



Biogenic hydrogen production from household food waste via lactate-driven dark fermentation: A comparative study of single-stage and two-stage configurations

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ABSTRACT

Lactate-driven dark fermentation (LD-DF) is a novel approach that integrates lactate production with its conversion to biohydrogen (H_2), offering a solution to the inhibition of hydrogen-producing bacteria by lactic acid bacteria while enhancing process stability. This study investigates the efficacy of LD-DF for continuous H_2 production by comparing the performance of a two-stage system (lactate fermentation followed by LD-DF) with a single-stage configuration. Household food waste (FW) was used as the substrate, and the impact of varying the hydraulic retention time (HRT; 12, 8, and 6 h) was assessed in both configurations. In the two-stage system, a progressive reduction in HRT resulted in the highest volumetric H_2 production rate (HPR) of 4.4 ± 0.4 L- H_2 /L-d and a hydrogen yield (HY) of 22.9 ± 2.1 mL- H_2 /g-VS_{added}. In contrast, the single-stage configuration exhibited a decline in HPR from 3.8 ± 0.6 – 1.4 ± 0.3 L H_2 /L-d when the HRT decreased from 12 h to 6 h, although it achieved the highest HY of 39.5 ± 6.0 mL- H_2 /g-VS_{added}. Principal component analysis identified a positive correlation between HPR and butyrate concentrations, a trend predominantly observed in the two-stage configuration. Conversely, the HPR negatively correlated with high levels of lactate, acetate, and propionate, which were more prevalent in the single-stage system. Interestingly, *Veillonella* and *Bacteroides* were identified as the main H_2 producers during LD-DF in both configurations. These findings demonstrate that lactic acid pre-fermentation enhances H_2 productivity in FW LD-DF systems and facilitates operation at lower HRTs compared to single-stage configurations.

1. Introduction

The pursuit of a sustainable economic and productive model at a global level has become a priority for nations worldwide. In this context, food supply chains and the generation of food waste (FW) play a significant role in sustainability discussions, particularly concerning climate change, as they contribute approximately 26 % of global greenhouse gas (GHG) emissions [1]. The European Union (EU) defines FW as any food that is discarded and no longer intended for consumption [2]. According to the EU, food encompasses “any substance or product, whether processed, partially processed, or unprocessed, intended to be, or reasonably expected to be, ingested by humans” [3]. Approximately one-third of all food produced globally is wasted rather than reaching consumers [4]. In this context, the EU generated approximately 58.4 million tons (Mt) of FW in 2020 [5]. In comparison, other regions,

including the Asia-Pacific, sub-Saharan Africa, North America, and Latin America, generated 465, 232, 168, and 127 Mt of FW per year, respectively [6]. This indicates that valorizing FW could significantly contribute to reducing GHG emissions [7].

FW-based integrated biorefineries could play a crucial role in strengthening the bioeconomy by promoting a sustainable circular approach to FW valorization [7,8]. Through these biorefineries, unavoidable FW can be processed into valuable products such as methane (CH_4), hydrogen (H_2), bioplastics, carboxylates, and other alternatives. In this context, H_2 is gaining increasing attention as a leading alternative to fossil-based fuels. The EU has outlined various actions to decarbonize its economy, emphasizing a transition toward an H_2 -based energy system [9]. H_2 offers significant advantages, including high gravimetric energy density (120 kJ/g) and the production of only water vapor during combustion, resulting in zero carbon emissions [10]. Although

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water electrolysis remains the most efficient technology for H₂ production [9], several biological processes offer an alternative by simultaneously producing bioH₂ and carboxylic acids during FW valorization. Of these, dark fermentation (DF) stands out as a particularly promising approach [11]. DF is a microbial-driven process classified under anaerobic digestion biotechnologies, in which diverse microbial communities break down complex organic molecules, primarily carbohydrates, into organic acids (OAs) such as acetate and butyrate, leading to the net release of bioH₂ [12]. Therefore, a well-performed DF biorefinery presents an attractive possibility for FW treatment.

While the potential of DF with various substrates has been demonstrated at a laboratory scale, its long-term implementation in continuous processes has been hindered by instability issues in bioH₂ production [13]. One of the primary causes of these instabilities is the excessive proliferation of lactic acid bacteria (LAB), which are widely distributed in FW [14]. LAB, capable of thriving across a wide range of environmental conditions (e.g., pH 3.5–10; temperature 5–45 °C) [15], compete with hydrogen-producing bacteria (HPB) for the carbohydrates present in the substrate. Their superior ability to degrade complex substrates, along with the release of species-specific antimicrobial compounds, ultimately displaces HPB and hinders the DF process over extended operational periods [13]. To mitigate LAB overgrowth, various pre-treatment methods have been explored. However, these approaches are generally effective only in the short term, as LAB tend to proliferate gradually over time [14]. Consequently, new methods must be developed to address LAB competition in DF systems.

On the other hand, LAB can exert positive effects in DF reactors. While many HPB ferment primarily carbohydrates, certain species, known as lactate-utilizing HPB (LU-HPB), can metabolize lactate. This ability allows LU-HPB to benefit from the superior hydrolytic capacity of LAB, enhancing substrate conversion efficiency. Additional advantages of LAB activity include biomass retention and residual oxygen consumption [16]. Linking lactate production with its subsequent conversion to H₂ harnesses the presence of LAB, fostering the development of a highly synergistic LAB–LU-HPB microbial consortium [16]. This process, commonly known as lactate-driven dark fermentation (LD-DF), eliminates the need for costly pre-treatment steps while ensuring greater process stability over time [14]. Previous studies have reported bioH₂ production using FW as a substrate through both conventional DF, in which carbohydrates undergo direct fermentation via acetic- and/or butyric-type pathways [17,18], and the LD-DF process [19–21]. The former achieves promising results in terms of bioH₂ productivity and stability. Therefore, the LD-DF aims to become an efficient alternative to cope with LAB proliferation in DF systems.

Optimizing the LD-DF process in a single-stage configuration is challenging due to imbalances in microbial activities, as LAB and LU-HPB coexist in the same reactor, often leading to competition for substrates and suboptimal process conditions. Recently, a novel two-stage LD-DF concept has been proposed and evaluated in continuous reactors using simulated FW [22]. The two-stage LD-DF configuration is designed to spatially separate LAB and LU-HPB activities into distinct reactors, with the first stage dedicated to lactate fermentation under optimized conditions for lactate production, while the second stage facilitates the targeted conversion of lactate to H₂ by LU-HPB. This two-stage concept was later investigated in batch mode using cafeteria-derived FW [23] and in continuous mode using simulated FW [22], further supporting the benefits of LAB and LU-HPB separation for enhanced H₂ production. The objective of this study is to comparatively evaluate the functional performance of single- and two-stage LD-DF systems using real FW. Additionally, the microbiology involved in each configuration was also studied. To the best of the authors' knowledge, this research constitutes the first study on the continuous performance of LD-DF using real household FW in both single-stage and two-stage configurations. By systematically assessing the impact of hydraulic retention time (HRT), a key operating parameter, this study provides valuable insights into optimizing mesophilic bioH₂ production from FW.

2. Materials and methods

2.1. Substrate

Household-FW was collected over 24 days (from 24 April to 19 May 2023) from the organic fraction of kitchen waste (after first discarding glass, packaging, and other inert components) of different households in the city of Valladolid, Spain. On the same day of collection, the inert components (i.e., bones, mollusc shells, and mixed non-organic debris) were manually removed. The resulting fraction (70.7 kg) was then shredded, mixed and subsequently stored in 2 kg zip-lock bags at –20 °C. The grinding process required the addition of tap water (16.8 % w/w) to facilitate the grinding of the substrate.

The concentration of total solids (TS) and volatile solids (VS) in the collected mixture was 214.7 and 200.9 g/L, respectively. The mixture obtained, with a pH value of 5.42, had the following composition: 65.7 ± 4.6 % of carbohydrates, 15.9 ± 4.8 % of proteins, 9.1 ± 0.4 % of lipids, and 7.0 ± 0.4 % of ashes. The elemental analysis revealed the following composition: 48.1 ± 0.1 % carbon (C), 6.7 ± 0.1 % hydrogen (H), 40.4 ± 0.7 % oxygen (O), 2.6 ± 0.8 % nitrogen (N) and 2.28 ± 0.1 % phosphorus (P). Sulfur was not detected.

