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# Continuous lactate-driven dark fermentation of restaurant food waste: Process characterization and new insights on transient feast/famine perturbations

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## Abstract

The effect of hydraulic retention time (HRT) on the continuous lactate-driven dark fermentation (LD-DF) of food waste (FW) was investigated. The robustness of the bioprocess against feast/famine perturbations was also explored. The stepwise HRT decrease from 24 to 16 and 12 h in a continuously stirred tank fermenter fed with simulated restaurant FW impacted on hydrogen production rate (HPR). The optimal HRT of 16 h supported a HPR of 4.2 L $H_2$/L-d. Feast/famine perturbations caused by 12-h feeding interruptions led to a remarkable peak in HPR up to 19.2 L $H_2$/L-d, albeit the process became stable at 4.3 L $H_2$/L-d following perturbation. The occurrence of LD-DF throughout the operation was endorsed by metabolites analysis. Particularly, hydrogen production positively correlated with lactate consumption and butyrate production. Overall, the FW LD-DF process was highly sensitive but resilient against transient feast/famine perturbations, supporting high-rate HPRs under optimal HRTs.

## Keywords:

Acidogenic fermentation, Biogenic hydrogen, Hydraulic retention time, Organic waste, Process disturbances, Robustness.

## 1. Introduction

Renewable hydrogen produced from biomass via biochemical conversion processes is gaining momentum in the European Union and beyond, since it fosters the decarbonisation of energy-intensive industrial processes and the transition towards a circular economy (European Commission, 2020). Hydrogen can not only be used as a clean energy carrier, but it can also be an industrial feedstock (European Commission, 2020; Dawood et al., 2020). Dark fermentation (DF) stands out as the most promising biological method to produce renewable hydrogen, owing to its potentially higher hydrogen production yields (HY) and rates (HPR), and good flexibility in using a wide range of different organic wastewaters and wastes (Cheng et al., 2022). In this context, food waste (FW) has attracted an increasing attention as a biorefinery feedstock due to its year-round availability and physicochemical features, conferring it a high potential for producing added-value compounds and energy carriers including hydrogen via microbial fermentation processes (Zabaniotou & Kamaterou, 2019; Battista et al., 2020; Talan et al., 2021; Shanmugan et al., 2023)). To date, one third of the food produced worldwide ends up as FW, which in turn causes serious environmental, social and health issues (Scherhaufer et al., 2018; Fattibene et al., 2020; Shanmugan et al., 2023). In the European Union, this figure accounted for 56.8 million tons in 2020, corresponding to about 127 kg of FW generated per inhabitant (Eurostat, 2022). The North America, Latin America and Caribbean, sub-Saharan Africa and Asia-Pacific regions generate yearly about 168, 127, 232 and 465 million tons of FW, respectively (García-Depraect et al., 2023b).

Although DF has long been proven as a feasible technology to produce hydrogen from FW (Yun et al., 2018; Habashy et al., 2021), its development and further scale up is still limited by several technical bottlenecks. Of them, the inhibition of hydrogen production caused either directly or indirectly by the overgrowth of lactic acid bacteria (LAB) in DF systems remains one of the most prevalent and deleterious issues (García-Depraect et al., 2021). Indeed, the proliferation of LAB has been considered the main cause of process collapse in the long-term operation of continuous DF processes. LAB often outcompete hydrogen-producing bacteria (HPB) due to their wider capacity to degrade complex substrates and because they can release antimicrobial compounds (Sikora et al., 2013; García-Depraect et al., 2021). Besides, LAB such as *Lactobacillus* and lactate-oxidizing-HPB such as *Clostridium butyricum,* share similar physical and chemical growth requirements, thus it is quite challenging to keep LAB away from the DF process by modulating process parameters such as the hydraulic retention time (HRT), organic loading rate (OLR), temperature, and pH (García-Depraect et al., 2021). Another aspect contributing to the common occurrence of LAB in dark fermenters is their ubiquitous nature, being part of the autochthonous microbiota of many organic feedstocks including FW. Indeed, the microbial community structure of FW is typically dominated by LAB during its storage (García-Depraect et al., 2023b).

Unfortunately, the effective elimination of LAB from the DF of FW still presents serious technical and economic issues, since pre-treatments intended to that purpose (heat shock for instance) are not only typically costly but have also been shown to be impractical and inefficient (Villanueva-Galindo et al., 2023). In this context, lactate-driven DF (LD-DF) is recently attracting scientific attention as a platform able to produce hydrogen from FW, while tackling the overgrowth of LAB (Regueira-Marcos et al., 2023). Although LD-DF is seen as a non-conventional hydrogen-producing pathway, its prevalence in dark fermenters seems thermodynamically efficient, which is desirable in shaping stable microbial communities in the long run operation (Fuess et al., 2018). It has been argued that the long-lasting functional ecosystem in LD-DF is driven by syntrophic (cross-feeding) interactions between LAB and some HPB, in which the former microbial group ferment carbohydrates into lactate, while the latter transforms lactate into hydrogen (García-Depraect et al., 2021). This process would benefit both microbial groups, as lactate can be consumed by certain HPB avoiding deleterious LAB growth by product inhibition, while preventing competition between LAB and HPB for the fermentable carbohydrates (Park et al., 2021; Pérez-Rangel et al., 2021; Ohnishi et al., 2022). Despite the potential of this syntrophic microbial association to boost hydrogen production, the fate of lactate in the DF process is usually overlooked in most studies (Detman et al., 2019; García-Depraect et al., 2021; Ohnishi et al., 2022). In the case of LD-DF of FW, this has not been extensively investigated, thus many relevant insights remain unrevealed yet. To the best of the authors' knowledge, the process performance of the LD-DF of FW under continuous operation has not been systematically investigated yet. In continuous DF systems, HRT is likely the most critical process parameter determining the hydrogen production efficiency, in terms of HY and HPR (Sivagurunathan et al., 2016).

Hence, this work investigated for the first time the influence of the HRT on the continuous LD-DF of simulated restaurant FW. Additionally, the robustness and resilience of this novel LD-DF technology was assessed by characterizing process response to short-term feast/famine perturbations, which are foreseen to occur in full scale DF facilities and might compromise hydrogen production. The results and discussion of this study can be helpful in the development and deployment of enhancement strategies aimed at harnessing the presence of LAB in dark fermenters.

