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► **To cite this version:**

Laura Álvarez-Fraga, Gabriel Capson-Tojo, Malo Sanglier, Jérôme Hamelin, Renaud Escudié, et al..
A meta-analysis of pathogen reduction data in anaerobic digestion. 2024. hal-04734450

HAL Id: hal-04734450

<https://hal.inrae.fr/hal-04734450v1>

Preprint submitted on 14 Oct 2024

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1 **A meta-analysis of pathogen reduction data in anaerobic digestion**

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25 26 **Highlights**

- 27 • Artificially spiking of pathogens leads to removal overestimation
- 28 • Current pathogen indicators accurately represent their respective microbial groups
- 29 • Temperature, pH, and batch duration affect pathogen reduction
- 30 • Spore-forming bacteria, including *Clostridium perfringens*, are not affected by AD
- 31 • Thermophilic AD coupled with heat post-treatment fulfills most legislation limits

32 33 **Abstract**

34 Anaerobic digestion (AD)-derived digestate can be used as an organic fertilizer or for soil
35 amendment. However, its utilization for resource recovery raises valid biosafety concerns.
36 Despite extensive research on the capacity of AD for pathogen reduction, the variability in results
37 poses challenges for drawing definitive conclusions. To address this lack of unification, results
38 from 121 scientific articles were compiled, and a comprehensive meta-analysis was conducted.
39 Findings indicate that artificial pathogen spiking leads to performance overestimation. Current
40 most common indicators represent accurately their respective microbial groups. *Clostridiaceae*
41 are barely affected by AD and may be favored by some pre-treatment technologies. The impact
42 of operational parameters and the coupling of pre- and post-treatments with AD on pathogen
43 reduction was also investigated. While an optimal batch duration was identified, the hydraulic
44 retention time in (semi)continuous systems did not affect the overall pathogen reduction. Heat-
45 based post-treatments coupled with thermophilic AD resulted in the highest pathogen reductions,
46 fulfilling legislations. Unprecedented statistical analyses allowed categorizing quantitatively key

47 parameters. Results confirmed that temperature is the most relevant parameter. Thermophilic
48 conditions resulted in the highest pathogen reductions, while psychrophilic and mesophilic
49 temperatures showed similar performances. The impact of pH on pathogen removal was
50 confirmed, with acidic and basic values enhancing pathogen reductions. More research
51 considering all AD products within a multicriteria optimization approach (e.g., pathogen
52 reduction, biogas production, and digestate quality) is needed to determine optimal conditions
53 considering all aspects. This study provides novel and relevant conclusions for AD at research
54 and industrial scale, drawing several R&D perspectives.

55

56 **Word count**

57 9,180 words

58

59 **Keywords**

60 Digestate, pathogen inactivation, fermentation, resource recovery, hygienization, pasteurization,
61 *Escherichia coli*, *Enterococcus* sp., *Clostridium perfringens*, virus

62

63 **Abbreviations and symbols**

64 ABP - Animal By-Product

65 AD - Anaerobic digestion

66 AnSBR - Anaerobic sequencing batch reactor

67 ANOVA - Analysis of variance

68 CFU - Colony forming unit

69 DNA - Deoxyribonucleic acid

70 EU - European Union

71 FBR - Fixed bed reactor

72 HRT - Hydraulic retention time

73 HSD - Post-hoc Tukey's Honesty Significant Difference test

74 IQR - Interquartile range

75 LR - Log reduction

76 MPN - Most probable number

77 n - Number of independent datapoints

78 N - Number of articles

79 N_0 - Number of colony forming units before AD, pre- or post-treatment

80 N_1 - Number of colony forming units after AD, pre- or post-treatment

81 OLR - Organic loading rate

82 PABFR - Panelled anaerobic baffle-cum-filter reactor

83 PFR - Plug flow reactor

84 PLS - Partial least squares

85 qPCR - Quantitative polymerase chain reaction

86 RNA - Ribonucleic acid

87 STR - Stirred tank reactor

88 TPAD - Temperature phased AD

89 TS – Total solids

90 US EPA - United States Environmental Protection Agency

91 VBNC - Viable but non-culturable cell

92 VFA - Volatile fatty acid

93 VS - Volatile solids

96 **1. Introduction**

97 The need to implement a more sustainable development of society calls for a shift from the
98 current linear economy to a more circular system. This approach prioritizes the recovery and
99 recycling of resources from waste, ensuring their reintroduction into the production-consumption
100 loop. To facilitate this transition, extensive research efforts have been dedicated to the
101 advancement and implementation of environmentally friendly and cost-effective waste
102 valorization technologies.

103 Anaerobic digestion (AD) is among the most widely applied technologies for the valorization of
104 organic waste streams. AD is a well-established biological process with a triple role: (i)
105 production of biomethane (used as an energy source), (ii) waste treatment and stabilization, and
106 (iii) generation of nutrient-rich digestate [1,2]. AD has become a primary technology for
107 generating renewable energy and facilitating resource recovery, with over 182,000 digesters
108 operating worldwide at various scales [3]. Thanks to supporting policies, the number of AD
109 plants has increased significantly in the last decades. In Europe, the power generation capacity
110 from biogas reached 209 TWh in 2018, representing 7.4% of the total net electricity generated.
111 Recently, the European Commission presented the ambitious REPowerEU action plan, which
112 anticipates a twelve-fold increase in AD capacity by 2030 [4].

113 This expansion of the AD capacity will require the effective management of larger quantities of
114 digestate. Currently, around 290-300 million tons/year are produced worldwide, a value that
115 could be increased twelve-fold by 2030 [5]. Digestate usually contains high concentrations of
116 easily available nutrients, slowly biodegradable organic matter, and trace elements, making it a
117 valuable resource applicable as organic fertilizer and for soil amendment [6]. The benefits of
118 applying digestate as fertilizer are significant compared to commonly used raw organic wastes
119 (e.g., manure). Digestate presents notable advantages when compared to raw substrates,
120 displaying lower pathogen concentrations, enhancing nutrient availability for plant absorption,
121 and reducing considerably the risk of water and soil pollution due to its slow-release nature [5].
122 The use of digestate as soil amendment holds the potential to replace 5-7% of the current total
123 inorganic fertilizer usage [7]. Despite the notable advantages associated with digestate utilization,
124 its application for resource recovery purposes raises reasonable concerns. The persistence of
125 pathogenic microorganisms, commonly found in AD feedstocks and thus potentially in the
126 digestate after the AD process, is one of them. If not managed properly, the agricultural usage of
127 digestate could lead to the dissemination of pathogens, posing serious threats to animal and
128 human health [8,9].

129 To effectively prevent and mitigate the risks associated with the use of digestate in agriculture, it
130 is imperative to develop and implement meticulous management and risk assessment protocols
131 throughout the entire AD lifecycle. These practices, regulated at a national and international
132 level, play a pivotal role in safeguarding both the environment and public health. For example,
133 the European Union (EU) has taken a proactive approach by providing comprehensive guidelines
134 (i.e., EC1069/2009 and EC142/2011) [10,11], which establish standard practices and protocols
135 for operating AD plants. These guidelines also incorporate sampling collection protocols and
136 microbiological standards (i.e., maximum allowed concentrations of pathogen indicators),
137 ensuring that the digestate is suitable for agricultural use. Fulfilling these standards for targeted
138 microorganisms is therefore crucial, as their presence could limit digestate application. Certainly,
139 other relevant legislations exist worldwide, such as those in China [12] or the United States [13].

140 Despite being more or less restrictive and allowing different digestate applications, they all share
141 the same objective: ensuring the safe utilization of recovered resources from digestate.
142 AD can effectively reduce the concentration of pathogens present in a wide range of feedstocks,
143 such as sewage sludge, manure or biowaste [14–17]. However, the pathogen reduction capacity
144 of AD (commonly referred to as hygienization) can be insufficient, resulting in concentrations of
145 microorganisms in the digestate exceeding biosafety levels. To enhance the microorganism
146 inactivation during AD, it is crucial to understand and optimize the factors influencing the
147 pathogen reduction performance. Different factors affecting pathogen removal have been
148 identified, including the type of pathogens present, the byproducts formed during the process
149 (e.g., volatile organic acids (VFAs) or ammonia nitrogen), and different operational parameters
150 (e.g., temperature or retention time). Despite previous efforts done to elucidate optimal pathogen
151 reduction conditions, the challenge remains, mostly due to the limited scope of many
152 experimental studies, which assess the inactivation of specific pathogens under specific
153 operational conditions, thereby resulting in data that cannot be extrapolated and even in
154 contradictory results.

155 To address this issue, it is essential to adopt a more comprehensive and holistic approach, for
156 example, by conducting a meta-analysis of data collected from existing literature. Only two
157 recent studies have undertaken such an approach, unifying and synthesizing existing data to
158 understand pathogen inactivation during AD. The first study presented a descriptive review,
159 limiting its statistical analyses to few factors [18]. It highlighted the considerable impact of
160 pathogen type, temperature, and reactor feeding mode on pathogen inactivation. Specifically,
161 thermophilic temperatures and batch mode appeared to be optimal conditions for achieving high
162 removal efficiencies. While this study provided valuable insights, it left multiple aspects
163 unexplored. For instance, the impact of the type of reactor lacked a comprehensive assessment,
164 and critical operational conditions, including pH and organic loading rate (OLR), were not
165 thoroughly examined. The study did not assess either the effect of coupling different pre- and
166 post-treatments to AD. The second study conducted a more extensive statistical analysis to
167 elucidate and quantify how AD operational conditions influence the inactivation of major
168 foodborne indicator-pathogens [17]. This meta-analysis demonstrated the effectiveness of AD for
169 efficiently reducing some pathogenic species, such as fecal coliforms, *Escherichia coli*, or
170 *Salmonella* spp. Noteworthy findings include the positive impacts of temperature, high
171 intermediate VFA concentrations, and pre-treatments on the pathogen reduction performance.
172 However, this study has significant limitations. Namely, it focused solely on specific pathogens
173 (i.e., Gram-negative microorganisms), and it analyzed each pathogen individually. The diverse
174 behaviors exhibited by different groups of microorganisms during AD (e.g., Gram-negative
175 bacteria, Gram-positive bacteria, Gram-positive spore-forming bacteria, viruses, or parasites)
176 jeopardize the extrapolation of these results from one group to others.

177 The present study aims at consolidating and analyzing the available experimental data, providing
178 a global view of the capacity of AD for pathogen removal. Specifically, the impact of different
179 operational conditions and reactor designs/types on the pathogen reduction performance was
180 evaluated. Opposed to previous studies, a wide range of reactors, substrates, and operational
181 conditions were considered, and all relevant microorganisms were included. For the first time, a
182 quantitative analysis of the data was conducted to identify the most influencing parameters for
183 pathogen removal. Additionally, an integrated assessment of the AD treatment line was
184 performed by investigating the impact of common pre-treatment and post-treatment processes
185 (either alone or coupled with AD) on pathogen reduction, aiming at identifying conditions
186 leading to the highest pathogen removal. Lastly, the resulting database was compared against two

187 relevant pathogen-related regulations to assess compliance with regulatory requirements.
188 Considering these diverse factors collectively allowed gaining deeper insights into the overall
189 effectiveness of AD for pathogen inactivation, and optimizing its pathogen reduction
190 performance. Increasing the current understanding of the pathogen reduction process is crucial
191 for developing more efficient waste management processes allowing safe resource recovery.
192 Ultimately, this research has the potential to contribute significantly to guaranteeing the
193 production of safe and high-quality digestate, crucial to boosting AD implementation.

194 195 **2. Material and methods**

196 **2.1. Article search strategy and selection process**

197 A comprehensive literature search was conducted from inception up to May 2023 using the Web
198 of Science (WoS) database. A set of specific keywords was chosen to identify the articles
199 focusing on the pathogen reduction capacity of AD. The Boolean string utilized was as follows:
200 (*“Anaerobic *digestion” OR biogas*) AND (*coliform* OR Enterococc* OR faecalis OR*
201 *perfringens OR botulinum OR Citrobacter OR Enterobacter* OR Escherichia OR coli OR*
202 *Klebsiella OR Salmonella OR Shigella OR Listeria OR Campylobacter OR Parvovirus OR*
203 *Ascaris OR helminth OR egg* OR pathogen* OR *virus**) AND (*temperature OR pH OR*
204 *“retention time” OR ammoni* OR volatile fatty acid* OR VFA* OR “organic load* rate” OR*
205 *biochar OR “conductive material*”*) AND (*reduction OR removal OR inactivation OR decrease*
206 *OR hygieni*ation OR sanitation OR “viable but *culturable*” OR VBNC**) AND (*sludge OR*
207 *manure OR slurry OR *waste OR slaughterhouse OR “animal by-product*” OR food*). The
208 asterisk (*) is used to represent any sequence of characters. References identified by previous
209 meta-analyses/reviews were also reviewed [14,15,17,18].

210 The eligibility criteria were as follows: (i) peer-reviewed articles published in English and
211 available in full text, (ii) original studies evaluating pathogen reduction during AD, (iii) original
212 studies evaluating pathogen reduction including different pre- and/or post-treatments and (iv)
213 availability of pathogen reduction data or data allowing its calculation. Data from book chapters,
214 systematic reviews, meta-analyses, conference papers, and letters to the editor were excluded.
215 Further exclusion criteria included: (i) absence of key inputs or outputs, (ii) reported units
216 incompatible with pathogen reduction calculation, or (iii) inconsistencies in the provided data
217 (e.g., unreasonable methane yields or unreasonable volatile solids (VS) reduction values).