2.2. Inoculum

The lactate-producing reactor (LR) was operated using the native (autochthonous) microorganisms present in the FW [6], as previously reported by Regueira-Marcos et al. (2024) [22]. The FW was enriched for 24 h at 37 °C in a 2.1 L glass flask with a closed atmosphere, initially composed of atmospheric air, and without pH control. The initial inoculum concentration, in TS and VS, was 70.6 and 65.3 g/L, while the final concentration was 62.2 and 56.7 g/L, respectively. Its pH value at the time of inoculation was 3.49. The inoculum of the bioH₂-producing reactor (HR) was prepared and enriched using the method described in Regueira-Marcos et al. (2023b) [20], also using the same inoculum source of this study. The dominant genera of the inoculum included *Lactobacillus*, *Klebsiella*, *Clostridium*, *Stenotrophomonas*, *Acinetobacter*, among others. The inoculum had a pH of 5.0 and concentrations of total suspended and volatile suspended solids of 0.34 and 0.31 g/L, respectively. No reinoculation was carried out in the HR during the switch from a two-stage to a single-stage configuration.

2.3. Experimental setup

As shown in Fig. 1, the LR was composed of a continuous stirred tank reactor (CSTR) with a total capacity of 1.1 L and a working volume of 0.9 L. The reactor body was constructed from glass, while the lid was fabricated from rigid transparent PVC. Conversely, the HR comprised a 1.2 L CSTR with a 0.8 L working volume. The HR body was constructed from PVC glass, while the lid was fabricated from propylene. Both reactors were equipped with a feed inlet port and an effluent outlet port, in addition to a pH probe, an alkali inlet for pH control, and a gas outlet via a sampler port. The stirring of the cultivation broths was carried out via magnetic stirring plates (LBX instruments, S20 series stirred plate). The feed and effluent flux were facilitated by peristaltic pumps, which were automatically regulated by a custom-built feeding and discharging system. The fermentative off-gas generated was quantified using a custom-designed wet gas flow meter, based on the water displacement method. The connections for both liquid and gas circulation consisted of tubing with low gas permeability (Marprene® and polyethylene Tubepack®). A pH controller (BSV, EVOPH-P-5, Spain) was utilized to ensure the maintenance of the operational pH. A 6 M NaOH solution was employed as an alkali for pH control in both LR and HR. The fermentation process was conducted in a temperature-controlled room at 37 ± 1 °C.

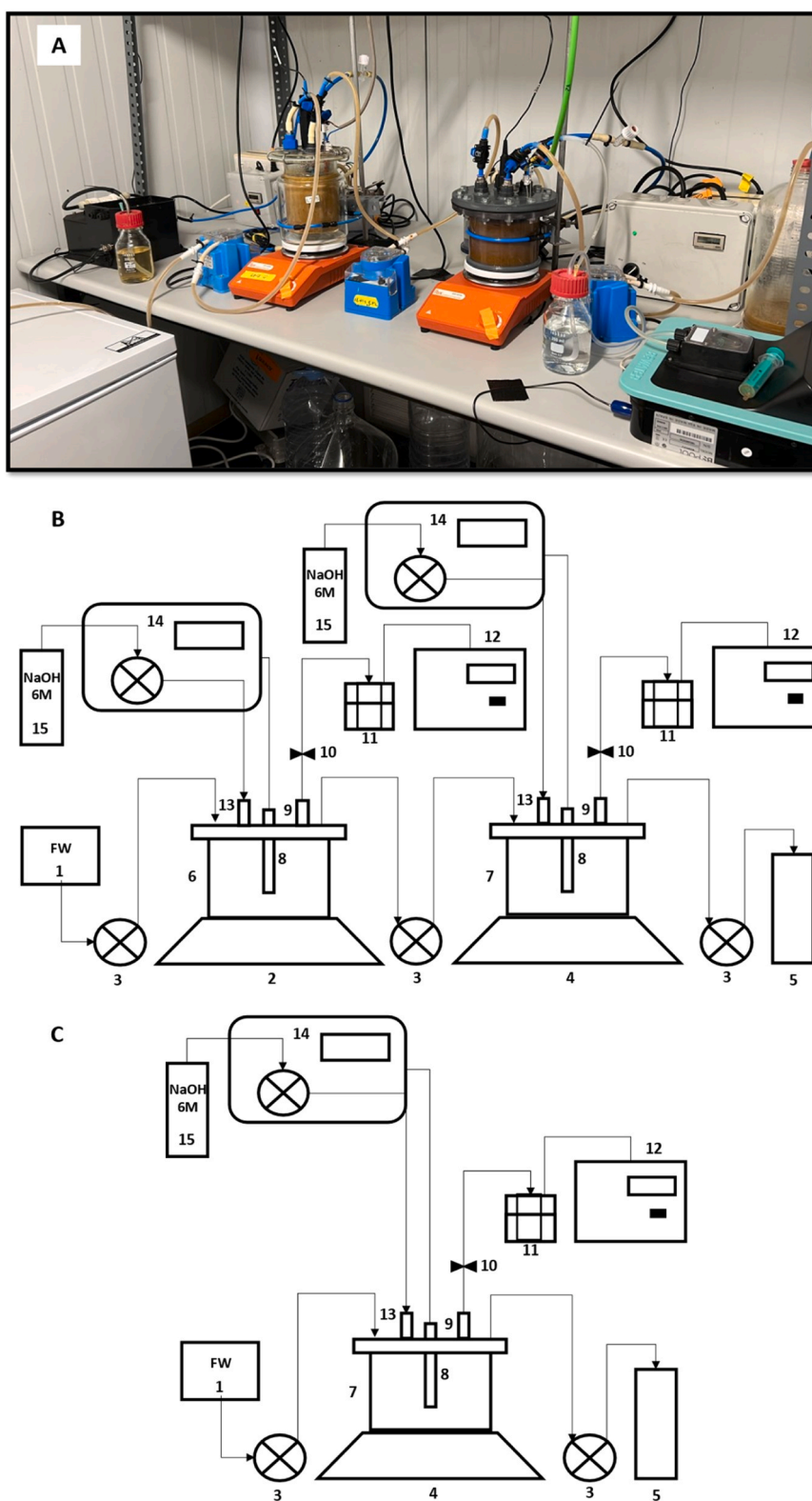


Fig. 1. A) Image of the two-stage reactor configuration employed in the hydrogen production test. B) Schematic diagram of the experimental two-stage reactor configuration. C) Schematic diagram of the single-stage reactor configuration. The numbers used in the diagram correspond to the following: feedstock tank (1), magnetic stirring plates (2 and 4), peristaltic pumps (3), effluent tank (5), LR (6), HR (7), pH probe (8), gas outlet (9), gas sampling port (10), water column (11), gas flow meter (12), alkali input (13), pH controller (14), and NaOH 6 M solution tank (15).

2.4. Process operation

The experiment spanned a total duration of 38 days, commencing with the setup of the HR reactor. The experimental approach involved two bioreactor configurations. In the two-stage configuration, the first stage (LR) is optimized for lactate fermentation, ensuring conditions favourable for lactate production. In the second stage, the HR is devoted to the efficient conversion of lactate into H_2 . In the single-stage configuration, the LR was omitted, and the FW was fed directly into the HR for H_2 production. The operation of both reactor configurations was subdivided into three distinct periods (P1, P2, and P3 for the two-stage configuration, and P4, P5, and P6 for the single-stage configuration). In both reactor configurations, the HRT in the HR was progressively shortened from 12 to 8 and 6 h over the operation time. The feeding was carried out in a semi-continuous mode by activating the pumps for a fixed amount of time (depending on the HRT exerted) every 30 min with the help of a timer. The summary of operational conditions applied in each reactor configuration is shown in Table 1.

During the process start-up, the LR was initiated 18 days before the inoculation of the HR to guarantee a stable lactate input concentration for bio H_2 production. Both reactors, LR and HR, were filled with FW substrate (90 % v/v) and inoculum (10 % v/v), following the steps outlined in Section 2.1. Additionally, both reactors were kept in batch mode for 24 h to initiate continuous operation at high production rates (of lactate and bio H_2 , respectively). The pH of LR was set at 4.5 based on the results described in Regueira-Marcos et al. (2024) [22], while the pH of HR was fixed at 6.5, based on the results presented in Regueira-Marcos et al. (2023) [24].

