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Highlights

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

Abstract

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

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

Word count

9,180 words

Keywords

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

Abbreviations and symbols

ABP - Animal By-Product
AD - Anaerobic digestion
AnSBR - Anaerobic sequencing batch reactor
ANOVA - Analysis of variance
CFU - Colony forming unit
DNA - Deoxyribonucleic acid
EU - European Union
FBR - Fixed bed reactor
HRT - Hydraulic retention time
HSD - Post-hoc Tukey's Honesty Significant Difference test
IQR - Interquartile range
LR - Log reduction
MPN - Most probable number
n - Number of independent datapoints
N - Number of articles
N_0 - Number of colony forming units before AD, pre- or post-treatment
N_1 - Number of colony forming units after AD, pre- or post-treatment
OLR - Organic loading rate
PABFR - Panelled anaerobic baffle-cum-filter reactor
PFR - Plug flow reactor
PLS - Partial least squares
qPCR - Quantitative polymerase chain reaction
RNA - Ribonucleic acid
STR - Stirred tank reactor
TPAD - Temperature phased AD
TS – Total solids
US EPA - United States Environmental Protection Agency
VBNC - Viable but non-culturable cell
VFA - Volatile fatty acid
VS - Volatile solids

WoS - Web of Science

1. Introduction

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

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

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

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

Despite being more or less restrictive and allowing different digestate applications, they all share the same objective: ensuring the safe utilization of recovered resources from digestate.

AD can effectively reduce the concentration of pathogens present in a wide range of feedstocks, such as sewage sludge, manure or biowaste [14–17]. However, the pathogen reduction capacity of AD (commonly referred to as hygienization) can be insufficient, resulting in concentrations of microorganisms in the digestate exceeding biosafety levels. To enhance the microorganism inactivation during AD, it is crucial to understand and optimize the factors influencing the pathogen reduction performance. Different factors affecting pathogen removal have been identified, including the type of pathogens present, the byproducts formed during the process (e.g., volatile organic acids (VFAs) or ammonia nitrogen), and different operational parameters (e.g., temperature or retention time). Despite previous efforts done to elucidate optimal pathogen reduction conditions, the challenge remains, mostly due to the limited scope of many experimental studies, which assess the inactivation of specific pathogens under specific operational conditions, thereby resulting in data that cannot be extrapolated and even in contradictory results.

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

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

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

2. Material and methods

2.1. Article search strategy and selection process

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

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

2.2. Data collection

Data were extracted from tables or text in articles. When data were not explicitly provided, values were extracted from graphs and/or manually calculated. Extracted data were organized in a spreadsheet using Microsoft Excel. Data encompassed crucial information regarding individual experiments, such as reactor type, feeding mode, reactor inoculum, feedstock, reactor operational conditions, and primary process outcomes such as pathogen reduction or methane yield. Categories were defined for different factors, including reactor types, feedstocks (including mixtures indicated as "co-digestion"), and microorganisms studied. The full database and a list of the categories considered can be found in Supplementary Material (Table S1). The database was also deposited in the research data repository Mendeley Data [19]. Assumptions were applied for data standardization (see Appendix A).

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

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

2.3. Statistical analysis and data representation

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

3. Results and discussion

3.1. Literature search and screening

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

3.2. Data overview

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

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

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

An evident disparity was observed in the scale of the studies, with a substantial majority conducted at laboratory scale (74.4%), followed by pilot-scale studies (17.3%) and industrial-scale studies (11.6%) (Figure S3A). Concerning AD feedstocks, sewage sludge (50.4%) and livestock waste & effluents (35.5%) were the most prevalent (Figure S3B). Mono-digestion 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

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

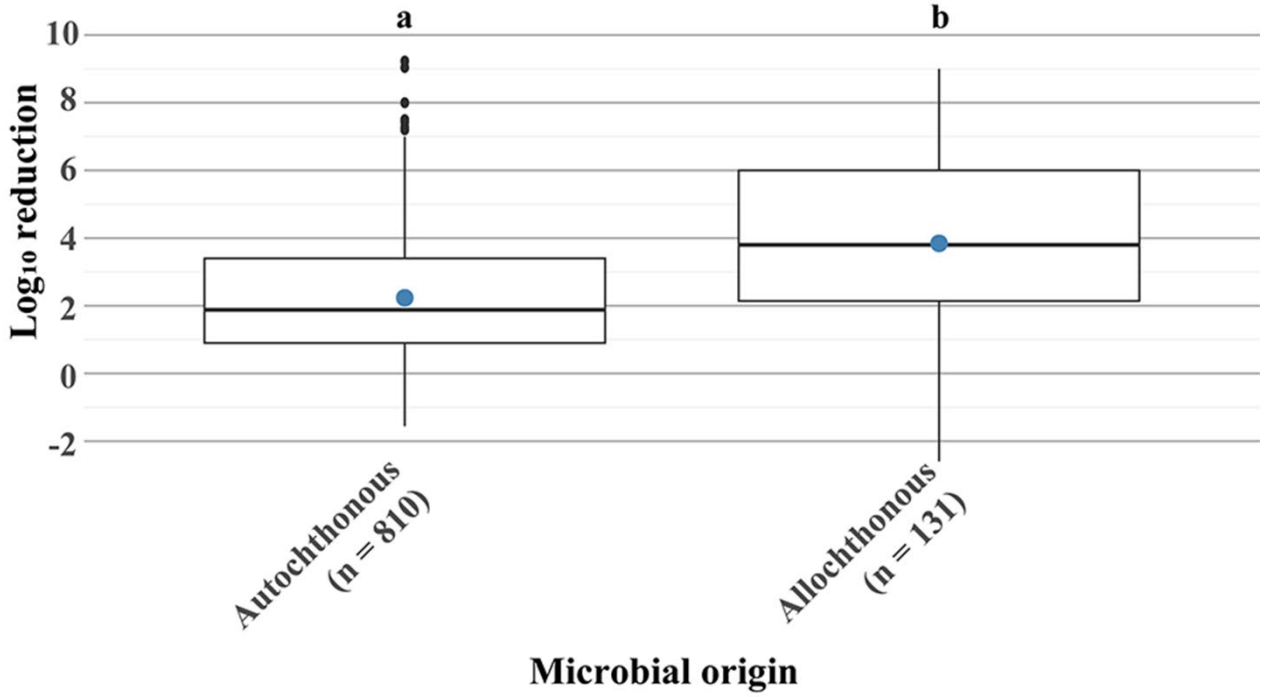


Figure 1. Microorganism Log_{10} reduction for experiments studying autochthonous pathogen reduction (naturally present in the feedstock) and for experiments in which allochthonous pathogens were inoculated. Mean values are represented by blue dots. Identical letters above boxplots indicate homogeneous groups. n stands for the number of independent datapoints.

The different pathogen reduction between autochthonous and allochthonous pathogens can be attributed to the adaptation of native microorganisms to the substrate and to the conditions occurring during its natural decay (potentially similar to those of AD). Autochthonous populations may also be protected when present in highly physically structured environments, such as granules or biofilms. Inoculated pathogens might lack these adaptations, potentially affecting their survival and persistence. Although the specific susceptibility of allochthonous pathogens to reduction during AD has not been explicitly compared with that of autochthonous pathogens, it appears evident that their behavior and fate in AD systems are clearly influenced by their origin. A similar trend was observed in previous studies where allochthonous viruses and bacteriophages experienced a rapid decline upon inoculation into sludge compared to the autochthonous microorganisms [23]. This rapid reduction in numbers was attributed to a matrix effect. In spiking experiments, the feedstock is also usually inoculated to an initial concentration of microorganisms higher than their natural levels in the substrate (approximately 1 log_{10} higher). The reduced resistance of allochthonous microorganisms, combined with higher artificial concentrations in the feedstock intended for pathogen reduction, may explain the observed augmentation in pathogen reductions.

