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Abstract

This study examined the combined effect of hydraulic retention time (HRT), organic loading rate (OLR) and heat pretreatment of manure (70 °C, 1 h) on the fate of *E. coli,* enterococci, *C. perfringens*, *C. difficile*, and on chemical parameters (volatile fatty acids and ammonia) that may inactivate pathogens. Semi-continuous mesophilic anaerobic reactors were fed with pig manure and horse feed. The operating conditions were 2, 3, 4 $COD.L^{-1}.d^{-1}$ (OLR), 24, 35, 46 days (HRT) and use or not of a thermal pretreatment. The levels of the chemical parameters did not reach concentrations capable of inactivating the four bacteria. Anaerobic digestion led to a Log_{10} removal > 3 (*E. coli*), 0.9-2.1 (enterococci), 0.1-0.6 (*C. perfringens*) and 0-2 (*C. difficile*). Increasing HRT only reduced the concentration of *E. coli* in the digestate. Increasing OLR reduced the Log_{10} removal of enterococci and *C. difficile*. The heat pretreatment led to non-detection of *E. coli* in the digestate, reduced the concentration of *C. perfringens* by 0.8-1.3 Log_{10} and increased the concentration of *C. difficile* by 0.04-0.7 Log_{10}. Enterococci, not detected in the heated manure, were present in the digestate. The distribution of genes encoding virulence factors of *C. difficile* (*tcdA* and *tcdB*) and *C. perfringens* (*cpa*, *cpb2* and *cpb*) was not impacted by anaerobic digestion or by the heat pretreatment. Enterococci, *C. perfringens*, *C. difficile* were present in the digestate at relatively stable concentrations regardless of the operating conditions, indicating that even with heat pretreatment, the biosafety of digestate cannot be guaranteed in mesophilic conditions.

Keywords: anaerobic digestion, thermal treatment, *E. coli*, enterococci, *C. perfringens*, *C. difficile*, operational parameters

1. Introduction

The biogas sector is among the objectives of the Energy Transition for Green Growth Law adopted in 2015 by the French Parliament, i.e., the reduction of greenhouse gas emissions, the development of renewable energies and of the circular economy. Decentralization of renewable energy has been promoted in France in the last decade and has led to a significant increase in the number of decentralized on-farm biogas plants (31 sites in 2010 vs 534 in 2020), and simultaneously to an increase in the volume of digestate intended for use as crop fertilizer. Spreading digestates on the land, the most common use of this by-product, enables recycling of nutrients and organic matter, creating an on-site circular economy. However, this practice may also involve the risk of disseminating pathogenic micro-organisms in field soil (Jiang et al., 2020; Orzi et al., 2015). French on-farm biogas plants, which commonly use pig or cattle manure as feedstock, are mainly operated within the mesophilic temperature range (35-42 °C) that has been reported to be less effective than thermophilic temperatures in reducing pathogenic micro-organisms (Jiang et al., 2020; Nag et al., 2019; Pandey and Soupir, 2011; Sahlstrom et al., 2004; Thomas et al., 2019). Pathogenic enteric bacteria such as *Salmonella* sp, *Listeria monocytogenes*, *Yersinia enterocolitica* or thermotolerant *Campylobacter* have been detected in digestate sampled from mesophilic digesters fed with pig or cattle manure (Le Marechal et al., 2019; Orzi et al., 2015) suggesting their persistence throughout the anaerobic digestion (AD) process. A low impact of mesophilic AD on pathogenic spore-forming

bacteria has also been reported (Le Marechal et al., 2019; Lloret et al., 2013; Orzi et al., 2015; Rounsefell et al., 2013).

In addition to the intrinsic characteristics of the bacteria, the main AD parameters that may affect the inactivation of pathogenic bacteria are feedstock composition, operating conditions (temperature, retention time, organic loading rate) and intermediate products (volatile fatty acids, ammonia) (Jiang et al., 2020). Among operational parameters, the impact of the digestion temperature (mesophilic *vs* thermophilic) on bacterial persistence is well established (Jiang et al., 2020; Zhao and Liu, 2019), but less research has focused on retention time and the organic loading rate (OLR). Using mesophilic reactors fed with sludge or with pig manure, Coelho et al. (2011) and Dennehy et al. (2018), observed no consistent relationship between the increase in retention time from 5 to 20 days or from 21 to 41 days, respectively, and the Log_{10} removal of total coliforms, *E. coli* or enterococci. In contrast, Chen et al. (2012) reported that the removal of *E. coli* and *Salmonella* sp. in mesophilic reactors fed with sludge, led to a respective increase of 1.08 and 1.79 Log_{10}, with an increase in retention time from 11 to 25 days. Only two studies, one performed in reactors fed with livestock manure under thermophilic conditions (Skillman et al., 2009) and the other under psychrophilic conditions (Rosenblum et al., 2015), tested the effect of OLR on the persistence of bacteria. Skillman et al. (2009) observed no change in *Clostridium perfringens* concentrations when OLR was increased from 2.2 to 6.4 g chemical oxygen demand (COD).L^{-1}.day^{-1}, whereas in the experiment conducted by Rosenblum et al. (2015), the reduction in the concentration of *E. coli,* fecal coliforms and *Enterococcus* sp. was significantly higher with an OLR of 0.3 than with an ORL of 1.3 g volatile solids (VS).L^{1}.day^{-1}. Due to the scarcity of

available data combined with the use of different experimental conditions, the role of retention time and OLR in the pathogen removal remains unclear.

To protect both animal and human health, the use of digestate as fertilizer is governed by European Regulation (EC) No 1069/2009 and its implementing Regulation (EC) No 142/2011. Among the operating conditions used in anaerobic digesters, only temperature, via thermal pretreatment, is accounted for in the EU Regulation. The regulation classifies animal by-products in three categories according to their potential health risks. Manure, which is category 2 material, must be pasteurized (particle size, 12 mm; 70 °C; 60 min). In France, competent authorities may, by derogation, authorize that a pasteurization unit shall not be mandatory for on-farm biogas plants. Most on-farm biogas plants benefit from this exemption. However, the current strengthening of French regulations governing digestate application will increase the number of pasteurization units in the coming years. It is thus important to evaluate the effect of this pretreatment, which has been shown to reduce the concentration of pathogenic vegetative bacteria, but appears to be unable to reduce spore forming bacteria (Sahlstrom et al., 2008).

Understanding the role of thermal pretreatment (70 °C, 1 hour), retention time and OLR in the persistence of enteric bacteria is essential to improve the deactivation of pathogens in the digestate. As the impact of these parameters is still poorly documented, in this study we used an experimental design to investigate the combined effect of operational parameters and thermal pretreatment on (i) the chemical parameters that may affect inactivation of pathogens (volatile fatty acids, ammonia), and (ii) the fate of four selected bacteria in digestate sampled from semi-continuous mesophilic reactors fed with pig manure. *E. coli* and enterococci (indicator bacteria commonly used to estimate the inactivation of enteric pathogenic bacteria)

were selected as EU Regulation No. 142/2011 stipulates their enumeration to assess the quality of the digestate. In addition, two spore-forming pathogens, *Clostridium perfringens and Clostridioides difficile,* were selected as they are important toxin-producing pathogens (Leuzzi et al., 2014; Uzal et al., 2018) and because of their presence in digestate sampled from farm biogas plants (Derongs et al., 2020; Froeschle et al., 2015; Le Marechal et al., 2019; Orzi et al., 2015).