Liquid samples were taken periodically in both reactors to measure the concentration of TS, VS, carbohydrates, and OAs, as well as to verify the correct measurement of the pH value by the controller with the help of an external pH meter. The off-gas volume and composition were periodically analyzed, along with the amount of alkali consumed, expressed as mL NaOH per gram of VS added and mL NaOH per liter per day (equivalent to 1 M NaOH). To assess system performance, several key parameters were measured, including the volumetric biogas production rate (BPR), volumetric hydrogen production rate (HPR), hydrogen yield (HY), hydrogen concentration in the acidogenic off-gas (% v/v), hydrogen production stability index (HPSI), and the organic acid (OA) profile. Additionally, substrate degradation efficiency was evaluated based on carbohydrate, volatile solids (VS), and chemical

oxygen demand (COD) removal. Energy output was also assessed through calculations of the energy production rate (kJ/L-d) and energy production yield (kJ/g-VS_{added}), following the methodology outlined by Regueira-Marcos et al. (2023) [24].

2.5. Analytical methods

The gas composition of the off-gas produced in both reactors (CO_2 , H_2 , O_2 , and N_2) was measured using an Agilent 8860 gas chromatograph (GC) (USA), as described by Regueira-Marcos et al. (2024) [22]. The OA concentration was obtained from a Shimadzu HPLC (Model LC-2050C; Oregon, USA), configured based on Regueira-Marcos et al. [22]. The elemental analysis (C, H, O, N, and S) was performed using an elemental analyser EA FLASH 2000 (Thermo Fisher Scientific), coupled with a TCD detector and a Mettler Toledo XP6 microscale, employing helium as a gas carrier at 140 mL/min for C, H, N, and S, while a reference gas (100 mL/min) at 1060 °C furnace temperature was used for O measurement. All analyses were based on the internal method of the Central Instrumental Laboratories of the University of Burgos, Spain.

Phosphorus (P) concentration was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES), following the internal method of the Laboratory of Instrumental Techniques at the University of Valladolid, Spain [22]. Protein content was calculated using a nitrogen-to-protein conversion factor of 6.25 [25]. Lipid concentration was analyzed using a gravimetric method performed by the Regional Service for Agri-food Research and Development (SERIDA, Spain) [22]. Carbohydrates were measured using the phenol-sulfuric method, which involved adding 1 mL of sample to 0.6 mL of phenol (5 % v/v) and 3.6 mL of sulfuric acid (95 % v/v). The resultant solution was measured in a Spectrophotometer Star Nano from BMG LACTECH to determine the carbohydrate concentration based on its absorbance at a wavelength of 480 nm [22]. Lastly, the TS, VS, and COD concentrations were measured following the standard procedures for wastewater analysis described in Eaton et al. (2005) [26]. Microbial community analysis was conducted through 16S rRNA gene amplicon sequencing, using triplicate samples for each set of optimal conditions. Each replicate was collected on a different day during the steady-state period corresponding to the optimal conditions of the LR, HR, and single-stage configurations. Additionally, the raw substrate was sequenced for comparative purposes. Inoculum data were obtained from García-Depraet et al. [25]. DNA extraction and sequencing procedures were carried out as

Table 1
Summary of the operating conditions tested during the two-stage LD-DF of FW.

Parameter	Two-Stage			Single-Stage		
	Lactate-producing reactor					
	P1	P2	P3	P4	P5	P6
Time (days)	0–7.1	7.1–14.0	14.0–22.3	-	-	-
HRT (h)	13.5	9.0	6.7	-	-	-
^a OLR (g VS/L-d)	83.2	124.8	166.4	-	-	-
^a OLR (g COD/L-d)	155.6	233.3	311.1	-	-	-
HRT cycles	12.6	18.4	29.5	-	-	-
Parameter	H ₂ -producing reactor					
	P1	P2	P3	P4	P5	P6
Time (days)	0–7.1	7.1–14.0	14.0–22.3	22.3–28.2	28.2–35.9	35.9–38.0
HRT (h)	12	8	6	12	8	6
^a OLR (g VS/L-d)	93.6	140.4	187.2	93.6	140.4	187.2
^a OLR (g COD/L-d)	175.0	232.5	350.0	175.0	232.5	350
HRT cycles	14.2	20.7	33.2	11.8	23.1	8.4
Parameter	Global					
	P1	P2	P3	P4	P5	P6
Time (days)	0–7.1	7.1–14.0	14.0–22.3	22.3–28.2	28.2–35.9	35.9–38.0
HRT (h)	25.5	17	12.75	12	8	6
^a OLR (g VS/L-d)	44.0	66.1	88.1	93.6	140.4	187.2
^a OLR (g COD/L-d)	82.4	123.5	164.7	175.0	232.5	350
HRT cycles	6.7	9.7	15.6	11.8	23.1	8.4

Note: ^a OLR calculated based on the initial content of VS or COD present in the FW.

described by Farveen et al. [27].

2.6. Data treatment

The biogas produced by both reactors was normalized to standard conditions (0 °C temperature and 1 atm pressure). HPSI was calculated based on Eq. 1, where HPSI refers to the bioH₂ production stability index, while HPR represents the bioH₂ productivity (NL H₂/L-d) during each operational period. For instance, an HPSI value of 1 indicates no variation in HPR, whereas a deviation in HPR equal to the average HPR results in an HPSI of 0. Steady-state conditions for each operational phase were considered when HPSI stayed above 80 % for a minimum of three consecutive HRT cycles, as established in previous research [20, 22].

$$HPSI = 1 - \frac{\text{Standard deviation HPR}}{\text{Average HPR}} \times 100 \quad (1)$$

The statistical analysis of the collected empirical data was carried out using Statgraphics Centurion software (version 19.2.01). A one-way ANOVA test was applied, followed by either a Tukey or Kruskal-Wallis test (significance level $p < 0.05$), depending on whether the dataset exhibited a normal or non-normal distribution, respectively. The Shapiro-Wilk test ($p < 0.05$) was employed to assess data normality. Additionally, a Principal Component Analysis (PCA) was conducted to identify potential directly or inversely proportional relationships between different performance indicator parameters.

The equivalent chemical oxygen demand (CODequiv.) of each OA was determined based on its stoichiometric combustion reaction (Rxn. 1) and calculated using Eq. 2, which relates the molecular weights of O₂ and OA to their respective stoichiometric coefficients. Where, OA refers to the specific organic acid being evaluated; “a,” “b,” “c,” and “d” represent the stoichiometric coefficients in the balanced combustion equation; and O₂MW and OA_{MW} denote the molecular weights of molecular oxygen and the OA, respectively.



$$\text{OA}_{\text{CODequiv.}} = \frac{\text{O}_{2\text{MW}} \bullet b}{\text{OA}_{\text{MW}} \bullet a} \quad (2)$$

3. Results and discussion

3.1. Two-stage lactate-driven dark fermentation

3.1.1. Process performance and hydrogen production

The system was operated in a two-stage reactor configuration for 22 days, applying different HRTs throughout the process (Table 1). The stability periods ranged between days 4.0 and 7.1 for P1, 10.9 and 14.0 for P2, and 15.0 and 18.3 for P3. During system operation, two operational incidents occurred due to clogging issues at the LR feed inlet, specifically on days 10 and 19 of operation (during P2 and P3, respectively). The system recovered steady-state HPR levels in less than one day after the P2 incident. However, the system required three days to restore HPR values to stability following the upset in P3. This recovery period was necessary to reestablish stable HPR values before modifying the system's configuration to a single LD-DF reactor.

Regarding the average stability values, the experiment exceeded the proposed stability limit of 80 % in HPR for all periods within the two-stage reactor configuration (Table 2). Particularly, the decrease in the HRT of the process impacted the two-stage system's productivity, increasing the HPR from 2.5 ± 0.2 L H₂/L-d at P1 to nearly identical values of 4.2 ± 0.2 and 4.4 ± 0.4 L H₂/L-d at P2 and P3, respectively (Table 2 and Fig. 2B). Due to its lower loading rate, this similar HPR resulted in a higher system yield during P2, with an HY of 29.0 ± 2.7 mL H₂/g VS_{added}. The HPR and HY during P3 corresponded to an energy recovery in the form of H₂ of 55.9 ± 4.9 kJ/L-d and 0.3 ± 0.03 kJ/g

Table 2

Primary parameters used to evaluate each HRT tested at both configurations, using steady-state values during continuous LD-DF process.

