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1 **Influence of operating conditions on the persistence of *E. coli*,**
2 **enterococci, *Clostridium perfringens* and *Clostridioides difficile* in**
3 **semi-continuous mesophilic anaerobic reactors**

4 Derongs Lorine¹, Druilhe Céline¹, Le Maréchal Caroline², Barbut Frédéric^{3, 4},
5 Heurtevent Lorette¹, Buffet Julie¹, Martin Laure², Ziebal Christine¹, Poezevara
6 Typhaine², Rouxel Sandra², Houard Emmanuelle², Syed Zaidi Rabab^{3, 4}, Couturier
7 Jeanne^{3, 4}, Pourcher Anne-Marie*¹

8 ¹ INRAE, OPAALE Research Unit, CS 64427, F-35044 Rennes, France

9 ² ANSES, Ploufragan-Plouzané Laboratory, Hygiene and Quality of Poultry and Pig
10 Products Unit, BP53, F-22440 Ploufragan, France

11 ³ National reference laboratory for *Clostridium difficile*, Saint-Antoine Hospital,
12 Assistance Publique- Hôpitaux de Paris, 34 rue Crozatier, 75012 Paris, France

13 ⁴ UMR INSERM S-1139, Faculté de Pharmacie de Paris, Université de Paris

14

15 *Corresponding author. Tel.:+ (33) 2 23 48 21 37

16 E-mail address: anne-marie.pourcher@inrae.fr

17

18 Abstract

19 This study examined the combined effect of hydraulic retention time (HRT), organic
20 loading rate (OLR) and heat pretreatment of manure (70 °C, 1 h) on the fate of *E.*
21 *coli*, enterococci, *C. perfringens*, *C. difficile*, and on chemical parameters (volatile
22 fatty acids and ammonia) that may inactivate pathogens. Semi-continuous mesophilic
23 anaerobic reactors were fed with pig manure and horse feed. The operating
24 conditions were 2, 3, 4 COD.L⁻¹.d⁻¹ (OLR), 24, 35, 46 days (HRT) and use or not of a
25 thermal pretreatment. The levels of the chemical parameters did not reach
26 concentrations capable of inactivating the four bacteria. Anaerobic digestion led to a
27 Log₁₀ removal > 3 (*E. coli*), 0.9-2.1 (enterococci), 0.1-0.6 (*C. perfringens*) and 0-2 (*C.*
28 *difficile*). Increasing HRT only reduced the concentration of *E. coli* in the digestate.
29 Increasing OLR reduced the Log₁₀ removal of enterococci and *C. difficile*. The heat
30 pretreatment led to non-detection of *E. coli* in the digestate, reduced the
31 concentration of *C. perfringens* by 0.8-1.3 Log₁₀ and increased the concentration of
32 *C. difficile* by 0.04-0.7 Log₁₀. Enterococci, not detected in the heated manure, were
33 present in the digestate. The distribution of genes encoding virulence factors of *C.*
34 *difficile* (*tcdA* and *tcdB*) and *C. perfringens* (*cpa*, *cpb2* and *cpb*) was not impacted by
35 anaerobic digestion or by the heat pretreatment. Enterococci, *C. perfringens*, *C.*
36 *difficile* were present in the digestate at relatively stable concentrations regardless of
37 the operating conditions, indicating that even with heat pretreatment, the biosafety of
38 digestate cannot be guaranteed in mesophilic conditions.

39

40

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

43

44 **1. Introduction**

45 The biogas sector is among the objectives of the Energy Transition for Green Growth
46 Law adopted in 2015 by the French Parliament, i.e., the reduction of greenhouse gas
47 emissions, the development of renewable energies and of the circular economy.
48 Decentralization of renewable energy has been promoted in France in the last
49 decade and has led to a significant increase in the number of decentralized on-farm
50 biogas plants (31 sites in 2010 vs 534 in 2020), and simultaneously to an increase in
51 the volume of digestate intended for use as crop fertilizer. Spreading digestates on
52 the land, the most common use of this by-product, enables recycling of nutrients and
53 organic matter, creating an on-site circular economy. However, this practice may also
54 involve the risk of disseminating pathogenic micro-organisms in field soil (Jiang et al.,
55 2020; Orzi et al., 2015). French on-farm biogas plants, which commonly use pig or
56 cattle manure as feedstock, are mainly operated within the mesophilic temperature
57 range (35-42 °C) that has been reported to be less effective than thermophilic
58 temperatures in reducing pathogenic micro-organisms (Jiang et al., 2020; Nag et al.,
59 2019; Pandey and Soupir, 2011; Sahlstrom et al., 2004; Thomas et al., 2019).
60 Pathogenic enteric bacteria such as *Salmonella* sp, *Listeria monocytogenes*, *Yersinia*
61 *enterocolitica* or thermotolerant *Campylobacter* have been detected in digestate
62 sampled from mesophilic digesters fed with pig or cattle manure (Le Marechal et al.,
63 2019; Orzi et al., 2015) suggesting their persistence throughout the anaerobic
64 digestion (AD) process. A low impact of mesophilic AD on pathogenic spore-forming