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

3.4. Impact of the targeted microbial group on pathogen reduction

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

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

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

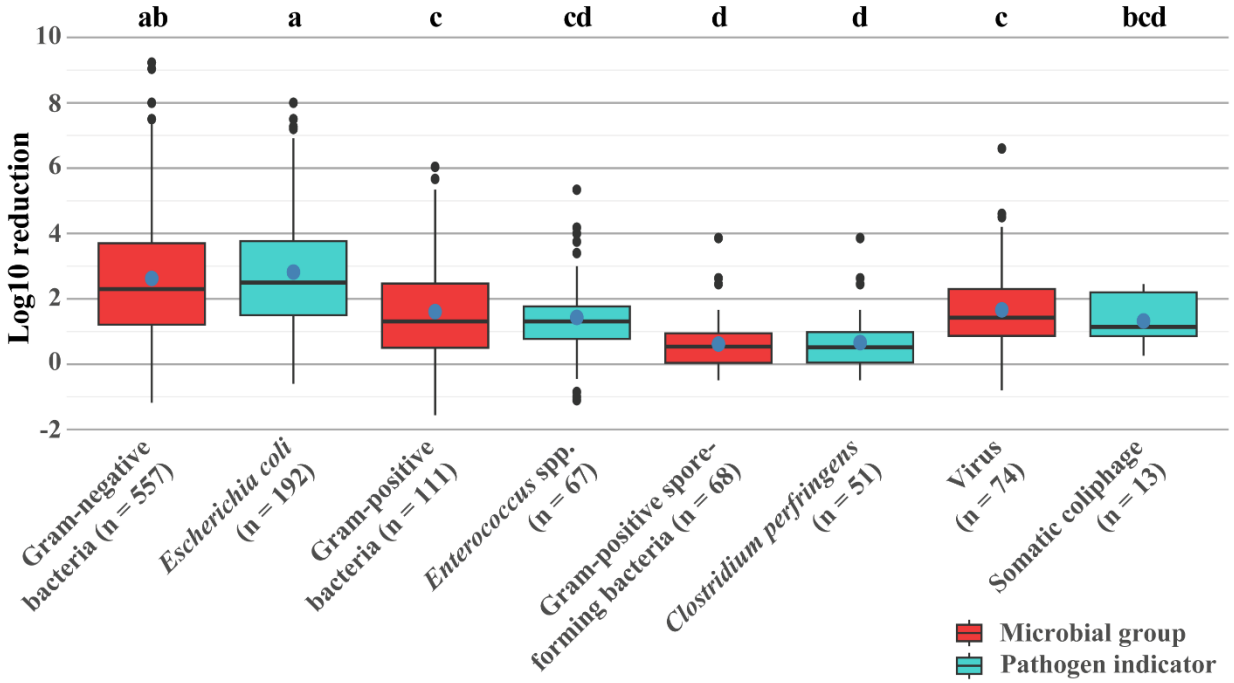


Figure 2. Microorganism Log_{10} reduction for different groups (red) of microorganisms and for their respective pathogen indicators (blue). Mean values are represented by blue dots. Only the microbial groups with three or more independent values ($n \geq 3$) are presented. Identical letters above boxplots indicate homogeneous groups. n stands for the number of independent datapoints.

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

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

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

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

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

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

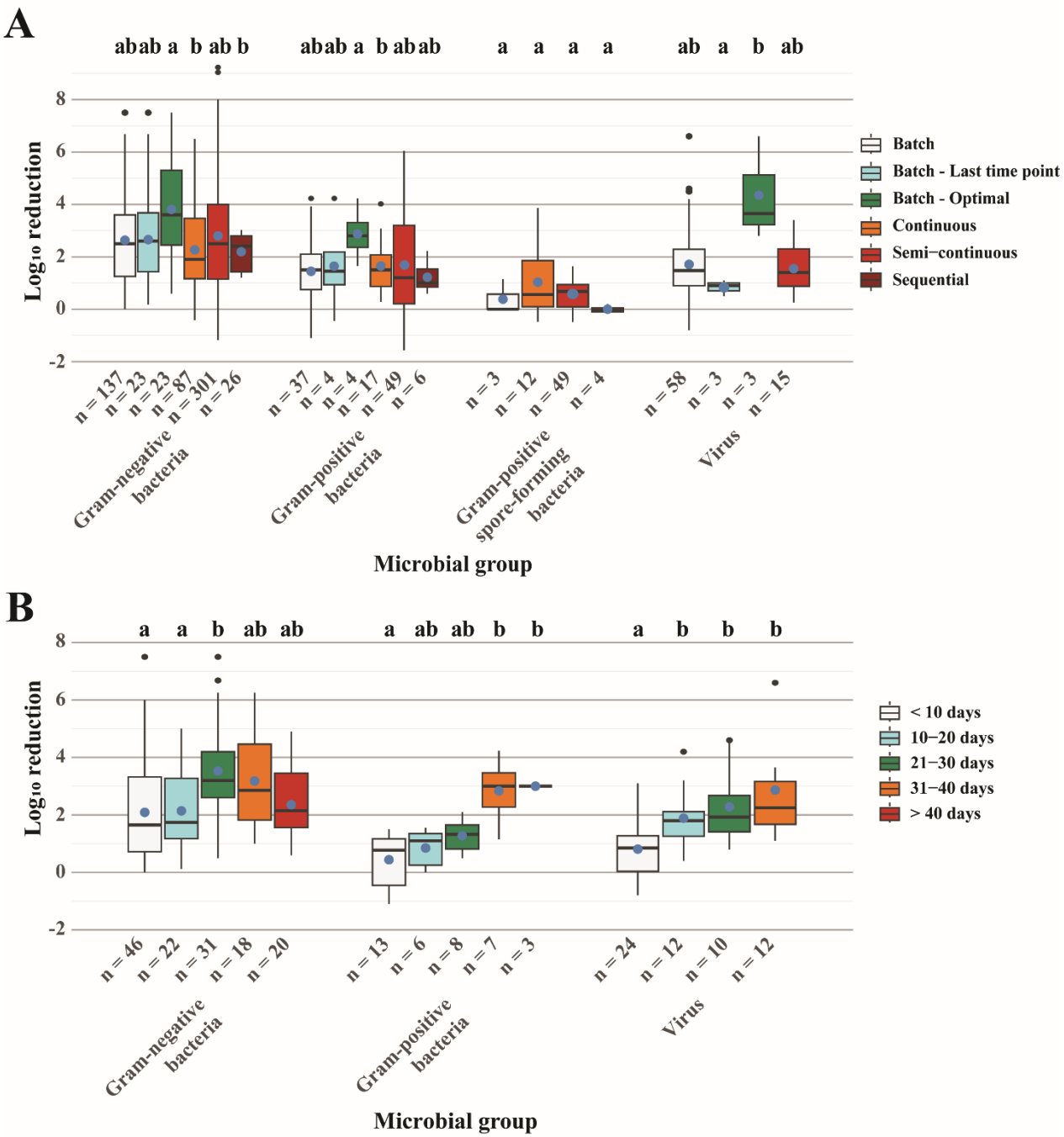


Figure 3. Microorganism Log_{10} reduction for A) different groups of microorganisms and different feeding modes and B) each microbial group in batch reactors with different durations. Mean values are represented by blue dots. Only the conditions with three or more independent values ($n \geq 3$) are presented. Identical letters above boxplots indicate homogeneous groups. n stands for the number of independent datapoints.

