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Exploring anaerobic fermentation stability against a temperature perturbation: process indicators and recovery strategies

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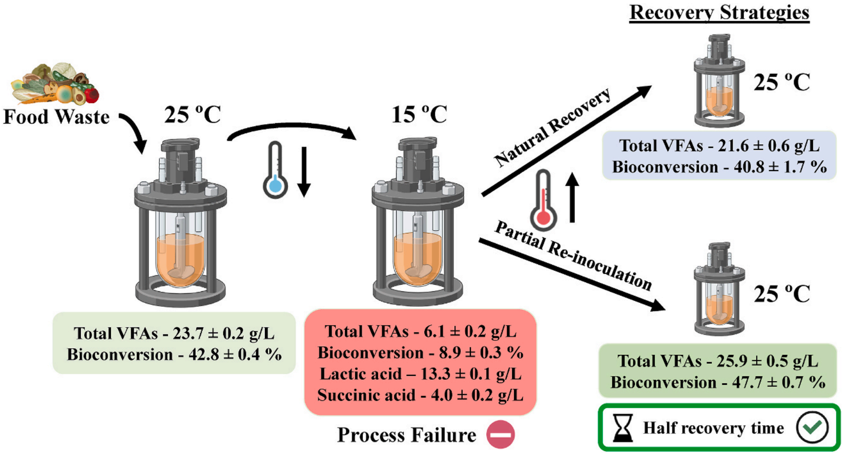
HIGHLIGHTS

- A temperature decrease to 15 °C limited the acidogenesis step.
- Lactic and succinic acids were identified as warning indicators of metabolic damage.
- Anaerobic fermentation was able to recover from a temperature drop to 15 °C.
- Partial re-inoculation boosted the recovery efficiency, reducing its time by half.

GRAPHICAL ABSTRACT



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ABSTRACT

Anaerobic fermentation (AF) efficiency is highly influenced by temperature, and operational disruptions in industrial settings can lead to severe process failure. In this study, AF of food wastes (FWs) was subjected to a temperature drop from 25 °C to 15 °C to elucidate AF behaviour and proper recovery strategies. The temperature decrease caused a marked decline in volatile fatty acids (VFAs) production from 23.7 ± 0.2 to 6.1 ± 0.2 g/L, reducing the bioconversion yields from 42.8 ± 0.4 to 8.9 ± 0.3 %. VFAs-producing pathways shifted toward lactic (HLact, 13.3 ± 0.1 g/L) and succinic (HSu, 4.0 ± 0.2 g/L) acids, indicating partial inhibition of the acidogenesis step. This shift was correlated with the enrichment with HLact-producing bacteria (LAB), namely *Enterococcus*, *Lactobacillus*, and *Lactococcus*. To recover VFAs production levels, two strategies were applied: (i) natural recovery by re-establishing the original operational conditions to 25 °C and (ii) a combined approach of

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re-establishing the operational conditions plus partial re-inoculation of the fermentation broth. While both strategies restored VFAs production and metabolite distribution, the re-inoculation strategy halved the recovery period. These findings underscore the severity of temperature-induced stress in AF of FWs and highlight the importance of effective interventions to recover the performance of a bioprocess under stress. Adopting appropriate countermeasures can effectively mitigate perturbations and safeguard the stability of industrial AF processes.

1. Introduction

Volatile fatty acids (VFAs) are carboxylic acids, commonly used as chemical building blocks in a wide range of industries (e.g. chemical, food-beverage, fragrances, textile). Traditionally, VFAs are produced by petrochemical routes, thereby being non-renewable (Agnihotri et al., 2022). In the last decade, the search for sustainable alternatives to produce those chemicals has led the development of biotechnological processes, such as anaerobic fermentation (AF). This process is a shorter version of the conventional anaerobic digestion (AD), where only the hydrolysis and acidogenesis steps take place while the methanogenesis is inhibited or lacking. In these steps the organic matter is solubilized and then metabolized into organic acids (e.g. VFAs) by hydrolytic and acidogenic bacteria, respectively. By suppressing the metabolic activity of VFAs-consumers (methanogens), the production of VFAs is maximized (Magdalena and González-Fernández, 2020). However, the state of development of this technology is still beyond industrialization, and requires further optimization to implement a commercial production.

To successfully reach industrialization of biotechnological processes, maximizing production yields and decreasing the operational costs is a must. Increasing the robustness of these processes is also a key to cope with possible instabilities caused by unforeseen events, such as equipment malfunctions or seasonal variations. Equipment malfunctions are unavoidable, and troubleshooting takes time. While the process is inactive for recovery, severe economic losses might take place. In the particular case of AF, it has been shown that when operational conditions are disturbed, microbial populations and their metabolisms can change, altering the products yield and consequently leading to a process failure (Gonçalves et al., 2024a). Out of the operational conditions that might affect AF, process temperature is crucial for efficient reactions. The membrane fluidity tends to change accordingly with the temperature, which affects the product release and the substrate uptake by the fermentative bacteria. Additionally, the bacterial enzymatic activity is also dependent on this parameter (Lü et al., 2016). Regardless of the specific enzyme, the enzymatic activity increases concomitantly with the temperature. However, there is a threshold from which, a temperature increase provokes an instability of the enzymes resulting in an inhibition by temperature (Nie et al., 2021). The optimal temperature for an enzyme's activity varies depending on the specific enzyme, being linked to the resistance and adaptation of the organism to those conditions (Marasco et al., 2023). In this sense, the absence of microbial adaptation to low temperature can decrease the enzymatic activity, thereby negatively influencing the solubilization of the organic matter content in feedstocks (Nie et al., 2021). Moreover, low temperatures directly affect bacterial growth as their intracellular reactions are also reduced, disrupting macromolecular interactions (Nie et al., 2021; Wang et al., 2017). To cope with that, fermenters need to be operated at appropriate temperature and avoiding oscillations by ensuring a proper homogenization of the fermentation broth to maintain a uniform temperature.