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

67 In addition to the intrinsic characteristics of the bacteria, the main AD parameters that
68 may affect the inactivation of pathogenic bacteria are feedstock composition,
69 operating conditions (temperature, retention time, organic loading rate) and
70 intermediate products (volatile fatty acids, ammonia) (Jiang et al., 2020). Among
71 operational parameters, the impact of the digestion temperature (mesophilic vs
72 thermophilic) on bacterial persistence is well established (Jiang et al., 2020; Zhao
73 and Liu, 2019), but less research has focused on retention time and the organic
74 loading rate (OLR). Using mesophilic reactors fed with sludge or with pig manure,
75 Coelho et al. (2011) and Dennehy et al. (2018), observed no consistent relationship
76 between the increase in retention time from 5 to 20 days or from 21 to 41 days,
77 respectively, and the Log₁₀ removal of total coliforms, *E. coli* or enterococci. In
78 contrast, Chen et al. (2012) reported that the removal of *E. coli* and *Salmonella* sp. in
79 mesophilic reactors fed with sludge, led to a respective increase of 1.08 and 1.79
80 Log₁₀, with an increase in retention time from 11 to 25 days. Only two studies, one
81 performed in reactors fed with livestock manure under thermophilic conditions
82 (Skillman et al., 2009) and the other under psychrophilic conditions (Rosenblum et
83 al., 2015), tested the effect of OLR on the persistence of bacteria. Skillman et al.
84 (2009) observed no change in *Clostridium perfringens* concentrations when OLR was
85 increased from 2.2 to 6.4 g chemical oxygen demand (COD).L⁻¹.day⁻¹, whereas in the
86 experiment conducted by Rosenblum et al. (2015), the reduction in the concentration
87 of *E. coli*, fecal coliforms and *Enterococcus* sp. was significantly higher with an OLR
88 of 0.3 than with an ORL of 1.3 g volatile solids (VS).L¹.day⁻¹. Due to the scarcity of

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

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

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

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

120 **2. Material and methods**

121

122 2.1. Inoculum and influent

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

128 The digestate used as inoculum was transferred into the reactors immediately after
129 sieving. The sieved manure was stored at 4 °C in 50 L canisters during the
130 experiment. Before the reactors were fed, the sieved manure was heated at 70 °C for
131 one hour, or not, depending on the experiment. The influent consisted of a mixture of
132 manure (unheated or heated) and a co-substrate added to increase the biomethane
133 potential of the influent. The co-substrate, made of lignocellulosic materials,
134 previously used by Peu et al. (2011), was pelleted horse feed, selected for its stable
135 composition and its high methane potential. The pelleted feed was crushed before
136 mixing with the manure. The ratio of manure and co-substrate, and the feed volume
137 were determined for each reactor according to the targeted organic loading rate
138 (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

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

188

189 2.5. Physicochemical analysis

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

195

196 2.6. Microbial analysis

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

203

204 *E. coli*

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

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

210

211 *Enterococci*

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

217

218 *Clostridium perfringens*

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

228

229 *Clostridioides difficile*

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

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

234

235 2.7. DNA extraction, detection of toxin genes and antimicrobial susceptibility