218 219 **2.2. Data collection**

220 Data were extracted from tables or text in articles. When data were not explicitly provided, values
221 were extracted from graphs and/or manually calculated. Extracted data were organized in a
222 spreadsheet using Microsoft Excel. Data encompassed crucial information regarding individual
223 experiments, such as reactor type, feeding mode, reactor inoculum, feedstock, reactor operational
224 conditions, and primary process outcomes such as pathogen reduction or methane yield.

225 Categories were defined for different factors, including reactor types, feedstocks (including
226 mixtures indicated as “co-digestion”), and microorganisms studied. The full database and a list of
227 the categories considered can be found in Supplementary Material (Table S1). The database was
228 also deposited in the research data repository Mendeley Data [19]. Assumptions were applied for
229 data standardization (see Appendix A).

230 Pathogen reduction was quantified in terms of Log reduction (LR), expressed as $\text{Log}_{10} (N_0 / N_1)$,
231 where N_0 represents the initial number of colony forming units (CFUs) or most probable number
232 (MPN) of microorganisms before AD, pre- or post-treatment and N_1 represents the number of
233 CFUs or MPN after AD, pre- or post-treatment.

234 Data obtained using molecular techniques, such as quantitative polymerase chain reaction
235 (qPCR), were also included in the database [19] and are briefly discussed in Section 4. However,
236 they were excluded from the meta-analysis due to the limited number of data points available.
237

238 2.3. Statistical analysis and data representation

239 Statistical analyses were performed using R Statistical Software (v4.3.2; R Core Team, 2023). To
240 assess significant differences among groups with normally distributed data and homogeneous
241 variances, analysis of variance (ANOVA) was employed. Post-hoc Tukey's Honest Significant
242 Difference (HSD) tests were then applied for pairwise comparisons (differences between groups
243 are indicated as letters on the top of the boxplots). The validity of the ANOVA assumptions was
244 evaluated through normality analysis using Shapiro-Wilk tests and homogeneity of variance
245 using Bartlett's tests. For cases involving non-normally distributed data, non-parametric tests
246 were employed. Specifically, the Kruskal-Wallis test was used, followed by Dunn's tests for
247 pairwise comparisons. A significance threshold of 95% ($p = 0.05$) was applied for all tests.
248 The provided boxplots display data points corresponding to the lowest datum within 1.5 times the
249 interquartile range (IQR) of the first quartile, the first quartile itself, the median, the third quartile,
250 and the highest datum within 1.5 times the IQR of the third quartile. Values falling below the
251 lowest datum or exceeding the highest datum within the boxplots were identified as outliers.
252 Partial least squares regression (PLS) analyses were performed to elucidate quantitatively which
253 parameters were affecting the pathogen reduction performances the most. To do so, the LR was
254 used as the output variable and the microorganism classification, temperature, pH, and either the
255 hydraulic retention time (HRT; for semi(continuous) reactors) or the batch duration (for batch
256 reactors) as input variables. The PLS was performed in R 4.3.2, using the packages pls (function
257 pls) and ggplot2 [21,22].
258

259 3. **Results and discussion**

260 3.1. Literature search and screening

261 In this meta-analysis, a rigorous literature search to identify relevant studies concerning the
262 pathogen reduction capacity of AD was performed, including articles assessing the impact of
263 different pre- and post-treatment technologies. Five hundred fifty entries using the previously
264 described Boolean string were retrieved. The screening process, guided by predefined inclusion
265 and exclusion criteria (see Section 2.1), was systematically applied. Initial screening of titles and
266 abstracts resulted in 214 entries eligible for further evaluation. Full-text screening identified 121
267 articles (N) meeting the inclusion criteria, subsequently included in the meta-analysis. A
268 complete list of the 121 articles meeting the inclusion criteria and another list including the 92
269 articles excluded after full-text review (along with the reasons for exclusions) can be found in
270 Supplementary material (Table S1 and Table S2) and in the Mendeley Data repository [19].
271 A total of 2,051 independent datapoints (n) were extracted from the 121 articles. Of these, 1,526
272 datapoints were dedicated to investigating pathogen reduction during AD, either alone or coupled
273 to pre- or post-treatment processes (Table S1). The remaining 525 datapoints corresponded to
274 data specifically focused on pathogen reduction during pre-treatment (n = 350) or post-treatment
275 (n = 175) processes alone (Table S1).
276

277 3.2. Data overview

278 To ensure that the resulting dataset was unbiased and that the results could be extrapolated to
279 general AD processes, a detailed analysis of the sources of the data was performed. The database
280 encompassed research findings from diverse regions across all five continents (Figure S1), with

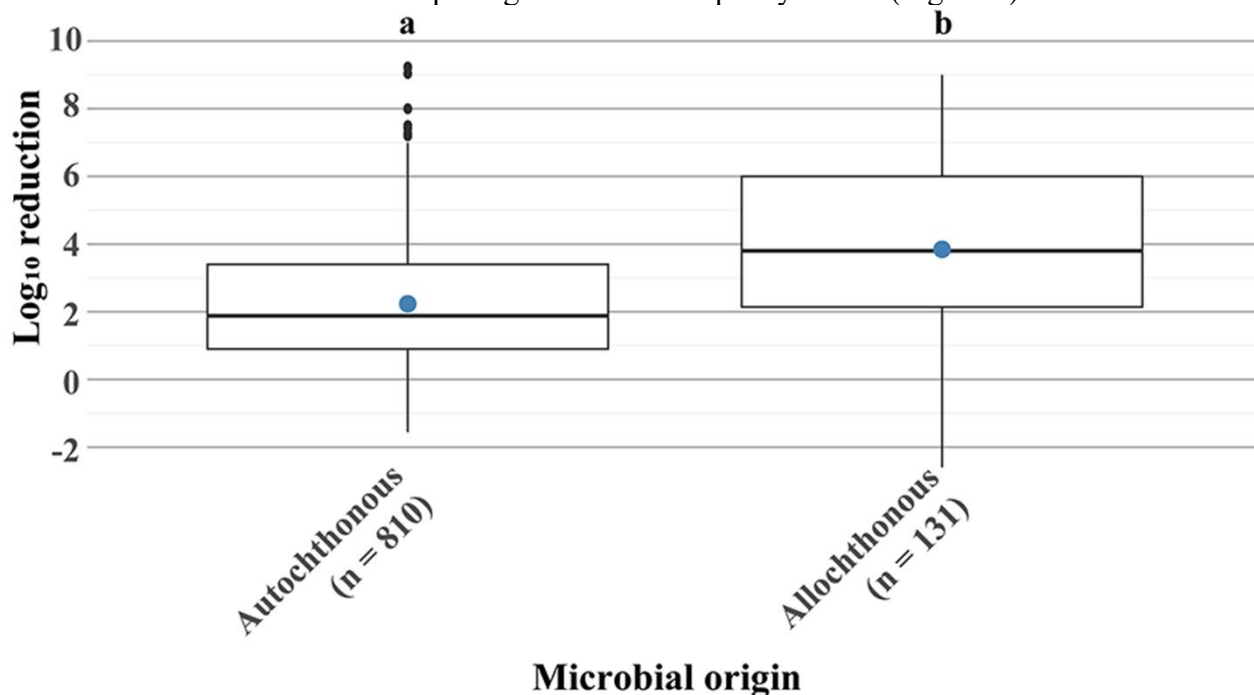
281 notable emphasis on America (N = 48) and Europe (N = 41). Among these, the USA (N = 24),
282 Spain (N = 15), and Canada (N = 11) emerged prominently. Noteworthy contributions also come
283 from China (N = 9) and Japan (N = 8). This global distribution provides a diverse perspective,
284 enhancing the robustness and global applicability of the presented findings.

285 Regarding publication years, data reveals a recent surge in studies (Figure S2). From 1997 to
286 2005, only an average of 2.7 studies per year focused on pathogen reduction during AD. Between
287 2006 and 2015, this average increased to 4.9 studies per year, reaching its peak after 2016 with an
288 average of 6.0 studies per year. This highlights the escalating interest within the scientific
289 community concerning AD and its associated pathogen dissemination risks.

290 An evident disparity was observed in the scale of the studies, with a substantial majority
291 conducted at laboratory scale (74.4%), followed by pilot-scale studies (17.3%) and industrial-
292 scale studies (11.6%) (Figure S3A). Concerning AD feedstocks, sewage sludge (50.4%) and
293 livestock waste & effluents (35.5%) were the most prevalent (Figure S3B). Mono-digestion
294 studies were predominant (88.4%), followed by agri/biowaste co-digestion (9.0%) (Figure S3C).

3.3. Impact of artificial spiking on pathogen reduction during AD

297 The first result of this analysis concerns a crucial aspect regarding the methodology employed in
298 the gathered studies. While most articles in the database assessed the reduction of autochthonous
299 pathogens, several articles assessed this reduction after artificially spiking pathogens into the
300 substrates. This raised a question concerning the potential impact of spiking pathogens artificially
301 into the substrates on the resulting pathogen reduction performances. To answer it, the database
302 was divided into two separate experimental groups, one comprising experiments in which the
303 naturally occurring autochthonous pathogens in the AD feedstock were assessed, and another one
304 comprising experiments where pathogens had been introduced in the feedstock before AD. When
305 comparing the performance of these two groups, it is clear that artificially inoculating pathogens
306 leads to an overestimation of the pathogen reduction capacity of AD (Figure 1).



307

308 **Figure 1.** Microorganism Log₁₀ reduction for experiments studying autochthonous pathogen
309 reduction (naturally present in the feedstock) and for experiments in which allochthonous
310 pathogens were inoculated. Mean values are represented by blue dots. Identical letters above
311 boxplots indicate homogeneous groups. n stands for the number of independent datapoints.
312

313 The different pathogen reduction between autochthonous and allochthonous pathogens can be
314 attributed to the adaptation of native microorganisms to the substrate and to the conditions
315 occurring during its natural decay (potentially similar to those of AD). Autochthonous
316 populations may also be protected when present in highly physically structured environments,
317 such as granules or biofilms. Inoculated pathogens might lack these adaptations, potentially
318 affecting their survival and persistence. Although the specific susceptibility of allochthonous
319 pathogens to reduction during AD has not been explicitly compared with that of autochthonous
320 pathogens, it appears evident that their behavior and fate in AD systems are clearly influenced by
321 their origin. A similar trend was observed in previous studies where allochthonous viruses and
322 bacteriophages experienced a rapid decline upon inoculation into sludge compared to the
323 autochthonous microorganisms [23]. This rapid reduction in numbers was attributed to a matrix
324 effect. In spiking experiments, the feedstock is also usually inoculated to an initial concentration
325 of microorganisms higher than their natural levels in the substrate (approximately 1 log₁₀ higher).
326 The reduced resistance of allochthonous microorganisms, combined with higher artificial
327 concentrations in the feedstock intended for pathogen reduction, may explain the observed
328 augmentation in pathogen reductions.

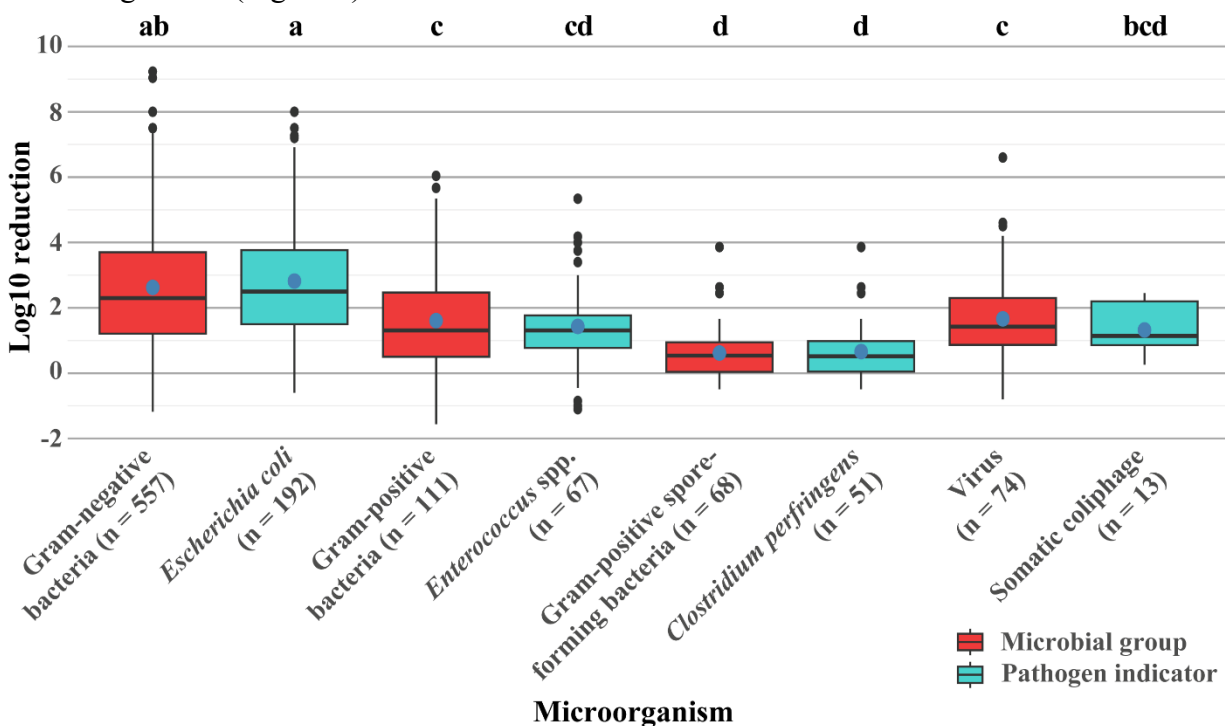
329 This finding has particularly significant research implications, as it implies that studies focusing
330 on artificially spiking of pathogens (17.3% of the total) may not represent accurately real-world
331 scenarios in terms of pathogen reduction. Thus, the obtained LR results might be biased, and
332 extrapolating the associated conclusions could lead to potentially dangerous overestimations of
333 pathogen reduction capabilities. Laboratory-scale studies potentially dosed with allochthonous
334 pathogens might be useful to study specific inactivation factors and/or certain microbial
335 processes, but the overall microbial reductions should not be extrapolated to scaled systems.
336 According to this result and to mitigate potential biases associated with the methodology
337 followed during the studies in the database, the subsequent analyses were conducted using only
338 data on the reduction of autochthonous pathogens.
339

340 3.4. Impact of the targeted microbial group on pathogen reduction

341 The first assessment of the overall pathogen reduction efficiency of AD involved a
342 comprehensive analysis of pathogen reduction across the entire database. The analysis performed
343 showed an average LR of 2.23 ± 1.81 (n = 810), confirming the well-established understanding
344 that AD can effectively reduce pathogens [14,17,18].