## 2. Materials and Methods

### 2.1 Microbial inoculum and feedstock

The inoculum source was digestate collected from a well-performing 100-L anaerobic digester fed with restaurant FW and operated under mesophilic conditions. A hydrogen-producing mixed culture was obtained by applying a heat shock pretreatment (90 °C for 20 min) followed by three consecutive subcultures, following the procedure reported by Martínez-Mendoza et al. (2022). The resulting acidogenic culture was composed of *Lactobacillus*, *Klebsiella*, *Clostridium*, *Stenotrophomonas*, *Acinetobacter*, among others (Regueira-Marco et al., 2023). Then it was stored at 4 °C and used on-demand as inoculum for LD-DF (Garcia-Depraect et al., 2022; Regueira-Marcos et al., 2023). Prior to use and with the aim of reactivating microorganisms, the bacterial inoculum was subjected to a 17-h fermentation step using a 2.1-L gas-tight anaerobic reactor filled with 0.1 L of inoculum and 0.9 L of a growth medium composed of (in g/L): 10.0 lactose, 2.40 $NH_4Cl$, 2.4 $K_2HPO_4$, 1.18 $MgCl_2$, 0.60 $KH_2PO_4$, 0.11 $CaCl_2$, and 0.024 $FeCl_2$ (Martínez-Mendoza et al., 2022). The incubation conditions applied were 37 ± 1 °C and ≈ 200 rpm.

The feedstock herein used was simulated FW prepared according to Neves et al. (2008), which mimics the composition of restaurant-derived FW. That formulation included 78% potatoes, 14% chicken, 4% pork lard, and 4% cabbage (on wet weight basis), as the source of carbohydrate, protein, lipid, and fiber, respectively. Potatoes and chicken breast were boiled in an autoclave at 120 ºC for 30 min to simulate cooked FW. The FW was initially blended by using a commercial blender (Sammic, XM-32, Spain) and subsequently stored at -20 °C to prevent any change in its composition. The microbial community structure of the recipe-based FW used has been reported previously by García-Depraect et al. (2023b). The blended FW was physiochemically characterized as follows: 51.0 ± 0.5% carbohydrates, 22.3 ± 1.6% proteins, 17.6 ± 1.5% lipids, and 4.0 ± 0.4% ash (on dry basis). Moreover, the blended FW had a pH of 6.2 ± 0.1, a chemical oxygen demand (COD) content of 286 ± 21.9 g $O_2$/kg, and a total (TS) and volatile (VS) solids content of 215.6 ± 1.7 g/kg and 207.0 ± 1.6 g/kg, respectively. Finally, the elemental analysis of the FW blend showed the following composition: 51.2 ± 0.2% carbon, 8.1 ± 0.1% hydrogen, 35.6 ± 0.9% oxygen, 3.4 ± 0.1% nitrogen, 2.0 ± 0.0% phosphorus and a negligible content of sulphur.

**2.2 Experimental set-up**

Continuous hydrogen production via LD-DF was performed in a 1.2-L continuously stirred tank reactor (CSTR) with 0.8 L of working volume. The body of the reactor was made of glass PVC, while polypropylene was used for the base and cover. The CSTR was equipped with sampling ports for both the liquid and gaseous phase (see Supplementary Material). The amount of acidogenic off-gas evolved was measured by using an in-house wet gas flow meter based on the water displacement method, which was interconnected to the reactor using low gas permeability Marprene® and polyethylene Tubepack® tubing. The CSTR was placed in a temperature-controlled room at 37 ± 1 °C and magnetically stirred at ≈ 200 rpm. A pH controller (BSV, EVOPH-P-5 model, Spain) was used to measure and maintain constant the operational pH at 6.5 ± 0.1 by pumping a 6 M NaOH solution on demand (Regueira-Marcos et al., 2023). Finally, continuous FW feeding and anaerobic broth withdrawal was carried out by using a time controlled peristaltic pump and a liquid level controlled peristaltic pump, respectively.

**2.3 Impact of HRT on the performance of continuous lactate-driven dark fermentation of food waste**

The CSTR was operated for 51 days to assess the influence of HRT and transient feast/famine perturbations on the FW LD-DF process performance. The response of the process to changes in the HRT was evaluated during the first 35.4 days of operation, which were divided into 3 operational stages, i.e., I–III (Table 1). The HRT was stepwise reduced from 24 to 16 and 12 h, leading to corresponding OLRs values of 99.5, 149.3 and 199 g COD/L-d for stage I, II and III, respectively. The CSTR reactor was initially operated in batch mode. To that aim, the reactor was filled with 720 mL of FW and 80 mL of inoculum with a volatile suspended solids (VSS) concentration of

0.41 ± 0.05 g/L. The initial TS content of the FW was 7.5% (*w/w*), as it was previously found to be optimal for FW LD-DF (Regueira-Marcos et al., 2023). The feeding regime of the reactor was switched to continuous mode during the exponential phase in regard to hydrogen production. The CSTR was thus continuously fed at a variable flow rate depending on the HRT tested. It is noteworthy that the FW supplied was refrigerated at 4 °C and had a constant TS concentration of 7.5% (*w/w*) throughout the operation.

Liquid samples were taken and analysed periodically during operation for the determination of pH and the concentration of carbohydrates, VS, COD, and organic acids. Likewise, the flow rate of the gas produced and its composition was periodically monitored. The volume of hydrogen was adjusted to standard temperature and pressure conditions (0 °C and 1 atm). The HPR, HY, acidogenic off-gas quality (hydrogen content), hydrogen production stability index (HPSI), and carboxylic acid profile were selected as the main process performance indicators. HPSI was calculated as reported previously by García-Depraect et al. (2020b). The process was also characterized based on VS, COD and total carbohydrates removal efficiencies, and the requirement of alkali (expressed in mL $OH^{-}$/g-$VS_{added}$ and mL $OH^{-}$/L-d). Energy production rate (kJ/L-d) and energy production yield (kJ/g-$VS_{added}$) were calculated as reported elsewhere (Martínez-Mendoza et al., 2023). Pseudo-steady state was considered to occur when HPR deviation remained within 20% of the mean value for at least three consecutive days.

See Supplementary material for a more detailed information about the equations used to evaluate operational parameters and process performance.

**2.4 Transient feast/famine perturbations on the performance of continuous lactate-driven dark fermentation of food waste**

Three consecutive transient feast/famine perturbations (i.e., FF1, FF2, FF3) were applied during stage IV, from the 35$^{th}$ day of operation onwards, to evaluate the robustness and resilience of the FW LD-DF process. All feast/famine perturbations consisted of 12 h (equivalent to 1 HRT) of famine followed by continuous feeding at 12 h of HRT (FW feast). Before any process perturbation, the CSTR always showed HPS indices ≥ 80% for at least 3 consecutive days. The response of the process to transient fest/famine perturbations was evaluated based on the HPR, HY, hydrogen content in the gaseous phase, and the organic acids profile. More specifically, the response to famine perturbations was assessed in four different states, as follows: i) pre-perturbation pseudo-steady state, which included process data collected prior to the perturbation; ii) transient perturbation state, which included those process data collected during famine conditions; iii) recovery state, which included process data collected following feeding restauration until the start of a new pseudo-steady state, and iv) post-perturbation pseudo-steady state, which included process data from the new steady state. Additionally, the process was also characterized based on the settling time, which was defined as the time required by the process to reach a new pseudo-steady state (HPR values within 20% tolerance deviation for at least 3 consecutive days) following perturbation. It is noteworthy to mention that resilience was herein defined as the ability of the process to return to its steady state following the temporary feast/famine disturbance, while robustness is referred to the ability of the process to remain stable and at high performance even under adverse perturbations (Stricker and Lanza, 2014). The higher the robustness of the DF system was, the lower the modification of process performance indicators by operational perturbations.