236 A total of 406 *C. perfringens* isolates were collected from the influents (n=220) and
237 the digestates (n=186). DNA was extracted as previously described by Derongs et al.
238 (2020) using the Nucleospin® Microbial DNA kit (Macherey-Nagel, Duren, Germany).
239 Genes *cpa*, *cpb*, *etx* and *iap*, encoding the four major toxins (α , β , ϵ , and ι) and genes
240 *cpe*, *netB* and *cpb2* encoding the enterotoxin CPE and the NetB and β 2 toxins, were
241 performed using real-time PCR as described by Derongs et al. (2020). The
242 proportion of *C. perfringens* toxinotypes in the influents and digestates was
243 compared using the Chi-square test in XLSTAT 2019 (Addinsoft, Bordeaux, France).
244 Antimicrobial susceptibility profiles of *cpa*-positive isolates from the influent (n=203)
245 and digestate (n=161) were determined by microdilution method (Sensititre™
246 Bovine/porcine MIC plate) according to the manufacturers' instructions
247 (ThermoFisher Diagnostics, France) as described by Derongs et al. (2020).
248 Susceptibility was tested against penicillin, ampicillin, ceftiofur, tylosine tartrate,
249 tilmicosin, tulathromycin, clindamycin, enrofloxacin, danofloxacin, tiamulin,
250 florfenicol, chlortetracycline and oxytetracycline.

251

252 A total of 184 *C. difficile* isolates were collected from the influents (n=96) and the
253 digestates (n=88). DNA was extracted as previously described by Le Marechal et al.
254 (2020). *tpi* (triose phosphate isomerase) gene fragments used to identify *C. difficile*,
255 *tcdA* (toxin A) and *tcdB* (Toxin B) were detected using real-time PCR with the primers
256 described by Barbut et al. (2019). PCR ribotyping, multiplex PCR and susceptibility to