345 Microbial physiology, morphology, and metabolism affect the survival of microorganisms under
346 different stress conditions. Thus, it is reasonable to hypothesize that they play a pivotal role in
347 shaping the fate of microorganisms during AD. In practical scenarios, analyzing all the potential
348 pathogens present in a digestate is impossible. Hence, the selection of pathogen indicators is
349 essential for effective quality/safety assessments. The EU regulation incorporates specific
350 indicators such as *Escherichia coli* (Gram-negative bacteria), *Enterococcus* spp. (Gram-positive
351 bacteria), and *Clostridium perfringens* (Gram-positive spore-forming bacteria) to monitor key
352 microbial groups in digestates [11,24], although they are not all required in every scenario and
353 regulatory conformity pathway (see Section 3.11).

354 Accordingly, microorganisms were categorized into large microbial groups (including Gram-
 355 negative bacteria, Gram-positive bacteria, Gram-positive spore-forming bacteria, and viruses),
 356 and subsequent analyses were conducted. The previously mentioned pathogen indicators from
 357 each microbial group were also considered. Somatic coliphages were also included in the analysis
 358 since they are used as viral indicators at a European level as fecal contamination indicators in
 359 drinking water [25]. The obtained results underline that microorganism resistance during AD is
 360 intricately linked to well-known survival mechanisms and adaptive traits inherent to each group
 361 of microorganisms (Figure 2).



362 **Figure 2.** Microorganism Log₁₀ reduction for different groups (red) of microorganisms and for
 363 their respective pathogen indicators (blue). Mean values are represented by blue dots. Only the
 364 microbial groups with three or more independent values (n ≥ 3) are presented. Identical letters
 365 above boxplots indicate homogeneous groups. n stands for the number of independent datapoints.
 366

367
 368 The mean reductions in pathogen concentrations observed during AD varied across microbial
 369 groups, with the most significant reductions observed for Gram-negative bacteria (mean LR of
 370 2.63 ± 1.83). Gram-negative bacteria are characterized by a cell wall featuring a lipid-rich outer
 371 membrane and a monolayer of peptidoglycan [26]. This structural composition provides limited
 372 protection against environmental stress factors encountered during AD, such as non-optimal
 373 temperature or pH values [27]. This is in agreement with previous studies [18]. After Gram-
 374 negative bacteria, viruses and Gram-positive bacteria exhibited the second highest reduction
 375 values, with mean LR of 1.66 ± 1.40 and 1.61 ± 1.57 , respectively. Gram-positive bacteria
 376 possess a robust cell wall consisting of multi-layered peptidoglycan interwoven with long anionic
 377 polymers known as teichoic acids [26]. This complex structure gives them more protection under
 378 stress conditions, surviving at a wide range of pH and temperature values or under higher NaCl
 379 concentrations (osmotic pressures) than Gram-negative bacteria [28]. Viruses rely on protein
 380 capsids as their primary resistance mechanism. Environmental factors such as temperature,
 381 humidity, solar light incidence, or air pollutants can significantly affect the viability and

382 infectivity of viruses [29]. The created dataset primarily accounted for non-enveloped viruses, a
383 category known for its high environmental persistence [30]. This consideration explains their
384 greater resistance to AD compared with Gram-negative bacteria. Finally, Gram-positive spore-
385 forming bacteria were the most resistant to AD, with a mean LR of 0.62 ± 0.74 . This result is not
386 surprising considering that certain spore-forming bacteria, such as pathogenic *Clostridium* spp.
387 can survive and even regrow under certain AD conditions [31]. This high resistance can be
388 explained by their ability to produce intracellular spores, which are a dormant form of vegetative
389 bacteria highly resistant to physical and chemical stresses [32]. The stimulation of spore
390 germination followed by inactivation of the resulting vegetative cells could potentially enhance
391 the pathogen reduction efficiency.

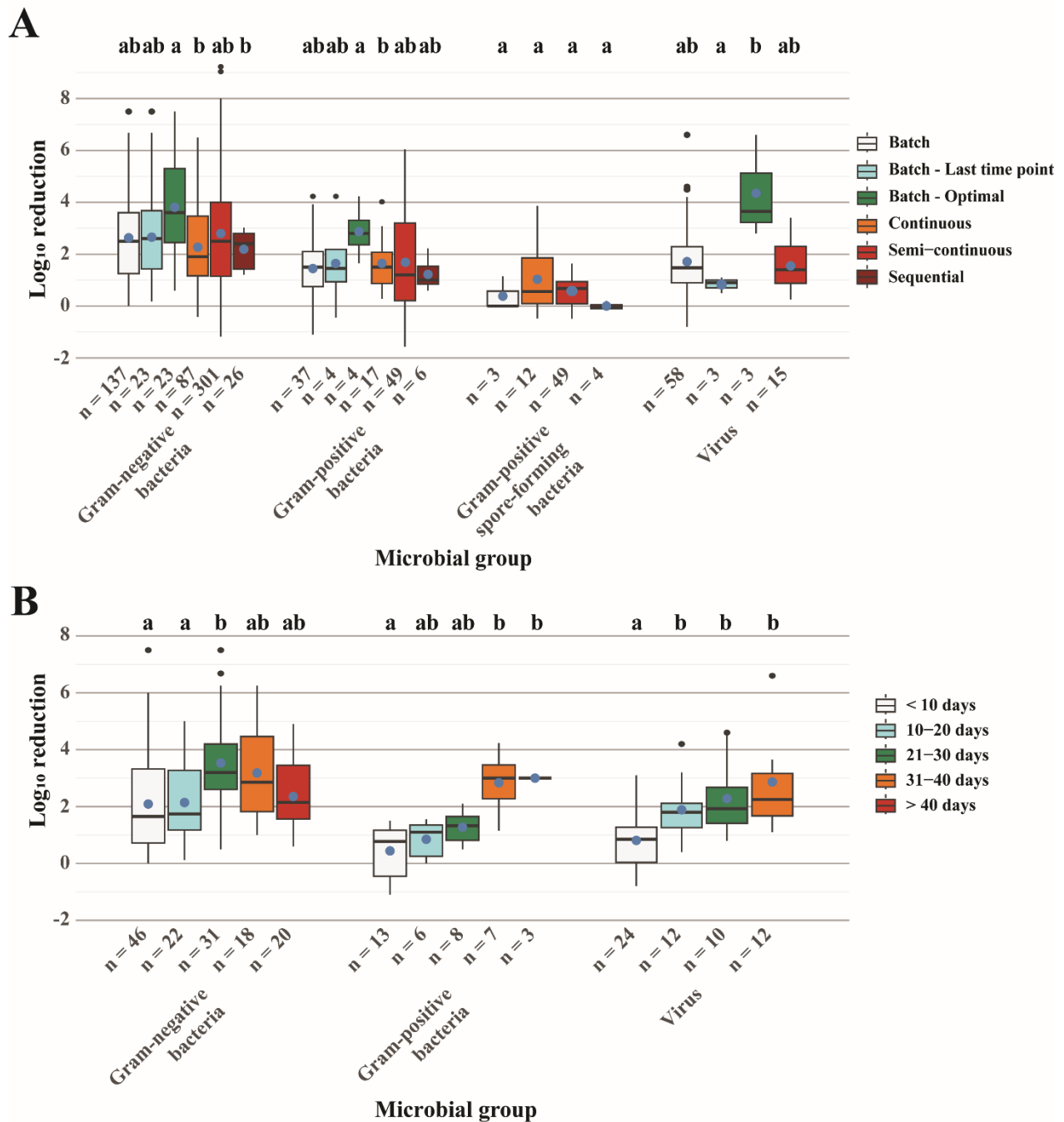
392 These results are in line with previous studies [18], where similar findings were pointed out. The
393 authors reported elevated LR values, such as 2.2-5.0 for Gram-negative bacteria and 1.8-3.0 for
394 Gram-positive bacteria (interquartile ranges). These values are higher than those presented in this
395 study (2.63 ± 1.83 and 1.61 ± 1.57 , respectively). These differences can be attributed to the
396 potential inclusion of data from studies considering the spiking of pathogens, which were
397 excluded from this analysis.

398 To confirm the representativeness of current pathogen indicators, their reductions (Figure 2, blue)
399 were compared with each corresponding group that they represent (Figure 2, red). Results
400 showed that the pathogen indicators represent accurately their respective groups (Figure 2). No
401 significant differences were found between each pair of group-indicators, confirming the validity
402 of extrapolating the removal of these indicators to each corresponding group.

403

404 3.5. Impact of the reactor type or feeding strategy on pathogen reduction

405 An analysis was performed to elucidate if the feeding modes and the type of reactors used in the
406 studies had an impact on the pathogen reduction performances. The feeding mode (categorized as
407 batch, semi-continuous, continuous and sequential) did not affect the overall LRs obtained
408 (Figure 3A).



409
 410 **Figure 3.** Microorganism Log₁₀ reduction for A) different groups of microorganisms and
 411 different feeding modes and B) each microbial group in batch reactors with different durations.
 412 Mean values are represented by blue dots. Only the conditions with three or more independent
 413 values ($n \geq 3$) are presented. Identical letters above boxplots indicate homogeneous groups. n
 414 stands for the number of independent datapoints.

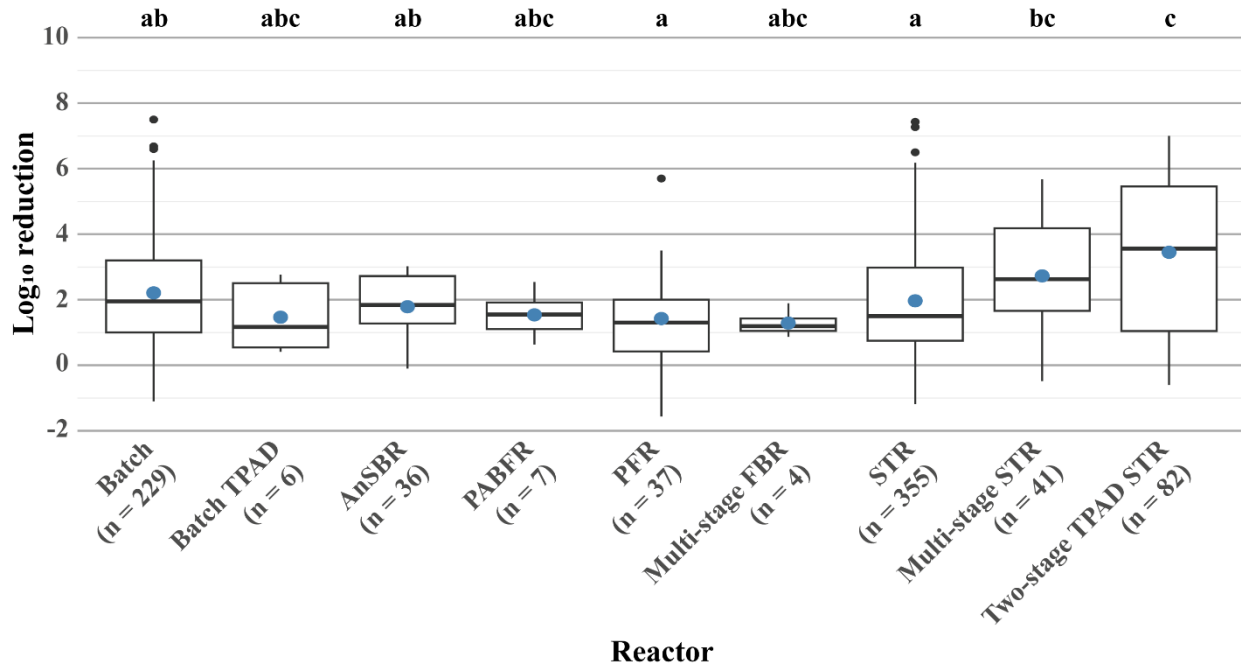
415
 416 Previous reviews have pointed out that, for some pathogens, batch reactors can lead to enhanced
 417 pathogen reduction [17,18]. This enhancement is generally attributed to transient VFA peaks
 418 during the batch tests [18]. Another possibility is that, while batch configurations ensure that all