When a perturbation is maintained for a prolonged period without troubleshooting, the failure of the process requires of recovery strategies to re-establish the AF's metabolisms, restoring the AF efficiencies and product profiles. When the damage of metabolisms is severe, the recovery period might be extremely prolonged, extending the non-productive period of the AF (Gonçalves et al., 2024a). Some strategies can be applied in the AF to speed up the process recovery. While in AF there are not many studies related to the recovery of disturbed processes, most common strategies in conventional AD entail the re-inoculation or bioaugmentation of the fermentation broth (Basak et al., 2021; Zhang et al., 2018). Alternatively, it has been also reported that the addition of trace elements might favour the recovery of the microbial activity (Zhang et al., 2018).

In this context, the novelty of this study was to elucidate the warning indicators of AF failure against a temperature perturbation in systems particularly targeting at VFAs production as valuable compounds, assessing the AF outcomes in terms of chemical features and microbial dynamics. To this end, the present investigation evaluated the effect of a long-term exposure of AF to low temperature (15 °C), mimicking the malfunction of an industrial heating system in cold environments. Furthermore, recovery strategies were also studied to evaluate the recovery capacity of the process after failure to provide a quick and effective re-establishment of the AF, providing solutions to cope with severe conditions caused by unforeseen events.

2. Materials and methods

2.1. Feedstock

The feedstock was a synthetic food waste mixture (SFW) composed of a blended mixture of minced meat (150 g/kg), panned fish (100 g/kg), natural yoghurt (100 g/kg), french fries (200 g/kg), bread (150 g/kg), vegetables (150 g/kg) and red berries (150 g/kg). This feedstock was selected according to the annual average organic waste composition provided by French retailers (Noguer et al., 2022), which allowed to replicate an industrial residue, while simultaneously preventing any compositional variation throughout the experiment. The SFW was stored in small batches at −20 °C to prevent its degradation.

The chemical characterization of the SFW included the analysis of pH, ammonium (NH_4^+-N), total and soluble chemical oxygen demand (TCOD and SCOD, respectively), total and volatile solids (TS and VS, respectively), the VFAs, succinic acid (HSu), ethanol (EtOH), and the percentage of carbohydrates, proteins, lipids and ash (Table 1).

Table 1
Chemical characterization of the SFW utilized as feedstock (mean ± standard deviation).

Parameter	SFW
pH	4.1 ± 0.1
TCOD (g/L)	253.7 ± 2.5
SCOD/TCOD (w/w %)	37.6 ± 1.3
TS (g/L)	233.7 ± 1.4
VS/TS (w/w %)	96.4 ± 0.8
NH_4^+-N (g N/L)	0.10 ± 0.01
HAc (g/L)	1.0 ± 0.1
HLact (g/L)	3.0 ± 0.3
EtOH (g/L)	0.8 ± 0.1
HSu (g/L)	< LD[a]
Ash (w/w %)	3.6 ± 0.1
Lipids (w/w %)	16.5 ± 1.7
Proteins (w/w %)	21.5 ± 2.0
Carbohydrates (w/w %)	58.5 ± 0.7

[a] Lower than the limit of detection (LD).

2.2. Experimental setup

The AFs were performed in continuous stirred tank reactors (CSTRs) with 1.5 L of volume (0.5 L headspace) homogenized with magnetic stirrers (Hei-PLATE Mix 20 L, Heidolph Instruments, Germany). The temperature was regulated by utilizing a water jacket connected to a thermostatic water bath (CORIO CD-200 F, Julabo GmbH, Germany). The CSTR was inoculated with sludge collected from a conventional anaerobic digester located in a wastewater treatment plant (El Soto, Móstoles-Spain). Initially, the CSTR was operated at a pH of 6.5, an organic loading rate (OLR) of 3 g VS/L, hydraulic retention time (HRT) of 20 days and 25 °C. Although studies related to AF normally use short HRTs (<5 days) (Noguer et al., 2022) to promote methanogens wash out, these values also compromise the hydrolytic step when highly particulate wastes are used as feedstock. In this case, methanogenic activity can be limited by combining long HRTs with slightly acidic pH, low temperature and an overloading. These operational conditions were selected according to previous studies demonstrating a high AF efficiency using a similar feedstock (Gonçalves et al., 2024a, 2025). Once the CSTR reached the stability (Control reactor) in terms of AF yields and effluent composition, a long-term temperature perturbation was applied by decreasing the temperature from 25 °C (Control CSTR) to 15 °C (15-CSTR). This perturbation simulated the failure of the heating system of a CSTR in AF of FWs during cold periods (e.g. winter season), which provokes a more severe impact on bioprocess performance due to the decrease in metabolic rates. The temperature range of 25 °C–15 °C was selected to suppress methanogenesis while still allowing hydrolysis and acidogenesis to proceed, thus promoting VFA accumulation. Operating in this range also reduces energy requirements by enabling near-ambient temperature processing, which is more cost-effective. Additionally, these conditions reflect real-world scenarios where equipment malfunctions are common challenges faced by plant operators on a daily basis. 15 °C was selected and maintained until AF exhibited stability to evaluate the AF response to low temperature and the severity of the metabolisms damage. Thereafter, process temperature was re-stablished to 25 °C and two recovery strategies were tested: (i) natural recovery with no further modifications (CSTR-R1) and ii) re-inoculation of the 15 % (v/v) of CSTR (CSTR-R2) with fresh anaerobic sludge collected from an AD reactor of a wastewater treatment plant (El Soto, Móstoles, Spain). The fresh anaerobic sludge utilized to re-inoculate the CSTR-R2 was the same one used as inoculum in the reactor start-up, without applying any conditioning step (e.g. pretreatment or dilution). The AF was considered to be recovered when the bioconversion efficiencies and the metabolites profile reached similar values in comparison with the control CSTR.