### 2.4 Analytical methods

The concentrations of TS, VS and total and soluble COD were measured following standard methods (Eaton, 2005). The characterization of the substrate, in term of CHN(O)SP, carbohydrate, protein and lipid contents, was performed according to the methods previously described by Regueira-Marcos et al. (2023). The content of hydrogen in the acidogenic off-gas was analysed by gas chromatography, employing a Varian CP-3800 gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a Varian CP-Molsieve 5A capillary column (15 m × 0.53 mm × 15 µm) interconnected with a Varian CP-PoreBOND Q capillary column (25 m × 0.53 mm × 10 µm) (Alcántara et al., 2015). Helium, at a flow rate of 13 mL/min, was used as the carrier gas. The GC-TCD was also capable of measuring methane, carbon dioxide and hydrogen sulphide. Finally, organic acids were analysed by high-performance liquid chromatography (HPLC) using an Alliance HPLC system (model e2695, USA), which was equipped with an ultraviolet detector (214 nm) and an Aminex chromatographic column (HPX-87H, Bio Rad, USA) maintained at 75 °C. A Micro-Guard Cation H + refill cartridge of 30 × 4.6 mm (Bio Rad, USA) was used as a pre-column. Sulfuric acid (25 mM), at a flow rate of 0.7 mL/min, was employed as the eluent. The target organic acids included lactate, acetate, formate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate.

### 2.4 Statistical analysis

One-way ANOVA followed by the Tukey or Kruskal-Wallis test (p-value < 0.05), depending on the case, was employed to compare the different process performance measures computed during the operation. Data normality distribution was assessed with the Shapiro-Wilk test (p-value ≤ 0.05). All the statistical analyses were carried out using the Statgraphics Centurion software (version 19.2.01).

## 3. Results and discussion

### 3.1 Influence of HRT on the continuous lactate-driven dark fermentation of food waste

#### 3.1.1 Hydrogen production

The key operational parameter tested *viz* HRT exerted a significant effect on process performance. The HPR achieved during pseudo-steady state at an HRT of 24, 16 and 12 h accounted for 3.5 ± 1.0, 4.2 ± 0.6 and 2.9 ± 0.6 L $H_2$/L-d, respectively (Table 2). As shown in Figure 1, the recorded HPR peaked at the beginning of the operation at 9.1 L

$H_2$/L-d before suddenly decreasing down to 0.7 L $H_2$/L-d. This behaviour in HPR is typically observed in healthy DF systems and tends to occur because hydrogen production kinetics are promoted batchwise (Regueira-Marcos et al., 2023). Interestingly, an HRT of 24 h (stage I) led to a much less stable process with fluctuating HPR values in the range of 1.9 and 4.7 L $H_2$/L-d and with a HPSI of 0.71. In stage II, a clear trend to increase HRP until day 17$^{th}$ was observed, wherein reactor feeding was unfortunately interrupted overnight due to tubing clogging. Following this unforeseen perturbation, the LD-DF reactor was able to recover, reaching a relatively high HPSI of 0.86. An in-depth analysis of transient feast/famine perturbations is presented in section 3.2. In stage III, the HPR showed a gradual decreasing trend when reducing the HRT from 16 to 12 h, reaching a new equilibrium state with less than 20% variation in HPR. This sharply decrease in HPR may be attributed to a transient organic overloading due to the high OLR (199 g COD/L-d) imposed in stage III, as previously observed Paudel et al. (2017). The DF process capacity found in this study was very similar to that reported by Martinez-Mendoza et al. (2023), who assessed the continuous DF of fruit-vegetable waste at HRTs ranging from 24 down to 6 h using the same biocatalyst. These authors reported the highest HPR (11.8 NL $H_2$/L-d) and HY (95.6 mL $H_2$/g-$VS_{added}$) at an HRT of 9 h with a corresponding OLR value of 136.5 g COD L-d, which is very similar to the OLR (149.3 g COD/L-d) associated with the best HRT in this study. Martinez-Mendoza et al. (2023) also observed a marked decrease in HPR at 6 h HRT and 182 g COD/L-d OLR, the latter parameter being quite similar to the OLR imposed herein at 12 h HRT (199 g COD/L-d).

A statistically significant negative correlation was found between HY and HRT (Table 2). More specifically, the corresponding HY data recorded in stage I, II and III were 48.5 ± 13.9, 38.8 ± 5.35 and 20.4 ± 4.1 mL $H_2$/g-$VS_{added}$, respectively. In terms of energy recovery, the energy production rate ranged between 37.4 and 53.4 kJ/L-d, while the computed energy yield was 0.62, 0.49 and 0.26 kJ/g-$VS_{added}$ in stage I, II and III, respectively. Regarding the quality of the off-gas produced, the hydrogen content was found to be similar in operational stages I and III (57% on average), which was significantly higher than that computed in stage II (53.0 ± 1.2%). No methane production was observed during the entire experiment, which suggested that the short HRT and pH values imposed washed out methanogenic communities. On the other hand, the removal efficiencies of VS remained on average at 50.9 ± 3.6% throughout the entire operation. Likewise, the pseudo-steady state carbohydrate and COD removal efficiencies averaged 90.7 ± 2.7 and 30.0 ± 7.0%, 86.2 ± 3.2 and 23.8 ± 1.1%, and 87.1 ± 1.0 and 28.0±1.2 % in stage I, II and III, respectively. These results indicated that the performance of the LD-DF process was not sufficiently described by the hydrogen content in the off gas and the removal of VS, COD and carbohydrates provided valuable information, which is in accordance with previous observations (Martínez-Mendoza et al., 2022; García-Depraect et al., 2023a). Finally, it was found that the shorter the HRT, the higher the amount of alkali required for the system to keep pH constant, which ranged from 317.7 ± 56.6 to 767.2 ± 98.0 mL $OH^-$/L-d (Table 2). Interestingly, the alkali requirements normalised to the amount of VS fed ranged between 4.40 and 5.33 mL $OH^-$/g-$VS_{added}$ (on average) during operational stages I–III.

There are not many reports in the literature dealing with the effect of HRT on the continuous DF process using FW as substrate. Besides, the conditions and methodologies applied markedly differ from each other, making the benchmarking of disclosed data difficult. Hydrogen productivity during the DF of FW has been commonly reported to be in the range of 0.2–1.4 L $H_2$/L-d (Villanueva-Galindo et al., 2023), although there are few recent studies reporting higher HPR (as high as 13 L $H_2$/L-d) (Algapani et al., 2019; Lee et al., 2014; Jung et al., 2022; Regueira-Marcos et al., 2023). Thus, the LD-DF process can be considered as a promising platform to produce hydrogen from FW based on the best HPR of 4.2 ± 0.6 L $H_2$/L-d achieved in this study. However, further process optimization with enhanced hydrogen productivities is still required.