419 pathogens stay in the reactor for the whole duration of the AD process, the HRT in
420 (semi)continuous system represents an average, which implies that some microorganisms might
421 leave the reactor due to short circuits, thus affecting their reduction. The overall data do not show
422 an enhanced performance for batch reactors, probably because of a main factor determining the
423 LRs in batch tests: the batch duration. As shown in Figure 3B, the batch duration impacts
424 considerably the pathogen reduction performance. Therefore, the sampling time for measuring
425 the pathogen concentration affects the resulting LR. Most previous studies consider the last point
426 to evaluate the LR in batch tests [18]. As shown in Figure 3, this is not necessarily the optimal
427 value. The overall LR in batch reactors (considering all the points over time) and the LR
428 considering only the last point are not significantly different. However, if the LR is calculated
429 considering the lowest pathogen concentrations (resulting in the higher LR; optimal point in
430 Figure 3A), batch mode reactors outperform other reactors. This agrees with the hypothesis
431 suggesting that transient VFA peaks enhance pathogen reduction, implying that once these VFA
432 are consumed, pathogens can regrow, reducing the overall LR [18]. This phenomenon can be
433 observed in Figure 3B for Gram-negative bacteria (the most vulnerable group to non-ionized
434 VFAs [18]). Optimal LRs were achieved at batch durations of 21-30 days, with decreasing values
435 at higher and lower durations. As vulnerable but fast-growing microorganisms, Gram-negative
436 bacteria first experience a reduction, followed by growth afterwards, once the VFAs have been
437 consumed. Gram-positive bacteria and viruses did not show this behavior, as they are more
438 resistant to high VFA concentrations and usually grow slower than Gram-negative bacteria. Some
439 of these results should be interpreted with caution due to the low number of data points available,
440 particularly concerning Gram-positive bacteria and viruses.

441 While batch mode reactors seem to offer a notable advantage in reducing pathogens compared to
442 semi-continuous systems, it is crucial to remember that the primary goal during AD is the
443 production of methane and the generation of a stabilized digestate. Because of this, most studies
444 take the last point in batch tests (usually a few days after the maximum methane yield has been
445 achieved, given by a gas “plateau”) for pathogen reduction calculation, which would not be equal
446 to the optimal LR value. This implies that reactors would not be stopped at the point of highest
447 pathogen reduction, but once the VFA would have been consumed (i.e., at the final point in
448 Figure 3). Thus, assuming that the transient VFA peaks are responsible for the improved batch
449 performance, the LRs obtained in (semi)continuous systems (operated at low VFA values) would
450 be similar to those from batch reactors. These are the overall LRs that are presented.

451 Novel fermentative biorefinery concepts aiming to generate other high value-added products such
452 as VFAs might indeed benefit from this improved pathogen reduction performance. In such
453 scenarios, (semi)continuous systems would also work at high VFA concentrations, meaning that
454 batch mode reactors would not necessarily be beneficial either. Research is needed to confirm the
455 latter. Kinetic studies should also be done following both methane production rates, cumulative
456 methane productivities, and pathogen reductions to confirm that VFAs are indeed responsible for
457 the enhanced performances in pathogen reduction and to elucidate if optimal conditions
458 considering both pathogen abatement and methane yields can be found.

459 Moving on to the reactor types, most of the reactors used did not show significant differences in
460 the obtained LRs (Figure 4).



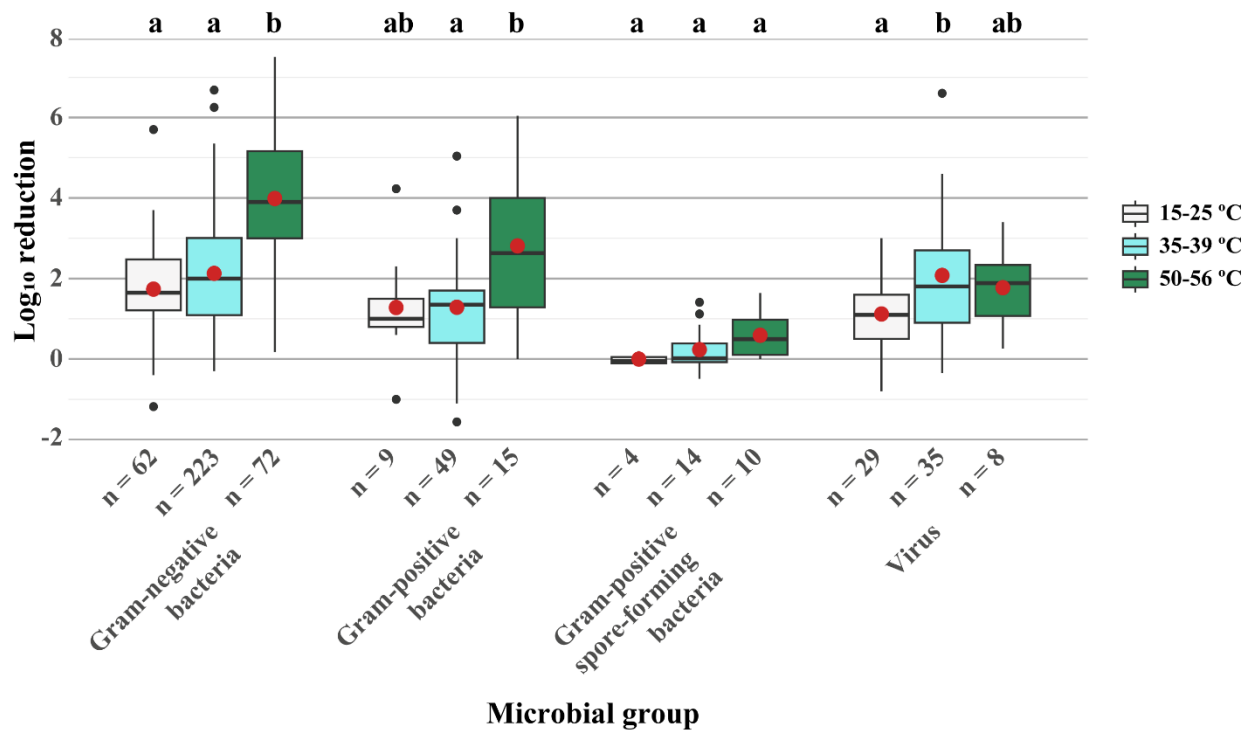
461
 462 **Figure 4.** Overall microorganism Log₁₀ reduction for different reactor types. Mean values are
 463 represented by blue dots. Only the reactors with three or more independent values (n ≥ 3) are
 464 presented. Identical letters above boxplots indicate homogeneous groups. TPAD stands for
 465 temperature phased anaerobic digestion, AnSBR for anaerobic sequencing batch reactor, PABFR
 466 for panelled anaerobic baffle-cum-filter reactor, PFR for plug flow reactor, FBR for fixed bed
 467 reactor, and STR for stirred tank reactor. n stands for the number of independent datapoints.

468
 469 Only multi-stage stirred tank reactors (STRs) and two-stage temperature phased AD (TPAD)
 470 STRs showed enhanced performances. As it will be further detailed in sections 3.6 and 3.7, this
 471 may be a consequence of the low pH values in the first stage of multi-stage STRs and of high
 472 temperatures in the first stage of two-stage TPAD STRs, which is always thermophilic (see
 473 Figure S4 for the separate LRs at different stages) [1,15]. As is further discussed below, both low
 474 pH values and thermophilic temperatures result in higher LR values.

475 3.6. Impact of temperature on pathogen reduction

476
 477 Temperature plays a crucial role in the inactivation of pathogens, guiding a complex and
 478 multifaceted process. The inactivation of pathogens induced by temperature entails the alteration
 479 of multiple cellular structures, including the outer and inner membrane, the peptidoglycan cell
 480 wall, the nucleoid, RNA, ribosomes, and diverse enzymes. Consequently, deciphering the
 481 specific mechanism leading to cell death poses a complex challenge [33].

482 The influence of temperature on pathogen reduction during AD has been widely studied. To
 483 confirm previous findings and to assess general trends, the database was categorized according to
 484 the three primary temperature ranges associated with AD: psychrophilic (15-25 °C), mesophilic
 485 (35-39 °C) and thermophilic (50-56 °C). Subsequently, a comprehensive analysis was conducted
 486 to assess the extent of pathogen reduction within each microbial group across these temperature
 487 ranges. Figure 5 illustrates the LR of reactors operated under psychrophilic, mesophilic, and
 488 thermophilic conditions.



489
490
491
492
493

Figure 5. Microorganism Log_{10} reduction for different groups of microorganisms and for different temperature ranges. Mean values are represented by red dots. Identical letters above boxplots indicate homogeneous groups. n stands for the number of independent datapoints.

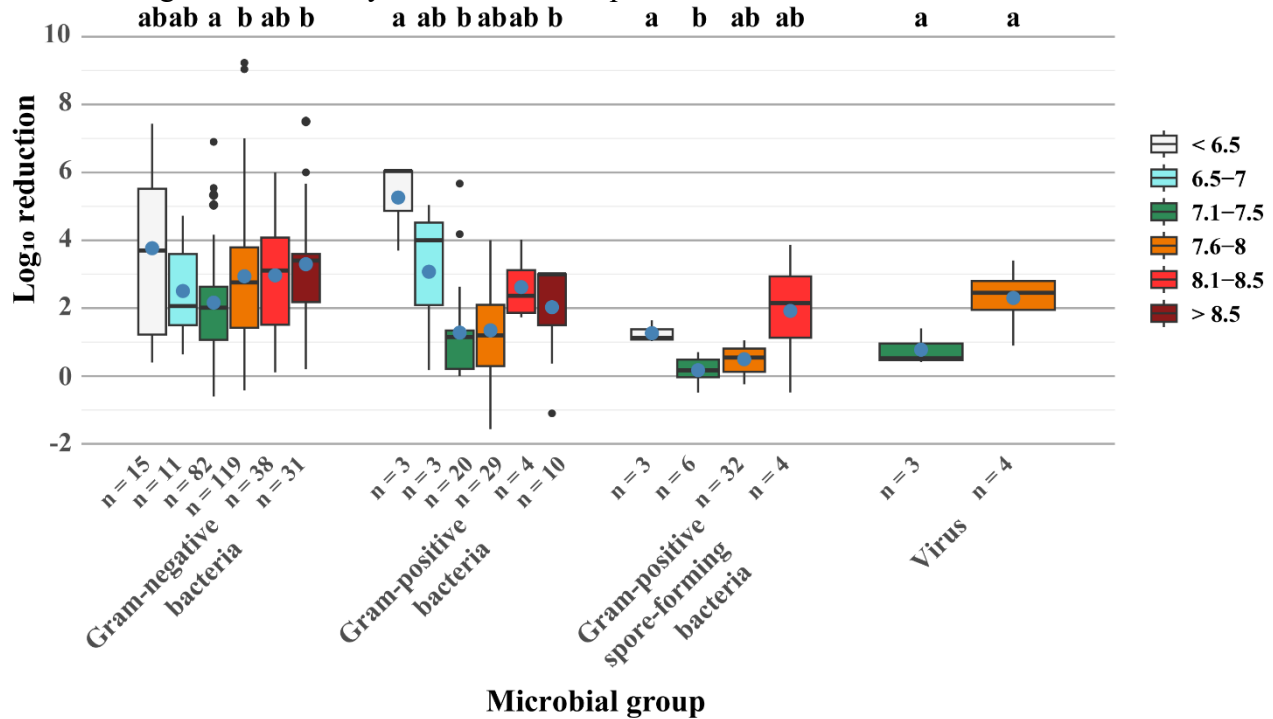
494 Thermophilic temperatures resulted in significantly higher LRs compared with psychrophilic and
495 mesophilic conditions for most groups. The analysis also revealed variations in the reduction of
496 pathogen concentrations among microbial groups across the different temperature ranges. The
497 most significant effect was observed for Gram-negative bacteria, showing a 2.25-fold higher LR
498 in thermophilic conditions compared to psychrophilic temperatures. Gram-negative
499 microorganisms were followed by Gram-positive bacteria (1.53-fold difference), viruses (0.65-
500 fold), and Gram-positive spore-forming bacteria (0.59-fold). These results are consistent with
501 previous research, confirming that thermophilic AD represents the most effective temperature
502 choice for pathogen removal [17,18].

503 These results agree with previous statements, further highlighting the impact of the targeted
504 microbial group on pathogen reduction performance. The general assumption that Gram-positive
505 bacteria exhibit higher resistance to heat compared to Gram-negative bacteria [34] is clearly
506 confirmed. Gram-positive spore-forming microorganisms were the least affected by temperature
507 variations, as spores can resist higher temperatures than vegetative cells. At lower temperatures,
508 a decreased LR or even complete persistence of pathogens such as *C. perfringens*, *C. botulinum* or
509 *C. difficile* was observed. A previous study even documented bacterial growth during AD at 27
510 °C, resulting in an increased concentration of *C. perfringens* and a lower proportion of spores in
511 the digestate compared to the initial substrate, suggesting germination [35].