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

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

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

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

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

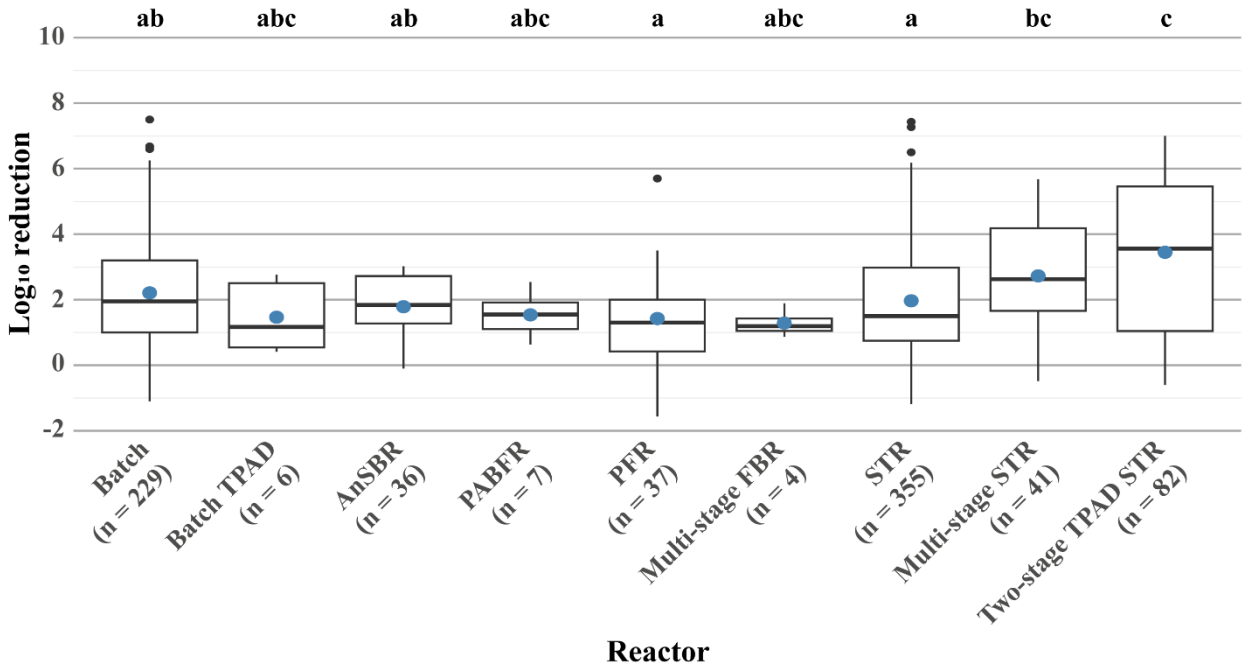


Figure 4. Overall microorganism Log_{10} reduction for different reactor types. Mean values are represented by blue dots. Only the reactors with three or more independent values ($n \geq 3$) are presented. Identical letters above boxplots indicate homogeneous groups. TPAD stands for temperature phased anaerobic digestion, AnSBR for anaerobic sequencing batch reactor, PABFR for panelled anaerobic baffle-cum-filter reactor, PFR for plug flow reactor, FBR for fixed bed reactor, and STR for stirred tank reactor. n stands for the number of independent datapoints.

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

3.6. Impact of temperature on pathogen reduction

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

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

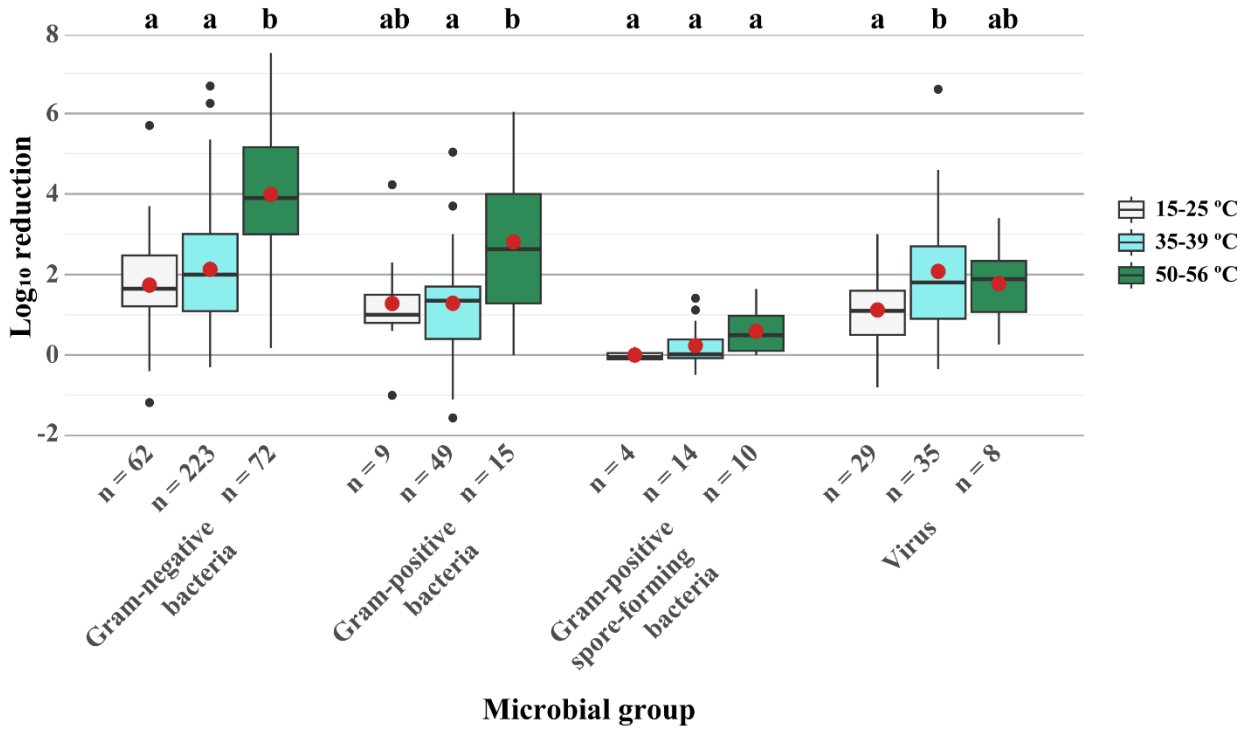


Figure 5. Microorganism Log_{10} reduction for different groups of microorganisms and for different temperature ranges. Mean values are represented by red dots. Identical letters above boxplots indicate homogeneous groups. n stands for the number of independent datapoints.

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

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

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

3.7. Impact of working pH on pathogen reduction

The pH is a well-known parameter affecting microbial growth. For example, pH variations affect the ionization of amino-acid functional groups, resulting in protein denaturation and activity decrease. Extremely acidic or basic pH can also cause DNA breakup and lipid hydrolysis, respectively. The pH also affects several biological processes, such as the proton motive force and many other reactions involving the turnover of protons. In AD systems, studying the impact of pH is extremely complex. Not only the pH affects the aforementioned process, but also the speciation of the most common inhibitors in digesters: VFAs and free ammonia (NH_3) [1]. These interactions go both ways, as pH affects the microbial activity, but metabolic processes also modify the pH. Both VFAs and NH_3 are microbial products that affect (and sometimes determine) the pH in digesters. Due to the difficulties of separating the pathogen reduction effects related to the pH itself from those of VFA or NH_3 (and due to the general lack of data), only the overall impact of the reported pH values in the media is discussed here. Discussions around the findings from individual articles on pathogen reduction related to VFA and/or NH_3 can be found elsewhere [14,15,18].