The performance of the AFs was evaluated in terms of particulate organic matter conversion into soluble one (hydrolysis efficiency, Eq. (1)), the content of soluble organic matter (SCOD) converted into VFAs (acidification percentage, Eq. (2)), the global AF efficiency by calculating the bioconversion yield of the total organic matter (TCOD) into VFAs (Eq. (2)), and the organic matter conversion into biogas as COD removal (Eq. (4)) using the following equations:

$$\mathrm{VS}_{\mathrm{removal}}(\%) = \left(VS_{in} - VS_{eff}\right) / VS_{in} \times 100 \quad \text{(Eq. 1)}$$

$$\text{Acidification } (\%) = COD_{VFAs\ eff} / SCOD_{eff} \times 100 \quad \text{(Eq. 2)}$$

$$\text{Bioconversion yield } (\%) = COD_{VFAs\ eff} / TCOD_{in} \times 100 \quad \text{(Eq. 3)}$$

$$\mathrm{COD}_{\mathrm{removal}}\ (\%) = \left(TCOD_{in}\ -\ TCOD_{eff}\right) / TCOD_{in} \times 100 \quad \text{(Eq. 4)}$$

In the equations, the $\mathrm{COD}_{\mathrm{VFAs\ eff}}$ represented the COD equivalent of the concentration of the VFAs in the effluent: acetic acid (HAc), propionic acid (HPro), butyric acid and its isomeric form (HBu and isoHBu, respectively), valeric acid and its isomeric form (HVal and isoHVal, respectively), and caproic acid (HCa). The COD equivalent for each acid was stoichiometrically calculated and corresponded to 1.067, 1.513, 1.820, 2.039 and 2.207 for HAc, HPro, n-HBu, n-HVal and HCa, respectively. The $\mathrm{TCOD}_{\mathrm{in}}$ was the total COD (g COD/L) present in the feedstock fed to the CSTR, while the $\mathrm{TCOD}_{\mathrm{eff}}$ and the $\mathrm{SCOD}_{\mathrm{eff}}$ represented the COD content present in the total and soluble fraction of the CSTR effluent, respectively. The $\mathrm{VS}_{\mathrm{in}}$ represented the VS of the feedstocks fed in the CSTR and the $\mathrm{VS}_{\mathrm{eff}}$ was the VS content in the CSTR effluent (g VS/L).

2.3. Analytical methods

The procedures described in Standard Methods (APHA et al., 2017) were utilized to determine the contents of TS, VS, ash and total Kjeldahl nitrogen (TKN) in the feedstocks. The concentration of $\mathrm{NH_4^+}$-N and, TCOD and SCOD were analyzed using commercial kits (ISO 000,683 and ISO 15705, respectively). To perform the analysis of the $\mathrm{NH_4^+}$-N and SCOD, the samples were filtered by 0.45 μm filters in order to obtain the soluble fraction. Carbohydrates of the SFWs were determined following the phenol-sulfuric method (Dubois et al., 1956) and the protein content was calculated by multiplying the TKN by the nitrogen-protein conversion factor of 6.25 (González-López et al., 2010). Lipid content (w/w) was calculated by the difference between 100 and the percentage of carbohydrates, proteins and ash. The pH was measured daily using a pH meter (GLP 21, Crison, Hach Lange) and adjusted to 6.5 by adding NaOH (5 M).