512 When comparing psychrophilic and mesophilic conditions, it can be observed that the LRs were
513 only higher for mesophilic conditions for viruses. For any other microbial group, the resulting
514 LRs were similar. This implies that pathogen removal is not worsened under psychrophilic
515 conditions, as mesophilic temperatures do not appear to be sufficient to provide an enhanced LR.
516

517 3.7. Impact of working pH on pathogen reduction
 518 The pH is a well-known parameter affecting microbial growth. For example, pH variations affect
 519 the ionization of amino-acid functional groups, resulting in protein denaturation and activity
 520 decrease. Extremely acidic or basic pH can also cause DNA breakup and lipid hydrolysis,
 521 respectively. The pH also affects several biological processes, such as the proton motive force
 522 and many other reactions involving the turnover of protons. In AD systems, studying the impact
 523 of pH is extremely complex. Not only the pH affects the aforementioned process, but also the
 524 speciation of the most common inhibitors in digesters: VFAs and free ammonia (NH₃) [1]. These
 525 interactions go both ways, as pH affects the microbial activity, but metabolic processes also
 526 modify the pH. Both VFAs and NH₃ are microbial products that affect (and sometimes
 527 determine) the pH in digesters. Due to the difficulties of separating the pathogen reduction effects
 528 related to the pH itself from those of VFA or NH₃ (and due to the general lack of data), only the
 529 overall impact of the reported pH values in the media is discussed here. Discussions around the
 530 findings from individual articles on pathogen reduction related to VFA and/or NH₃ can be found
 531 elsewhere [14,15,18].

532 Optimal pH values for most microorganisms correspond to neutral values (i.e., around 7). As
 533 shown in Figure 6, AD ecosystems are no exception.



534 **Microbial group**
 535 **Figure 6.** Microorganism Log₁₀ reduction for different groups of microorganisms and for
 536 different pH ranges. Mean values are represented by blue dots. Only conditions with three or
 537 more independent values (n ≥ 3) are presented. Identical letters above boxplots indicate
 538 homogeneous groups. n stands for the number of independent datapoints.

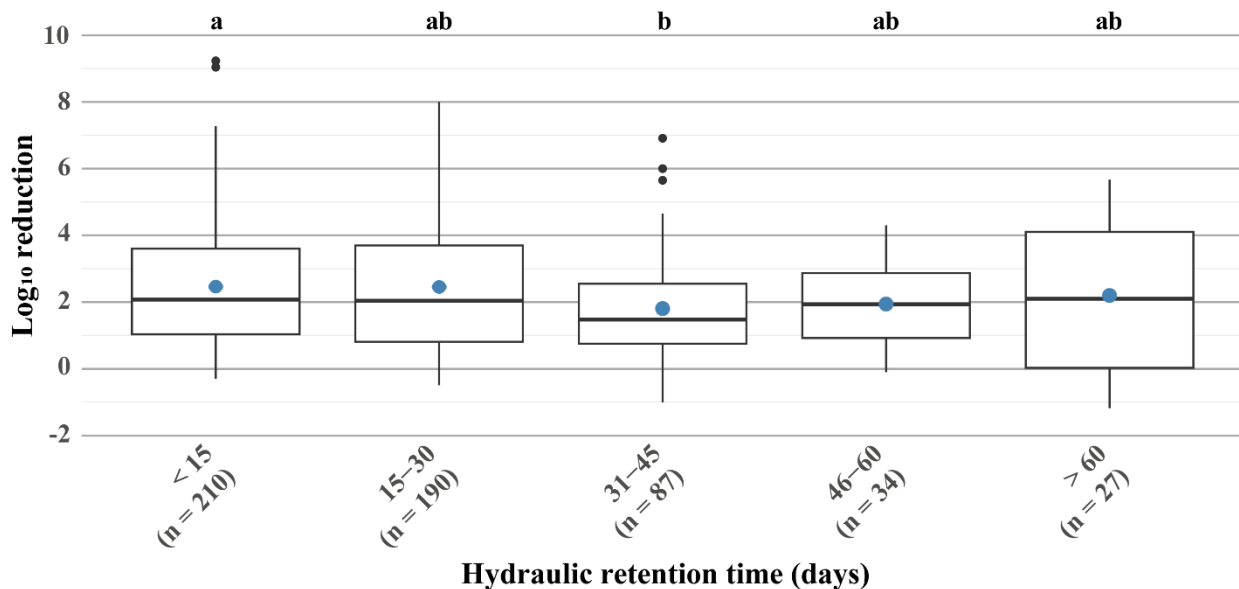
540 For all bacterial groups, the lowest LRs were reported at neutral pH ranges (7.1-8.0). Other than
 541 the neutrophilic nature of the microorganisms, pH values close to 7 result in low concentrations
 542 of both non-ionized VFAs (the toxic form) and NH₃, thus reducing their toxicity. pH ranges
 543 above or below neutrality resulted in enhanced pathogen reduction performances. Both Gram-
 544 negative and Gram-positive bacteria follow a similar trend, with increased reductions at pH

545 values below 7.0 and above 8.0. The high LR_s for Gram-positive at low pH values are
 546 particularly noteworthy, but the low number of data points also must be considered when
 547 extrapolating this observation. As for the temperature, the most resistant bacterial group to non-
 548 optimal pH ranges are Gram-positive spore-forming bacteria, for the same reasons stated above.
 549 Some pathogenic spore-forming Gram-positive bacteria are fermenters (e.g., *Clostridium*
 550 *perfringens*), who are acid resistant and survive at low pH values. This is illustrated in Figure 6,
 551 where this group of microorganisms shows the least noticeable impact of the pH on the LR_s,
 552 particularly at low values. The little amount of data for viruses jeopardizes the unbiased analysis
 553 of the obtained results.

554 Variable and/or non-reported VFA/NH₃ concentrations in pathogen reduction studies preclude
 555 the identification of the precise phenomena responsible for the increased LR_s. The overall trend
 556 of pathogen reduction data follows a similar trend as the one shown in Figure 6, with neutral pH
 557 ranges (i.e., 6.5-8.0) providing the lowest LR_s (Figure S5).
 558

3.8. Impact of hydraulic retention time and organic loading rate on pathogen reduction

560 The effect of the HRT on the pathogen reduction performance of (semi)continuous AD is
 561 controversial. While some studies claim that the HRT plays a main role (see [17] for individual
 562 examples for different pathogens), others have not observed any effect [18]. Putting all the
 563 available data together (Figure 7), it is clear from the created dataset that the HRT by itself does
 564 not impact the overall obtained LR_s.



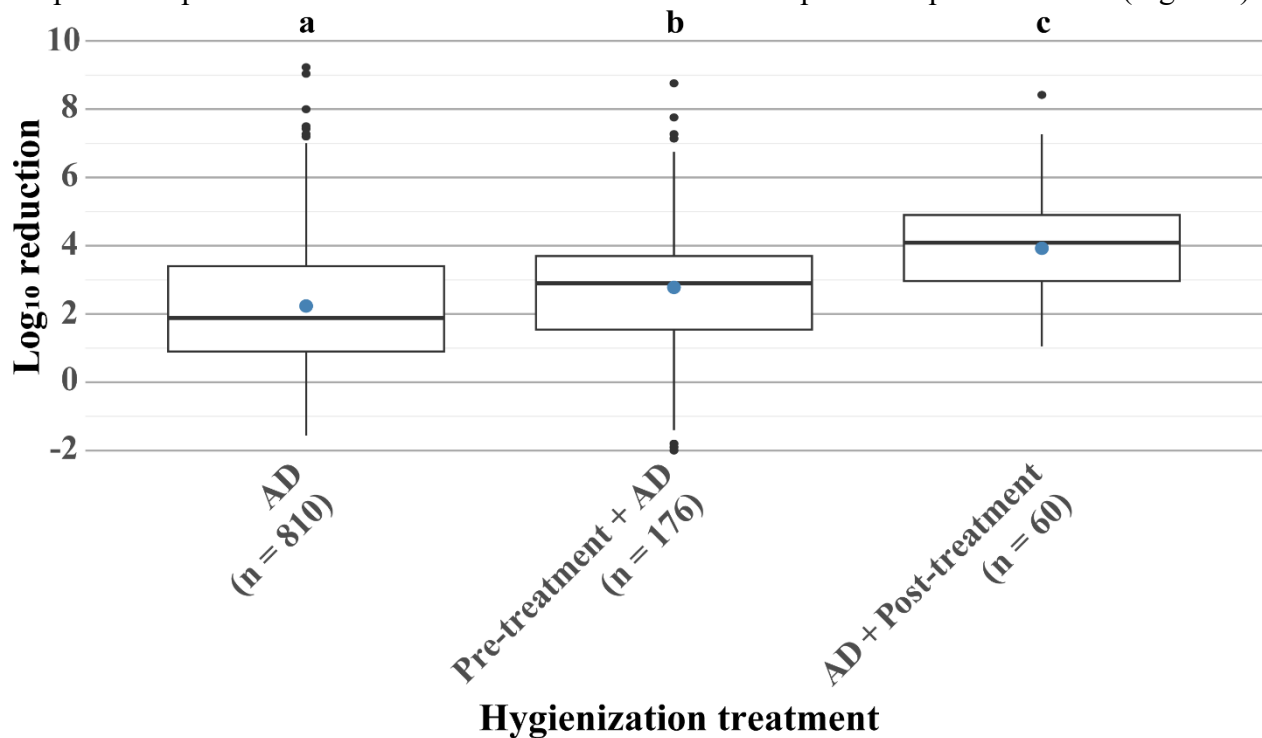
565 **Figure 7.** Overall microorganism Log₁₀ reduction for different hydraulic retention time (HRT)
 566 ranges. Mean values are represented by blue dots. Identical letters above boxplots indicate
 567 homogeneous groups. n stands for the number of independent datapoints.
 568

569 It is particularly noteworthy that, in agreement with the lower reduction of Gram-negative
 570 bacteria at long batch test durations, long HRTs did not result in enhanced LR_s. This is because,
 571 as long as the HRT is large enough to allow a stable and effective AD without considerable VFA
 572 accumulation, longer HRTs will not result in a higher pathogen reduction. For the same reasons
 573 as for the HRT, the applied OLR did not have a significant impact on the resulting LR_s (Figure
 574 S6), confirming the negligible effect of these two parameters. In agreement with the previous
 575

576 statements, the lowest OLR range assessed (≤ 2 g VS/L/d) did not result in enhanced pathogen
 577 reductions. In fact, the lowest average LR was obtained for this range, suggesting that low loads
 578 (or long retention times) do not enhance pathogen reduction.
 579 Although this conclusion goes against some experimental articles [36,37], this overall assessment
 580 agrees with what has been observed in a previous meta-analysis [18], validating it and suggesting
 581 that it is not a result of sampling biases. The main inactivation mechanisms appear to be related
 582 to other factors, such as the working temperature or pH. The inactivation times associated with
 583 the effect of these parameters are much shorter than common AD retention times (e.g., in the
 584 ranges of minutes-hours), meaning that the extra time provided does not result in any tangible
 585 benefit.

3.9. Pre- and post-treatments for enhancing pathogen reduction

588 Several methods for pre- and post-treatment (e.g., alkaline, heat-based, microwave, ultrasonic,
 589 ozonation, filtration, or irradiation) have been assessed for digestate pathogen reduction [17].
 590 This section presents a systematic comparison between the different approaches that exist,
 591 considering the LR as a single performance indicator. Coupling pre- or post-treatment with AD
 592 results in enhanced pathogen reduction performances with a 1.24-fold increase in LR when
 593 coupled with pre-treatment and a 1.76-fold increase when coupled with post-treatment (Figure 8).



594 **Figure 8.** Overall microorganism Log_{10} reduction during AD, either alone or coupled with pre- or
 595 post-treatment processes. Mean values are represented by the blue dots. Identical letters above
 596 boxplots indicate homogeneous groups. n stands for the number of independent datapoints and
 597 AD for anaerobic digestion.

600 Interestingly, post-treatment led to significantly higher LR values than pre-treatment. In
 601 agreement with the findings above, this might be due to the re-growth of pathogens during AD,
 602 which is obviously avoided when applying post-treatments. This hypothesis is further supported

603 by similar LRs for pre- and post-treatments individually, without considering the AD step (Figure
604 S7).

605 A more in-depth examination of the LRs for the different pre- and post-treatments coupled to AD
606 was conducted, focusing on specific treatment parameters. Pre-treatment conditions exhibited
607 considerable diversity across studies. For instance, alkali treatment involved pH levels ranging
608 from 10 to 12. Heat treatment spanned temperatures between 60 and 160 °C, with durations
609 varying from five minutes to one hour. Pasteurization conditions (70 °C for one hour) tended to
610 be prevalent in this type of pre-treatment. Ultrasound and microwave energy used during
611 treatment also showed variability, ranging from 2.4 to 27 kJ/g total solids (TS). Despite these
612 diverse conditions, no significant differences were observed between the performances of most of
613 the pre-treatment processes studied (i.e., alkali, heat, microwave, ozonation, ultrasound, and
614 ultrasound combined with heat) (Figure S8). Only results from ozonation (from two studies from
615 the same group) resulted in higher LRs. These findings must be approached with caution due to
616 the limited data for certain treatments, with only a single study in some cases, jeopardizing the
617 extrapolation of unbiased outcomes.