Parameter	Two-Stage			Single-Stage		
	P1	P2	P3	P4	P5	P6
HRT (h)	12	8	6	12	8	6
Stability period (HRT cycles)	6.0	9.0	16.0	9.6	6.9	8.0
HPSI (%)	90.7	90.9	90.9	87.5	86.4	81.5
LR-BPR (L off-gas/L-d)	1.2	1.6	2.0	-	-	-
HPR (L H ₂ /L-d)	2.5 ± 0.2	4.2 ± 0.2	4.4 ± 0.2	3.8	2.2	1.4
HY (mL H ₂ /g VS _{added})	25.9	29.0	22.9	39.5	15.2	7.5
H ₂ content (%)	47.1 ± 1.6	43.9 ± 2.0	41.1 ± 0.3	40.3	40.3	36.3
H ₂ Energy Recovery	0.33	0.37	0.3	0.5	0.19	0.09
Yield (kJ/g VS _{added})	± 0.03	± 0.03	± 0.03	± 0.1	± 0.03	± 0.02
H ₂ Energy Recovery Rate (kJ/L-d)	31.9	53.0	55.9	48.2	27.8	17.6
	± 3.0	± 4.9	± 4.8	± 6.0	± 3.8	± 3.3

VS_{added}, respectively. The H₂ content of the off-gas produced gradually declined as the HRT was reduced, from 47.1 ± 1.6 % during P1 to 40.9 ± 0.3 % during P3. Additionally, no H₂ production was detected in the LR, preventing the loss of reducing power in gaseous form during this stage of the process. Furthermore, the general performance of the LR was kept stable under the three operational conditions tested, excluding it as a source of variation in the productivity of the HR. The OA profile of this reactor is discussed in the “3.1.3 Organic acids” subsection.

Framing the results here obtained in relation to other studies, most continuous DF systems using FW as a substrate achieve HPR values ranging from 0.2 to 1.4 L H₂/L-d [28]. In addition, although high HPRs have been reported with 1–12 h of HRT during DF, fermenters generally struggle to operate effectively at HRTs shorter than 6 h without biomass retention [16]. Within this range, Paudel et al. (2017) [29] achieved an HPR of 1.35 L biogas/L-d (32.3 % H₂ content) at an HRT of 8 h and an organic loading rate (OLR) of 106 g VS/L-d in a CSTR operating at 37 °C, treating a blend of real FW slurry and brown water in a 7:3 ratio. Villanueva-Galindo et al. (2024) [23] achieved a maximum cumulative H₂ production of 1.74 L H₂/L_{reactor} in lactate-enriched fed DF batch experiments, using a 68:32 mixture of *Megaesphaera elsdenii* and *Clostridium beijerinckii* as inoculum. Algapani et al. [30] reported an HPR of 3 L H₂/L-d (60.8 % H₂ content) at an HRT of 5 days (OLR of 18 g VS/L-d) in a two-stage continuous system for H₂ and CH₄ production at 37 °C from fermenter digestate. Martínez-Mendoza et al. [19] successfully operated a single-stage DF reactor, achieving an HPR of 11.8 L H₂/L-d (65.1 % H₂ content) at an OLR of 188.1 g VS/L-d and an HRT of 6 h in a CSTR processing fruit and vegetable waste via LD-DF. Likewise, García-Depraet et al. [31] explored a two-stage LD-DF system using tequila vinasse, obtaining an optimal HPR of 12.3 L H₂/L-d at 6 h HRT and an OLR of 169 g COD/L-d (104 g VS/L-d). The same optimal HRT of 6 h was obtained by Regueira-Marcos et al. (2024) [22], resulting in an optimal HPR of 9.6 ± 0.9 L H₂/L-d (≈ 40 % H₂ content) and an HY of 49.3 mL H₂/g VS_{added}, applying an identical methodology to the present study while feeding their system with simulated FW. Here it is important to note that while the two-stage LD-DF configuration may involve higher capital and operational costs, their improved metabolic control and potential for enhanced hydrogen productivity could offset these expenses under optimized conditions. In this context, a detailed techno-economic analysis will be essential in future studies to fully evaluate the cost-benefit balance of such configurations.

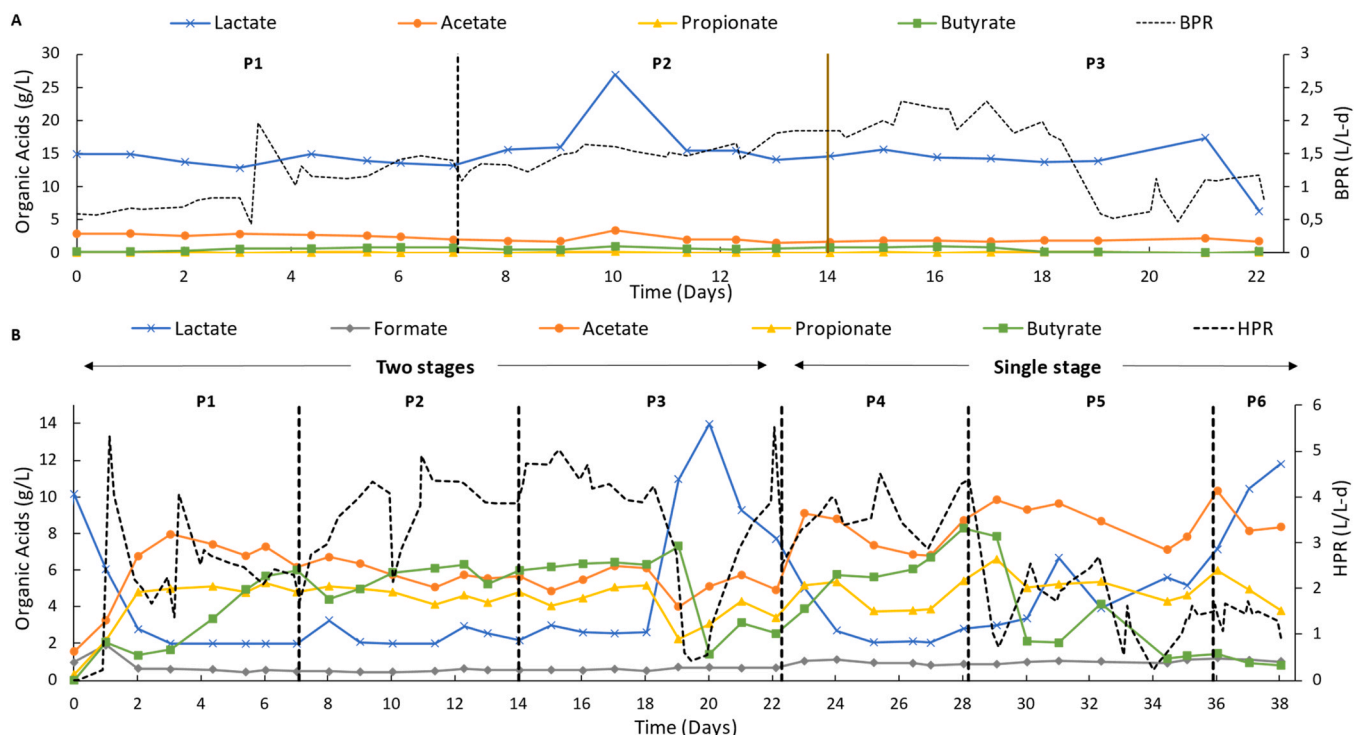


Fig. 2. Time course of the organic acid profile during the LD-DF process along with HPR values in A) the lactate producing reactor (LR) and B) the hydrogen producing reactor (HR).

