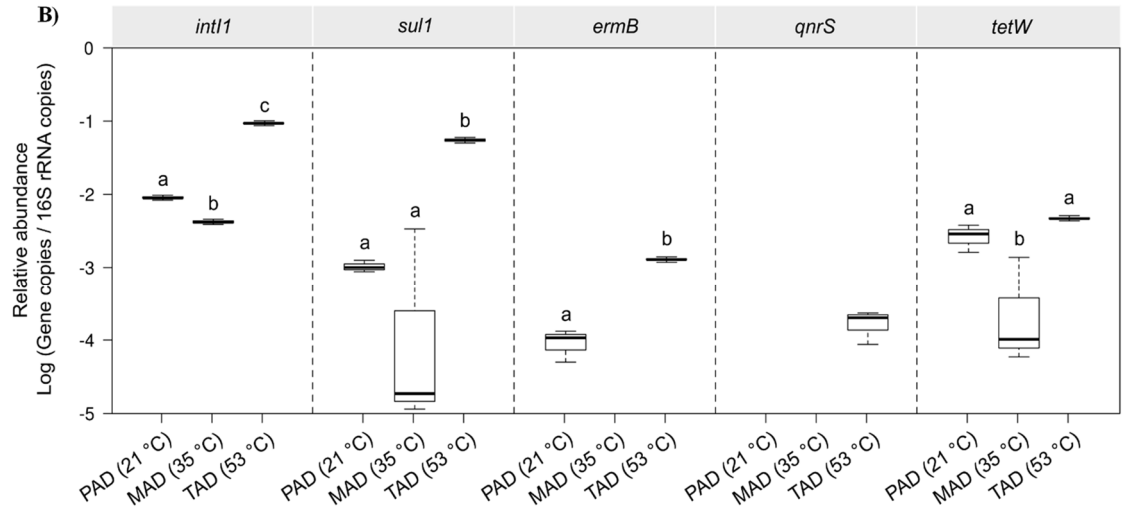


Figure 3. Relative abundance of ARGs in A) inoculum and SWW and B) anerobic effluents at different temperatures (21ºC, 35ºC and 53ºC). Different superscripts indicate significant difference ($p<0.05$).

Marked differences were observed between the inoculum and the SWW in all ARGs studied (Figure 3A), with SWW presenting higher abundances for all the ARGs. The *sul1* and *tetW* genes which confer resistance to sulfonamides and tetracyclines were the most abundant. The relative abundance of the *sul1* gene was ≈-3.0±0.4 log [*sul1* copies/16S rRNA copies] in inoculum and ≈-1.7±0.1 log [*sul1* copies/16S rRNA copies] in SWW and the relative abundance of the *tetW* gene was ≈-3.3±0.1 log [*tetW* copies/16S rRNA copies] in inoculum and ≈-0.9±0.1 log [*tetW* copies/16S rRNA copies] in SWW, respectively. Moreover, the *ermB* gene which confers resistance to macrolide-lincosamide-streptogramin antibiotics, showed a lower presence (≈-4.4±0.1 log [*ermB* copies/16S rRNA copies] in inoculum and ≈-2.2±0.0 log [*ermB* copies/16S rRNA copies] in SWW), while genes conferring resistance to fluoroquinolones (*qnrS*) were only detected in SWW (≈-2.19±0.1 log [*qnrS* copies/16S rRNA copies].

Regarding the anaerobic effluents (Figure 3B), genes conferring resistance to tetracyclines (*tetW*) and sulfonamides (*sul1*) were the most abundant in the anaerobic effluents, follow by macrolide-lincosamide-streptogramin antibiotics (*ermB*). These ARGs have been consistently reported as the most abundant ARGs detected in the anaerobic effluents from slaughterhouse sludge and pig manure (Gros et al., 2019; Huang et al., 2019; Zhang et al., 2020).

BMPs conducted under mesophilic temperature showed higher removals ($p<0.05$) of *ermB*, *tetW* and *intI1*genes than psychrophilic conditions. The *intI1* gene has implications to human health being linked to possible horizontal gene transfer between environmental bacteria and human pathogens, impacting the potential evolution and

selection of new antibiotic resistance phenotypes (Quintela-Baluja et al., 2019). *IntI1* gene has been reported to present a positive correlation with the overall abundance of ARGs (Zheng et al., 2020). In fact, a relationship between *intI1* and *sul1* was described in Gillings et al., (2015). On the other hand, the relative abundances of ARGs and *intI1* gene at 53 ºC were much higher (p<0.05) than at 35 ºC or 21 ºC. In fact, the *qnrS* gene was only detected at 53 ºC. This is very relevant since thermophilic (50-55 ºC) systems are usually considered more effective than mesophilic (35-40 ºC) in the removal of pathogens and ARGs (Gros et al., 2020; Oliver et al., 2020; Sui et al., 2016; Zhang et al., 2015). Huang et al. (2019) and Sun et al. (2019) also found an increase on the ARGs at higher temperatures in AD of swine and cattle waste. Huang et al. (2019) concluded that the increase in the relative abundance of ARGs at 55 ºC compared to lower temperatures (25 ºC and 37 ºC) was due to a change in the microbial communities, enhancing the abundance of a *Streptococcus* pathogen (22.12 %) at 55 ºC, while the relative abundance of the same microorganism at 37 ºC and 25 ºC was less than 3.5. Unfortunately we can not confirm that this was the case in our study since the abundance of *Streptococcus* was not quantified. Other reasons that could also explain the high values of ARGs at 53 ºC vs the other temperatures tested would be the higher ammonium levels in the TAD. It is reported in literature that ARGs, particularly *ermB* were augmented due to the increase levels of stress caused by free ammonia (FA) (Zhang et al., 2020).

In short, the results presented show that increasing temperature increases the quantity of ARGs and *intI1* gene during anaerobic digestion. More studies are needed to unravel the reason behind this observation which will help to clarify the potential of anaerobic digestion to attenuate ARGs in continuous feeding mode and different substrates.

4. Conclusions

In this study, the effect of psychrophilic, mesophilic and thermophilic temperatures on the performance of AD of SWW, including biogas production and the occurrence of 30 PhACs and ARGs was assessed. The main conclusions obtained are:

- BMPs conducted at 35 ºC resulted in the highest methane yield followed by the tests conducted at 53 ºC. The CH_4 obtained at 21 ºC was the lowest.
- The presence of pharmaceutical compounds was predominant in the solid fraction as compared to the soluble part, highlighting the importance of quantifying PhACs in this fraction for an accurate assessment of their occurrence.
- In general, BMP tests conducted at 21 ºC resulted in lower PhACs removal as compared to the tests conducted at 35 ºC. The highest removals detected were in the tests conducted at 53 ºC.
- The relative abundances of ARGs and *intI1* gene in SWW were much higher than the ones found in the inoculum. At TAD, higher relative copy numbers of most of the ARGs and *intI1* gene were detected as compared to MAD, indicating that higher temperatures diminishes the removal of the measured ARGs.

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