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

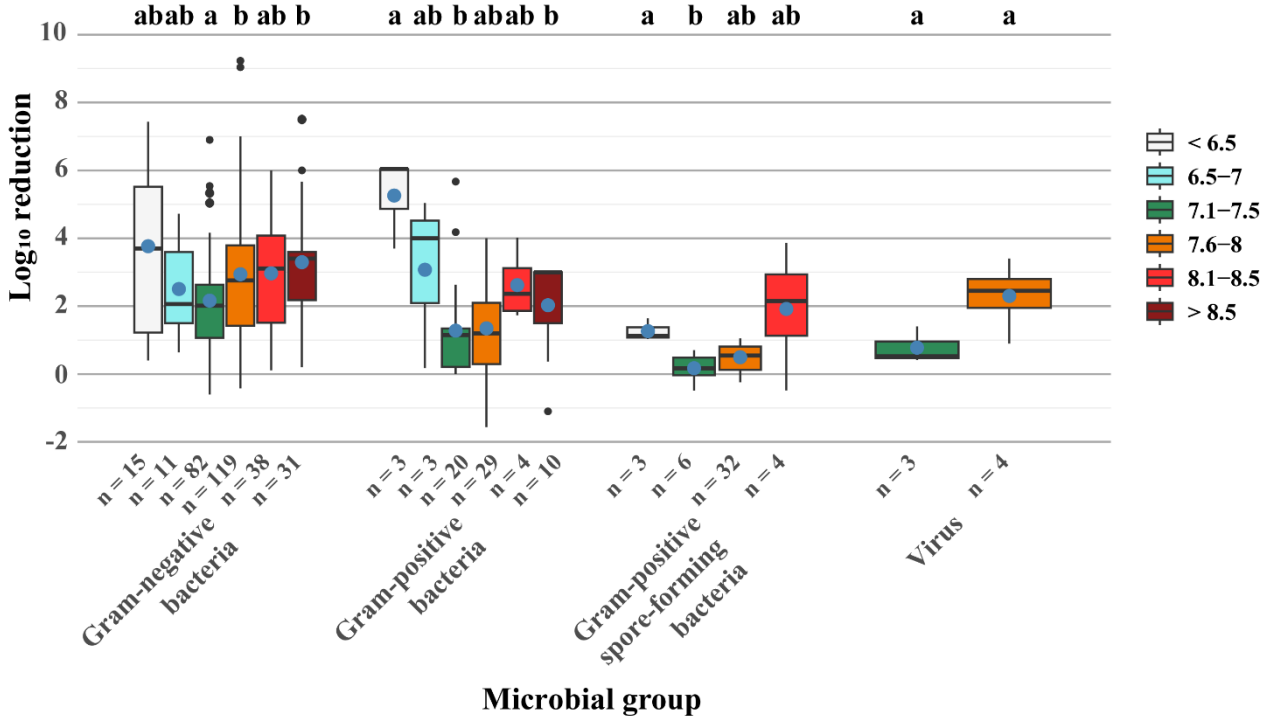


Figure 6. Microorganism Log_{10} reduction for different groups of microorganisms and for different pH ranges. Mean values are represented by blue dots. Only conditions with three or more independent values ($n \geq 3$) are presented. Identical letters above boxplots indicate homogeneous groups. n stands for the number of independent datapoints.

For all bacterial groups, the lowest LRs were reported at neutral pH ranges (7.1-8.0). Other than the neutrophilic nature of the microorganisms, pH values close to 7 result in low concentrations of both non-ionized VFAs (the toxic form) and NH_3, thus reducing their toxicity. pH ranges above or below neutrality resulted in enhanced pathogen reduction performances. Both Gram-negative and Gram-positive bacteria follow a similar trend, with increased reductions at pH

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

Variable and/or non-reported VFA/NH_3 concentrations in pathogen reduction studies preclude the identification of the precise phenomena responsible for the increased LRs. The overall trend of pathogen reduction data follows a similar trend as the one shown in Figure 6, with neutral pH ranges (i.e., 6.5-8.0) providing the lowest LRs (Figure S5).

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

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

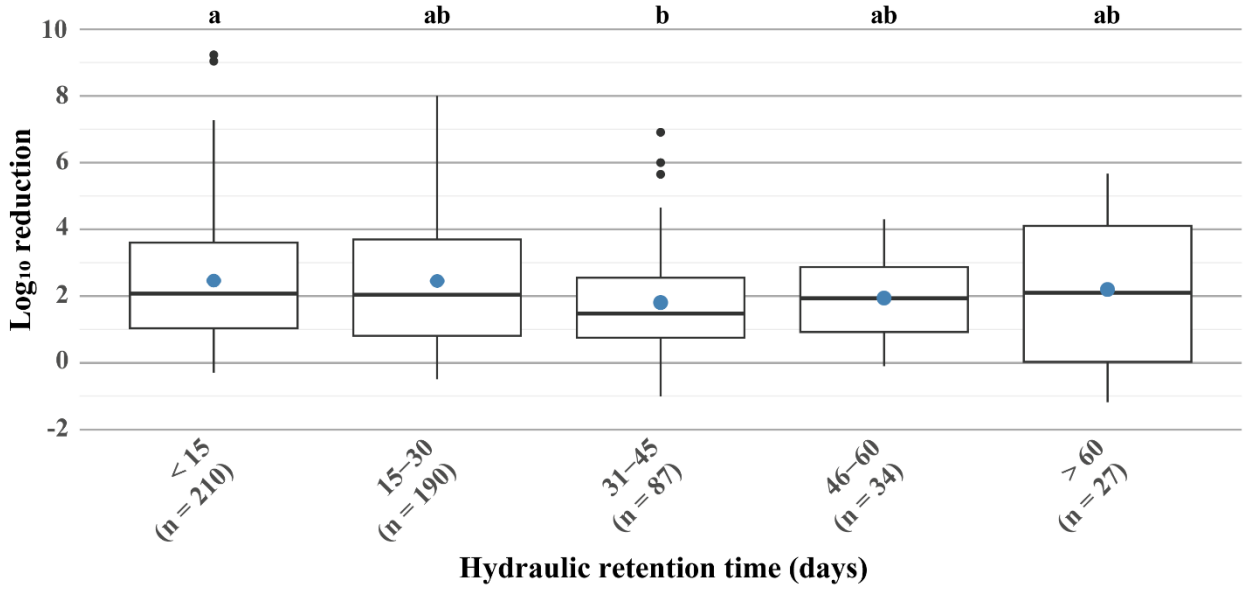


Figure 7. Overall microorganism Log_{10} reduction for different hydraulic retention time (HRT) ranges. Mean values are represented by blue dots. Identical letters above boxplots indicate homogeneous groups. n stands for the number of independent datapoints.

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

statements, the lowest OLR range assessed (≤ 2 g VS/L/d) did not result in enhanced pathogen reductions. In fact, the lowest average LR was obtained for this range, suggesting that low loads (or long retention times) do not enhance pathogen reduction.

Although this conclusion goes against some experimental articles [36,37], this overall assessment agrees with what has been observed in a previous meta-analysis [18], validating it and suggesting that it is not a result of sampling biases. The main inactivation mechanisms appear to be related to other factors, such as the working temperature or pH. The inactivation times associated with the effect of these parameters are much shorter than common AD retention times (e.g., in the ranges of minutes-hours), meaning that the extra time provided does not result in any tangible benefit.