### 3.1.2 Metabolic profile

The profile of carboxylic acids experienced well-defined trends throughout the entire experiment (Figure 2). Particularly, butyrate and acetate remained at relatively high concentrations (5.43 ± 0.96 g/L and 5.40 ± 2.17 g/L, respectively) during operational stages I–III. However, low concentrations of butyrate were detected when HPR slow down by the day 17$^{th}$ and during the putative transient overloading in the beginning of stage III. According to the PCA analysis, butyrate concentration was found to be positively correlated with both the HPR and HY, which were clustered together, but it had a negative association with lactate and acetate (Figure 3). Notably, the concentration of lactate in the broth skyrocketed from a few milligrams to about 9.8 g/L while HPR and butyrate plummeted from 6.1 to 1.1 L $H_2$/L-d and from 9.1 to 4.3 g/L during the operational failure that occurred on day 17. This lactate-butyrate-HPR correlation was confirmed at the beginning of stage III. Therefore, higher HPR values were closely associated with low titers of lactate and acetate (specially of the former) and high concentrations of butyrate in the fermentation broth, which reinforced the hypothesis of hydrogen production via LD-DF (García-Depraect et al., 2021). It has been reported that during LD-DF, lactate serves as the electron donor while acetate acts as an oxidant agent (Tao et al., 2016), explaining their close association. The putative LD-DF mechanism starts with the oxidation of lactate to acetate and carbon dioxide (or a derivative thereof), and the subsequent formation of butyrate by condensation of two moles of acetate to produce an intermediate compound that is ultimately reduced to butyrate (García-Depraect et al., 2021). The behaviour of propionate, which was detected throughout the operation at average broth concentrations of 2.24 ± 0.99 g/L, was also unexpected. It is commonly recognized that propionate acts as a hydrogen sink during DF, which can be produced either from the fermentation of carbohydrates or lactate (Grause et al., 2012; Fuess et al., 2018). Here it seems that propionate was negatively related to lactate (Figure 3). It has been reported that propionate can be produced from lactate, thereby leading to lower hydrogen production (Chen et al., 2019; Sim et al., 2022). Sim et al. (2019) avoided the conversion of lactate to propionate by *Megasphaera elsdenii* by bioaugmenting the process with *Clostridium butyricum* which can oxidase lactate to hydrogen. Other soluble metabolite identified over the course of the process was iso-valerate, with concentrations ranging from 2.1 g/L in stage I to 0.15 g/L in stage III, regardless of the behaviour of hydrogen production, suggesting that the production of iso-valerate did not significantly affect the electron flux toward hydrogen.

### 3.2 Influence of feast-famine perturbations on the continuous lactate-driven dark fermentation of food waste

#### 3.2.1 Hydrogen production during transient feast/famine perturbations

Feast/famine perturbations did not cause a severe deterioration on the degradation efficiency of carbohydrates and VS, which varied between 71.3 and 93.4 % and between 43.2 and 52.0 %, respectively. Hydrogen content in the acidogenic off-gas was slightly reduced only during famine conditions, from 52.3 ± 2.5% down to 41.5 ± 1.7%, but it rapidly returned to previous values after FW feeding resumption. However, all the three feast/famine perturbations intentionally applied led to a similar process's behaviour, which was characterized by a significant reduction in HPR during famine operation followed by an up-down HPR response following perturbation and subsequent HPR stabilization (Figure 4). It is also worth noting that the HY response to feast/famine perturbations showed a similar trend to the one observed for HPR, pointing out that the LD-DF process had the ability to bounce back from perturbation (Figure 4). Previous studies have also pointed out the resilience of dark fermenters against different types of stresses such a sudden pH acidification/alkalinization, organic overloading, substrate composition, and starvation (Park et al. 2015; Monroy et al., 2018; García-Depraect et al., 2020a).

Four different stages were defined using HPR as target process indicator, i.e., i) initial pseudo-steady state, ii) transient perturbation state, iii) recovery state, and iv) post-perturbation pseudo-steady state in order to have a deeper in-sight in the feast/famine perturbation effect. The preceding steady state HPR value of 2.9, 2.8, and 3.7 L $H_2$/L-d was used as reference for FF1, FF2 and FF3, respectively. A ratio between the HPR recorded during the four different stages and the reference HPR higher than 1 indicates a global process enhancement, while a global HPR reduction can be inferred from a ratio lower than 1 (Table 3). On average, the HPR recorded under starvation conditions (transient perturbation state) decreased to 0.7 ± 0.3 L $H_2$/L-d. Interestingly, when comparing the HPR achieved during transient perturbation state with the steady-state HPR computed prior to perturbation, a more pronounced decrease in HPR was observed as perturbations were applied, pointing out that the process became more susceptible to starvation as feast/famine operation progressed. During the recovery state (which lasted for 2.13, 1.31 and 1.35 for FF1, FF2, and FF3, respectively) HPR suddenly peaked to 10.1, 19.2 and 7.7 L $H_2$/L-d and rapidly levelled off reaching a new steady state. The underlying mechanisms of the observed behaviour need to be further investigated. However, the r/K selection theory could explain in some extent the sudden increase in HPR recorded. In this regard, lactate-oxidizing-HPB are classified as r-strategists with high growth rate and a competitive advantage in resource-rich environments. In contrast, LAB are suggested to be K-strategists with a high biomass specific uptake rate for fermentable carbohydrates (Kim et al., 2021). Thus, it was hypothesized that temporary starvation and subsequent feeding somewhat improved the activity of lactate-oxidizing -HPB, which could explain the HPR patterns observed during the transient perturbation state and the recovery state. The assumption of an enhanced hydrogen-producing activity could also endorse the behaviour of HPR identified during the post-perturbation pseudo-steady state. The HPR computed after perturbation was similar to the reference HPR value in FF1, and 33 and 17% higher in FF2 and FF3, respectively. This entailed an increase in hydrogen productivity after three feast/famine stresses despite an HRT of 12 h was not the best operating condition, as discussed previously in section 3.1.