3.1.2. Alkali usage and removal of volatile solids and carbohydrates

VS removal remained largely unaffected by HRT variations, with average values between 35.0 % and 37.0 % (Table 3, Fig. 3A). The LR accounted for the majority of removal within the system, achieving a share of 78.1 %, 71.8 %, and 76.8 % of the total VS removed during P1, P2, and P3, respectively. Similarly, the average carbohydrate removal efficiencies slightly decreased from 62.9 ± 3.5 % in P1 to 56.0 ± 2.6 % in P3 (Fig. 3C). In this particular case, the LR also played a key role in the removal efficiency, balancing with the HR as the HRT was reduced, likely due to the system's inability to maintain effective process performance at such a short HRT (or high OLR). Specifically, the LR capitalized on 74.9 %, 71.3 %, and 53.6 % of total carbohydrate removal during P1, P2, and P3, respectively, demonstrating its high efficiency in consuming carbohydrates and thus promoting lactate and biomass production (as discussed in detail in Section 3.1.3). The lack of significant variation between periods for both VS and carbohydrate removal confirms that these parameters are not reliable indicators for predicting LD-DF performance, as observed in previous studies [6,20,22,32]. In a prior two-stage LD-DF process with simulated FW, Regueira-Marcos

et al. (2024) reported higher removal efficiencies for both VS (~ 50 %) and carbohydrates (~ 70 %) [22], suggesting a lower degradability of real FW compared to the simulated FW used in that study.

The alkali consumption required to maintain the set pH in the system (Table 3; Fig. 3B) increased as HRT decreased, rising by 34.5 % from P1 (394.8 ± 51.8 mL $\text{OH}_{\text{equiv}}/\text{L-d}$) to P2 (531.1 ± 52.8 mL $\text{OH}_{\text{equiv}}/\text{L-d}$) and by 51.0 % from P2 to P3 (802.2 ± 205.8 mL $\text{OH}_{\text{equiv}}/\text{L-d}$). The highest alkali consumption occurred in the HR, accounting for 58.7 %, 61.0 %, and 60.0 % of the total alkali consumed in the overall process (including both LR and HR) during P1, P2, and P3, respectively, with similar fractions across operational stages. The alkali demand should directly correlate with the degradation of carbohydrates and their subsequent breakdown into OAs. Therefore, the increased feeding rate resulting from the reduction in HRT leads to a significant rise in alkali consumption to maintain a constant pH. A higher alkali demand in the LR would be expected due to its role in carbohydrate degradation, coupled with the low pKa of lactate (3.8) compared to other OAs such as acetate or butyrate (4.8) [33]. However, the lower operating pH in the LR could have contributed to the reduced alkali consumption observed

Table 3

Secondary parameters used to evaluate each HRT tested at both configurations, using steady-state values during continuous LD-DF process.

Parameter	Two-Stage			Single-Stage		
	P1	P2	P3	P4	P5	P6
LR-VS _{removal} (%)	28.1 ± 0.8	25.3 ± 3.0	28.2 ± 3.0	0	0	0
HR-VS _{removal} (%)	7.9 ± 1.3	9.9 ± 3.8	8.6 ± 1.0	35.1 ± 2.9	27.8 ± 1.1	26.6 ± 1.1
Global-VS _{removal} (%)	36.0 ± 2.1	35.2 ± 0.8	36.7 ± 3.9	35.1 ± 2.9	27.8 ± 1.1	26.6 ± 1.1
LR-CH _{removal} (%)	47.1 ± 3.2	42.0 ± 14.2	30.0 ± 10.7	0	0	0
HR-CH _{removal} (%)	15.8 ± 2.6	16.9 ± 10.0	26.0 ± 12.6	53.6 ± 5.1	51.4 ± 13.6	57.8 ± 0.3
Global-CH _{removal} (%)	62.9 ± 3.5	58.9 ± 7.7	56.0 ± 2.6	53.6 ± 5.1	51.4 ± 13.6	57.8 ± 0.3
LR-H _{equiv} ⁺ (mL/L-d)	163.0 ± 36.1	207.0 ± 48.7	320.9 ± 79.4	0	0	0
HR-H _{equiv} ⁺ (mL/L-d)	231.8 ± 21.2	324.1 ± 22.3	481.2 ± 194.0	371.4 ± 136.6	549.9 ± 224.6	785.2 ± 151.4
Global-H _{equiv} ⁺ (mL/L-d)	394.8 ± 51.8	531.1 ± 52.8	802.2 ± 205.8	371.4 ± 136.6	549.9 ± 224.6	785.2 ± 151.4
LR-H _{equiv} ⁺ (mL/g VS _{added})	1.7 ± 0.4	1.4 ± 0.3	1.7 ± 0.4	0	0	0
HR-H _{equiv} ⁺ (mL/g VS _{added})	2.4 ± 0.2	2.3 ± 0.2	2.5 ± 1.0	4.6 ± 2.7	3.5 ± 1.95	4.1 ± 0.8
Global-H _{equiv} ⁺ (mL/g VS _{added})	4.1 ± 0.5	3.7 ± 0.4	4.2 ± 1.1	4.6 ± 2.7	3.5 ± 1.95	4.1 ± 0.8

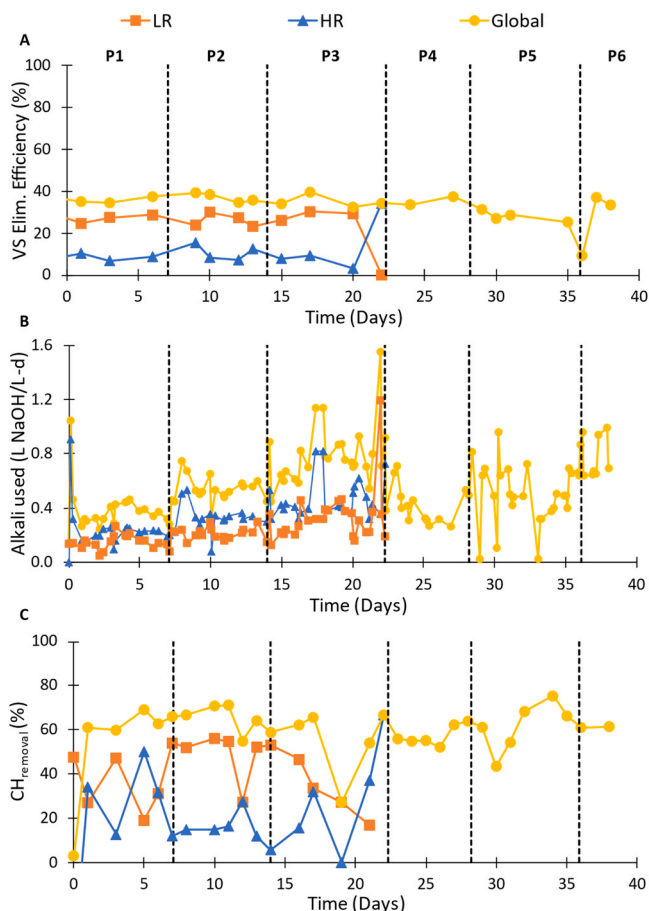


Fig. 3. Time course of A) volatile solids removal (VS_{removal} ; %), B) Alkali consumption (mL/L-d), and C) Carbohydrate removal (CH_{removal} ; %) in the lactate producing reactor (LR), the hydrogen producing reactor (HR), and in the overall LD-DF process.

in this reactor, which may also explain the higher demand in the HR, as it requires a pH increase from 4.5 to 6.5. In a previous study using simulated FW [22], the overall alkali consumption was lower at 6 h HRT (4.2 mL OH-equiv/g VS_{added}) compared to this study, with a more balanced distribution between both reactors (~50 %).