2. Material and methods

2.1. Inoculum and influent

Manure and digestate were collected from a large-scale mesophilic biogas plant already studied by Le Marechal et al. (2019) and Derongs et al. (2020). This biogas plant (referred to as 'BP1' in previous studies) treated pig manure and vegetables as co-substrate. Samples were collected two to four times depending on the HRT value. Both digestate and manure were sieved through a 5-mm mesh sieve.

The digestate used as inoculum was transferred into the reactors immediately after sieving. The sieved manure was stored at 4 °C in 50 L canisters during the experiment. Before the reactors were fed, the sieved manure was heated at 70 °C for one hour, or not, depending on the experiment. The influent consisted of a mixture of manure (unheated or heated) and a co-substrate added to increase the biomethane potential of the influent. The co-substrate, made of lignocellulosic materials, previously used by Peu et al. (2011), was pelleted horse feed, selected for its stable composition and its high methane potential. The pelleted feed was crushed before mixing with the manure. The ratio of manure and co-substrate, and the feed volume were determined for each reactor according to the targeted organic loading rate (OLR) and the hydraulic retention time (HRT) (Table S1) defined in the experimental

139 design. The physical characteristics of sieved manure and co-substrate are listed in
140 Table S2. Microbial analysis of the co-substrate, confirmed the absence of the four
141 targeted bacteria.

142

143 2.2. Experimental design

144 Screening experiments were performed using a two-level factorial design to evaluate
145 the factors that have significant effects on the bacterial and physicochemical
146 responses. Two quantitative factors (HRT, expressed in days and OLR, expressed in
147 COD) and one qualitative factor (heat pretreatment of manure) were investigated.
148 The experimental design was conducted in one block, and central points were
149 performed in duplicate. All factors were evaluated at two levels. The two levels and
150 the central point of HRT and OLR were 24, 46 and 35 days, and 2, 4 and 3 g COD.
151 L^{-1}. d^{-1}, respectively. The two levels of the qualitative factor were a heat pretreatment
152 (70 °C, 1 h) or no heat pretreatment. The experiment comprised 12 trials to cover the
153 entire experimental domain, as detailed in Table 1.

154

155 Table 1. Factors and levels for experimental design

Trial	HRT (days)	OLR (**g COD. L $^{-1}$. d^{-1}**)	Heat pretreatment
1	24 (-1)[a]	2 (-1)	without (-1)
2	24 (-1)	2 (-1)	with (+1)
3	24 (-1)	4 (+1)	without (-1)
4	24 (-1)	4 (+1)	with (+1)
5	35 (0)	3 (0)	without (-1)
6	35 (0)	3 (0)	with (+1)
7	35 (0)	3 (0)	without (-1)
8	35 (0)	3 (0)	with (+1)
9	46 (+1)	2 (-1)	without (-1)
10	46 (+1)	2 (-1)	with (+1)
11	46 (+1)	4 (+1)	without (-1)
12	46 (+1)	4 (+1)	with (+1)

156 [a] normalized value Statgraphics

157 The levels of HRT were selected to represent a compromise between the usual HRT
158 used in large-scale mesophilic biogas plants and a reasonable duration of the
159 experimental design. The reactors were operated for 2.5 HRT before the experiment
160 began to allow the steady state to be reached. The OLR were selected to enable
161 observation of biogas production while avoiding a malfunction in the anaerobic
162 reactors.
163 Analysis of variance (ANOVA) at the 95% confidence level was used to analyze the
164 results. The experimental design and the statistical analysis of the results were
165 carried out using Statgraphics Centurion XVI® software.
166
167 2.3. Experimental procedure in the semi-continuous reactor
168 Four parallel reactors with an effective 4.05 L working volume (Figure S1) were used.
169 The reactors were stirred continuously and operated in a semi-continuous mode. The
170 temperature, maintained at 39 ± 1 °C by circulating water through the reactor jacket,
171 was monitored with a Pt100 probe inserted into the reactor through the lid. To
172 maintain the operating volume of the liquid, each reactor was equipped with a funnel
173 and a feed valve in the lid and a discharge valve located at the upper side of the
174 reactor.
175 Feeding and discharge were performed three times a week. Once the steady state
176 was reached, influent and digestate from each reactor were sampled once a week for
177 one month and their chemical and microbial parameters analyzed.
178
179 2.4. Heat pretreatment of the manure
180 Heat pretreatment was performed in a 10-L double wall glass cell filled with 2.5 L of
181 sieved manure previously maintained at 4 °C. The cell was equipped with a Pt100

probe to measure the temperature and with a central shaft with a propeller to ensure continuous homogenization of the manure. A heat transfer fluid circulated in the double wall of the cell, the temperature of the fluid was controlled by a thermostat (Ministat 40, Huber, France). The temperature rise time (from 4 to 70 °C) was 68 minutes. The manure was maintained at 70 °C for 1 hour and then cooled to 40 °C before mixing with the co-substrate. The influent was then fed into the reactors.

2.5. Physicochemical analysis

VS, COD and total ammonia nitrogen (TAN) contents and pH were determined using standard methods (APHA, 2012). Volatile fatty acids (VFA) were analyzed by high performance liquid chromatography (HPLC, Varian©, U3000). Samples were first centrifuged (17 700 rcf, 4 °C, 20 min) and the resulting supernatants were used for analysis.

2.6. Microbial analysis

For *E. coli*, enterococci and *C. perfringens*, a 25-g sample (influent or digestate) was homogenized in 225 mL of sterile buffered peptone water (BPW; Thermo Fisher Diagnostics SAS, France). Serial 10-fold dilutions were then prepared in BPW. For *C. difficile*, a 1-g sample was 10-fold diluted in Brain Heart Infusion Broth (BHI, Biokar Diagnostics, France) supplemented with 0.1% taurocholate, cefoxitin (8 mg/L), and cycloserine (250 mg/L).

E. coli

One milliliter of each dilution was transferred to a sterile Petri dish and 15 mL of tryptone bile X-glucuronide medium (TBX; Thermo Fisher Diagnostics SAS, France)

were added. The plates were incubated at 44 °C for 24 h. Characteristic blue colonies (glucuronidase-positive) were counted. The results are expressed in colony forming units per gram wet weight (cfu.g^{-1}).

Enterococci

A 0.1-mL aliquot of the ten-fold dilution was plated on Slanetz–Bartley agar (Biokar Diagnostics, France) and incubated at 37 °C for 48 h. Colonies were transferred onto Bile-Esculin-Azide (BEA) agar (Biokar Diagnostics, France) and incubated at 44 °C for 2 h. Black colonies on BEA (esculin-positive) were counted as enterococci. Results are expressed as cfu.g^{-1}.