### 3.2.2 Metabolic profile during transient feast/famine perturbations

The transient feast/famine perturbations impacted not only the hydrogen production performance but also the profile of organic acids (Figure 3). Compared to an HRT of 16 h, an HRT of 12 h was found to be more conducive to the accumulation of lactate and acetate, but not of butyrate and propionate. Interestingly, all perturbation events applied resulted in a sudden and drastic decrease in lactate and acetate levels, and increases in butyrate, propionate and, to a lesser extent formate, after feeding restoration, which agrees with previous observations (Monroy et al., 2018). Hence, feast/famine perturbations likely triggered bacterial acidogenic activity, leading to a peak in the alkali requirement (Figure 4). In the LD-DF process, hydrogen production is closely related to the evolution of lactate. The low levels of lactate (electron donor) and acetate (acceptor donor) observed during the recovery state seems to reinforce the assumption that transient feast/famine cycles fostered, at least temporarily, the activity of lactate-oxidizing-HPB (Tao et al., 2016; Fuess et al., 2018). As discussed previously, the operational failure on day 17 is very illustrative for that case (Figure 2). In LD-DF, it has been argued that low concentrations of lactate in the broth are an indicator of an efficient process (García-Depraect et al., 2021). Thus, the fact that the broth concentrations of lactate and acetate slowly returned to high concentrations during the post-perturbation pseudo-steady state was likely attributed to the suboptimal HRT/OLR condition imposed (Paudel et al., 2017). Therefore, it can be hypothesized that 12 h of HRT boosted the activity of LAB over HPB, which would lead to a gradual accumulation of lactate under long term operation.

In brief, the present LD-DF study targeted an enhanced hydrogen production from FW. This alternative hydrogen-producing approach can cope with the inhibition issues related to the overgrowth of LAB, which typically outcompete HPB for fermentable carbohydrates (García-Depraect et al., 2021; Canto-Robertos et al., 2023). This study also helped filling the knowledge gap of continuous LD-DF of organic waste, which has been commonly studied in batch mode (e.g., Martínez-Mendoza et al., 2022; Lois-Regueira et al., 2023). The LD-DF process herein evaluated resulted in relatively high hydrogen productivities and confirmed the key role of HRT on stable process performance. Additionally, the LD-DF process was tested against temporary feast/famine disturbances, mimicking a stress that could occur in large DF plants. Despite the process rapidly deteriorated during famine periods, leading to low hydrogen production, the LD-DF exhibited a high resilience. Although the Readiness Technology Level (RTL) of DF is 5-6, it is expected that some unforeseen and/or foreseen perturbations can exist in further large-scale systems. In this line, further studies should focus on the impact of different operational perturbations, e.g., shocks in pH, organic load, temperature, and long starvation, etc, on process performance and the structure of the microbial populations. One of the weaknesses of this study was the use of synthetic organic recipe, which was used in the proof of concept of the continuous hydrogen production from FW via LD-DF. It is thus highly recommended to conduct further validation studies using real FW, which is expected to be more complex than the recipe-based FW. Real FW would present a high autochthonous bacteria load, be highly heterogenous and pre-acidified at some extent, all of which can impact the process. Finally, it must be pointed out that the peak in HPR observed during the transient state following famine conditions along with the associated profile of lactate strongly suggested that an adequate balance between LAB and lactate-oxidizing-HPB is crucial to support a high hydrogen production rate. Further studies should not only perform molecular analyses to get an in-depth knowledge of the microbiome involved but also to bridge the gap between process design and strategy and microbial and ecological aspects. The development of novel strategies aiming at improving the syntrophic association between LAB and HPB can further boost the high HPR herein achieved.

## 4. Conclusions

The influence of the HRT on the continuous LD-DF of FW was investigated for the first time. The best hydrogen production performance was found at 16 h of HRT, leading to a more stable operation and a HPR of 4.2 L $H_2$/L-d. Lactate consumption was identified as the main hydrogen-producing pathway rather than the one-step fermentation of carbohydrates. Further operation under temporary feast/famine perturbations evidenced a poor robustness of the process, but it exhibited a prominent resilient capacity following the disturbed operation. Overall, the LD-DF process supported promising results for the further optimization of continuous hydrogen production from real FW.

E-supplementary data for this work can be found in the e-version of this paper online.

## Acknowledgments

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**Figure captions:**

**Fig. 1**. Time course of A) hydrogen production rate (HPR), hydrogen content, and volatile solids (VS) and total carbohydrates (CH) removal, and B) hydrogen yield (HY) and alkali requirement during the continuous LD-DF of FW. Vertical dotted lines stand for the shifts in HRT (24, 18 and 12 h for stage I, II and III, respectively).

**Fig. 2**. Time course of organic acids concentration and HPR during the continuous LD-DF of FW at decreasing HRTs (24, 18 and 12 h for stage I, II and III, respectively).

**Fig. 3**. Principal component analysis (PCA) based on the evolution in HPR, HY and the concentration of lactate, acetate, propionate and butyrate at decreasing HRTs (24, 18 and 12 h for stage I, II and III, respectively).

**Fig. 4.** Time course of A) hydrogen production rate (HPR), hydrogen content, and volatile solids (VS) and total carbohydrates (CH) removal, and B) hydrogen yield (HY) and alkali requirement during the feast/famine perturbations applied in the continuous LD-DF of FW. Vertical shaded bars indicate the length of famine condition.

**Fig. 5**. Time course of organic acids concentration and HPR during the feast/famine perturbations applied in the continuous LD-DF of FW. Vertical shaded bars indicate the length of famine condition.

**Table 1.** Summary of the operating conditions tested during the continuous LD-DF of FW.

| **Parameter** | **Stage I** | **Stage II** | **Stage III** | **Stage IV** |
|---|---|---|---|---|
| **Time (days)** | 0–11.4 | 11.4–26.0 | 26.0–35.4 | 35.4–51.0 |
| **HRT (h)** | 24 | 16 | 12 | 12 |
| **OLR (g VS/L-d)** | 72.0 | 108.0 | 144.0 | 144.0 |
| **OLR (g COD/L-d)** | 99.5 | 149.25 | 199.0 | 199.0 |
| **HRT cycles (-)** | 11.4 | 21.9 | 18.8 | 31.2 |

**Note:** Stages I to III aimed at investigating the effect of HRT on the continuous LD-DF of FW, while stage IV was devoted to the study process performance under transient feast/famine perturbations. HRT: Hydraulic retention time; OLR: Organic loading rate; COD: Chemical oxygen demand.