618 Considering the similar performances, the choice of technology may be guided by other factors,
619 such as economic considerations (e.g., reduced costs due to energy requirements) and/or
620 biological aspects (e.g., enhanced substrate biodegradability after pre-treatment). Thermal pre-
621 treatments emerge as a promising option, showcasing the potential for positive energy balances
622 through increased biogas production with on-site heat generation from biogas combustion. They
623 offer the additional advantage of scalability, having been successfully implemented at full-scale
624 for treating sewage sludge, municipal solid wastes, and animal by-products (ABPs) [38].

625 However, careful consideration must be given to the fate of spore-forming microorganisms,
626 which may be favored by these treatments.

627 Regarding post-treatments, this analysis focused on heat-related processes. Treatment conditions
628 varied across studies, with temperatures ranging from 60 to 80 °C and durations spanning from
629 two minutes to 96 hours. Once again, pasteurization conditions were prevalent. Pasteurization
630 was indeed the main driver for the overall increase in LR values depicted in Figure 8.

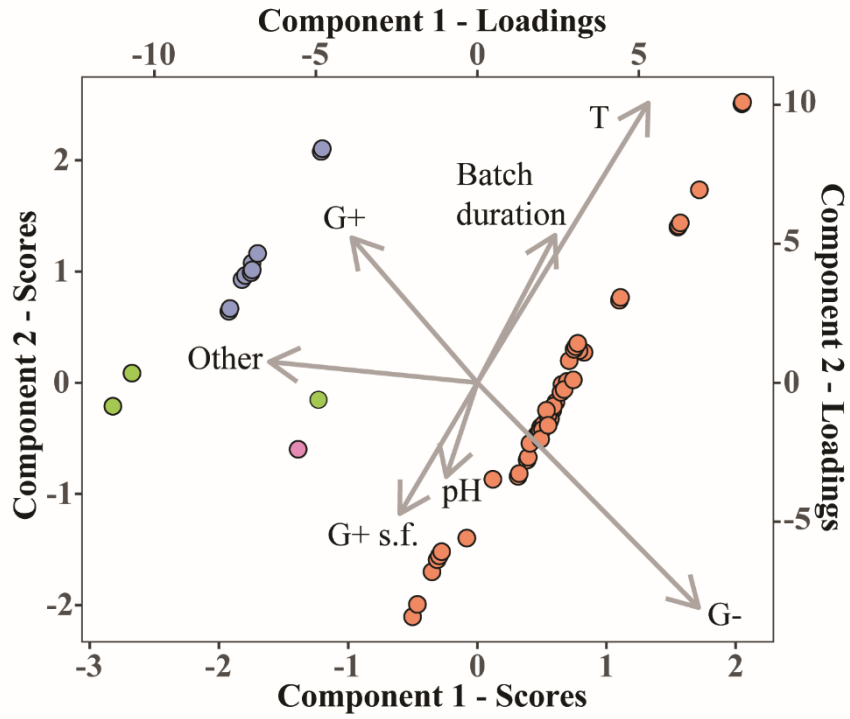
631 Specifically, when focusing on heat-related treatments, which constitute the majority of the
632 collected data points, the benefits of post-treatment coupled with AD (mean LR 3.92 ± 1.43)
633 compared with pre-treatment (mean LR 2.78 ± 2.05) become evident. Thus, pasteurization of the
634 digestate is preferable to pasteurization of the input substrates (considering pathogen reduction as
635 the sole criterion). The energy requirements of the latter are obviously lower.

636

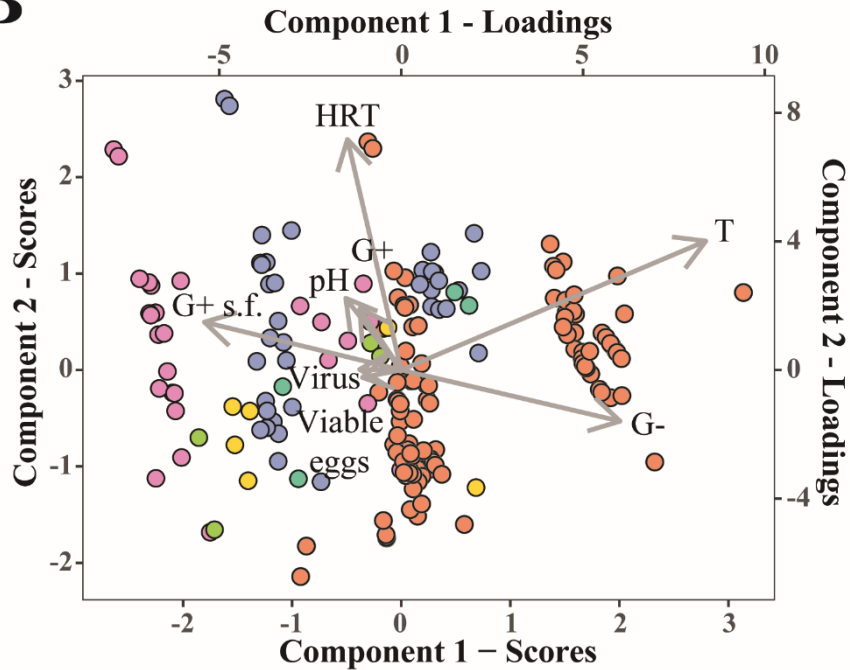
637 3.10. Overall assessment of process parameters on the pathogen reduction performance

638 To perform a quantitative analysis of the data and to confirm the overall trends discussed above,
639 PLS analyses were performed using the LR as the output variable and the microorganism
640 classification, temperature, pH, and either the HRT (for (semi)continuous reactors) or the batch
641 duration (for batch reactors) as input variables. The goal here was not to develop a predictive
642 model (reason why there is no validation dataset), but to evaluate jointly which parameters were
643 the most relevant for pathogen removal.

644 The corresponding score plots support the previous findings (Figure 9). The classification of
645 microorganisms played a major role in defining the obtained LRs. This is clearly seen for batch
646 reactors (Figure 9A), where the samples for Gram-negative bacteria, Gram-positive bacteria, and
647 “other microorganisms” are grouped separately in the plot. Gram-negative were directly
648 proportional to the LR, while Gram-positive, particularly spore-forming bacteria, impacted the
649 LR negatively due to their higher resistance during AD (see PLS coefficients in Table S3).

A

Microorganism classification ● G- ● G+ ● G+ s.f. ● Other

B

Microorganism classification ● Eggs ● G+ ● Viable eggs
● G- ● G+ s.f. ● Virus

651 **Figure 9.** PLS score plots for (A) batch reactors and (B) (semi)continuous reactors. LR values
652 were used as predicted variable and temperature (T), pH, batch duration, hydraulic retention time
653 (HRT), and the microorganism classification (e.g., Gram-negative bacteria (G-), Gram-positive
654 bacteria (G+), Gram-positive spore-forming bacteria (G+ s.f.), virus, eggs, viable eggs, or others)
655 as input variables. The two first components explained 39% (A) and 33% (B) of the total
656 variance. PLS stands for partial least squares and LR for log reduction.

657
658 The same can be observed in the results for (semi)continuous reactors, although two separate sub-
659 groups can be found for the aforementioned microbial groups (vertical dot groups, parallel to the
660 y-axis). This was due to the temperature parameter, which, as mentioned above, affected the most
661 the pathogen reduction performance. These sub-groups for (semi)continuous reactors (Figure 9B)
662 correspond to psychrophilic-mesophilic (vertical group positioned to the left) and thermophilic
663 systems (vertical group positioned to the right), clearly denoting that thermophilic systems have a
664 totally different behavior, affecting positively the obtained LRs (Table S3). These two groups can
665 be clearly found for Gram-negative bacteria, Gram-positive bacteria, and Gram-positive spore-
666 forming bacteria, confirming the similar observation regardless of the microbial group. The
667 different positions of these microbial groups are related to their resistance to pathogen reduction
668 (more resistant to the left, less resistant to the right; in agreement with the statement from Section
669 3.4). The temperature PLS coefficients were always the largest (Table S3), implying that this
670 parameter had the highest impact on the LR (using the two first components, comprising 72% of
671 the total variance). The parallel distribution of points for batch reactors with the temperature
672 vector underlines the crucial importance of this parameter.

673 Continuing with the batch duration, although it affected the LR less than the temperature, it
674 clearly impacted the resulting LR. As mentioned in Section 3.5, optimal LR values are obtained
675 at intermediate batch durations, when the pathogen reduction has been done but before the re-
676 growth of Gram-negative bacteria has occurred. The parallelism of the temperature and the batch
677 duration vectors in Figure 9A is a construct of the database. Apparently, tests at higher
678 temperatures lasted longer. The reason for this remains unknown, as there is no particular reason
679 to run thermophilic tests for a longer period of time. This phenomenon exacerbated the parallel
680 distribution of points around the vectors of these two parameters, which were the most relevant
681 for batch reactors.

682 Regarding the HRT in (semi)continuous reactors, this parameter impacted the predicted LR
683 values. This might appear in contradiction with the negligible effect described in Section 3.8, but
684 when looking at the data distribution along the HRT vector and at the HRT scores in the first two
685 components (Table S3), this finding can be explained. For component 1 (explaining 22% of the
686 variance; Table S4), the coefficient of the HRT was negative, while for component 2 (explaining
687 11%), the coefficient was positive (and higher in absolute value than for component 1).

688 Therefore, the overall trend (Figure 7) resulted in a negligible impact of the HRT, as in some
689 cases longer HRTs resulted in higher LRs and in others the opposite occurred. This dichotomy
690 agrees with the literature, where both conclusions have been proposed [17,18].

691 The pH was found to affect the resulting LRs negatively, which is in agreement with the positive
692 effect of acid pH values on the pathogen reduction performance. In any case, the overall impact
693 of the pH on the LR was much lower than that of the microorganism type or the temperature.

694 The outcomes from these analyses confirm the statements made in previous sections, giving also
695 numerical outputs (e.g., PLS coefficients) that can be used to compare quantitatively the relative
696 importance of each of the tested parameters on the pathogen reduction capacity of AD.

697

698 3.11. Anaerobic digestion for reducing the level of pathogens below regulation limits
699 To assess compliance with regulatory requirements, the created database was compared against
700 two relevant pathogen-related regulations in the field of organic waste AD (used for
701 benchmarking): the United States Environmental Protection Agency (US EPA) Class A biosolids
702 regulation (EPA/600/R-22/194) [13] and the EU ABP regulation (CE 142/2011) [11]. This
703 analysis is purely comparative, as the feedstocks, treatment lines, and analytical methods
704 employed in the studies from the database did not necessarily follow the regulation guidelines for
705 waste digestion, digestate sampling, or pathogen quantification.
706 Table 1 presents the limits from the legislations used for the benchmarking exercise. The
707 regulation CE 142/2011 is applied only to ABP material as defined by the regulation CE
708 1069/2009, and offers two options for complying: (i) dedicated protocols are followed and *E.*
709 *coli*, *Salmonella* sp., and *Enterococcaceae* are below given limits; or (ii) if other standard
710 protocols are followed (standard processing method 7 in CE 142/2011), *Enterobacteriaceae* and
711 *C. perfringens* are also below limits. The US EPA Class A biosolids regulation claims explicitly
712 that “the implicit goal of the Class A pathogen requirements is to reduce all the pathogens present
713 in sewage sludge [...] to below detectable levels”. Class A biosolids are post-treated to reach
714 these criteria, thus allowing for “unrestricted use”. The European criteria are less restrictive than
715 those from the US EPA because they do not imply unrestrictive use of the material. Several other
716 EU and regional/national regulations add further innocuity criteria depending on the digestate use
717 and status.

718
719 **Table 1.** Summary of the limits given in the regulations used for benchmarking.