Clostridium perfringens

C. perfringens were counted according to ISO 7937 (International Standards Organisation ISO, 2005) as previously described by Derongs et al. (2020). According to the ISO method, the concentration of *C. perfringens* was calculated based on the total counts of black colonies on tryptose sulfite cycloserine (TSC) agar and on the number of confirmed colonies ($n \leq 5$) on lactose sulfite broth (gas production and blackening of the culture medium) at a given dilution. The results are expressed in cfu.g^{-1}. At each sampling date, confirmed colonies (lactose sulfite positive) were purified and suspended in 50% glycerol / 50% BHI (Thermo Fisher Scientific, Courtaboeuf, France) before being stored at -80 °C.

Clostridioides difficile

C. difficile were counted as previously described by Le Marechal et al. (2020). The results are expressed in MPN.g^{-1}. At each sampling date, characteristic colonies of *C.*

difficile isolated on BHI agar were incubated at 37 °C for 48 h in an anaerobic chamber. The isolates were then stored at -80 °C.

2.7. DNA extraction, detection of toxin genes and antimicrobial susceptibility

A total of 406 *C. perfringens* isolates were collected from the influents (n=220) and the digestates (n=186). DNA was extracted as previously described by Derongs et al. (2020) using the Nucleospin® Microbial DNA kit (Macherey-Nagel, Duren, Germany). Genes *cpa*, *cpb*, *etx* and *iap*, encoding the four major toxins (α, β, ε, and ı) and genes *cpe*, *netB* and *cpb2* encoding the enterotoxin CPE and the NetB and β2 toxins, were performed using real-time PCR as described by Derongs et al. (2020). The proportion of *C. perfringens* toxinotypes in the influents and digestates was compared using the Chi-square test in XLSTAT 2019 (Addinsoft, Bordeaux, France). Antimicrobial susceptibility profiles of *cpa*-positive isolates from the influent (n=203) and digestate (n=161) were determined by microdilution method (Sensititre™ Bovine/porcine MIC plate) according to the manufacturers' instructions (ThermoFisher Diagnostics, France) as described by Derongs et al. (2020). Susceptibility was tested against penicillin, ampicillin, ceftiofur, tylosine tartrate, tilmicosin, tulathromycin, clindamycin, enrofloxacine, danofloxacin, tiamulin, florfenicol, chlortetracyline and oxytetracycline.

A total of 184 *C. difficile* isolates were collected from the influents (n=96) and the digestates (n=88). DNA was extracted as previously described by Le Marechal et al. (2020). *tpi* (triose phosphate isomerase) gene fragments used to identify *C. difficile*, *tcdA* (toxin A) and *tcdB* (Toxin B) were detected using real-time PCR with the primers described by Barbut et al. (2019). PCR ribotyping, multiplex PCR and susceptibility to

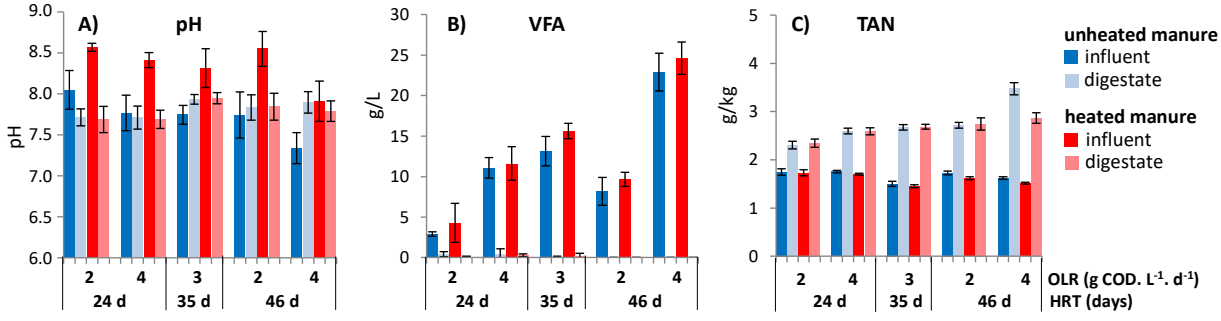
six antibiotics were performed on a selection of isolates from the digestates as previously described (Le Marechal et al., 2020). Antimicrobial susceptibility to erythromycin (15 IU), clindamycin (2 IU), moxifloxacin (5 µg) and tetracycline (30 IU) was determined by the disk diffusion method. Vancomycin and metronidazole susceptibility was tested using the MIC method recommended by the Antibiogram Committee of the French Microbiology Society.

3. Results

Three parameters (HRT, OLR with and without Heat pretreatment) were investigated in laboratory-scale anaerobic reactors fed with a mixture of pig manure and co-substrate to determine their effect on pH, VFA and TAN content, and on the concentration of four bacteria. Tables 2 and 3 present the statistical results of the experimental design on the effects of these three factors on the physicochemical parameters in both the influents and digestates and on the bacterial concentrations in the digestates. Factors with p-values less than 5% ($p < 0.05$) were considered significant.

3.1. Physicochemical parameters

The variation in HRT and OLR influenced the pH and VFA content of the influent (Figure 1A, 1B, Table 2).



277 Figure 1. Average pH (A), and average concentrations of VFA (B) and TAN (C) in the
278 influent (manure heated or not) and in the digestate at three HRT (24, 35 and 46
279 days) and with three OLR (2, 3 and 4 g COD. L^{-1}. d^{-1}). Errors bars represent standard
280 deviations of the four samples (24- and 46-day HRT) and of the eight samples (35-
281 day HRT) analyzed for each experimental condition.

282

283 The increase in HRT or OLR and their interaction resulted in a significant increase in
284 VFA content, from respectively 2.9 $g.L^{-1}$ (lowest HRT and OLR) to 24.6 $g.L^{-1}$ (highest
285 HRT and OLR), and in a 0.7 unit decrease in pH. These results were attributed to the
286 change in the composition of the influent depending on the HRT and OLR values
287 (Table S1). The highest HRT and OLR corresponded to the highest proportion of co-
288 substrate in which the VFA content was higher than in the manure. The VFA contents
289 in the manure and in the co-substrate were ≤ 0.26 $g.L^{-1}$ and 125.1 $g.L^{-1}$, respectively.
290 It is worth noting that the TAN content of the co-substrate was very low (0.09 $g.kg^{-1}$)
291 compared to that of manure (1.7-2 $g.kg^{-1}$), thereby explaining the absence of
292 influence of HRT and OLR on TAN content of the influent (Figure 1C, Table 2).

293

294 Table 2. Variance analysis of the influence of three factors (hydraulic retention time
295 (HRT), organic loading rate (OLR) and thermal pretreatment (Th.Pr)) and of their
296 interactions on pH, VFA and TAN contents of influent and digestate.