**Table 2.** Summary of the process performance indicators collected during the continuous FW LD-DF process.

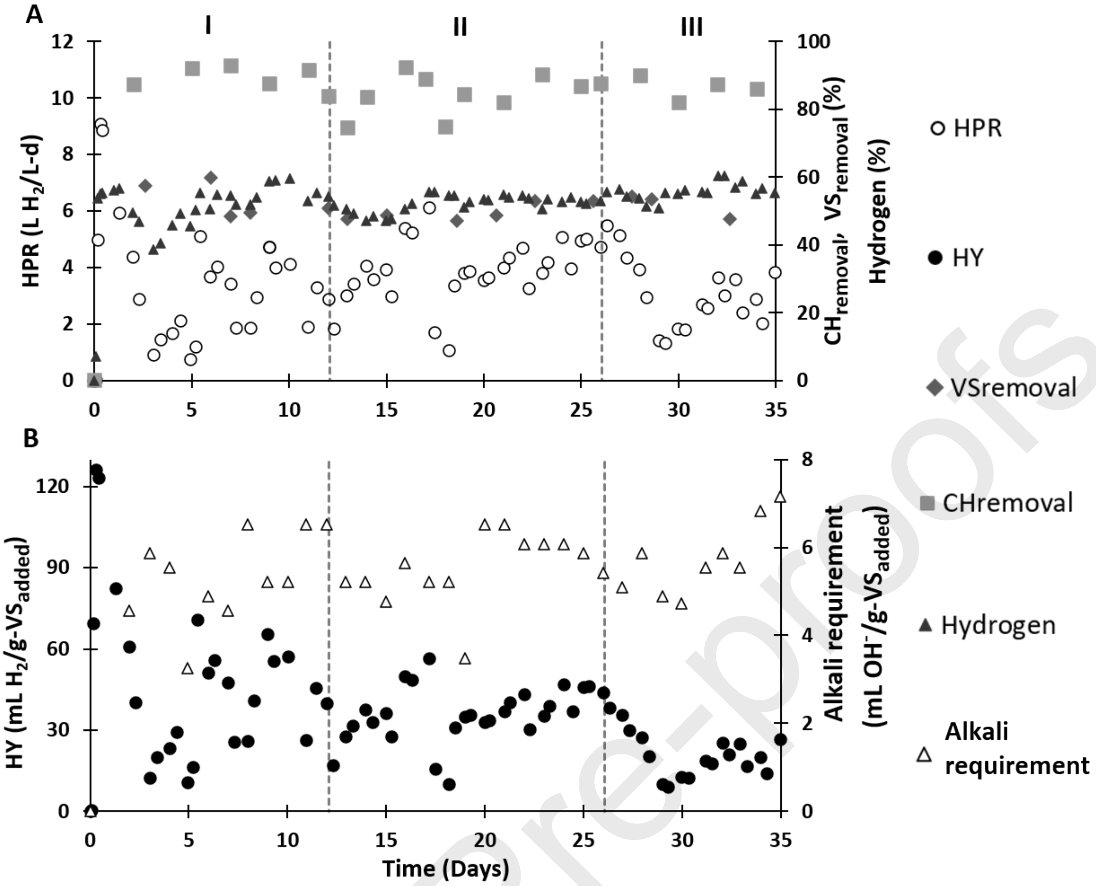
| Parameter | Stage I | II | III |
|---|---|---|---|
| **HRT** | 24 | 16 | 12 |
| **HPR (L $H_2$/L-d)** | 3.5 ± 1.0 ab | 4.2 ± 0.6 a | 2.9 ± 0.6 b |
| **HY (mL $H_2$/g-$VS_{added}$)** | 48.5 ± 13.9a | 38.8 ± 5.35b | 20.4 ± 4.1c |
| **$H_2$ content (%)** | 56.5 ± 2.9a | 53.0 ± 1.2b | 56.9 ± 2.2a |
| **$VS_{removal}$ (%)** | 52.5 ± 6.3 | 49.8 ± 2.5 | 51.8 ± 3.6 |
| **$CH_{removal}$ (%)** | 90.7 ± 2.7 | 86.2 ± 3.2 | 87.1 ± 1.0 |
| **$COD_{removal}$ (%)** | 30.0 ± 7.0 | 23.8 ± 1.1 | 28.0 ± 1.2 |
| **Alkali requirement (mL $OH^-$ L/L-d)** | 317.1 ± 56.6a | 507.7 ± 66.8b | 762.2 ± 98.1c |
| **Alkali requirement (mL $OH^-$/g-$VS_{added}$)** | 4.4 ± 0.8a | 4.7 ± 0.6ab | 5.3 ± 0.7b |
| **Energy production rate (kJ/L-d)** | 44.5 ± 12.7ab | 53.4 ± 7.4a | 37.4 ± 7.5b |
| **Energy yield (kJ/g-$VS_{added}$)** | 0.62 ± 0.18a | 0.49 ± 0.07b | 0.26 ± 0.05c |

**Note:** HRT: Hydraulic retention time; HPR: Hydrogen production rate; HY: hydrogen yield. Mean and deviation data is reported during pseudo-steady state, which lasted for 3.1, 7.0 and 4.3 days in stage I, II and III, respectively. Means with the same letter in the same raw do not differ significatively ($p \leq 0.05$). The sample size was 6, 15 and 10 for stage I, II and III, respectively.

**Table 3**. Summary of hydrogen productivities achieved from the LD-DF of FW during the different stages assessed under feast/famine perturbations.

| Condition | Settling period (Days) | TPS ($L\ H_2$/L-d) | TPS/ reference | RS ($L\ H_2$/L-d) | RS/ reference | PPSS ($L\ H_2$/L-d) | PPSS/ reference |
|---|---|---|---|---|---|---|---|
| FF1 | 2.13 | 1.03 | 0.68 | 7.2 | 2.46 | 2.8 | 0.96 |
| FF2 | 1.31 | 0.65 | 0.23 | 10.80 | 3.90 | 3.7 | 1.33 |
| FF3 | 1.35 | 0.39 | 0.11 | 5.5 | 1.48 | 4.3 | 1.17 |
| Average | 1.6 ± 0.5 | 0.69 ± 0.32 | 0.3 ± 0.3 | 7.9 ± 2.7 | 2.6 ± 1.2 | 3.6 ± 0.8 | 1.2 ± 0.2 |

**Note:** *Settling time* is defined as the time required by the process to reach a new pseudo-steady state following perturbation. *TPS* (transient perturbation state): HPR recorded along the 12-h starvation period. *RS* (recovery state): HPR computed during the settling period. *PPSS* (post-perturbation pseudo-steady state): average HPR achieved during a new steady state after perturbation. *TPS/reference*, *RS/reference* and *PPSS/reference* indicate the ratio between the value of HPR attained in TPS, RS or PPSS and the immediately preceding pseudo-steady HPR value. The steady-state HPR data recorded prior to FF1, FF2 and FF3 were used as the reference. For interpretation of the obtained ratios, the reader is kindly referred to section 3.2.1.