The AFs effluents were monitored twice a week by analysing the TCOD, SCOD, TS, VS and $\mathrm{NH_4^+}$-N following the methods described above. The concentration of metabolites (VFAs, HLact, HSu and EtOH) was analyzed by high-performance liquid chromatography (1260 HPLC, Agilent). The HPLC was equipped with a pre-column and an ion exclusion column (Cation H Refill Cartridge Microguard column, Biorad and Aminex HPX-97-H 300 × 7.8 mm, I. D., Biorad, respectively) and a refractive index detector. The mobile phase, flow rate and temperature of the HPLC's oven and detector were set as described by Gonçalves et al. (2024b). In order to maintain the integrity of the columns and of the HPLC, the samples were filtered by 0.22 μm. The composition of the biogas samples was analyzed twice a week through gas chromatography (GC) equipped with a thermal conductivity detector (Claurus 580 GC, PerkinElmer) and two coupled packed columns (HSN6-60/80 Sulfinert P 7′ × 1/8″ O.D. and MS13X4-09SF2 40/60 P 9′ × 1/8″ O.D., PerkinElmer). The temperature of the injector, detector and over were 80, 200 and 62 °C, respectively, and helium was used as a gas carrier.

2.4. Microbial community analysis

In order to assess the effect of a temperature decrease to 15 °C and each recovery strategy on the microbial community composition, the microbiome was characterized at the control reactor, at the steady state of 15-CSTR and after each recovery strategy (CSTR-R1 and CSTR-R2). The identification of the microorganisms was performed by analysing the 16S rRNA gene. DNA was extracted from 1 mL samples by using a commercial kit (FastDNA SPIN Kit for Soil, MP Biomedicals, LCC) and its quantity and quality were determined using nanodrop (Omega Spectrostar BMC Labtech). The amplification of the hypervariable regions V3 and V4 of the 16S rRNA gene was performed using the primers 341 F (F – CCTACGGGNGGCWGCAG) and 805R (R – GACTACHVGGGTATCTAATCC) to identify the bacterial and archaeal populations. The DNA sequencing was performed using a MiSeq (Illumina) sequencer by FISABIO (Valencia, Spain). The raw sequences were processed bioinformatically following the guidelines described by Greses et al. (2017).

3. Results and discussion

3.1. AF response against long-term temperature perturbation

Once the Control CSTR reached the stability, the AF exhibited a total

VFAs accumulation of 23.7 ± 0.2 g/L, which corresponded to a bioconversion yield of 42.8 ± 0.4 %. Moreover, a hydrolysis and acidification percentages of 42.5 ± 0.5 % and 97.1 ± 4.0 % were attained, respectively (Table 2). These results were in line with the high AF yields observed in the literature when carbohydrate-rich FWs were subjected to AF under slightly acidic pHs and operating at 25 °C, evidencing the high metabolic activity of the fermentative bacteria at those operational conditions. The slightly acid pH was in the optimal range (5.5–6.5) for fermentative bacteria, whereas methanogenic activity may be limited. As a matter of fact, low temperature (25 °C) mainly affected to slow-growing microorganisms (methanogenic archaea) since fermentative one exhibits higher robustness (Greses et al., 2022a). These operational conditions allowed to limit methanogenic metabolisms and boost acidogenic ones, thereby maximizing the acidification yield. Furthermore, the composition of the SFW also contributed to reach high AF efficiencies due to the high content of readily biodegradable carbohydrates (58.5 ± 0.7 %) that can be easily converted into VFAs via direct glycolysis. For instance, Greses et al. (2022b) found a VFAs production of 29.7 ± 0.4 g/L corresponding to a bioconversion yield of 41.5 ± 0.4 % when a carbohydrate-rich FWs (77.7 ± 2.4 %) was subjected to AF at a pH of 5.8 ± 0.1 and 25 °C. Similarly, Gonçalves et al. (2024a) reached a 29.2 g/L of VFAs production (48.1 and 92.7 % of bioconversion and acidification yields, respectively) in the AF of a carbohydrate-rich FWs (63.3 ± 5.4 % w/w) at 25 °C and a pH of 6.2. Thus, the high efficiencies obtained herein confirmed the effectiveness of the operational conditions to promote VFAs production when carbohydrate-rich waste is used as feedstock.

Besides, the conditions also hampered the methanogenesis activity in the control CSTR, which was evidenced by the low $COD_{removal}$ observed (11.5 ± 3.9 %) together with the absence of methane in the gas stream. While acidic pHs and longer HRTs promoted the efficiency of the fermentative stage, the combination of those conditions with 25 °C also favoured the inhibition of the methanogenesis step and, consequently, the VFAs consumption.

Regarding the VFA distribution, the conditions applied in Control CSTR promoted the production HAc and HBu, which together accounted for over 63 % of the total metabolites profile (Fig. 1, S1). The VFAs distribution with the predominance of even carbon chain VFAs might be correlated with the high abundance of carbohydrates in the feedstock (58.5 ± 0.7 % w/w). Similar metabolite profiles were previously found when carbohydrate-rich FWs were subjected to AF under similar conditions, confirming the correlation between even carbon chain VFAs production with the high carbohydrate content in feedstock (Aboudi et al., 2023; Greses et al., 2021, 2022b).

Control, 15-CSTR, CSTR-R1 and CSTR-R2 represent the periods of the Control CSTR, the end of the fermentation under 15 °C, the end of the natural recovery and the end of the re-inoculation recovery, respectively.

Table 2
Chemical characterization of the AF effluents collected at the steady state of each period studied.