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

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

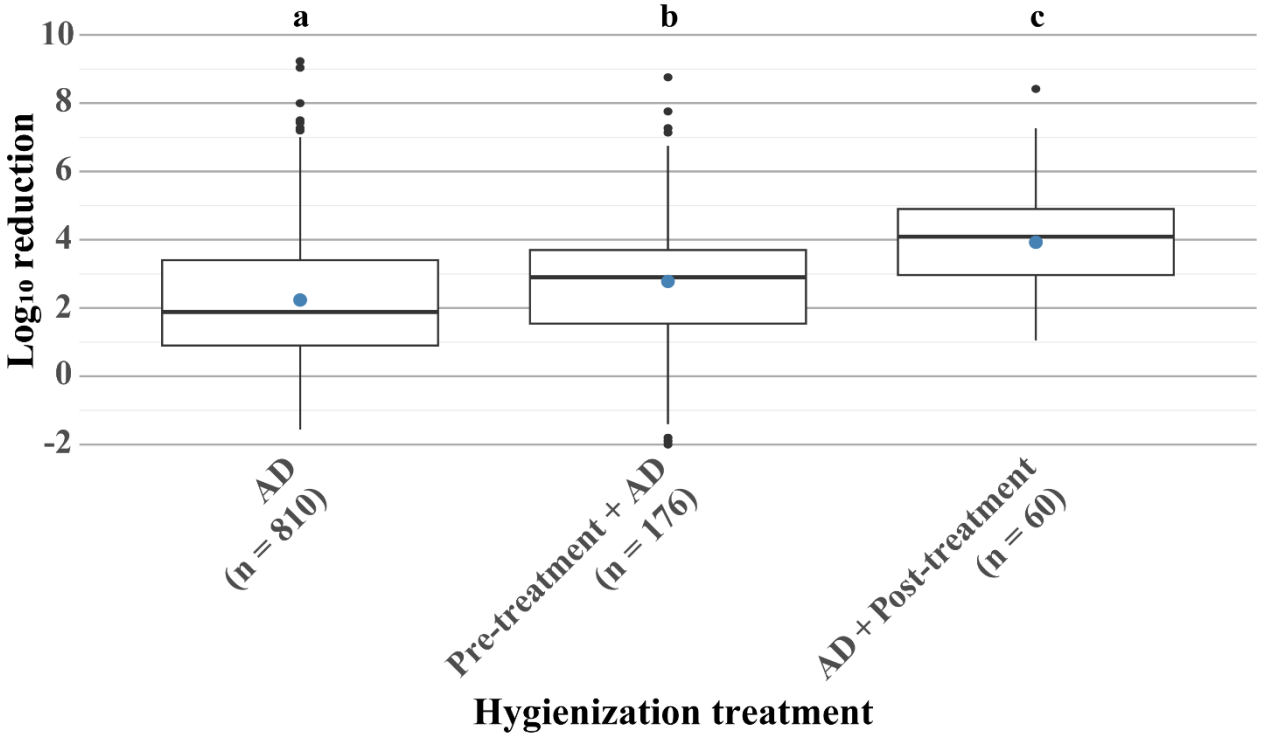


Figure 8. Overall microorganism Log_{10} reduction during AD, either alone or coupled with pre- or post-treatment processes. Mean values are represented by the blue dots. Identical letters above boxplots indicate homogeneous groups**.** n stands for the number of independent datapoints and AD for anaerobic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

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

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

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

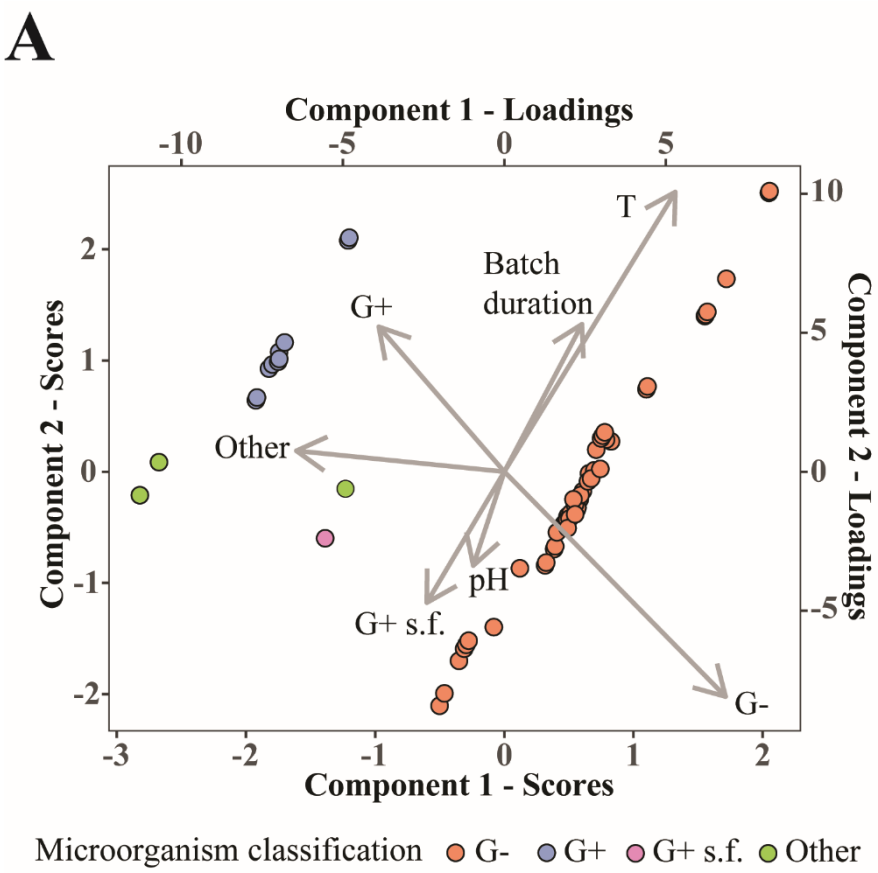
Considering the similar performances, the choice of technology may be guided by other factors, such as economic considerations (e.g., reduced costs due to energy requirements) and/or biological aspects (e.g., enhanced substrate biodegradability after pre-treatment). Thermal pre-treatments emerge as a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

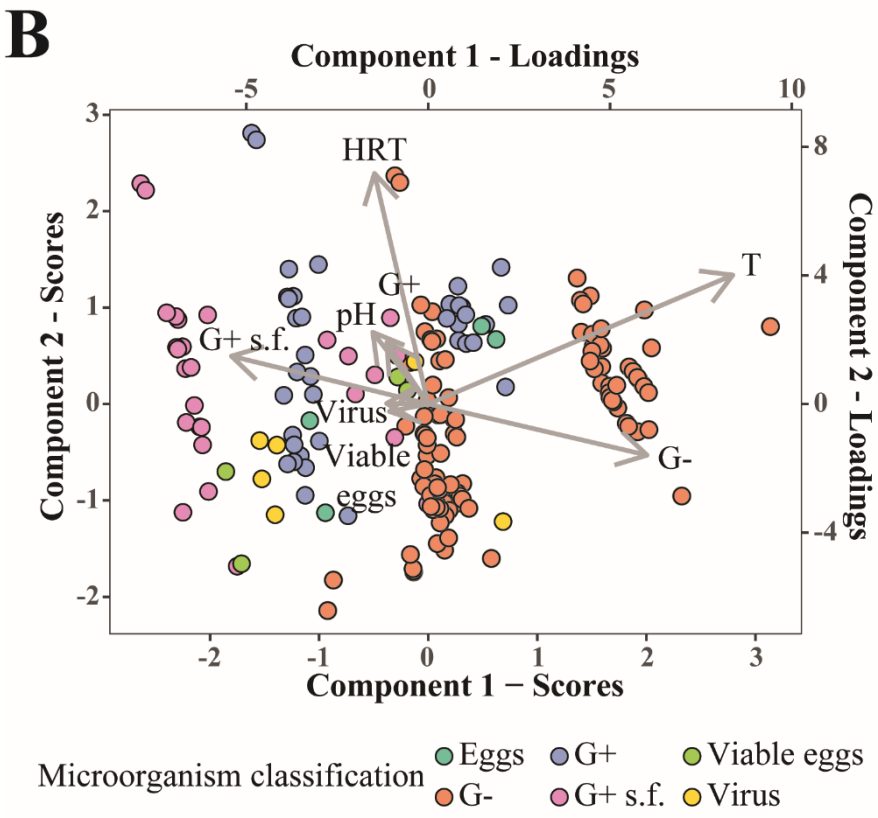
Regarding post-treatments, this analysis focused on heat-related processes. Treatment conditions varied across studies, with temperatures ranging from 60 to 80 ºC and durations spanning from two minutes to 96 hours. Once again, pasteurization conditions were prevalent. Pasteurization was indeed the main driver for the overall increase in LR values depicted in Figure 8. Specifically, when focusing on heat-related treatments, which constitute the majority of the collected data points, the benefits of post-treatment coupled with AD (mean LR 3.92 ± 1.43) compared with pre-treatment (mean LR 2.78 ± 2.05) become evident. Thus, pasteurization of the digestate is preferable to pasteurization of the input substrates (considering pathogen reduction as the sole criterion). The energy requirements of the latter are obviously lower.