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

263

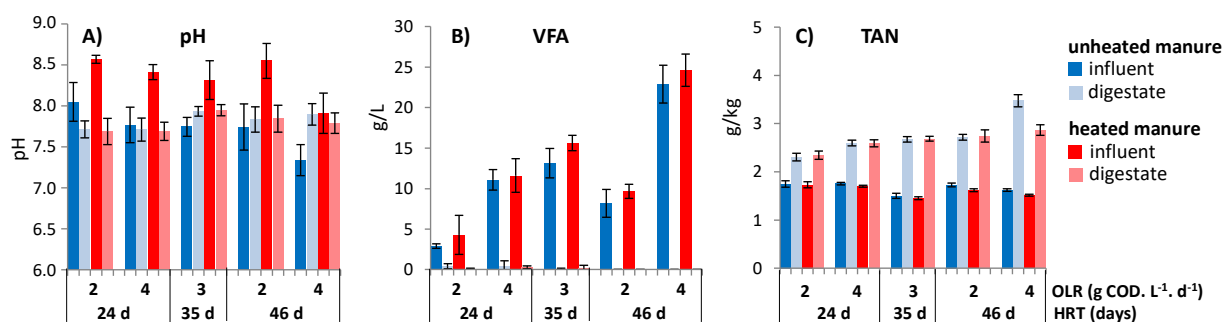
264 3. Results

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

273 3.1. Physicochemical parameters

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

276



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

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

299

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

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

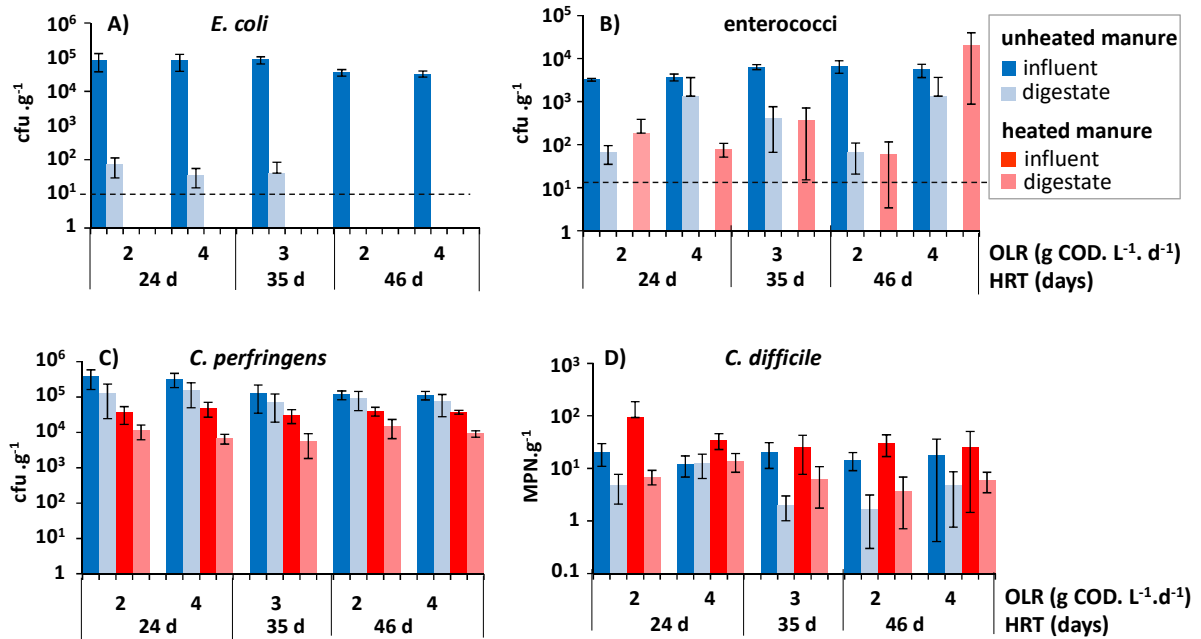
312

313 3.2. Microbial parameters

314 The concentrations of the four bacteria in the influent remained stable throughout the
 315 experiment. In the absence of heat pretreatment, the concentrations ranged from 3.3
 316 10⁴ to 8.2 10⁴ cfu.g⁻¹ (*E. coli*, Figure 2A), from 3.3 10³ to 6.8 10³ cfu.g⁻¹ (enterococci,

317 Figure 2B), from $1.1 \cdot 10^5$ to $3.8 \cdot 10^5$ cfu.g⁻¹ (*C. perfringens*, Figure 2C) and from 12 to
 318 20 MPN.g⁻¹(*C. difficile*, Figure 2D).

319



320

321

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

328

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

333

334 Table 3. Variance analysis of the influence of three factors (hydraulic retention time
 335 (HRT), organic loading rate (OLR) and heat pretreatment (Th.Pr)) and of their
 336 interactions on the concentrations of bacteria (expressed in Log₁₀ cfu g⁻¹ or MPN g⁻¹)
 337 in the digestates.

Factor		<i>E. coli</i>	enterococci	<i>C. perfringens</i>	<i>C. difficile</i>
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

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

341

342 3.2.1. Impact of the HRT and of the OLR on bacteria

343 Without heat pretreatment, the AD led to a decrease of more than 3 Log₁₀ in *E. coli*
 344 and of 0.9 to 2.1 Log₁₀ in enterococci (Table 4, Figure 2A and 2B). The changes in
 345 OLR did not influence the level of *E. coli* whereas the 46-day HRT resulted in no
 346 detection of this bacterium (Table 3, Figure 2A). The OLR significantly impacted the
 347 concentrations of enterococci in the digestate (Table 3). Indeed, doubling the OLR
 348 decreased the Log removal by ca. 1 Log₁₀ (Table 4). HRT had no significant effect on
 349 the concentration of enterococci (Table 3).

350

351 Table 4. Log₁₀ reduction in bacterial concentrations between the influent and
 352 digestate, calculated over the four weeks of the experiment.

Manure treatment	HRT (days)	OLR (g COD. L ⁻¹ . d ⁻¹)	<i>E. coli</i> Mean ± SD	Enterococci Mean ± SD	<i>C. perfringens</i> Mean ± SD	<i>C. difficile</i> 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

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

356

357 Concentrations of *C. perfringens* were slightly affected by the AD with a Log₁₀
 358 reduction ranging from 0.1 to 0.6 without heat pretreatment, and from 0.5 to 0.8 with
 359 heat pretreatment, independently of the HRT and the OLR (Table 4, Figure 2C). The
 360 Log₁₀ reduction in *C. difficile*, which ranged from 0 to 2, is more difficult to interpret,
 361 given the low concentrations of this pathogen in the influent. However, a significant
 362 impact of the OLR was observed on the concentrations of *C. difficile* (Table 3). The

363 lowest OLR led to the highest Log₁₀ 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 Log₁₀ (Figure
370 2C) while the concentration of *C. difficile* increased slightly by 0.1 to 0.7 Log₁₀ (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

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

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

389 ^a Number of isolates, regardless of the HRT and OLR

390

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

403

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

Genes	Unheated manure		Heated manure		
	Influent n=48 ^a	Digestate n=42	Influent n=48	Digestate n=46	Total n=184
<i>tpi</i>	100	100	100	100	100
<i>tcdA</i>	100	97.6	100	95.6	98.4
<i>tcdB</i>	100	97.6	100	95.6	98.4