	Control CSTR	15-CSTR	CSTR-R1	CSTR-R2
pH	6.4 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	6.4 ± 0.1
TCOD (g/L)	74.7 ± 3.8	73.2 ± 2.8	73.2 ± 2.7	74.3 ± 2.6
SCOD/TCOD (%)	49.6 ± 5.1	49.3 ± 1.9	49.3 ± 1.5	51.9 ± 2.3
TS (g/L)	53.2 ± 1.0	51.4 ± 0.8	47.7 ± 1.1	55.1 ± 0.8
VS/TS (%)	65.4 ± 0.7	62.8 ± 0.1	61.9 ± 0.1	62.4 ± 0.2
NH_4^+-N (g N/L)	0.24 ± 0.01	0.02 ± 0.01	0.18 ± 0.01	0.21 ± 0.02
Total VFAs (g VFAs/L)	23.7 ± 0.2	6.1 ± 0.2	21.6 ± 0.6	25.9 ± 0.5
Bioconversion (%)	42.8 ± 0.4	8.9 ± 0.3	40.8 ± 1.7	47.7 ± 0.7
Acidification (%)	97.1 ± 4.0	28.1 ± 1.0	96.9 ± 3.5	99.0 ± 4.0
$VS_{removal}$ (%)	42.5 ± 0.5	46.7 ± 0.5	51.6 ± 2.1	43.3 ± 1.0
$COD_{removal}$ (%)	11.5 ± 3.9	13.9 ± 2.4	14.6 ± 2.4	9.4 ± 1.7

When the AF was subjected to a temperature drop from 25 °C to 15 °C, the VFA production decreased to 6.1 ± 0.2 g/L, corresponding to a bioconversion yield of 8.9 ± 0.3 %. Although the literature regarding the application of cold perturbations in AF is scarce, previous studies related to conventional AD observed a decrease in the VFA accumulation and biogas when decreasing the temperature from 25 °C to 15 °C. Komemoto et al. (2009), confirming the effect of a low temperature on both methanogenesis and acidogenesis steps. Additionally, together with the bioconversion yields, the acidification percentage also decreased over time down to 28.1 ± 1.0 % when the reactor reached stability. The total decrease in the AF yields evidenced severe damage to the acidogenic activity in the reactor. This trend was in accordance with the fact that low temperatures can decrease metabolic rates, and lead to inefficient acidogenesis (Akindolire et al., 2022). In this regard, several enzymes involved in VFAs production have been identified as sensitive to low temperatures, such as acetate kinase, phosphotransacetylase and butyryl-CoA dehydrogenase (Vigil et al., 2023; Yuan et al., 2016; Zheng et al., 2025). Hence, the reduced enzymatic activity might hamper the acidogenic metabolisms, ultimately leading to a decrease in VFAs production yields. Surprisingly, the hydrolysis efficiencies of the fermentation were not affected (46.7 ± 0.5 %, Table 2). This indicated that acidogenic bacteria were sensitive to low temperatures, while the hydrolytic ones presented high robustness to this condition, maintaining their activity. This behaviour was unexpected as the hydrolysis step is normally considered the bottleneck of bioprocesses when highly particulate organic residues are used as feedstock (Menzel et al., 2020). These results suggested that operating AF at optimal pH for hydrolysis can offset the effect of a temperature decrease on the enzymatic activity. The composition of SFW also played a key role since the particulate organic matter presented a high content of biodegradable carbohydrate, which reduced the complexity related to the hydrolytic step. However, the hydrolysis of each macromolecular compound (carbohydrate, protein and lipid) was altered when compared to Control CSTR. The nitrogen fate revealed that the proteolytic activity (hydrolysis of proteins) was affected. During the microbial degradation of nitrogen-rich organic matter (protein-rich feedstocks), the organic nitrogen present in the feedstocks is converted into inorganic forms (e.g. NH_4^+) (Hagemann et al., 2016), allowing to evaluate the proteolytic activity by following the accumulation of NH_4^+. When the reactor was operated at 15 °C, NH_4^+-N concentration decreased from 0.24 ± 0.01 (at 25 °C) to 0.02 ± 0.01 g/L, which evidenced a decrease in protein degradation (Bareha et al., 2018). The high hydrolysis efficiencies of the AF (46.7 ± 0.5 % VS removal) at this point indicate that the hydrolytic bacteria followed the carbohydrate degradation routes. Since the simple carbohydrates present in FW are easier to degrade than proteins, favouring this hydrolytic route suggested a resilience mechanism of the bacteria to overcome adverse conditions. Previous studies suggested that, in order to survive and acclimate to stressful environments, microorganisms tend to follow the most energetically advantageous metabolisms (Gonzalez and Aranda, 2023).