660

661 **Figure 1**

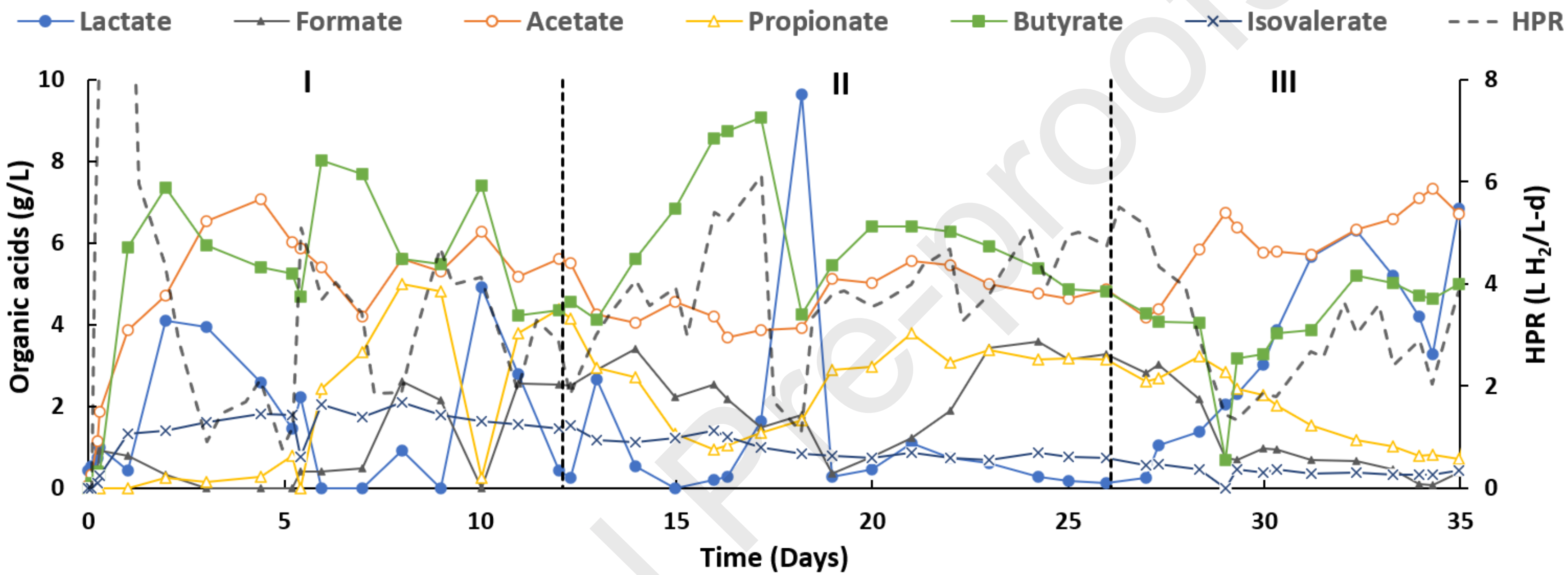
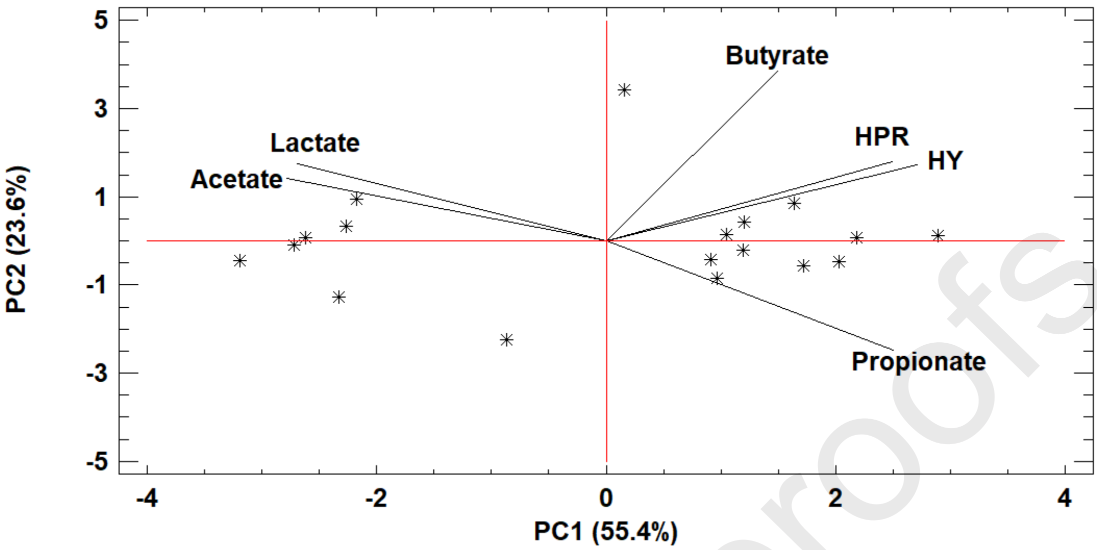
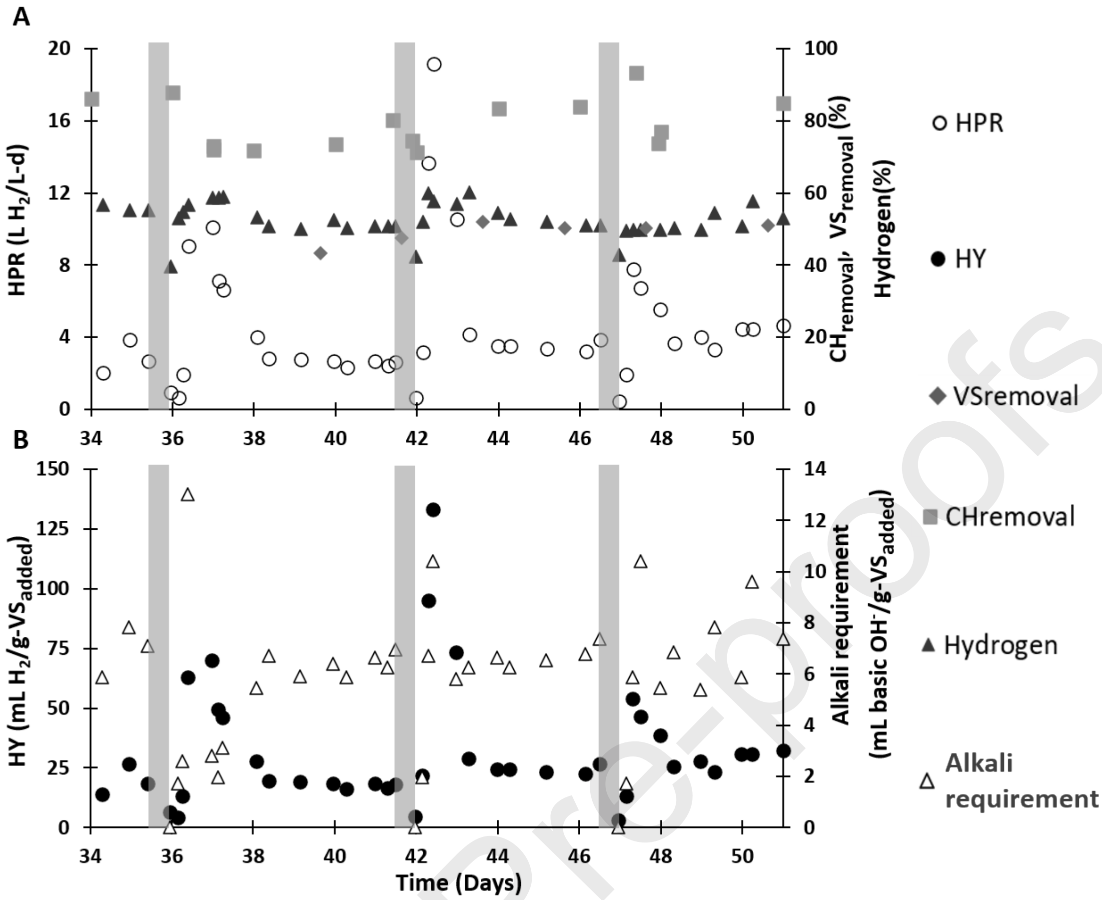