Factor		pH		VFA		TAN	
		Influent	Digestate	Influent	Digestate	Influent	Digestate
A: HRT	F	48.8	2.43	40.09	12.90	1.18	39.57
	p-value	**0.0009 (-)** [a]	0.179	**0.0014 (+)**	**0.0157 (-)**	0.327	**0.0015 (+)**
B: OLR	F	67.4	0.00	64.95	0.41	0.26	20.77
	p-value	**0.0004 (-)**	0.958	**0.0005+**	0.550	0.633	**0.0061 (+)**
C: Th.pr	F	279.3	0.07	2.13	0.28	0.62	1.86

	p-value	**0.0000 (+)**	0.796	0.204	0.620	0.466	0.231
AB	F	11.1	0.00	6.42	0.41	0.21	1.19
	p-value	**0.0208 (-)**	0.958	0.052	0.550	0.665	0.325
AC	F	1.49	0.03	0.06	1.96	0.13	3.96
	p-value	0.277	0.874	0.822	0.221	0.732	0.103
BC	F	0.44	0.11	0.01	0.26	0.01	4.46
	p-value	0.535	0.752	0.917	0.635	0.945	0.081

[a] values in bold followed by the symbol (-) or (+) indicate a negative or positive significant effect or interaction ($p < 0.05$).

While the variation in HRT and OLR and their interaction did not impact the pH of the digestate, which ranged from 7.7 to 7.9 (Figure 1A), increasing the two factors significantly increased TAN contents (Table 2, Figure 1C). At the lowest HRT and OLR, TAN contents in the digestates were 2.31 - 2.35 $g.kg^{-1}$, whereas at the highest HRT and OLR values, they ranged from 2.9 to 3.5 $g.kg^{-1}$, depending on whether the heat pretreatment was applied or not. The VFA contents of the digestates were very low (less than 0.42 $g.L^{-1}$, Figure 1B), confirming the correct performance of the pilots. Even if the concentrations were low, the increase in HRT significantly reduced the VFA contents in the digestate (Table 2), with no detection of VFA with an HRT of 46 d (<10 $mg.L^{-1}$).

The heat pretreatment of the manure only significantly affected the pH of the influent, which increased by 0.6 units when the manure was preheated (Figure 1A, Table 2).

3.2. Microbial parameters

The concentrations of the four bacteria in the influent remained stable throughout the experiment. In the absence of heat pretreatment, the concentrations ranged from 3.3 10^4 to 8.2 10^4 $cfu.g^{-1}$ (*E. coli*, Figure 2A), from 3.3 10^3 to 6.8 10^3 $cfu.g^{-1}$ (enterococci,

Figure 2B), from 1.1 10^5 to 3.8 10^5 cfu.g^{-1} (*C. perfringens*, Figure 2C) and from 12 to 20 MPN.g^{-1}(*C. difficile*, Figure 2D).

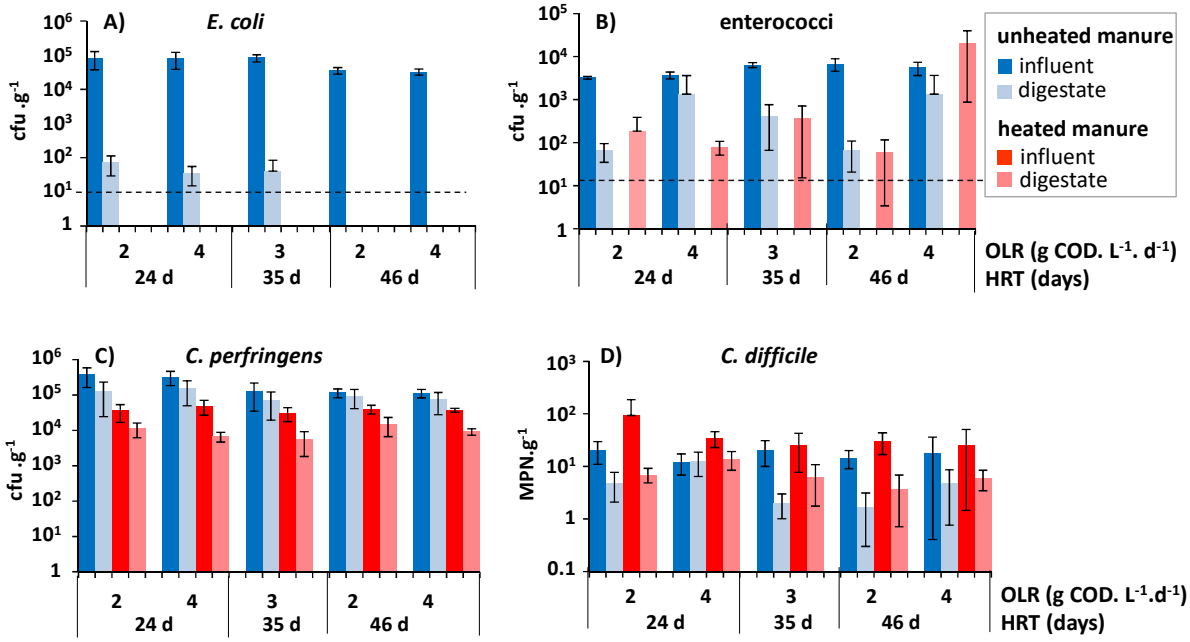


Figure 2. Average concentrations of *E. coli* (A), enterococci (B), *C. perfringens* (C) and *C. difficile* (D) in the influent (manure heated or not) and in the digestate at three HRT (24, 35 and 46 days) and with three OLR (2, 3 and 4 g COD. L^{-1}. d^{-1}). Error bars represent standard deviations of the four samples (24 and 46-days HRT) and the eight samples (35-days HRT) analyzed for each experimental condition. The dashed line represents the limit of detection.

As shown in Table 3, the concentrations of the bacteria in the digestate were significantly affected by one or two of the operational parameters studied. It is noteworthy that no effect of interactions between HRT, OLR and the heat pretreatment was observed.

Table 3. Variance analysis of the influence of three factors (hydraulic retention time (HRT), organic loading rate (OLR) and heat pretreatment (Th.Pr)) and of their interactions on the concentrations of bacteria (expressed in Log_{10} cfu g^{-1} or MPN g^{-1}) in the digestates.

Factor		*E. coli*	enterococci	*C. perfringens*	*C. difficile*
A: HRT	F	15	1.19	1.33	2.07
	p-value	**0.0117 (-)** [a]	0.325	0.301	0.209
B: OLR	F	0.27	7.32	0.60	12.25
	p-value	0.628	**0.043 (+)**	0.475	**0.0173 (+)**
C: Th.pr	F	193.3	0.05	42.9	8.04
	p-value	**0.0000 (-)**	0.833	**0.0012 (-)**	**0.0365 (+)**
AB	F	0.11	3.67	1.09	0.67
	p-value	0.758	0.114	0.344	0.451
AC	F		1.19	1.76	0.00
	p-value	nr[b]	0.325	0.241	0.975
BC	F		0.08	0.41	1.96
	p-value	nr	0.794	0.551	0.220

[a] values in bold followed by the symbol (-) or (+) indicate a negative or positive significant effect or interaction ($p < 0.05$). [b] The interpretation of the statistical test was not relevant.