3.1.3. Organic acids

Regarding the production of OAs (Table 4; Fig. 2A), lactate was the predominant OA in the LR during the whole operation, accounting for

81.6, 85.5, and 84.3 % of the total acids present in the culture broth, during P1 (14.2 ± 1.0 g/L), P2 (14.9 ± 0.6 g/L) and P3 (14.1 ± 0.4 g/L), respectively. Therefore, the LR succeeded in selectively producing lactate as the main OA, minimizing the allocation of reducing power towards the synthesis of alternative by-products. The concentration remained largely stable at around 14–15 g/L throughout the operation, with the exception of the peak concentration on day 10 when, following the restoration of the feed after a clogging event, the concentration spiked to 26.9 g/L, stabilizing the following day. Considering a feed TS concentration of 50 g/L (with 65.9 % carbohydrate), this peak implies a high conversion ratio of 0.82 g lactate/g carbohydrate, almost doubling from the values of 0.43–0.46 g lactate/g carbohydrate obtained during stability periods. This steady-state conversion ratios were higher than those observed in a previous two-stage system with simulated FW, where values of 32.7–39.9 g lactate/g carbohydrate were achieved [22]. On the other hand, Pau et al. (2024) obtained the same high yield of 0.82 g lactate/g carbohydrate at 14 days HRT in the lactate fermentation process from FW [34]. This high conversion ratio could probably be derived from a starvation stress process, where a sudden feed overloading after a short famine period could have enhanced the degradation rate of the microbial community [20,35,36]. The accumulation of other acids was considerably lower in this reactor, although it remained constant throughout the process, with acetate being the predominant OA within this pool, followed by butyrate. The presence of these OAs, along with the biogas production, indicates the onset of the oxidative decarboxylation pathway in this process [37,38]. Contrary to the concentrations of these OAs, biogas production in LR rose slightly between P1 and P3 (1.2 ± 0.2 – 2.0 ± 0.2 L/L-d), the composition of which consisted only of CO_2 . No H_2 was detected, even though the stoichiometry of the production of these OAs imposes an excess of reducing power which can be released as this gaseous compound [38,39]. In general, the concentration of OAs remained almost unaltered by the stepwise reductions in HRTs. Compared to the literature, Regueira-Marcos et al. (2024) also observed small variations in OA composition during a stepwise reduction of HRT (from 12 to 6 h) in a two-stage system fed with simulated FW [22]. In this regard, De Groof et al. concluded that low HRTs and high OLRs favor the accumulation of lactate over other organic acids [40]. Based on this, it is possible that the range of operating conditions applied in the present study was not significantly wide enough to show impactful differences in the OA profile within the context of a lactate fermentation system. In other words, higher lactate yields from FW may be achieved at increased TS concentrations, provided that appropriate microbial communities and operating conditions are maintained.

On the other hand, the HR exhibited a more diverse OA profile (Table 4; Fig. 2B). Lactate from the LR was degraded, leading to the formation of new OAs [41]. Based on the average concentrations under

Table 4

Steady-state organic acids concentrations measured in the lactate-producing reactor (LR) and in the hydrogen-producing reactor (HR).

Reactor	Organic acid (g/L)	Two-Stage			Single-Stage		
		P1	P2	P3	P4	P5	P6
LR	Lactate	14.2 ± 1.0	14.9 ± 0.6	14.5 ± 0.8	0	0	0
	Formate	0.17 ± 0.03	0.12 ± 0.0	0.11 ± 0.0	0	0	0
	Acetate	2.3 ± 0.4	1.8 ± 0.2	1.8 ± 0.1	0	0	0
	Propionate	0	0	0.1 ± 0.04	0	0	0
	Isobutyrate	0	0	0	0	0	0
	Butyrate	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.4	0	0	0
	Isovalerate	0	0	0	0	0	0
	Valerate	0	0	0	0	0	0
HR	Lactate	2.2 ± 0.6	2.4 ± 0.4	2.7 ± 0.2	2.8 ± 1.1	5.3 ± 1.9	11.1 ± 1.0
	Formate	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.1
	Acetate	6.9 ± 0.5	5.5 ± 0.3	5.7 ± 0.6	7.9 ± 1.0	9.2 ± 0.7	8.2 ± 0.2
	Propionate	5.0 ± 0.2	4.4 ± 0.3	3.9 ± 0.5	4.6 ± 0.8	5.3 ± 0.1	4.4 ± 0.8
	Isobutyrate	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
	Butyrate	4.9 ± 1.1	5.9 ± 0.5	6.3 ± 0.1	6.0 ± 1.4	3.1 ± 1.5	0.9 ± 0.1
	Isovalerate	0	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0
	Valerate	0	0	0	0	0	0

steady state for each period, the HR degraded 84.5 %, 83.9 %, and 82.3 % of the lactate pumped from the LR during P1, P2, and P3, respectively. Despite these high removal efficiencies, lactate could not be completely depleted in the HR, as observed in the previous experiment with simulated FW operating between 12 and 6 h of HRT [22]. Lactate degradation in the HR was primarily directed toward the production of acetate, propionate, and butyrate, with smaller amounts of formate, isobutyrate, and isovalerate. Based on the evolution of OA concentrations, data indicate that during P1, the levels of acetate (6.9 ± 0.5 g/L) and propionate (5.0 ± 0.2 g/L) were higher than those of butyrate (4.9 ± 1.1 g/L). However, when the HRT was reduced (P3), this pattern shifted, with butyrate concentrations increasing (6.7 ± 0.5 g/L) and a concomitant decrease in acetate (5.2 ± 1.1 g/L) and propionate (3.9 ± 1.5 g/L) levels. Thus, higher HPRs were associated with increased butyrate concentrations relative to acetate and propionate, which was consistent with previous studies [20,22]. This effect is particularly relevant for propionate, whose formation from pyruvate or lactate requires an investment in reducing power by microorganisms, consequently lowering bioH₂ yield [39,41]. During the clogging incident on day 19, lactate levels rose rapidly, reaching 14.0 g/L on day 20. These levels decreased to 7.7 g/L by the end of P3 (day 21) and did not return to previous values of around 2.0 g/L until day 24 of operation. The increase in lactate concentration was accompanied by a reduction in butyrate levels, which also did not recover to previous levels until day 24.

An analysis of the COD equivalents of the measured OAs (Table 5) indicates that the total concentration across periods for each reactor exhibited minimal variation. The values ranged between approximately 18.0 and 19.0 g COD/L in the LR, whereas the HR reached concentrations of around 27 g COD/L. Based on these values, the transition from LR to HR resulted in an increase in COD equivalents of OAs by 41.3 %, 39.7 %, and 48.9 % for P1, P2, and P3, respectively. This increase can be

attributed to the additional carbohydrate degradation occurring in the HR. However, it is important to highlight that, although 50–70 % of the initial carbohydrates fed into the system reached the HR, only 15–26 % of this fraction was degraded, while nearly all the lactate supplied (80–90 %) was removed, a phenomenon previously reported by Regueira-Marcos et al. (2024) [22]. This suggests a preferential utilization of lactate over carbohydrate degradation by the HPBs prevailing in the HR, as observed by Fuentes-Santiago et al. (2023) [42] and Villanueva-Galindo et al. (2024) [23]. Alternatively, this trend may be explained by the high presence of partially recalcitrant carbohydrate-rich compounds in the FW, a phenomenon also observed in previous studies analyzing the anaerobic degradation of kitchen FW, where complex polysaccharides such as cellulose and hemicellulose exhibited limited biodegradability [43,44].

3.2. Single-stage lactate-driven dark fermentation

3.2.1. Process performance and hydrogen production

Following the removal of the LR, the system was operated in a single-stage reactor configuration for 15.7 days, with HRT values modified as described in Table 1. The stability periods used for evaluating each condition ranged from 23.4 to 28.2 days for P4, 30.0–32.3 days for P5, and 36.1–38.0 days for P6. In this configuration, a clogging event occurred on day 33 of operation during P5. The system required approximately three days to recover a stable HPR value following the short starvation period caused by the unforeseen operational upset, after which the condition was adjusted to P6. In contrast to the two-stage reactor configuration, the average stability values (Table 2 and Fig. 2B) indicate that HPR decreased as HRT was reduced, declining from 3.8 ± 0.6 L H₂/L-d at P4 to 1.4 ± 0.3 L H₂/L-d at P6, during which system productivity significantly collapsed. The system at P4 exhibited the highest HY value recorded in this comparative study, reaching 39.5 ± 6.0 mL H₂/g VS_{added}. In terms of energy output, the productivity recorded during P4 resulted in an energy recovery, in the form of bioH₂, of 48.2 ± 7.3 kJ/L-d and 0.5 ± 0.1 kJ/g VS_{added}. Regarding the H₂ content of the produced gas, it remained around 40 % during P4 and P5 but decreased to 36 % during P6. As observed in the two-stage reactor configuration, the system successfully exceeded the 80 % stability threshold for all HRT conditions tested in this test series.