The metabolites profile was also severely influenced by the temperature decrease. HAc became the predominant VFA, comprehending 74.0 ± 2.8 % (w/w) of the total VFA pool. The failure of the VFAs-producing AF was also linked to the production of HLact and HSu (13.3 ± 0.1 and 4.0 ± 0.2 g/L, respectively) (Fig. 1, S2). HLact and HSu are intermediate metabolites in various metabolisms devoted for the production of VFA. The high accumulation of HLact during the conventional AD of FWs under 15 °C was previously reported by Komemoto et al. (2009), who detected the depletion of HLact when the temperature increased from 15 °C to 25 °C with a concomitant production of VFAs. Thus, the similar AF behaviour observed herein, in terms of VFA profile change, confirmed the acidogenic step limitation. Likewise, HSu is an intermediate metabolite of the succinate pathway for the production of HPro (Oliphant and Allen-Vercoe, 2019). Following this metabolism via two distinct pathways (the Wood-Werkman cycle or the sodium pumping pathway), HSu is consumed to ultimately produce HPro (Gonzalez-Garcia et al., 2017). The simultaneous accumulation of HSu

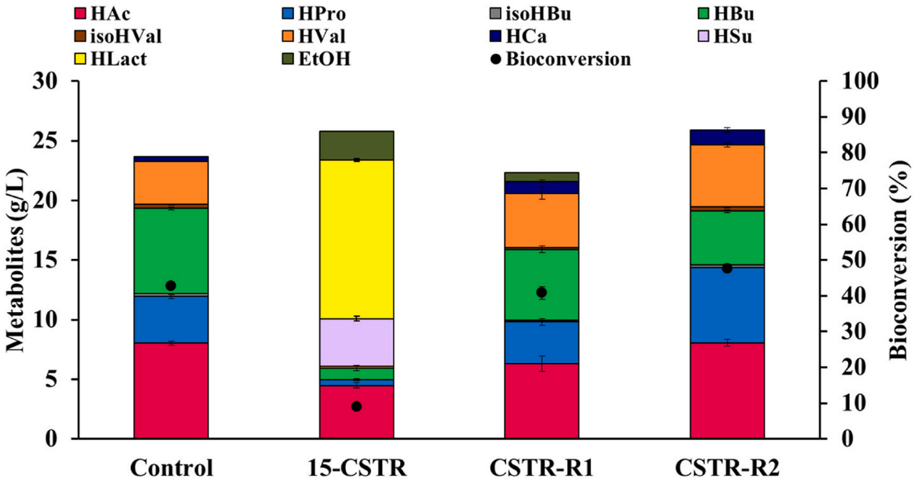

Fig. 1. – Metabolites distribution at the steady state of each period of the experiment.

and the absence of HPro production in the AF evidenced the acidogenesis perturbation, resulting in the partial inhibition of the VFAs production metabolism. The accumulation of these metabolites was previously observed in AF of FWs subjected to alternative stressful perturbations (Gonçalves et al., 2024b, 2025). Therefore, these metabolites (HLact and HSu) can be identified as warning indicators of AF damage, which can help detecting and troubleshooting an operational perturbation before reaching a non-return threshold.

3.2. Effect of the recovery strategies on the process efficiencies and product outcome

To face the AF failure due to a decrease in the operational temperature to 15 °C, two different recovery strategies were applied (CSTR-R1 and CSTR-R2). The development of an efficient recovery strategy is of major importance in a continuous biotechnological production since it will reduce the recovery periods, minimizing the non-active period of the process. The CSTR-R1 (natural recovery in which temperature was restored to 25 °C) reached a total VFA concentration of 21.6 ± 0.6 g/L (40.8 ± 1.7 % of bioconversion yield), while the CSTR-R2 (15 % v/v re-inoculation) achieved 25.9 ± 0.5 g/L (47.7 ± 0.7 % of bioconversion yield) (Table 2, Fig. 1). Although both recovery strategies exhibited high acidogenic bacterial activity (Table 2), the recovery time was remarkably shorter in CSTR-R2 than CSTR-R1 (Fig. S3). The CSTR-R1 lasted 24 days and the CSTR-R2 strategy only required 12 days. These results indicated that CSTR-R2 strategy ensured high acidogenic activity by re-seeding fermentative bacteria without metabolic perturbation, as those microorganisms had not been exposed to harsh stressing conditions. As a matter of fact, a slight increase in bioconversion yield was also observed when CSTR-R2 was compared to CSTR-R1 and Control CSTR (Table 2), confirming the benefits of replacing 15 % of the microbiome with highly active fermentative bacteria. Although both strategies were able to recover the AF to similar values than the ones observed at the control CSTR, this study evidenced the advantages of partially re-inoculating the AF to replace the microbiome, when AF was subjected to a temperature decrease perturbation. As AD microbiome presents the common metabolisms required to perform AF, the decline in metabolic rates due to low temperature can be addressed following this strategy. However, severe AF perturbations related to toxics or inhibitors presence should be specifically evaluated to find the most proper approach.

In terms of VFAs composition, the profiles were similar in both CSTRs at the steady state of the AF after the recovery strategies. However, when compared to Control CSTR, long-chain VFAs slightly increased in the reactors subjected to both recovery strategies. In this regard, HVal increased from 15.6 % (Control CSTR) to 21.0 % and HCa from 1.4 % to 4.9 % (Fig. 1). The depletion of HLact from the metabolites pool and the restoring of the VFAs production indicated that HLact was metabolized by secondary fermenters in the VFA-producing pathways. Furthermore, HLact can be also an intermediate in the HVal and HCa metabolisms (Dong et al., 2023; Veras et al., 2020), suggesting that HLact depletion has promoted those pathways, increasing the production of the longer VFAs. Indeed, the consumption of HSu, an intermediary of HPro production metabolism, together with the accumulation of HVal, suggested the re-establishment of a propionate metabolism from the succinate pathway (Oliphant and Allen-Vercoe, 2019).