3.2.1. Impact of the HRT and of the OLR on bacteria

Without heat pretreatment, the AD led to a decrease of more than 3 Log_{10} in *E. coli* and of 0.9 to 2.1 Log_{10} in enterococci (Table 4, Figure 2A and 2B). The changes in OLR did not influence the level of *E. coli* whereas the 46-day HRT resulted in no detection of this bacterium (Table 3, Figure 2A). The OLR significantly impacted the concentrations of enterococci in the digestate (Table 3). Indeed, doubling the OLR decreased the Log removal by *ca.* 1 Log_{10} (Table 4). HRT had no significant effect on the concentration of enterococci (Table 3).

Table 4. Log_{10} reduction in bacterial concentrations between the influent and digestate, calculated over the four weeks of the experiment.

Manure treatment	HRT (days)	OLR (g COD. L^{-1}. d^{-1})	*E. coli* Mean ± SD	Enterococci Mean ± SD	*C. perfringens* Mean ± SD	*C. difficile* Mean ± SD
unheated	24	2	3.1 ± 0.2	1.7 ± 0.2	0.6 ± 0.4	0.6 ± 0.3
	24	4	3.2 ± 0.8	0.9 ± 0.7	0.4 ± 0.2	0.0 ± 0.2
	35	3	3.5 ± 0.5	1.3 ± 0.3	0.3 ± 0.3	1.0 ± 0.2
	46	2	≥3.6	2.1 ± 0.4	0.1 ± 0.3	1.0 ± 0.4
	46	4	≥3.6	1.1 ± 0.8	0.3 ± 0.3	0.6 ± 0.7
heated	24	2	-[a]	≥-0.8 ± 0.5[b]	0.5 ± 0.4	0.9 ± 0.4
	24	4	-	≥-0.6 ± 0.2	0.8 ± 0.1	0.5 ± 0.0
	35	3	-	≥-1.0 ± 0.5	0.8 ± 0.3	0.6 ± 0.3
	46	2	-	≥-0.4 ± 0.5	0.5 ± 0.2	1.0 ± 0.2
	46	4	-	≥-2.9 ± 0.4	0.6 ± 0.0	0.5 ± 0.5

[a] no *E. coli* were detected in the influent or in the digestate; [b] negative values correspond to an increase in Enterococci concentrations during the course of digestion.

Concentrations of *C. perfringens* were slightly affected by the AD with a Log_{10} reduction ranging from 0.1 to 0.6 without heat pretreatment, and from 0.5 to 0.8 with heat pretreatment, independently of the HRT and the OLR (Table 4, Figure 2C). The Log_{10} reduction in *C. difficile*, which ranged from 0 to 2, is more difficult to interpret, given the low concentrations of this pathogen in the influent. However, a significant impact of the OLR was observed on the concentrations of *C. difficile* (Table 3). The

363 lowest OLR led to the highest Log_{10} reduction under both 24 and 46-day HRTs with
364 and without heat pretreatment (Table 4, Figure 2D).
365
366 3.2.2. Impact of heat pretreatment on bacteria
367 The heat pretreatment impacted the bacterial concentrations in both the influent and
368 the digestate. Indeed, no *E. coli* and enterococci were detected in the influent (Figure
369 2A and 2B), the concentration of *C. perfringens* decreased by 0.5 to 1 Log_{10} (Figure
370 2C) while the concentration of *C. difficile* increased slightly by 0.1 to 0.7 Log_{10} (Figure
371 2D).
372 In the digestates, regardless of the HRT and of the OLR, the heat pretreatment led to
373 no detection of *E. coli* and reduced the concentration of *C. perfringens,* whereas the
374 concentrations of *C. difficile* were slightly higher than those observed in the digestate
375 sampled from the reactors fed with unheated manure (Table 3, Figure 2).
376 Surprisingly, the heat treatment, which reduced enterococci to an undetectable level
377 in the influent, did not affect their concentration in the digestate.
378
379 3.2.3. Characterization of spore-forming bacteria
380 Among the 406 lactose sulfite positive isolates of *C. perfringens*, 369 (90.9%) carried
381 the *cpa* gene. Type A represented 99.2% of the *cpa* positive isolates, among which
382 5.0% carried the *cpb2* gene. Three isolates (0.8%) were type C (*cpb* gene) (Table 5).
383 There was no significant difference (Chi-square test $p > 0.67$) between the proportion
384 of the three genes in the influent and the digestate whether the manure was
385 preheated or not.
386

Table 5. Proportion (%) of toxinotypes of *C. perfringens* isolates in the influent and in the digestate of the reactors fed with unheated manure and heated manure.

Type (genes)	Unheated manure		Heated manure		
	Influent	Digestate	Influent	Digestate	Total
	n=100 [a]	n=99	n=108	n=62	n=369
A (*cpa*)	95.0	92.9	100.0	93.5	94.3
A (*cpa + cpb2*)	4.0	7.1	3.9	4.8	4.9
C (*cpa + cpb*)	1.0	0.0	1.0	1.6	0.8

[a] Number of isolates, regardless of the HRT and OLR

The antimicrobial resistance profiles of 364 isolates varied considerably regardless of their origin (manure or digestate) and of the operating conditions (Table S3, Figure S2). With the exception of tulathromycin (MIC 50, 64 µg.mL^{-1}), most of the isolates were susceptible to β-lactam and macrolides. Enrofloxacin and danofloxacin had low MIC 50 values, 0.5 and 1 µg.mL^{-1}, respectively. Most of the isolates were sensitive to chlortetracycline with a MIC 50 value of 1 µg.mL^{-1}. A few isolates in the influent (n=5) and in the digestate (n=4) were highly resistant to tilmicosin (MIC > 64 µg.mL^{-1}). One isolate from the digestate differed by its high resistance to tylosin (MIC > 32 µg.mL^{-1}). All the 184 *C. difficile* isolates carried the *tpi* gene, confirming their identification (Table 6). With the exception of 1.6% isolates in the digestates (one from the reactor fed with untreated manure and two from the reactor fed with preheated manure), all the isolates carried both *tcdA* and *tcdB* genes.

Table 6. Proportion (%) of *tpi*, *tcdA* and *tcdB* genes in *C. difficile* isolates taken from the influent and the digestate of the reactors fed with unheated manure and heated manure.

Genes	Unheated manure		Heated manure		
	Influent	Digestate	Influent	Digestate	Total
	n=48 [a]	n=42	n=48	n=46	n=184
tpi	100	100	100	100	100
tcdA	100	97.6	100	95.6	98.4
tcdB	100	97.6	100	95.6	98.4

[a] Number of isolates, regardless of the HRT and OLR

The PCR-ribotype and antimicrobial susceptibility of six antibiotics were determined in 10 isolates (one per digestate in each condition tested). All the isolates belonged to PCR ribotype 078 (Table S4). They were resistant to clindamycin and erythromycin and susceptible to moxifloxacin, vancomycin and metronidazole. One strain displayed resistance to tetracycline (Table S4).