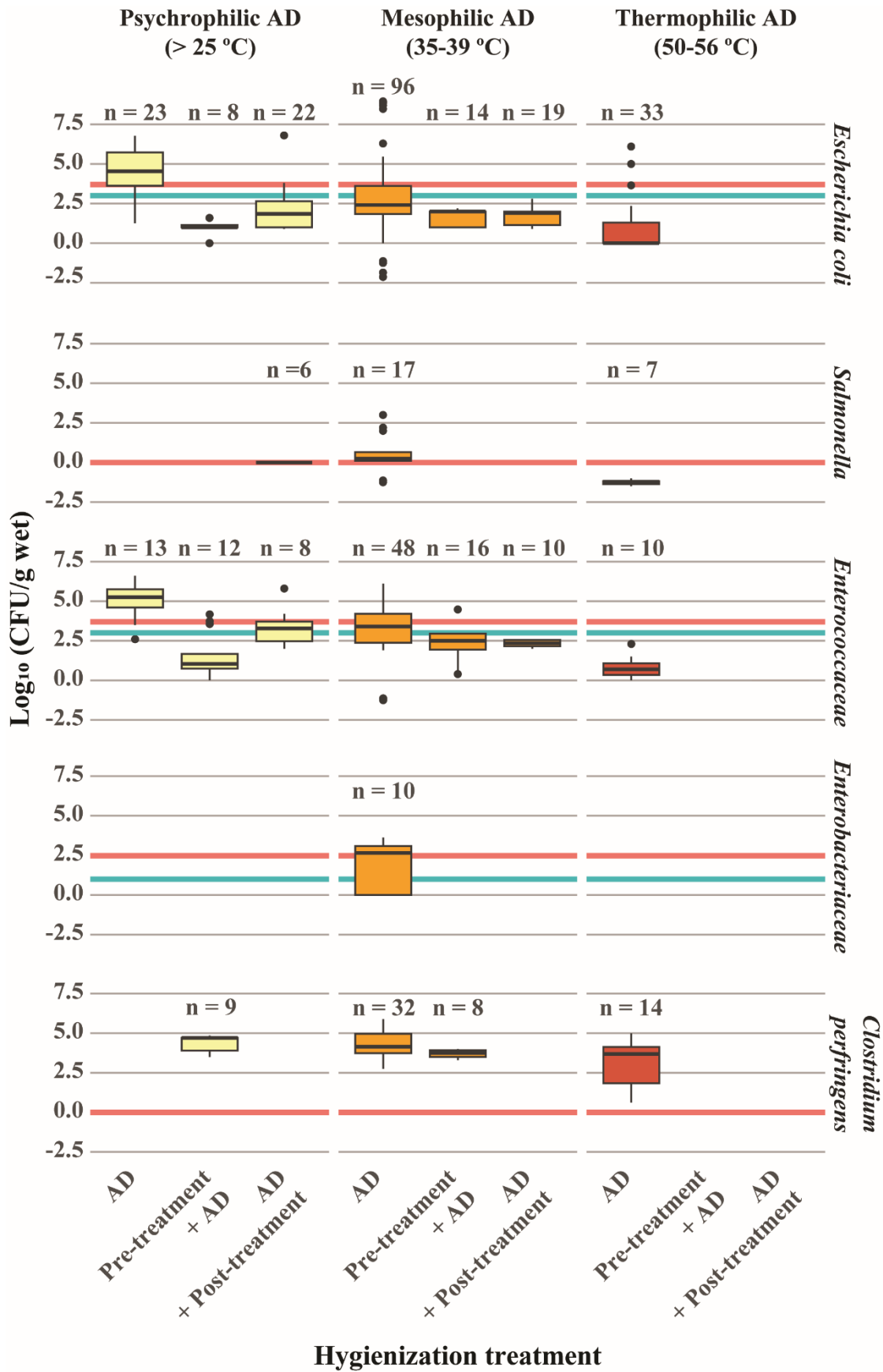
Indicator	Regulation	Implications	Limit ^a	Included pathogens retrieved in the database
<i>Escherichia coli</i>	CE 142/2011	Requirement for any digestion residue produced from authorized ABP material	Lower limit: ≤1,000 CFU in 1 g Upper limit: <5,000 CFU in 1 g	<i>Escherichia coli</i>
<i>Salmonella</i>	CE 142/2011	Requirement for any digestion residue produced from authorized ABP material	= 0 CFU in 25 g	<i>Salmonella</i> spp., <i>Salmonella typhimurium</i> , <i>Salmonella typhi</i>
<i>Enterococcaceae</i>	CE 142/2011	Requirement for any digestion residue produced from authorized ABP material	Lower limit: ≤1,000 CFU in 1 g Upper limit: <5,000 CFU in 1 g	<i>Enterococcus</i> spp.
<i>Enterobacteriaceae</i>	CE 142/2011	Further requirement when other standard procedures are followed (standard processing method 7).	Lower limit: ≤10 CFU in 1 g Upper limit: <300 CFU in 1 g	<i>Enterobacteriaceae</i>
<i>Clostridium perfringens</i>	CE 142/2011	Further requirement when other standard procedures are followed (standard processing method 7).	= 0 CFU in 1 g	<i>Clostridium perfringens</i>
Fecal coliforms	EPA/600/R-22/194	Requirement for Class A biosolids (sewage sludge). Unrestricted use of digestate.	<1000 MPN in g TS	Fecal coliforms
<i>Salmonella</i> sp.	EPA/600/R-22/194	Requirement for Class A biosolids (sewage sludge). Unrestricted use of digestate.	<3 MPN in 4 g TS	<i>Salmonella</i> spp., <i>Salmonella typhimurium</i> , <i>Salmonella typhi</i>

720 ^a The CE142/2011 regulation establishes the number of replicates to be analyzed (usually 5) and two microbial limits. The lower
721 limit represents the threshold value for the number of bacteria. The result is considered satisfactory if the number of bacteria in all
722 replicates does not exceed this limit. In addition, the regulation also establishes the number of replicates that can be between the
723 lower and the upper limit (maximum value for the number of bacteria). The result can also be considered satisfactory if none of
724 the replicates exceed the upper limit, even if a given number of replicates are between the lower and upper limits.

725 * MPN stands for most probable number, CFU for colony forming unit, ABP for animal by-product, and TS for total solids.

726

727 In Figure 10 (CE 142/2011 benchmarking), the general mandatory requirements in the EU
728 regulation for ABP-derived digestates (i.e., *E. coli*, *Salmonella*, and *Enterococcaceae*) are
729 compared with the gathered database (for any feedstock and reactor type).

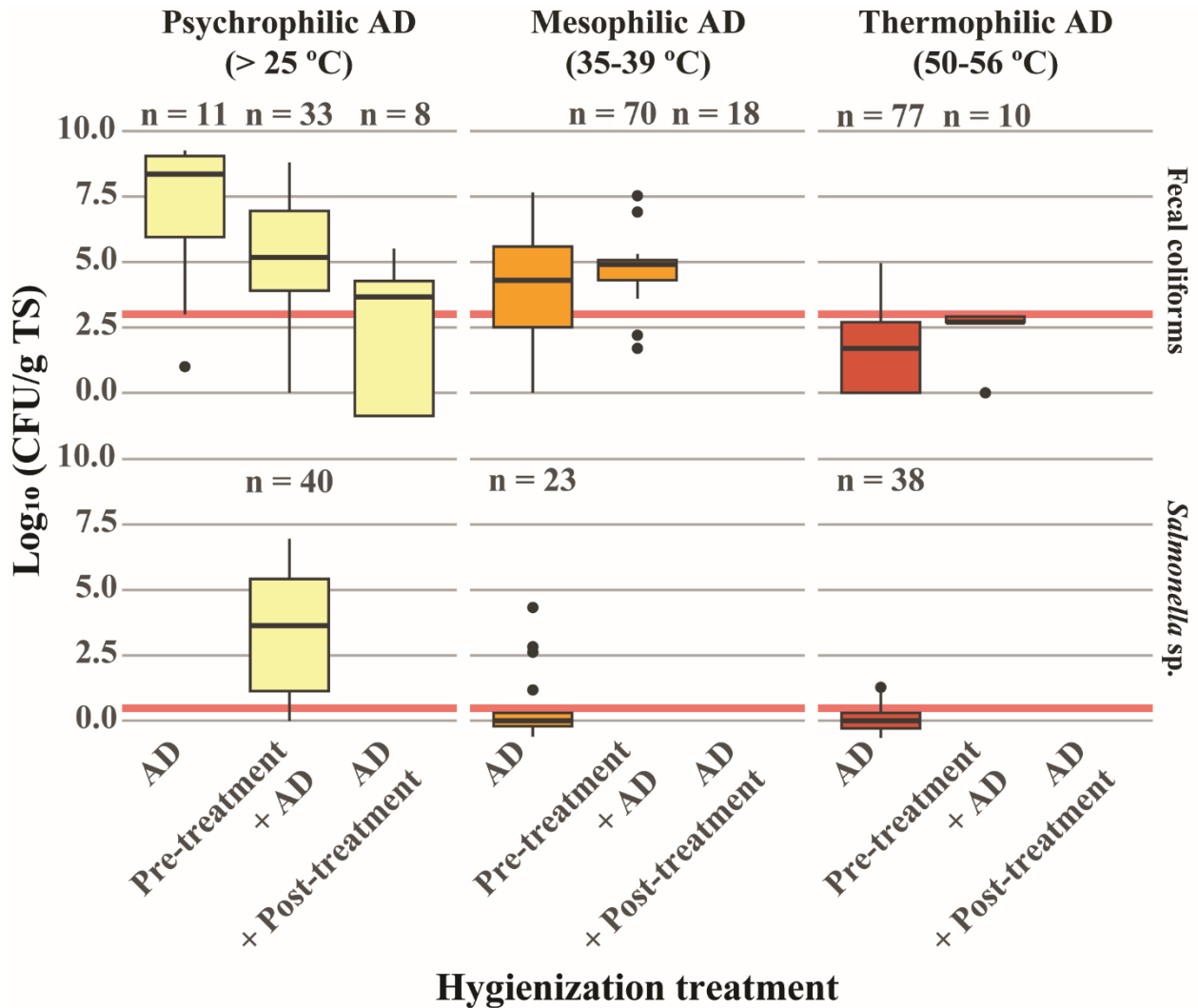


731 **Figure 10.** Database comparison against the EU ABP regulatory limits (CE142/2011). The
732 concentration in the digestate of each pathogen indicator is shown for different AD temperatures
733 and considering additional treatments (i.e., pre- or post- treatment). The red line represents the
734 upper limit and the blue line the lower limit when applicable. Limits as absence (zero CFUs/g
735 wet) were adopted as below 1 for graphical purposes. Only conditions with three or more
736 independent values ($n \geq 3$) are presented. *Escherichia coli*, *Salmonella* sp., and *Enterococcaceae*
737 are mandatory for ABP digestates, while *Enterobacteriaceae* and *Clostridium perfringens* are part
738 of a particular non-mandatory conformity pathway. CFU stands for colony forming units, AD for
739 anaerobic digestion, ABP for animal by-product, and n stands for the number of independent
740 datapoints.

741
742 Most of the concentrations for *E. coli* were below acceptable limits. Only psychrophilic AD and a
743 few values for mesophilic AD, both without any pre- or post-treatment, resulted in values above
744 limits. Thermophilic AD resulted, as expected, as the most effective process to obtain
745 concentrations below limits. The integration of pre- or post-treatments with AD ensured
746 digestates with *E. coli* concentrations below limits, regardless of the AD temperature.
747 Thermophilic digestates seem to present lower *Salmonella* levels, which is coherent with results
748 for Gram-negative bacteria (see Section 3.6). However, *Salmonella* contamination is punctual,
749 meaning that *Salmonella* reduction by itself should not be an exclusion criterion for a given
750 process, as the presence of this pathogen might occur very rarely. Thus, *Salmonella* must be
751 monitored, and eventual contaminated batches of digestates and by-products should be
752 eliminated. Concerning *Enterococcaceae*, they follow the previously observed trend for the
753 reduction of Gram-positive bacteria, with increasing reduction at higher temperatures. As for *E.*
754 *coli*, thermophilic AD and mesophilic AD coupled to pre- or post-treatments resulted in
755 concentrations below detection limits. Regarding the two indicators applied when other standard
756 but derogatory methods are used (*Enterobacteriaceae* and *C. perfringens*), it can be observed that
757 few data were available for both. *Enterobacteriaceae* as an indicator ($n = 10$) was only available
758 at mesophilic temperatures. *Enterobacteriaceae* being a large family of Gram-negative bacteria
759 (including *E. coli*), acceptable limits could be expected to be easily achieved by switching to
760 thermophilic AD and/or by engineered pathogen reduction processes if necessary. Concerning *C.*
761 *perfringens*, none of the available data resulted in acceptable values since its absence is required.
762 *C. perfringens* is a recognized fermentative bacterium capable of competing for substrates with
763 other *Clostridia* commonly found during AD. Therefore, special attention must be paid in
764 reactors where its presence is detected, as it may persist in the system rather than being a
765 transient occurrence [39]. Consequently, *C. perfringens* (along with other pathogenic *Clostridium*
766 species such as *C. botulinum* or *C. difficile*) represents a raising concern that, being a spore-
767 forming Gram-positive bacteria, seems to be poorly removed during AD [40]. As it can be
768 observed, the literature lacks data on the effects of post-treatments on the removal of this
769 pathogen.

770 Given the large number of studies that did not provide TS concentrations in the digestates, it was
771 not possible to calculate the concentrations of indicators for the benchmarking exercises. This
772 reduced considerably the number of points in the database (n). To overcome this issue, a second
773 benchmarking analysis was performed, assuming that, for the studies with unknown TS contents:
774 (i) wet AD had TS values of 5%, and (ii) dry AD had TS values of 15%. This allowed to extend
775 considerably the number of data points (Figure S9). The observed trends in Figure 10 were
776 confirmed by this second analysis, further validating the given conclusions. The increase in data

777 concerning *C. perfringens* is particularly relevant, as the database was significantly enlarged and
 778 still the obtained concentrations were always unsatisfactory.
 779 The results for the US EPA Class A biosolids benchmarking (limits for high quality and
 780 unrestricted use) are shown in Figure 11. Data indicate that most thermophilic digestates would
 781 be conforming to fecal coliforms and *Salmonella* sp. criteria. Most psychrophilic and mesophilic
 782 digestates in the database, with or without pre- or post-treatments, would fail to comply with this
 783 high-quality standard.
 784



785 **Hygienization treatment**
 786 **Figure 11.** Database comparison against the US EPA Class A biosolids regulatory limits
 787 (EPA/600/R-22/194). The concentration in the digestate of each pathogen indicator is shown for
 788 different AD temperatures and considering additional treatments (i.e., pre- or post- treatment).
 789 The red line represents the limit. Only conditions with three or more independent values (n ≥ 3)
 790 are presented. CFU stands for colony forming units, TS for total solids, AD for anaerobic
 791 digestion, and n for the number of independent datapoints.