3.3. Microbial community behaviour against temperature drop and AF recovery

In order to better understand the AF response against temperature perturbation and the recovery strategies, the microbial populations were characterized at the steady state of each period. The microbial distribution at the steady state of the control CSTR was specialized in degrading organic matter for VFAs production. Specifically, at phylum level, the microbial populations were dominated by Firmicutes (52.2 % relative abundance), Bacteroides (34.1 %) and Actinobacteria (11.5 %) (Fig. 2a).

Firmicutes is a versatile phylum, composed of several hydrolytic and acidogenic bacteria, usually found in the AF of carbohydrate-rich feedstocks for VFAs production (Greses et al., 2022b; Li et al., 2019), such as Clostridiales (26.3 %), *Bulleidia* (6.6 %) and Lachnospiraceae (4.8 %). Clostridiales have been previously associated with the hydrolysis of complex carbohydrates and the fermentation of simple sugars into HAc, HPro and HBu via glycolysis (Blasco et al., 2020; Tracy et al., 2012). The genera *Bulleidia*, and the bacteria belonging to Lachnospiraceae (Fig. 2b) have been also described as sugars fermenters, which are capable to transform organic matter into VFAs (Gulhane et al., 2017; Yin et al., 2022). The phylum Bacteroides was mostly composed of the genus *Bacteroidetes* (32.6 %) which have been identified as degraders of complex carbohydrates and proteins into HAc and HPro (Tukanghan et al., 2021).

The presence of bacteria with high hydrolytic and acidogenic activity justified the high AF performance in terms of hydrolysis efficiency (42.5 ± 0.5 % $VS_{removal}$) and bioconversion yield (42.8 ± 0.4 %), confirming the proper selection of the operational conditions to promote VFAs production in Control CSTR. Moreover, LAB belonging to the genus *Lactobacillus* (5.7 %) and the family Bifidobacteriaceae (6.6 %) were also identified, which have been found as HLact and HAc producers from the AF of glucose and fructose (Castillo-Martinez et al., 2013). Their presence and the lack of HLact confirmed the total conversion of the primary metabolites (HLact) into VFAs, resulting in a well-performed AF.

The complete change observed in the microbiome in 15-CSTR with

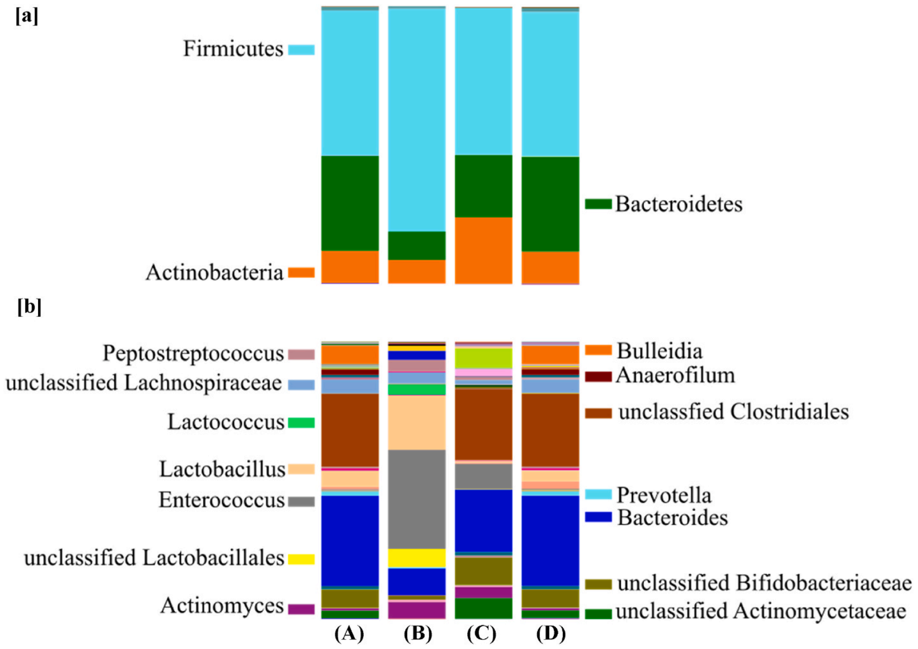

Fig. 2. - Relative abundance of microorganisms identified throughout the experiment at [a] phylum and [b] genus level ((A) Control CSTR, (B) 15-CSTR, (C) CSTR-R1, (D) CSTR-R2). Microorganisms with relative abundance lower than 1 % have been excluded from the plot legend.