4. Discussion

Most French agricultural biogas plants treat livestock manure at mesophilic temperatures. However, several studies have demonstrated that the efficiency of mesophilic anaerobic digestion (MAD) with regard to fecal indicators (*E. coli*, fecal coliforms, total coliforms or enterococci) is lower than that of thermophilic anaerobic digestion (Amani et al., 2011; Astals et al., 2012; Lopez et al., 2020; Pandey and Soupir, 2011; Watcharasukarn et al., 2009; Ziemba and Peccia, 2011). In order to

422 improve the inactivation efficiency of MAD, it is important to identify effective
423 operational strategies. The aim of this study was thus to compare the effect of
424 increasing the HRT and the OLR and of including a heat pretreatment on the
425 persistence of four selected bacteria (vegetative and spore forming bacteria).
426 With no heat pretreatment, sensitivity to the MAD process ranked *E. coli* >
427 enterococci > clostridia (*C. perfringens* and *C. difficile*). This is in agreement with the
428 results of previous studies performed under mesophilic conditions at the lab scale or
429 using field scale reactors (Arias et al., 2020; Bonetta et al., 2011; Chiapetta et al.,
430 2019; Costa et al., 2017; Lopez et al., 2020; Orzi et al., 2015; Watcharasukarn et al.,
431 2009).
432
433 4.1. Overall impact of MAD on the four bacteria
434 In our study, while the two clostridia were almost not affected, the Log_{10} reduction of
435 *E. coli* and enterococci ranged from 3.1 to ≥3.6 and from 0.9 to 2.1, respectively,
436 depending on the values of the HRT and the OLR. Although all the above studies
437 reported greater inactivation of *E. coli* than of enterococci under mesophilic
438 temperatures, the reduction in their concentrations varied with the experimental
439 conditions. In batch experiments, the Log_{10} reduction of *E. coli* and enterococci were
440 reported to be, respectively, 4.9 and 3.1 after six days of incubation (Watcharasukarn
441 et al., 2009) whereas in another study, they were 4 and < 1 after 75 days (Arias et al,
442 2020). At field scale, in semi-continuous stirred tank reactors, the Log_{10} reduction of
443 *E. coli* and enterococci were 3.1 and 0 (Bonetta et al., 2011), 2.1 and 0.8 (Chiapetta
444 et al., 2019), 2.8 and 1.4 (Costa et al., 2017), respectively. Orzi et al. (2015)
445 compared the sanitation efficiency of eight biogas plants (BGPs) fed with manure and
446 reported a Log_{10} reduction in *E. coli* and enterococci of between 0->3.7 and 0.3-3.1,

447 respectively, depending on the feedstock used, on the initial concentration of the two
448 indicator bacteria, and on the operating parameters.
449 The isolates of *C. perfringens* belonged to two toxinotypes. Most were type A, which
450 has been reported to be the dominant toxin type in farm animals (Fohler et al., 2016;
451 Li et al., 2020; Ngamwongsatit et al., 2016). Only three isolates were type C, a type
452 frequently associated with necrohemorrhagic enteritis of neonatal animals (Freedman
453 et al., 2015; Uzal et al., 2018). The *cpb2* gene encoding the β2 toxin, which is
454 associated with diarrheal diseases (Freedman et al., 2015; Uzal et al., 2018), was
455 found in 5% of the isolates. As previously observed in the manure and digestate of
456 three biogas plants (Derongs et al., 2020), the antimicrobial resistance profiles of *C.*
457 *perfringens* isolates were not impacted by anaerobic digestion. It is also noteworthy
458 that none of the three operating conditions (HRT, OLR and heat pretreatment)
459 affected the distribution of the toxinotypes or that of the antimicrobial resistance
460 profiles in the digestate.
461 All *C. difficile* isolates were PCR-ribotype 078, which is commonly isolated from pigs
462 (Andres-Lasheras et al., 2017; Krutova et al., 2018; Stein et al., 2017) and often
463 associated with *C. difficile* infection in both humans and animals (Connor et al.,
464 2019). Regardless of the operating conditions, the same PCR-ribotype was detected
465 in digestates and resembled the one previously detected in manure and digestate
466 sampled from this biogas plant (Le Marechal et al., 2020), suggesting their
467 persistence and stable profile throughout the MAD. The resistance profiles of the
468 isolates to the six antibiotics is in agreement with the proportion of antimicrobial
469 resistance in isolates observed by Andres-Lasheras et al. (2017) and Knight et al.
470 (2017). Indeed, both authors reported that all isolates of pig origin were susceptible
471 to vancomycin and metronidazole. Moreover, the resistance to clindamycin and

472 erythromycin displayed by the isolates, is widespread among human and animal *C.*
473 *difficile* strains (Alvarez-Perez et al., 2017). As we also observed in our study, MAD
474 has been reported to have no impact or only a weak impact on clostridia (Arias et al.,
475 2020; Costa et al., 2017; Watcharasukarn et al., 2009). Our results are in agreement
476 with those of Fontana et al. (2020), who investigated the effect of the MAD process
477 on the *Clostridium* consortia, and observed a slight decrease in cultivable clostridial
478 spores (less than 0.7 Log_{10}), mainly represented by *C. perfringens*, in laboratory
479 reactors (46-day HRT) fed daily with a mixture of agricultural substrate. The Log_{10}
480 reduction in *C. perfringens* in the eight BGPs studied by Orzi et al. (2015) ranged
481 from 0 to 3.9 (six of the eight BGPs presented a Log_{10} reduction ≤ 1) again
482 underlining the variability of persistence associated with BGP characteristics.
483 The impact of MAD on *C. difficile* has rarely been studied. Our results suggest a
484 limited impact of MAD on *C. difficile,* in agreement with the results of one study
485 carried out in flasks by Xu et al. (2016). These authors reported no reduction in the
486 concentration of spores of five isolates of *C. difficile* ribotype 078 in digested sludge
487 incubated at 36 °C for 53 days, and only one of the isolates was inhibited at 42 °C.
488 There is a consensus that *E. coli* is more sensitive than enterococci and that
489 clostridia are not or only slightly affected by the anaerobic digestion. However,
490 according to data in the literature, it is clear that their fate depends on the operational
491 parameters of the MAD process.