Excluding the HY value achieved at P4, the transition from a two-stage to a single-stage system resulted in a noticeable decline in system performance. This reduction may be attributed to system overload caused by an excessive OLR (175.0–350.0 g COD/L-d), which could explain why the successive reduction in HRT (from 12 to 6 h) led to an even greater decrease in HPR, contrary to observations in the two-stage system. Although the HRTs applied in the HR were identical in both configurations, in the two-stage system, the overall organic load was distributed between two reactors, whereas in the single-stage system, the HR processed the entire OLR alone. Most studies conducted on DF of various substrates indicate that optimal OLR values range between 100 and 200 g COD/L-d [16]. Accordingly, both P3 (164.7 g COD/L-d) and P4 (175.0 g COD/L-d), the most productive conditions in each configuration, were operated under relatively similar OLRs. In contrast, the OLRs in P5 and P6 exceeded 200 g COD/L-d, which may have negatively impacted system performance. These findings showed that both OLR and HRT play critical roles in optimizing HPR in LD-DF systems, with OLR influencing system overload and substrate availability, while HRT affects microbial adaptation and retention time for fermentation processes. In this context, a comparable HPR of 4.2 L H₂/L-d (53 % H₂ content) and an HY of 38.8 mL H₂/g VS_{added} were reported by Regueira-Marcos et al. (2023) [20] under a longer HRT of 16 h and a slightly lower OLR of 149.3 g COD/L-d (equivalent to 108 g VS/L-d) in a CSTR processing simulated FW at 37°C. An alternative explanation for the decline in productivity following the configuration change may be the adaptation of the HR microbiota from a lactate-rich digested substrate. Not all HPB are capable of directly metabolizing lactate from the

Table 5

Steady-state COD-equivalent of the organic acid concentrations (based on a combustion reaction stoichiometry) measured in the lactate-producing reactor (LR) and in the hydrogen-producing reactor (HR).

Reactor	Organic Acid (g/L)	Two-Stage			Single-Stage		
		P1	P2	P3	P4	P5	P6
LR	Lactate	15.1 ± 1.1	15.9 ± 0.6	15.0 ± 0.6	0.0	0.0	0.0
	Formate	0.1 ± 0.0	0.0	0.0	0.0	0.0	0.0
	Acetate	2.5 ± 0.4	1.9 ± 0.2	1.9 ± 0.1	0.0	0.0	0.0
	Propionate	0.0	0.0	0.2 ± 0.1	0.0	0.0	0.0
	Isobutyrate	0.0	0.0	0.0	0.0	0.0	0.0
	Butyrate	1.3 ± 0.4	1.1 ± 0.2	1.1 ± 0.7	0.0	0.0	0.0
	Isovalerate	0.0	0.0	0.0	0.0	0.0	0.0
	Total	18.9 ± 1.9	18.9 ± 1.0	18.2 ± 1.3	0.0	0.0	0.0
	Lactate	2.3 ± 0.6	2.6 ± 0.4	2.7 ± 0.0	3.0 ± 1.2	5.6 ± 2.0	11.8 ± 1.1
	Formate	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
HR	Acetate	7.4 ± 0.5	5.9 ± 0.3	5.5 ± 1.2	8.4 ± 1.1	9.8 ± 0.7	8.7 ± 0.2
	Propionate	7.6 ± 0.3	6.7 ± 0.5	5.9 ± 2.3	7.0 ± 1.2	8.0 ± 0.2	6.7 ± 1.2
	Isobutyrate	0.4 ± 0.2	0.2 ± 0.9	0.2 ± 0.9	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0
	Butyrate	8.9 ± 2.0	10.7 ± 0.9	12.2 ± 0.9	10.9 ± 2.5	5.6 ± 2.7	1.6 ± 0.2
	Isovalerate	0.0 ± 0.0	0.2 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.0 ± 0.0
	Valerate	0.0	0.0	0.0	0.0	0.0	0.0
	Total	26.7 ± 3.7	26.4 ± 2.3	27.1 ± 4.6	30.4 ± 6.2	30.2 ± 5.8	29.4 ± 2.7

culture medium [41]. Consequently, the shift to a feedstock rich in undigested complex carbohydrates (such as untreated FW) would require microbial adaptation in the HR. Given the prior interconnection between the LR and the HR in the two-stage reactor configuration, it is reasonable to assume that LAB from the LR were also present in the HR. Thus, the microbial composition of the HR would likely adapt rapidly to facilitate lactate fermentation alongside DF. In this regard, it is important to emphasize that the previous two-stage operation provides critical context for interpreting the single-stage results. Although LAB are ubiquitous in FW and their competitive displacement of HPB is well documented [16], it would be inappropriate to assume that the HR in a standalone single-stage configuration would exhibit the same microbial succession or metabolite profile under the tested conditions. Here, it is worth mentioning that no reinoculation was carried out in the HR during the switch from a two-stage to a single-stage configuration. In this context, conducting parallel experiments using identical inoculum, substrate, setup, and operating conditions is recommended for future studies aiming to characterize the performance of LD-DF in single- and two-stage configurations. This approach would minimize the influence of differing operational histories on the fermentative microbial community, thereby allowing for a more accurate comparison of system performance.

3.2.2. Alkali usage and removal of volatile solids and carbohydrates

VS removal during P4 remained at an average value of $35.1 \pm 2.9\%$, comparable to the results obtained in the two-stage configuration (Fig. 3A, Table 3). VS removal then declined to $27.8 \pm 1.1\%$ in P5 and $26.6 \pm 15.1\%$ in P6. In the case of P6, the low VS removal was linked to a marked performance decline during the phase transition. However, the system recovered, reaching 37% in subsequent measurements, suggesting a transient effect rather than a sustained inefficiency. With respect to carbohydrates (Fig. 3C), the average values remained similar to those observed in the two-stage reactor configuration ($59.3 \pm 4.6\%$ and $54.6 \pm 6.3\%$ for the two-stage and single-stage configurations, respectively). Interestingly, the highest carbohydrate removal was recorded during P6, averaging $57.8 \pm 0.3\%$. For pH control (Fig. 3B), alkali consumption in the single-stage configuration followed a similar pattern to that observed in the two-stage system, increasing as HRT decreased. Thus, NaOH consumption rose by 48% from P4 to P5 and by 42.8% from P5 to P6. The total alkali usage was comparable between both configurations, with slightly lower consumption in the single-stage system at 12 and 6 h HRT (5.9% and 2.1% lower, respectively), yet marginally higher at 8 h HRT (0.4% higher).

3.2.3. Organic acids

The shift in configuration resulted in an increase in total OA concentrations from 19 to 20 g/L to 22 – 26 g/L, despite a slight reduction in the carbohydrate removal rate (Table 4). This rise in total OA concentration did not necessitate a higher alkali dosage, as previously described. Additionally, the decrease in HRT resulted in an increase in total OA concentration from 22.7 ± 4.5 g/L at P4 to 25.8 ± 2.2 g/L at P6. The OA profile followed a well-defined trend throughout the configuration. Lactate concentration increased as HRT was reduced, rising from 2.8 ± 1.1 g/L at P4 to 11.1 ± 1.0 g/L at P6. Conversely, butyrate concentration decreased from 6.0 ± 1.4 g/L at P4 to 0.9 g/L at P6. The variation in butyrate concentration closely aligned with changes in HPR in both configurations. Acetate and propionate levels peaked at P5, reaching 9.2 ± 0.7 g/L and 5.3 ± 0.1 g/L, respectively, though their fluctuations across periods were notably smaller compared to those observed for lactate and butyrate. While propionate levels remained similar to those in the two-stage reactor configuration, acetate concentrations increased overall by 43.8% upon switching to the single-stage system. The concentrations of formate, isobutyrate, and isovalerate showed minimal variation between periods, except for the complete disappearance of isovalerate during P6.

Concerning the COD equivalents (Table 5), the transition from the

two-stage to the single-stage configuration resulted in an overall increase in energy retention in the form of OAs, with values reaching approximately 30 g COD/L in the single-stage configuration. Although the reduction in HRT led to an increase in total OA concentration (from 22.7 ± 4.5 g/L at P4 to 25.8 ± 2.2 g/L at P6), in terms of COD, the amount of retained energy was slightly higher in P4 than in P6, with values of 30.4 ± 6.2 g COD/L and 29.4 ± 2.7 g COD/L, respectively. This minor difference can be attributed to the higher energy density of butyrate (1.82 g COD/g) compared to lactate (1.07 g COD/g), which offsets the balance despite its lower concentration in the culture broth.