regard to Control CSTR (Fig. 2) supported the metabolite profile shift detected, corroborating the bioprocess perturbation when imposed 15 °C. Regarding the microbiome collected in the 15-CSTR, Firmicutes also dominated at phylum level, comprehending 80.4 % of the total microbiome. However, the bacterial composition of Firmicutes changed (Fig. 2b) since the abundance of the acidogenic bacteria belonging to the order Clostridiales sharply decreased and the phylum became dominated by LAB. The genera *Enterococcus* (35.8 %), *Lactobacillus* (19.5 %), *Lactococcus* (3.8 %), and the order Lactobacillales (6.5 %) comprised over 65 % of the Firmicutes population. The genus *Enterococcus* has been described to be able to ferment complex carbohydrates into HLact, HAc and HBu (Ramsey et al., 2014). *Lactobacillus*, *Lactococcus* and bacteria belonging to Lactobacillales shared similar metabolisms, being also able to produce HLact from homolactic or heterolactic pathways (Castillo-Martinez et al., 2013; Feng et al., 2018; Sant'Ana et al., 2017). The dominance of these bacteria justified the high concentrations of HLact determined at the steady state of the 15-CSTR. The presence of HAc (Fig. 1) was correlated with the heterolactic fermentation, performed by most of the LAB present in the reactor (Castillo Martinez et al., 2013). The dominance of these bacteria was related to their higher resistance to stress conditions than other acidogenic bacteria commonly found in AF microbiomes (Akindolire et al., 2022; Gonçalves et al., 2024a). As a matter of fact, these bacteria have been reported for their cellulase-producing capacities, advantageous for the degradation of complex carbohydrates (Zhao et al., 2021). The dominance of LBA corroborated the decrease of the proteolytic activity in the AF, which was indicated by the remarkable reduction of NH_4^+-N production (Table 2).

With regard to Bacteroidetes, this phylum decreased from 32.6 % (Control CSTR) to 9.7 % (15-CSTR). Considering that Bacteroidetes was mainly composed of proteolytic bacteria (*Bacteroides*), this bacterial decrease also confirmed the reduction of the proteolytic activity in the AF, thereby limiting the protein degradation in the 15-CSTR. Additionally, the accumulation of HSu also evidenced the metabolic perturbation of this genus. *Bacteroides* have been found as fermenter of organic matter into HPro, following the succinate pathway (den Besten et al., 2013). Taking into account that HSu is an intermediate metabolite of this pathway, the accumulation of HSu in 15-CSTR denoted the partial inhibition of this metabolism. Indeed, previous studies also observed the accumulation of HSu when *Bacteroides* was subjected to stressful conditions. For instance, Gonçalves et al. (2024b) observed the accumulation of HSu when the AF of FWs at 25 °C was subjected to a pH shock (decrease from 6.1 to 4.5), associating the AF response to *Bacteroides* perturbation. Similarly, Ikeyama et al. (2020) concluded that *Bacteroides* under stressful conditions led to the downregulation of transporters for the vitamin B_{12}, responsible for the conversion of HSu to HPro.

When the recovery strategies were applied to the 15-CSTR, the microbial populations tended to recover back to the composition identified in the Control CSTR. The CSTR-R1 re-established the abundance of major contributors to AF, namely Clostridiales, *Bacteroides*, and Bifidobacteriaceae (Fig. 2b). As aforementioned, these bacteria were responsible for the high VFAs accumulation, which was corroborated by the reestablishment of the VFAs production efficiencies. Furthermore, the LAB presence considerably decreased when compared to 15-CSTR. The increase in acidogenic bacteria and non-growth of LAB were crucial to recover the AF yields to those values attained in the Control CSTR. The success of this recovery strategy (CSTR-R1) can be confirmed by the VFA profile attained since all the intermediate metabolites (HLact and HSu) were consumed (Fig. S3b). When observing the microbial populations developed at the steady state CSTR-R2, the microbiome was similar to the consortium obtained for the control CSTR (Fig. 2). The abundance of Clostridiales, *Bacteroides* and Bifidobacteriaceae increased, justifying the high AF efficiencies. Furthermore, the increase in *Bacteroides* to their initial abundance (similar to Control CSTR) supported the high hydrolytic activity in CSTR-R2. Specifically, the increase in the proteolytic activity detected by the increase of NH_4^+-N, which was mainly related to the presence of *Bacteroides* (Tukanghan et al., 2021). Therefore, bacteria belonging to the order Clostridiales, the genus *Bacteroides* and the family Bifidobacteriaceae can be associated with optimal acidogenic performance, being the main bacteria recovered from the fresh anaerobic sludge used in the re-inoculation strategy. Likewise, HSu and HLact were identified as warning indicators of AF perturbation, being LAB dominance as microbial indicator of AF failure. Although setting back the temperature to 25 °C promoted a functional VFA-producing consortium, the strategy entailing the re-inoculation of the fermentation broth (15 % v/v) was proven to be more effective than CSTR-R1 the recovery period was considerable reduced. This improvement was due to the addition of bacteria of interest that were not subjected to harsh conditions.

4. Conclusions

This investigation examined the effects of a long-term temperature perturbation on AF of FWs, leading to partial inhibition of VFA production and accumulation of HSu and HLact. These intermediate metabolites served as reliable early indicators of AF damage. From the tested recovery strategies, both approaches successfully restored process stability, but re-inoculation significantly reduced the recovery time due to the reintroduction of critical bacterial populations. The findings emphasize the importance of maintaining stable conditions in AF and highlight the advantage of identifying intermediate metabolites as warning indicators for process perturbations. This approach could serve as a practical intervention to enhance the robustness of AF in diverse applications.

Ethics of experimentation statement

Not applicable.

CRediT authorship contribution statement

Manuel João Afecto Gonçalves: Writing – original draft, Investigation, Data curation. **Cristina González-Fernández:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization. **Silvia Greses:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Disclosure statement

The authors report there are no competing interests to declare.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2025.144669.

Data availability

Data will be made available on request.

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