492 4.2. Impact of HRT and OLR

493 As noted in the review by Jiang et al. (2020) on inactivation of pathogens in AD
494 process, many of the authors reported the persistence of indicators and spore
495 forming bacteria in batch experiments, and all reported a reduction in *E. coli* and
496 enterococci with an increase in retention time. The impact of HRT in continuous

497 stirred reactors is less clear. In our study, increasing the HRT from 24 to 46 days had
498 no impact on enterococci and clostridia. Only the concentration of *E. coli*, no longer
499 detected with the 46-day HRT, was impacted by increasing this parameter. However,
500 it is noteworthy that at a HRT of 24 days, *E. coli* already showed a relatively high
501 abatement of 3.1 Log_{10} leading to an average concentration in the digestate (ranging
502 from 35 to 70 $cfu.g^{-1}$) close to the limit of detection of the method (10 $cfu.g^{-1}$). Our
503 results showed that the sanitary efficiency of the MAD process was not increased, or
504 only slightly increased, by increasing the HRT by 22 days. This is in agreement with
505 the results of a field study conducted by Orzi et al. (2015). According to the
506 concentrations observed in the influents and in the digestates of the eight BGPs
507 studied by these authors, there was no impact of the HRT (which ranged between 20
508 and 70 days) on *E. coli* and enterococci. Moreover, in semi-continuous stirred
509 reactors fed with sewage sludge, Lopez et al. (2020) observed no consistent trend in
510 the removal efficiency of *E. coli* and enterococci using four HRT ranging from 10 to
511 20 days corresponding to an OLR of 1.90 and 1.03 kg VS. $m^{-3}.day^{-1}$, respectively.
512 Using semi-continuous tank reactors fed with manure, Dennehy et al. (2018) also
513 reported that *E. coli* and enterococci counts in the digestate were not impacted by
514 HRT ranging from 21 to 41 days, corresponding to an OLR of 3 to of 1 kg VS. $m^{-3}.day^{-1}$.
516 The impact of the HRT on *E. coli* reflects their lower resistance than that of Gram
517 positive bacteria to anaerobic treatment (Jiang et al., 2020). The difference in
518 persistence between *E. coli* and the three other bacteria may be attributed to their
519 metabolic characteristics (i.e., their ability to compete for nutrients, to develop
520 resistance to antagonistic bacteria, to survive under anaerobic conditions) and to
521 their cell wall structure, Gram-positive bacteria being surrounded by layers of

522 peptidoglycan thicker than that of *E. coli* (Silhavy et al., 2010). Two of the four
523 targeted bacteria (enterococci and *C. difficile*) were affected by the increase in the
524 OLR. The impact of this parameter has mainly been studied in biogas production,
525 and very few studies reported its effect on the persistence of indicator or pathogenic
526 bacteria. In our study, increasing the OLR reduced the abatement of the two bacteria.
527 The same trend was reported by Rosenblum et al. (2015), who tested different OLR
528 in continuous stirred tank reactors fed with cattle manure under psychrophilic
529 conditions. At three OLR (0.3, 0.8 and 1.3 kg $VS.m^{-3}.day^{-1}$) corresponding to three
530 HRT (188.3, 70.6, 43.3 days, respectively), these authors observed that the higher
531 the OLR, the lower the reduction in the concentration of *E. coli* and enterococci.
532 However, as increasing OLR reduced HRT, it is difficult to decide if the observed
533 effects were due to one of the two parameters, or to a combination of the two. In a
534 study conducted by Skillman et al. (2009) in continuous stirred tank reactors fed with
535 piggery wastewater and maintained at 55 °C, with a short HRT of two days, the
536 reduction in the concentration of *C. perfringens* was -0.1, 0.2 and 0.1 Log_{10} with OLR
537 of 2.2, 3.5 and 6.4 g $COD.L^{-1}.d^{-1}$. The absence of an effect of OLR on *C. perfringens*
538 is in agreement with our results.

539 As reported by Rosenblum et al. (2015), the quantities of nutrients, which depend on
540 the organic load used, may impact the concentrations of enteric bacteria during
541 anaerobic digestion. In our study, the increase in OLR led to an increase in the level
542 of enterococci and *C. difficile* in the digestate. The increase in the concentration of
543 enterococci may be due to their metabolic flexibility and their ability to utilize diverse
544 carbohydrates for their growth (Gaca and Lemos, 2019). Different results were
545 obtained for *C. perfringens* and *C. difficile*. *C. difficile* is indeed phylogenetically
546 distant from the genus *Clostridium* and has been reclassified in the family

547 *Peptostreptococcaceae* (Lawson et al., 2016). This may explain why *C. difficile* react
548 differently under anaerobic digestion conditions. Despite its very low concentrations
549 in the manure, *C. difficile* was present at the same concentrations in the digestate,
550 suggesting that this bacterium is well adapted to the environment found in the
551 anaerobic digesters, as previously suggested by Froeschle et al. (2015) who showed
552 that *C. difficile* can survive anaerobic digestion for a long time. Moreover, it has been
553 demonstrated that *C. difficile* has great metabolic flexibility in the gut environment,
554 not only feeding on sugars and proteins but potentially also on CO_2 and H_2 (Kopke et
555 al., 2013). The increase in OLR may change the metabolic pathways, thereby
556 improving its growth.
557 It is noteworthy that the increase in OLR at a given HRT led to a limited increase in
558 the ratio of co-substrate to manure (Table S1) in the feed mixture. The fact the co-
559 substrate contained more organic matter than the manure (Table S2) could favor the
560 growth of both enterococci *and C. difficile*.
561 The absence of an effect of OLR on *C. perfringens* is difficult to explain as this
562 hydrogen producer secretes several hydrolytic enzymes that degrade extracellular
563 substrates (Petit et al., 1999). However, the absence of an effect of variations in the
564 OLR on *C. perfringens* may reflect competition with other spore formers, as reported
565 by Skillman et al. (2009).
566
567 4.3. Indirect impact of HRT and OLR
568 Both HRT and OLR can influence the concentrations of ammonia and volatile fatty
569 acids, two important intermediate products that may have a toxic effect on micro-
570 organisms (Mahdy et al., 2020). Mahdy et al. (2020) compared the effect of different
571 HRT and OLR on the accumulation of TAN and VFA during AD of chicken manure

572 and reported that the increase in HRT led to a decrease in VFA and to an increase in
573 TAN contents in the digestates. It is noteworthy that free VFA and free ammonia
574 (FAN) are more toxic than ionized VFA and ammonium ions (NH_4^+) due to their
575 lipophilic properties that facilitate their passage through the cell membrane (Jiang et
576 al., 2020). Free VFA and FAN contents depend on pH and temperature. In our study,
577 the average pH of the digestate ranged from 7.7 to 7.9 (Figure 1) thus favoring the
578 ionized VFA and NH_4^+ form.
579 Data on the impact of ammonia on enteric bacteria are scarce and available data
580 mainly focus on *Salmonella*. The effect of ammonia content depends on the type of
581 bacteria concerned. Ottoson et al. (2008) observed that *Salmonella* Typhimurium
582 inoculated in dairy manure incubated at 14 °C was more sensitive to 187-190 mM kg^{-1}
583 of free NH_3 than *Enterococcus faecalis*. Park and Diez-Gonzalez (2003) reported
584 that *E. coli* O157:H7 was less susceptible than S. Typhimurium DT104. Neither of the
585 bacteria inoculated in LB broth incubated at 37 °C were inhibited at a concentration
586 of NH_3 < 5 mM. After six hours of incubation, *Salmonella* was completely inhibited at
587 > 40 mM ammonia whereas 180 mM was needed to obtain > 5 Log_{10} reduction in *E.*
588 *coli* O157:H7. Jiang et al. (2018a) investigated the factors responsible for *Salmonella*
589 inactivation during the MAD process, and compared the minimum inhibitory
590 concentrations (MIC) of ammonia for three strains of *Salmonella.* The MIC values
591 ranged from 646 to 841 mM at pH 7.0 and from 690 to 720 mM at pH 8, depending
592 on the serotype inoculated. In our study, the levels of free ammonia in the digestate
593 ranged from 164 to 375 N-NH_3 mg. kg^{-1} (*i.e.,* 11.7 mM and 26.8 mM) (Table S5) and
594 did not reach the concentration that could inhibit the four targeted bacteria.
595 VFA contents were also low, ranging from <10 to 416 $mg.L^{-1}$ (Figure 1), propionate
596 being the main VFA (data not shown). Jiang et al. (2018b) studied the fate of