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

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

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


A
Component 1 - Loadings
-10
-5
0
5
T
Batch
duration
G+
Other
pH
G+ s.f.
G-
Component 2 - Scores
Component 2 - Loadings
Component 1 - Scores
Microorganism classification
G-
G+
G+ s.f.
Other


B
Component 1 - Loadings
HRT
T
G+
pH
G+ s.f.
Virus
Viable
eggs
G-
Component 2 - Scores
Component 2 - Loadings
Component 1 – Scores
Microorganism classification
Eggs
G+
Viable eggs
G-
G+ s.f.
Virus

650

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

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

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

Regarding the HRT in (semi)continuous reactors, this parameter impacted the predicted LR values. This might appear in contradiction with the negligible effect described in Section 3.8, but when looking at the data distribution along the HRT vector and at the HRT scores in the first two components (Table S3), this finding can be explained. For component 1 (explaining 22% of the variance; Table S4), the coefficient of the HRT was negative, while for component 2 (explaining 11%), the coefficient was positive (and higher in absolute value than for component 1). Therefore, the overall trend (Figure 7) resulted in a negligible impact of the HRT, as in some cases longer HRTs resulted in higher LRs and in others the opposite occurred. This dichotomy agrees with the literature, where both conclusions have been proposed [17,18].

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

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

3.11. Anaerobic digestion for reducing the level of pathogens below regulation limits

To assess compliance with regulatory requirements, the created database was compared against two relevant pathogen-related regulations in the field of organic waste AD (used for benchmarking): the United States Environmental Protection Agency (US EPA) Class A biosolids regulation (EPA/600/R-22/194) [13] and the EU ABP regulation (CE 142/2011) [11]. This analysis is purely comparative, as the feedstocks, treatment lines, and analytical methods employed in the studies from the database did not necessarily follow the regulation guidelines for waste digestion, digestate sampling, or pathogen quantification.

Table 1 presents the limits from the legislations used for the benchmarking exercise. The regulation CE 142/2011 is applied only to ABP material as defined by the regulation CE 1069/2009, and offers two options for complying: (i) dedicated protocols are followed and *E. coli, Salmonella* sp., *and Enterococcaceae* are below given limits; or (ii) if other standard protocols are followed (standard processing method 7 in CE 142/2011), *Enterobacteriaceae* and *C. perfringens* are also below limits. The US EPA Class A biosolids regulation claims explicitly that "the implicit goal of the Class A pathogen requirements is to reduce all the pathogens present in sewage sludge […] to below detectable levels". Class A biosolids are post-treated to reach these criteria, thus allowing for "unrestricted use". The European criteria are less restrictive than those from the US EPA because they do not imply unrestrictive use of the material. Several other EU and regional/national regulations add further innocuity criteria depending on the digestate use and status.

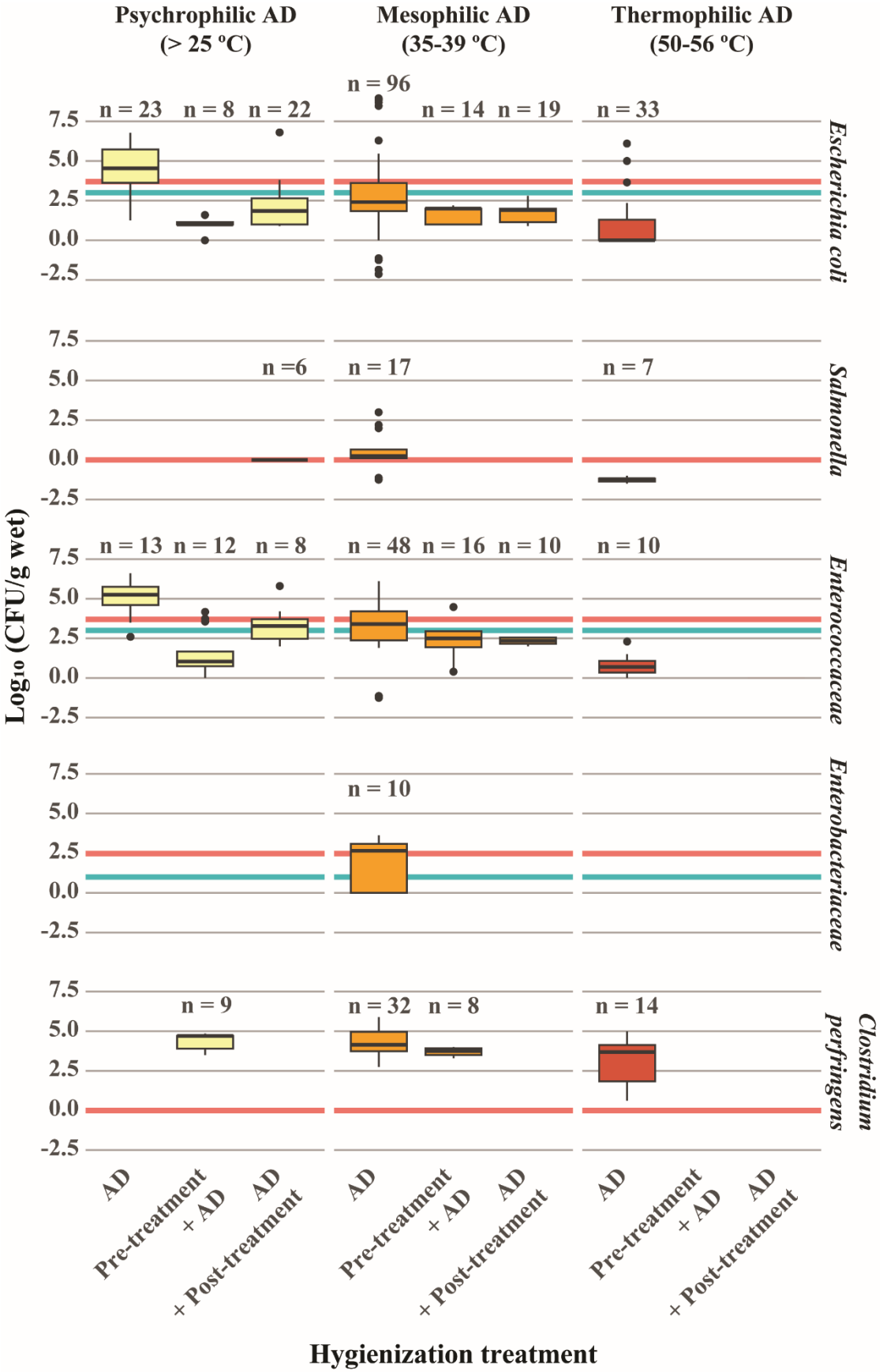
Table 1. Summary of the limits given in the regulations used for benchmarking.