792
 793 As for the comparison against the EU legislation, the US EPA benchmarking was also repeated
 794 assuming the TS contents mentioned above (5% for wet AD and 15% for dry AD (Figure S10)).

795 This analysis further confirmed the observations extracted from Figure 11, showing the same
796 trends and similar conditions providing effective pathogen reduction.
797 While AD does not always reduce the levels of pathogens below regulation limits, a large
798 fraction of data points fulfills the most restrictive regulation thresholds. In agreement with
799 previous findings, thermophilic AD and post-treatments allowed fulfilling limits more than any
800 other working conditions or treatment trains.

801 802 **4. Implications for technology implementation**

803 The first two novel points to underline concern how tests for assessing pathogen reduction
804 performances are done: (i) spiking of pathogens leads to removal overestimation, and (ii) current
805 pathogen indicators accurately represent their respective microbial groups. Both findings are
806 crucial, not only for research but also for effective digestate quality/safety assessment and for
807 optimizing pathogen reduction performances in digesters.

808 As a general trend, the pathogen reduction effect of AD seems clear. Thus, the agricultural
809 application of digestates appears to be safer than the direct use of feedstocks (e.g., manure).
810 Cases where pathogen indicators increase after AD are rare [31]. Pathogen reduction during AD
811 depends on several factors, including the microbial group of the pathogen (i.e., Gram-negative
812 bacteria, Gram-positive bacteria, Gram-positive spore-forming bacteria, or viruses). For instance,
813 on the one hand, Gram-positive spore-forming bacteria showed virtually no removal after
814 psychrophilic or mesophilic AD. On the other hand, Gram-negative bacteria were effectively
815 removed by AD (e.g., thermophilic conditions with an interquartile range of 3-5 Log₁₀ reduction).
816 Operational parameters also affect the pathogen reduction performance. The most relevant is the
817 temperature. Thermophilic digesters resulted in the highest removals, while mesophilic and
818 psychrophilic digesters resulted in similar overall reductions for most pathogens. This implies
819 that, from a pathogen reduction point of view, increasing the temperature from psychrophilic to
820 mesophilic ranges does not improve the performances. The pH also affects the pathogen
821 reduction performance, with neutral ranges (commonly found in digesters) resulting in the lowest
822 pathogen reductions. More research is needed to investigate the effects at both basic and acidic
823 pH values and to differentiate the impact of the pH itself from that of the concentrations of VFAs
824 and/or NH₃. Assessing these factors separately can lead to a deeper understanding of the
825 multifactorial process leading to pathogen reduction during AD, particularly at high loads. Long-
826 term (semi)continuous studies should also be performed to account for the possibility of pathogen
827 adaptation. Novel fermentative biorefinery concepts aiming to generate other high value-added
828 products such as VFAs might also benefit from the enhanced pathogen reduction performance at
829 low pH values. In this case, (semi)continuous systems would also work at high VFA
830 concentrations, implying that the performance of batch reactors would not necessarily be
831 enhanced compared to continuous reactors. Further research is needed to confirm this.
832 In link with the previous statement, the batch duration affected the pathogen reduction
833 performance. Optimal reductions were obtained after 20-30 days, while too long batches (over
834 30-40 days) resulted in the re-growth of fast-growing organisms (i.e., Gram-negative bacteria).
835 Importantly for (semi)continuous reactors, neither the HRT (ranges from two hours to 120 days)
836 nor the OLR (ranges from 0.12 to 26.9 g VS/L/d) had a significant impact on pathogen removal,
837 implying that these parameters can be optimized according to another criteria (e.g., maximization
838 of biogas production) without affecting the pathogen reduction performance.
839 AD combined with pre- or post-treatments tends to enhance overall pathogen removals. Most of
840 the used pre-treatment processes perform similarly, suggesting that the process selection could be
841 done considering other factors (e.g., economic and/or energetic). Post-treatment processes (e.g.,

842 digestate pasteurization) seem to be more effective than pre-treatments, which could be observed
843 even with the high noise of the pooled data. Looking at details, some studies suggest that in
844 certain cases, pre-treatment could select thermotolerant bacteria that might regrow as part of the
845 fermentative consortium during AD [41]. The results presented here show that regulators should
846 aim at post-treatment as a simple solution (e.g., post-pasteurization) instead of favoring both pre-
847 and post-treatments equally (as is the general case, for example, with ABPs AD in the EU).
848 Digestate valorization through post-treatments allowing some extent of resource recovery is a
849 topic of great scientific and industrial interest, as it can be a lever for ensuring economic
850 performance of AD. The effect of novel post-treatments (e.g., nitrogen stripping, struvite
851 recovery, (vacuum-)evaporation, or enhanced thermal drying) on overall pathogen removal
852 should be more often taken into consideration as a potential additional benefit of these
853 technologies. A good indicator of this lack of research activity is that no study in the present
854 meta-analysis database was part of any digestate post-treatment valorization approach such as
855 those mentioned above.

856 Regardless of the pathogen reduction treatments used, benchmarking the final digestate pathogen
857 concentrations to two very distinct quality criteria allowed to conclude that most thermophilic
858 digestates were conforming to the highest standards, while a post-treatment (e.g., pasteurization)
859 is highly recommended for mesophilic/psychrophilic digestates. Thermophilic conditions lead to
860 higher energy requirements, but this might be balanced out by enhanced biogas productivities
861 [42] and by a safe land application of digestates. Pathogen reduction-wise, two-stage systems are
862 not recommended, as pathogen removal only occurs significantly in the thermophilic stage.
863 The absence of studies using molecular methods (e.g., quantitative polymerase chain reaction
864 (qPCR)) analyzing pathogen reduction during AD precludes their inclusion in the meta-analysis.
865 This lack of research can be attributed to relevant pathogen-related legislations, which establish
866 culture-based methods as the standard for studying pathogen concentrations in digestates. Despite
867 this limitation, the potential of molecular methods as an alternative to culture-based methods
868 cannot be overlooked. Molecular methods offer the advantage of exploring a wider spectrum of
869 microorganisms, yet they also have the disadvantage of potentially detecting non-viable
870 microorganisms (e.g., free genetic material present in the media). Although the pathogen
871 reduction trend was found to be similar between culture-based and molecular methods in the
872 database (data not shown), it is important to highlight that the LRs observed when qPCR was
873 employed were generally lower (probably due to sequencing of genetic material from dead cells).
874 Further research is needed to extrapolate findings from different methodological approaches to
875 full scale plants.

876 Overall, the systematic analysis of pathogen reduction allowed drawing several perspectives for
877 R&D. For certain microbial groups, AD can be optimized through conventional process levers
878 (e.g., temperature) to enhance pathogen removal if they become limiting for digestate application.
879 This is the case of Gram-negative bacteria. Other pathogens, such as *C. perfringens*, represent a
880 challenge that must be addressed specifically.

881 It seems worthwhile, therefore, to investigate the levers of the AD process for pathogen control
882 through case-by-case studies according to specific contexts of interest (i.e., a given set of
883 feedstock, digestate, and pathogen group). Despite the generally acknowledged positive impact of
884 AD, it must be noticed that, particularly for agricultural scenarios, the practical AD input/output
885 perspective (selecting inflows simply based on economic considerations) overlooks the overall
886 impact of an AD plant (and its associated sanitary risks) on the evolution of common operational
887 practices, such as flow pooling and interchange. In this context, the impact of AD can vary, being

888 either positive or negative, depending on the baseline practices, their evolution, and adherence to
889 regulations. These crucial aspects go beyond the scope of the present study.
890 Finally, it must be mentioned that, given the lack of data from full scale plants, the results
891 presented here should be extrapolated with caution to large scale installations. The trends
892 concerning the impact of variables such as pH and temperature and/or microbial groups should be
893 similar regardless of the scale. However, results from batch and (semi)continuous reactors might
894 indeed be different already at laboratory, pilot, and industrial scales (results not shown), so it is to
895 be expected that extrapolating LRs from batch laboratory-scale reactors to full scale processes
896 (usually (semi)continuous) will result in overestimations of the reduction capacities (even if
897 allochthonous pathogens were not spiked). As a work based on an analysis of available data, the
898 conclusions from this study are limited by the amount of data that could be gathered, their
899 accuracy, and their repeatability. Similarly, it was not possible to differentiate between specific
900 scenarios, as the amount of data for each case would not be sufficient, leading to biased
901 conclusions.

903 5. Conclusions

904 The performed meta-analysis has resulted in novel and relevant conclusions for AD at both
905 research and large scale. The large amount of collected data and the systematic data analysis done
906 have resulted in a global view of the pathogen reduction capacity of AD. When designing
907 experiments to assess AD pathogen reduction performance, artificial pathogen spiking leads to
908 performance overestimation, and thus results cannot be extrapolated to scaled systems.
909 Importantly, current pathogen indicators accurately represent their respective groups.
910 *Clostridiaceae* are barely affected by AD and may be favored by some pre-treatment
911 technologies. Concerning operational parameters, temperature is the parameter that most
912 significantly affects pathogen reduction performance. Thermophilic AD resulted in enhanced
913 pathogen removal, with both psychrophilic and mesophilic conditions resulting in significantly
914 lower performances. The pH also affected pathogen removal, with both acidic and basic values
915 enhancing LRs. This is probably due to a combination of the effect of the pH itself and of the
916 concentrations of inhibitory compounds also affecting pH (e.g., VFAs or $\text{NH}_3/\text{NH}_4^+$). An optimal
917 batch duration was identified, but the HRT in (semi)continuous systems did not enhance the
918 overall pathogen reduction, implying that the HRT/OLR values can be set according to the
919 desired methane production rates. Heat-based post-treatments coupled to thermophilic AD
920 resulted in the best pathogen reduction performances. These conditions fulfilled most legislation
921 limits. Further research should focus on multifactorial process optimization, considering the links
922 between different factors (e.g., pH, VFA, and NH_3 concentrations) and developing mathematical
923 models that allow optimization and scenario evaluations. The impact of novel post-treatments
924 allowing resource recovery (e.g., nitrogen stripping, evaporation, or enhanced thermal drying) on
925 overall pathogen removal should also be further studied.

927 Data availability

928 The complete database used in this meta-analysis is available on the research data repository
929 Mendeley data under the digital object identifier (DOI): 10.17632/3m9ph7j578.2.

931 Appendices

932 Appendix A: Assumptions considered

- 933 - If not specified, room temperature was assumed to be 25 °C.
934 - If not specified, mesophilic conditions were assumed to be 35 °C.

- 935 - If not specified, thermophilic conditions were assumed to be 55 °C.
936 - If not specified, the type of reactor was assumed to be stirred tank reactor (STR).
937 - If not specified, the feeding mode was assumed to be semi-continuous.
938 - Sewage sludge refers to the mixture of primary sludge and waste activated sludge.
939 - If not specified, sludge was assumed to be sewage sludge.
940 - If not specified, grams of “dry solids” was assumed to be grams of total solids (TS).
941 - If not specified, the “reactor volume” was assumed to be the working volume.
942 - If not specified, CH₄ volume (L) was assumed to be given at standard pressure and
943 temperature (273.15 K and 0.987 atm).
944 - Except for dry anaerobic digestion (AD), reactors were assumed to be stirred (if not
945 specified).
946 - If not specified, chemical oxygen demand (COD) concentrations were assumed to be total
947 values (i.e., raw samples).
948 - When reporting concentrations of TS or volatile solids (VS) as weight for weight (w/w), it
949 was assumed to be equivalent to weight for volume (w/v).
950 - When studying pre-treatment coupled to AD, the initial TS, VS and COD concentrations
951 pertain to the pre-treated substrate.
952 - When studying pre-treatment coupled to AD, the initial TS and COD concentrations pertain
953 to the substrate before pre-treatment.
954 - When studying pre-treatment coupled to AD, the initial VS concentration pertain to the
955 substrate after pre-treatment.
956 - When heat treatment was performed, the time of treatment represents the time after reaching
957 the desired temperature (without taking into account the heating ramp).
958 - When the Colony Forming Unit (CFU) value was <X, CFU was assumed to be to be
959 equivalent to X.
960 - When the CFU value was ≥X, CFU was assumed to be equal to X.
961 - When microbial concentration was reported as Most Probable Number (MPN), it was
962 assumed to be equivalent to Colony Forming Units (CFU).
963 - The reporting of N₀ and N values displayed variability, with instances presented on a wet
964 weight basis (CFU/g), on a dry matter basis (CFU/g TS), or in volumetric units (CFU/mL).
965 When possible, the values were converted to CFU/g TS using the TS concentration of the
966 feedstock or digestate. A density of 1 g/mL was assumed for volume/mass conversions.

967 **Acknowledgements**

968 Laura Álvarez-Fraga acknowledges the European Union's Horizon 2020 research and innovation
969 programme for their support through the Marie Skłodowska-Curie grant agreement No
970 101108532. The authors would like to acknowledge INRAE Bio2E Facility [43].
971

972 **Formatting of funding sources**

973 This work was supported by the project Biogaz RIO (No. 24001371) funded by the Région
974 Occitanie and the European Regional Development Fund (ERDF).
975

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