indicator bacteria during dry co-digestion of food waste and pig manure and observed a greater impact of free VFA on *E. coli* than on enterococci. These authors reported that in addition to the residence time, which was the most significant factor in *E. coli* reduction, the free VFA concentration of 35 $mg.L^{-1}$ might be enough to inactivate *E. coli* whereas it did not affect enterococci.

Considering that propionate was the only VFA in our reactors, the VFA content in digestate was less than *ca.* 5.6 mM. Wrigley (2004) reported no inhibition of a *C. perfringens* strain inoculated in a culture medium at pH 7 supplemented with 10 mM propionate. Jeong et al. (2019) observed that the growth *E. faecalis* was not inhibited in presence of propionate at a concentration of 15 mM, the MIC being 1 000 mM. Jiang et al. (2018a) reported MIC values of propionate for three strains of *Salmonella* ranging from 190 to 236 mM at pH 7.0, and from 381 to 416 mM at pH 8. These data suggest that the VFA contents observed during our experiment were probably not toxic.

4.4. Impact of the heat pretreatment

The drastic effect of the heat pretreatment (70 °C, 1 h.) on the concentration of *E. coli* and enterococci in pig manure we observed is in agreement with the results of previous studies. However, while all the studies conducted on sludge or on manure agree that heat treatment at 70 °C inactivates Gram negative bacteria such as *E. coli*, fecal coliforms or *Salmonella* (Bonjoch and Blanch, 2009; Lang and Smith, 2008; Ruiz-Espinoza et al., 2012; Sahlstrom et al., 2008; Watcharasukarn et al., 2009; Yin et al., 2016), the results obtained for enterococci are less consistent. Although two studies reported that heat treatment at 70 °C for 1 h is not sufficient to completely inactivate *Enterococcus faecalis* (Watcharasukarn et al., 2009) or enterococci

622 (Bonjoch and Blanch, 2009), it is usually acknowledged that this temperature reduces
623 the concentration of enterococci to undetectable levels (Luste et al., 2012; Martinez
624 et al., 2003; Sahlstrom et al., 2008; Yin et al., 2016). Yin et al. (2016) pointed out that
625 the degree of inactivation of the heat treatment decreased with an increase in the
626 concentration of total solids (TS) in the effluent. At 70 °C, the time needed for
627 complete inactivation of both fecal coliforms and enterococci in the sludge was 60, 80
628 and 100 minutes at a TS content of 2%, 4% and 8%, respectively. In our study, the
629 homogeneity of the pig manure, ensured by continuous stirring, and the low TS
630 content (2.1-2.7%) were favorable conditions for heat transfer and consequently for
631 the inhibition of *E. coli* and enterococci. However, the presence of enterococci in the
632 digestate at a concentration close to that observed without heat treatment could be
633 explained by the fact that a small fraction of the bacterial population was heat
634 resistant, as reported by Watcharasukarn et al. (2009). Moreover, it has been
635 reported that the heat resistance of enterococci is species and strain dependent
636 (McAuley et al., 2012). Another hypothesis is that the enterococci originally supplied
637 by the inoculum (the digestate of the biogas plant where the manure was collected)
638 used to fill the reactors, were acclimated to the substrates and operating conditions
639 of the reactors, as suggested by Luste et al. (2012). Indeed, these authors also
640 observed the presence of enterococci in digestate fed with preheated manure (70 °C,
641 1 h). Although it is not possible to distinguish their origin (i.e., heat-resistant fraction
642 or inoculum), it is important to underline that enterococci were able to establish in the
643 mesophilic digester even after a heat pretreatment.

644 Both *C. perfringens* and *C. difficile* were generally only slightly impacted by heat
645 pretreatment of the manure, leading to a relatively small decrease in their
646 concentrations in the digestate (0.5 to 0.8 and 0.5 to 1 Log_{10} units, respectively).

After heat pretreatment, the concentrations of *C. perfringens* in the influent decreased by an average of 0.7 Log_{10} units whereas those of *C. difficile* increased slightly by *ca.* 0.4 Log_{10}. It is unlikely that the spores were strongly inactivated as *C. difficile* and *C. perfringens* are relatively resistant to a temperature of 71 °C (Rodriguez-Palacios et al., 2010; van Asselt and Zwietering, 2006). Sahlstrom et al. (2008) also measured an average reduction of 0.4 Log_{10} in the number of *C. perfringens* after heating mixed biowaste from a BGP at 70 °C for one hour. The same trend was reported by Watcharasukarn et al. (2009), who observed weak abatement (less than 1 Log_{10}) in a *C. perfringens* isolate inoculated in cow manure heated at 70 °C for one hour. Pickering et al. (2019) heated spores of five *C. difficile* strains of different PCR-ribotypes (including PCR-ribotype 078) at 70 °C for one hour in phosphate buffered saline and reported that PCR-ribotype 078 was hardly affected (abatement of 0.3 Log_{10}). It should be noted that in our study, the heated manure was cooled to 40 °C before being mixed with the co-substrate. It is possible that the spores were induced to germinate during the 40-minute cooling period. The different responses of the two clostridia to heat pretreatment of the manure could be explained by their different ability to germinate under such conditions.

4. Conclusion

Despite the variability of bacterial responses, we observed that increasing the HRT improves the sanitary quality of the digestates by significantly increasing the removal of *E. coli*. Even if the effect on enterococci is not significant, lengthening the HRT (> 46 days) is recommended. We also observed that two of the four bacteria (enterococci and *C. difficile*) are more inactivated at low OLR. From an operational

point of view, at field scale, increasing the HRT is generally associated with a decrease of the OLR thereby favoring inactivation of most enteric bacteria.

Heat pretreatment (70 °C, 1 h) cannot guarantee the biosafety of digestate, as enterococci and clostridia, particularly *C. difficile*, which is considered as an emerging pathogen, were still present in the digestate at a relatively stable level even when the manure was pretreated.

Currently, using a thermal treatment before the digester, is being questioned by stakeholders as post heat treatment is more attractive from the point of view of energy consumption. Although re-growth of pathogenic bacteria cannot be excluded following post heat treatment (Sahlstrom, 2003), this pathway should be further explored to determine if it is more effective than preheat treatment in inhibiting the vegetative cells of Gram-positive bacteria such as enterococci.

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