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

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

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

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


Psychrophilic AD
(> 25 ºC)
Mesophilic AD
(35-39 ºC)
Thermophilic AD
(50-56 ºC)
Escherichia coli
Salmonella
Enterococcaceae
Enterobacteriaceae
Clostridium perfringens
Log10 (CFU/g wet)
n = 23
n = 8
n = 22
n = 96
n = 14
n = 19
n = 33
n =6
n = 17
n = 7
n = 13
n = 12
n = 8
n = 48
n = 16
n = 10
n = 10
n = 9
n = 32
n = 8
n = 14
7.5
5.0
2.5
0.0
-2.5
AD
Pre-treatment + AD
AD + Post-treatment
Hygienization treatment

730

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

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

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

concerning *C. perfringens* is particularly relevant, as the database was significantly enlarged and still the obtained concentrations were always unsatisfactory.

The results for the US EPA Class A biosolids benchmarking (limits for high quality and unrestricted use) are shown in Figure 11. Data indicate that most thermophilic digestates would be conforming to fecal coliforms and *Salmonella* sp. criteria. Most psychrophilic and mesophilic digestates in the database, with or without pre- or post-treatments, would fail to comply with this high-quality standard.

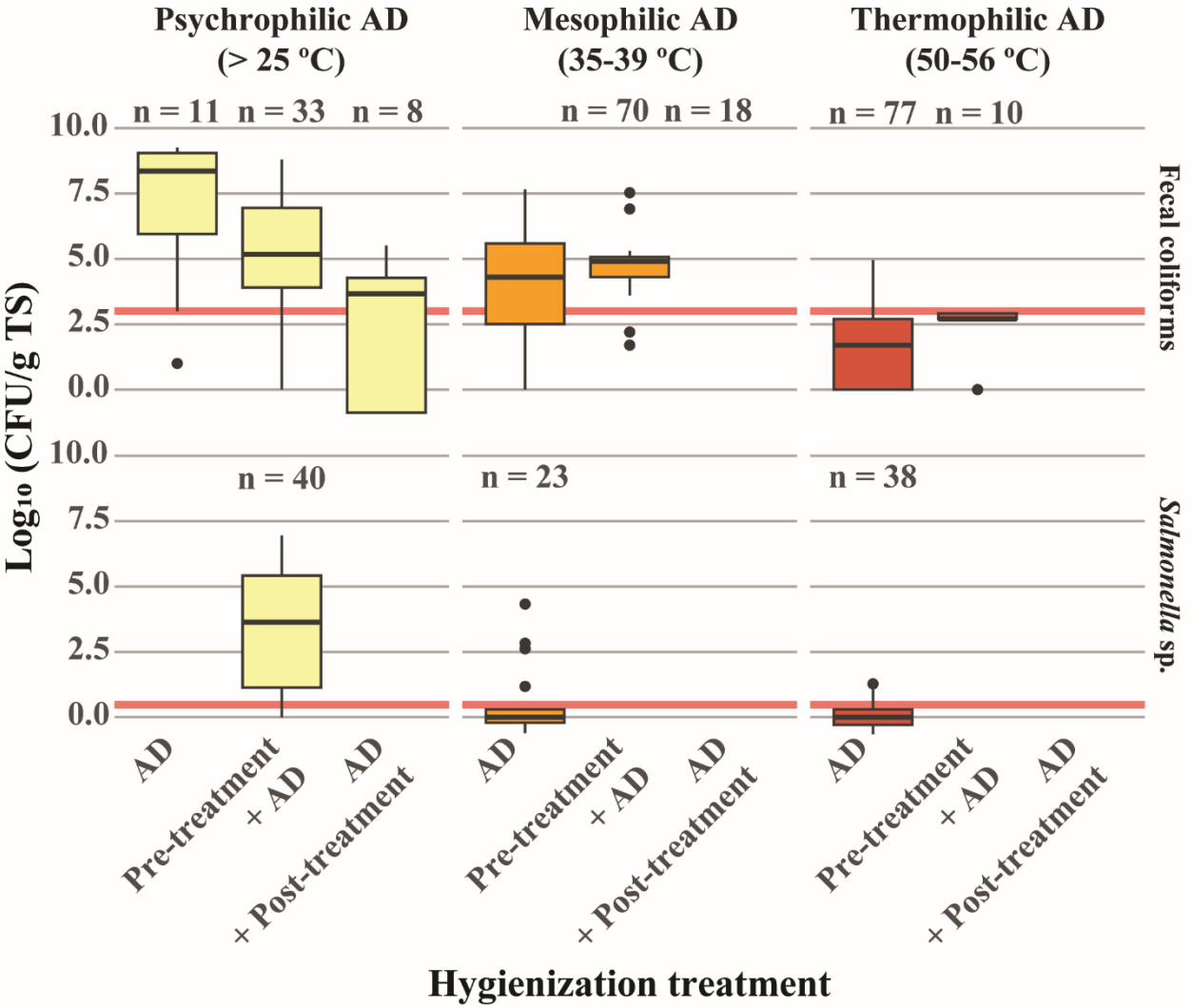


Figure 11. Database comparison against the US EPA Class A biosolids regulatory limits (EPA/600/R-22/194). The concentration in the digestate of each pathogen indicator is shown for different AD temperatures and considering additional treatments (i.e., pre- or post- treatment). The red line represents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

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

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

4. Implications for technology implementation

The first two novel points to underline concern how tests for assessing pathogen reduction performances are done: (i) spiking of pathogens leads to removal overestimation, and (ii) current pathogen indicators accurately represent their respective microbial groups. Both findings are crucial, not only for research but also for effective digestate quality/safety assessment and for optimizing pathogen reduction performances in digesters.
As a general trend, the pathogen reduction effect of AD seems clear. Thus, the agricultural application of digestates appears to be safer than the direct use of feedstocks (e.g., manure). Cases where pathogen indicators increase after AD are rare [31]. Pathogen reduction during AD depends on several factors, including the microbial group of the pathogen (i.e., Gram-negative bacteria, Gram-positive bacteria, Gram-positive spore-forming bacteria, or viruses). For instance, on the one hand, Gram-positive spore-forming bacteria showed virtually no removal after psychrophilic or mesophilic AD. On the other hand, Gram-negative bacteria were effectively removed by AD (e.g., thermophilic conditions with an interquartile range of 3-5 Log_{10} reduction).
Operational parameters also affect the pathogen reduction performance. The most relevant is the temperature. Thermophilic digesters resulted in the highest removals, while mesophilic and psychrophilic digesters resulted in similar overall reductions for most pathogens. This implies that, from a pathogen reduction point of view, increasing the temperature from psychrophilic to mesophilic ranges does not improve the performances. The pH also affects the pathogen reduction performance, with neutral ranges (commonly found in digesters) resulting in the lowest pathogen reductions. More research is needed to investigate the effects at both basic and acidic pH values and to differentiate the impact of the pH itself from that of the concentrations of VFAs and/or NH_3. Assessing these factors separately can lead to a deeper understanding of the multifactorial process leading to pathogen reduction during AD, particularly at high loads. Long-term (semi)continuous studies should also be performed to account for the possibility of pathogen adaptation. Novel fermentative biorefinery concepts aiming to generate other high value-added products such as VFAs might also benefit from the enhanced pathogen reduction performance at low pH values. In this case, (semi)continuous systems would also work at high VFA concentrations, implying that the performance of batch reactors would not necessarily be enhanced compared to continuous reactors. Further research is needed to confirm this.
In link with the previous statement, the batch duration affected the pathogen reduction performance. Optimal reductions were obtained after 20-30 days, while too long batches (over 30-40 days) resulted in the re-growth of fast-growing organisms (i.e., Gram-negative bacteria). Importantly for (semi)continuous reactors, neither the HRT (ranges from two hours to 120 days) nor the OLR (ranges from 0.12 to 26.9 g VS/L/d) had a significant impact on pathogen removal, implying that these parameters can be optimized according to another criteria (e.g., maximization of biogas production) without affecting the pathogen reduction performance.
AD combined with pre- or post-treatments tends to enhance overall pathogen removals. Most of the used pre-treatment processes perform similarly, suggesting that the process selection could be done considering other factors (e.g., economic and/or energetic). Post-treatment processes (e.g.,

digestate pasteurization) seem to be more effective than pre-treatments, which could be observed even with the high noise of the pooled data. Looking at details, some studies suggest that in certain cases, pre-treatment could select thermotolerant bacteria that might regrow as part of the fermentative consortium during AD [41]. The results presented here show that regulators should aim at post-treatment as a simple solution (e.g., post-pasteurization) instead of favoring both pre- and post-treatments equally (as is the general case, for example, with ABPs AD in the EU).