Lactate
Formate
Acetate
Propionate
Butyrate
Isovalerate
HPR
I
II
III
Organic acids (g/L)
HPR (L H2/L-d)
Time (Days)
0
5
10
15
20
25
30
35
0
2
4
6
8
10
0
2
4
6
8

**Figure 2**



Butyrate
Lactate
Acetate
HPR
HY
Propionate
PC2 (23.6%)
PC1 (55.4%)
5
3
1
-1
-3
-5
-4
-2
0
2
4


**Figure 3**




668

669 Figure 4

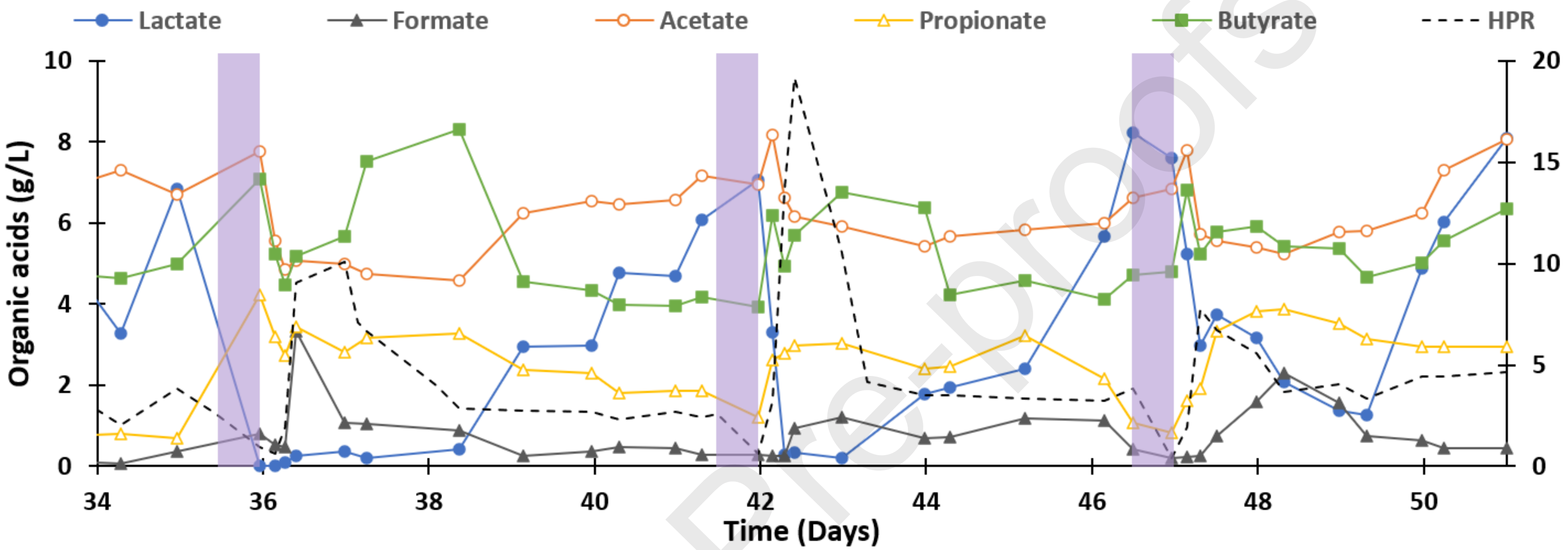


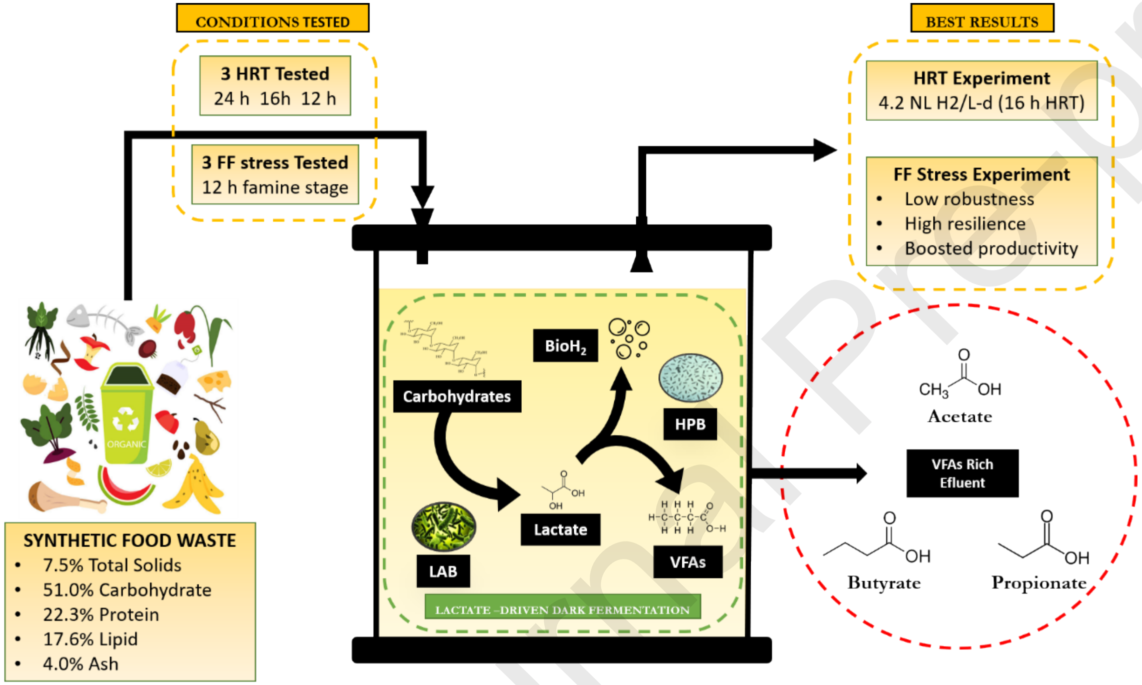

**Figure 5**

**Declaration of interests**

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

## Graphical Abstract




## Highlights

- LD-DF showed high potential for continuous $H_2$ production from simulated FW
- HRT impacted on LD-DF, with maximum HPR of 4.2 ± 0.6 NL $H_2$/L-d at 16 h
- Feast/Famine stress resulted in low robustness of HPR against the perturbation
- Fast stability recovery after perturbation highlighted LD-DF as resilient process
- High HPRs correlated with lactate/acetate consumption and butyrate production