407 ^a Number of isolates, regardless of the HRT and OLR

408

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

414

415 **4. Discussion**

416 Most French agricultural biogas plants treat livestock manure at mesophilic
 417 temperatures. However, several studies have demonstrated that the efficiency of
 418 mesophilic anaerobic digestion (MAD) with regard to fecal indicators (*E. coli*, fecal
 419 coliforms, total coliforms or enterococci) is lower than that of thermophilic anaerobic
 420 digestion (Amani et al., 2011; Astals et al., 2012; Lopez et al., 2020; Pandey and
 421 Soupir, 2011; Watcharasukarn et al., 2009; Ziembra 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 Log₁₀ 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 Log₁₀ 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 Log₁₀ 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 Log₁₀ 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 β 2 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 Log₁₀), mainly represented by *C. perfringens*, in laboratory
479 reactors (46-day HRT) fed daily with a mixture of agricultural substrate. The Log₁₀
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 Log₁₀ 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 Log₁₀ 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.
515 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 Log₁₀ 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 CO₂ and H₂ (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 $\text{NH}_3 < 5 \text{ mM}$. After six hours of incubation, *Salmonella* was completely inhibited at
587 $> 40 \text{ mM}$ ammonia whereas 180 mM was needed to obtain $> 5 \text{ Log}_{10}$ 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 $\text{N-NH}_3 \text{ mg. kg}^{-1}$ (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

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

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

611

612 4.4. Impact of the heat pretreatment

613 The drastic effect of the heat pretreatment (70 °C, 1 h.) on the concentration of *E. coli*
614 and enterococci in pig manure we observed is in agreement with the results of
615 previous studies. However, while all the studies conducted on sludge or on manure
616 agree that heat treatment at 70 °C inactivates Gram negative bacteria such as *E.*
617 *coli*, fecal coliforms or *Salmonella* (Bonjoch and Blanch, 2009; Lang and Smith, 2008;
618 Ruiz-Espinoza et al., 2012; Sahlstrom et al., 2008; Watcharasukarn et al., 2009; Yin
619 et al., 2016), the results obtained for enterococci are less consistent. Although two
620 studies reported that heat treatment at 70 °C for 1 h is not sufficient to completely
621 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 Log₁₀ units, respectively).

647 After heat pretreatment, the concentrations of *C. perfringens* in the influent
648 decreased by an average of 0.7 Log₁₀ units whereas those of *C. difficile* increased
649 slightly by ca. 0.4 Log₁₀. It is unlikely that the spores were strongly inactivated as *C.*
650 *difficile* and *C. perfringens* are relatively resistant to a temperature of 71 °C
651 (Rodriguez-Palacios et al., 2010; van Asselt and Zwietering, 2006). Sahlstrom et al.
652 (2008) also measured an average reduction of 0.4 Log₁₀ in the number of *C.*
653 *perfringens* after heating mixed biowaste from a BGP at 70 °C for one hour. The
654 same trend was reported by Watcharasukarn et al. (2009), who observed weak
655 abatement (less than 1 Log₁₀) in a *C. perfringens* isolate inoculated in cow manure
656 heated at 70 °C for one hour. Pickering et al. (2019) heated spores of five *C. difficile*
657 strains of different PCR-ribotypes (including PCR-ribotype 078) at 70 °C for one hour
658 in phosphate buffered saline and reported that PCR-ribotype 078 was hardly affected
659 (abatement of 0.3 Log₁₀). It should be noted that in our study, the heated manure was
660 cooled to 40 °C before being mixed with the co-substrate. It is possible that the
661 spores were induced to germinate_during the 40-minute cooling period. The different
662 responses of the two clostridia to heat pretreatment of the manure could be
663 explained by their different ability to germinate under such conditions.

664

665 **4. Conclusion**

666 Despite the variability of bacterial responses, we observed that increasing the HRT
667 improves the sanitary quality of the digestates by significantly increasing the removal
668 of *E. coli*. Even if the effect on enterococci is not significant, lengthening the HRT (>
669 46 days) is recommended. We also observed that two of the four bacteria
670 (enterococci and *C. difficile*) are more inactivated at low OLR. From an operational

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

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

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

683

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