Digestate valorization through post-treatments allowing some extent of resource recovery is a topic of great scientific and industrial interest, as it can be a lever for ensuring economic performance of AD. The effect of novel post-treatments (e.g., nitrogen stripping, struvite recovery, (vacuum-)evaporation, or enhanced thermal drying) on overall pathogen removal should be more often taken into consideration as a potential additional benefit of these technologies. A good indicator of this lack of research activity is that no study in the present meta-analysis database was part of any digestate post-treatment valorization approach such as those mentioned above.

Regardless of the pathogen reduction treatments used, benchmarking the final digestate pathogen concentrations to two very distinct quality criteria allowed to conclude that most thermophilic digestates were conforming to the highest standards, while a post-treatment (e.g., pasteurization) is highly recommended for mesophilic/psychrophilic digestates. Thermophilic conditions lead to higher energy requirements, but this might be balanced out by enhanced biogas productivities [42] and by a safe land application of digestates. Pathogen reduction-wise, two-stage systems are not recommended, as pathogen removal only occurs significantly in the thermophilic stage.

The absence of studies using molecular methods (e.g., quantitative polymerase chain reaction (qPCR)) analyzing pathogen reduction during AD precludes their inclusion in the meta-analysis. This lack of research can be attributed to relevant pathogen-related legislations, which establish culture-based methods as the standard for studying pathogen concentrations in digestates. Despite this limitation, the potential of molecular methods as an alternative to culture-based methods cannot be overlooked. Molecular methods offer the advantage of exploring a wider spectrum of microorganisms, yet they also have the disadvantage of potentially detecting non-viable microorganisms (e.g., free genetic material present in the media). Although the pathogen reduction trend was found to be similar between culture-based and molecular methods in the database (data not shown), it is important to highlight that the LRs observed when qPCR was employed were generally lower (probably due to sequencing of genetic material from dead cells). Further research is needed to extrapolate findings from different methodological approaches to full scale plants.

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

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

either positive or negative, depending on the baseline practices, their evolution, and adherence to regulations. These crucial aspects go beyond the scope of the present study.

Finally, it must be mentioned that, given the lack of data from full scale plants, the results presented here should be extrapolated with caution to large scale installations. The trends concerning the impact of variables such as pH and temperature and/or microbial groups should be similar regardless of the scale. However, results from batch and (semi)continuous reactors might indeed be different already at laboratory, pilot, and industrial scales (results not shown), so it is to be expected that extrapolating LRs from batch laboratory-scale reactors to full scale processes (usually (semi)continuous) will result in overestimations of the reduction capacities (even if allochthonous pathogens were not spiked). As a work based on an analysis of available data, the conclusions from this study are limited by the amount of data that could be gathered, their accuracy, and their repeatability. Similarly, it was not possible to differentiate between specific scenarios, as the amount of data for each case would not be sufficient, leading to biased conclusions.

5. Conclusions

The performed meta-analysis has resulted in novel and relevant conclusions for AD at both research and large scale. The large amount of collected data and the systematic data analysis done have resulted in a global view of the pathogen reduction capacity of AD. When designing experiments to assess AD pathogen reduction performance, artificial pathogen spiking leads to performance overestimation, and thus results cannot be extrapolated to scaled systems. Importantly, current pathogen indicators accurately represent their respective groups. *Clostridiaceae* are barely affected by AD and may be favored by some pre-treatment technologies. Concerning operational parameters, temperature is the parameter that most significantly affects pathogen reduction performance. Thermophilic AD resulted in enhanced pathogen removal, with both psychrophilic and mesophilic conditions resulting in significantly lower performances. The pH also affected pathogen removal, with both acidic and basic values enhancing LRs. This is probably due to a combination of the effect of the pH itself and of the concentrations of inhibitory compounds also affecting pH (e.g., VFAs or NH_3/NH_4^+). An optimal batch duration was identified, but the HRT in (semi)continuous systems did not enhance the overall pathogen reduction, implying that the HRT/OLR values can be set according to the desired methane production rates. Heat-based post-treatments coupled to thermophilic AD resulted in the best pathogen reduction performances. These conditions fulfilled most legislation limits. Further research should focus on multifactorial process optimization, considering the links between different factors (e.g., pH, VFA, and NH_3 concentrations) and developing mathematical models that allow optimization and scenario evaluations. The impact of novel post-treatments allowing resource recovery (e.g., nitrogen stripping, evaporation, or enhanced thermal drying) on overall pathogen removal should also be further studied.

Data availability

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

Appendices

Appendix A: Assumptions considered

- If not specified, room temperature was assumed to be 25 ºC.
- If not specified, mesophilic conditions were assumed to be 35 ºC.

- If not specified, thermophilic conditions were assumed to be 55 ºC.
- If not specified, the type of reactor was assumed to be stirred tank reactor (STR).
- If not specified, the feeding mode was assumed to be semi-continuous.
- Sewage sludge refers to the mixture of primary sludge and waste activated sludge.
- If not specified, sludge was assumed to be sewage sludge.
- If not specified, grams of "dry solids" was assumed to be grams of total solids (TS).
- If not specified, the "reactor volume" was assumed to be the working volume.
- If not specified, CH_4 volume (L) was assumed to be given at standard pressure and temperature (273.15 K and 0.987 atm).
- Except for dry anaerobic digestion (AD), reactors were assumed to be stirred (if not specified).
- If not specified, chemical oxygen demand (COD) concentrations were assumed to be total values (i.e., raw samples).
- When reporting concentrations of TS or volatile solids (VS) as weight for weight (w/w), it was assumed to be equivalent to weight for volume (w/v).
- When studying pre-treatment coupled to AD, the initial TS, VS and COD concentrations pertain to the pre-treated substrate.
- When studying pre-treatment coupled to AD, the initial TS and COD concentrations pertain to the substrate before pre-treatment.
- When studying pre-treatment coupled to AD, the initial VS concentration pertain to the substrate after pre-treatment.
- When heat treatment was performed, the time of treatment represents the time after reaching the desired temperature (without taking into account the heating ramp).
- When the Colony Forming Unit (CFU) value was <X, CFU was assumed to be to be equivalent to X.
- When the CFU value was ≥X, CFU was assumed to be equal to X.
- When microbial concentration was reported as Most Probable Number (MPN), it was assumed to be equivalent to Colony Forming Units (CFU).
- The reporting of N_0 and N values displayed variability, with instances presented on a wet weight basis (CFU/g), on a dry matter basis (CFU/g TS), or in volumetric units (CFU/mL). When possible, the values were converted to CFU/g TS using the TS concentration of the feedstock or digestate. A density of 1 g/mL was assumed for volume/mass conversions.

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