3.3. Principal component analysis

The PCA analysis was conducted by selecting the most relevant indicators of system performance based on the results of both configurations, explaining approximately 72% of the total variance between the two components (Fig. 4). The analysis confirmed the positive correlation between HPR, HY, and butyrate concentration. Conversely, lactate, acetate, and propionate levels, along with alkali consumption, exhibited a negative correlation with HPR. The positive relationship between HPR and HY is solid, as both parameters increase when reducing power is redirected into H_2 -producing pathways. The negative correlation of propionate with HPR is reasonable, given that its formation from lactate or acetate requires reducing power, thereby potentially lowering $bioH_2$ production [39,45,46]. Furthermore, an accumulation of lactate in the culture broth (i.e., P5 and P6) would imply that the consumption of this OA by LU-HPB is being hindered by the conditions imposed on the systems, thereby limiting the amount of substrate redirected to H_2 -producing pathways. An over-proliferation of LAB over LU-HPB could also explain this outcome. However, from a stoichiometric perspective, acetate production (4 mol H_2 /mol glucose) should theoretically enhance $bioH_2$ formation compared to butyrate production (2 mol H_2 /mol glucose) [38,39]. Despite this, the observed relationship between HPR and acetate and butyrate concentrations has been reported in previous studies [19,20,22]. Regarding this association, studies on OA concentration dynamics in the human gut suggest that high H_2 concentrations in the gas phase favor butyrate production over acetate, as this shift reduces H_2 output [38]. However, none of the aforementioned studies reported significant differences in H_2 concentration in the gas phase [19,20,22]. Another perspective is that since each mole of butyrate produced requires the same electron investment as two moles of acetate [37], further increases in acetate levels relative to butyrate could lead to a reduction in carbohydrate metabolism through

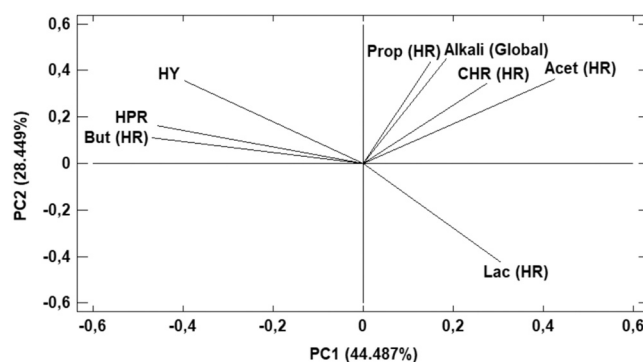


Fig. 4. PCA analysis carried out for different process performance indicators evaluated for both the two-stage and single-stage configurations. Details in brackets indicate for which reactor the parameter is referred to or if the parameter refers to the global process. LR: lactate-producing reactor; HR: H_2 -producing reactor; Global: both reactors; HPR: hydrogen production rate; HY: hydrogen yield; Lac: lactate concentration; Acet: acetate concentration; Prop: propionate concentration; But: butyrate concentration; Alkali: volume of alkali consumed to keep pH controlled; CHR: carbohydrate removal; VS: volatile solids removal.

bioH₂-producing pathways. This shift could result from excessively low HRT or, more likely in this context, system overload due to an excessively high OLR. Additionally, it is important to highlight that excessive acetate accumulation in the culture medium could lead to product inhibition processes [33].

3.4. Microbial characterization

The microbiological results reveal differences in the microbial communities across both configurations, partly due to the presence or absence of specific taxa but primarily in terms of their relative abundance (Fig. 5). The raw substrate was characterized by a low prevalence of any dominant genus, exhibiting high microbial diversity, with *Lactobacillus*, *Bacillus*, *Lactiplantibacillus*, and *Leuconostoc* being the most notable genera, although their combined relative abundance did not exceed 15 % of the total. It is noteworthy that the diluting effect observed in the analysis was due to the high relative abundance of “unidentified_chloroplast” and “unidentified_mitochondria,” which together accounted for over 50 % of the total abundance. These categories reflect the presence of chloroplasts and mitochondria from the plant and animal cells present in the FW matrix. It is also noteworthy that the high relative abundance assigned to the “Others” classification includes genera with a relative abundance of < 1 %, which in total accounted for around 35 % of the total abundance. On the other hand, the inoculum was mainly composed of *Lactobacillus* (55.2 %), *Klebsiella* (28.0 %), *Clostridium* (10.9 %), *Stenotrophomonas* (3.0 %), and *Acinetobacter* (1.8 %), as described in Section 2.1.

Concerning the reactors, the LR succeeded in selecting LAB, where about 80 % of the entire RA was composed of the genus *Lactobacillus* (53.7 %) and the genus *Bacillus* (26.8 %). These genera, and more specifically the genus *Lactobacillus*, are emblematic within the LAB group, being predominant in lactic fermentations due to their capacity to tolerate acidic pH levels [47]. *Bacillus* spp. can present lactate and/or H₂ producing species in the genera [39,47], but as no H₂ was produced at LR, it can be assumed that the detected *Bacillus* species was related to lactate production. Conversely, the transition from LR to HR had no impact on the relative abundance of *Lactobacillus*, which remained virtually unchanged at a relative abundance of 54.6 %. Nonetheless, the *Bacillus* genus was reduced to 3 %, thus allowing passage to other genera capable of carrying out lactic acid fermentation such as *Olsenella* (6.4 %) or *Bifidobacterium* (3.8 %), which were less adapted to the acidic pH (4.5) in LR [40,48]. Beyond LAB, the notable presence of the genera *Veillonella* (16.2 %) and *Bacteroides* (5.5 %) is particularly relevant, as both are known to contribute to H₂ production from organic substrates [49,50]. Specifically, these genera possess the metabolic capacity to

uptake lactate from the medium and convert it into H₂ and other OAs [41]. *Veillonella* especially excels in this role, and its H₂ production from lactate-rich substrates has been well documented previously. It is also a genus closely related to *Megaesphaera eldesnii*, as part of the *Veillonaceae* family [39], a well-studied species based on its aptitude to consume lactate during DF [51]. The presence of the *Bacteroides* genus is frequently less reported in the LD-DF process, even though its capability of producing H₂ from raw wastes has been previously documented [49].

Switching to a single-stage configuration resulted in a significant reduction in the prevalence of the *Lactobacillus* genus (20 %), facilitating the emergence of other genera capable of lactic fermentation, including *Olsenella* (19.5 %) and *Lactiplantibacillus* (5 %) [40]. This decline in *Lactobacillus* prevalence can be primarily attributed to its previous association with the LR, which continuously supplied these organisms to the HR. Upon decoupling, the reduction of *Lactobacillus* permitted the rise of other lactic acid-producing genera better suited to less acidic pH levels (i.e., 6.5). Additionally, among H₂ producers, the genus *Veillonella* flourished (28.4 %), while the presence of *Bacteroides* decreased to 2 % compared to the prior two-stage configuration. Notably, none of the key high-performance bacteria (HPB) present in the inoculum, such as *Klebsiella* and *Clostridium*, maintained dominance in the HR reactor under the tested conditions. Although these genera are well-established contributors to the conventional DF process [39], their effectiveness, particularly for *Klebsiella*, in the LD-DF process appears to be less competitive. This may be due to factors such as a limited capacity to uptake lactate from the medium, as observed in certain *Klebsiella* species [41], or a diminished ability to compete with *Veillonella* and *Bacteroides* for substrate utilization under the specific operational conditions applied [39,41].

4. Conclusions

This study demonstrated that a two-stage LD-DF system enhances H₂ productivity compared to a single-stage system when using real FW as a substrate. By progressively reducing the HRT, the two-stage system achieved the highest HPR of 4.4 ± 0.4 L H₂/L-d at a 6 h HRT, whereas the single-stage system exhibited a decline in performance under shorter HRT conditions. Nevertheless, the highest HY of 39.5 ± 6.0 mL H₂/g VS_{added} was observed in the single-stage configuration at a 12 h HRT. PCA confirmed a positive correlation between HPR and butyrate production, while lactate, acetate, and propionate negatively impacted HPR. Microbial characterization showed *Veillonella* and *Bacteroides* as the main HPBs during LD-DF in both configurations. These findings underscore the advantages of a two-stage configuration in LD-DF systems, enabling higher productivity at reduced HRTs. Overall, the

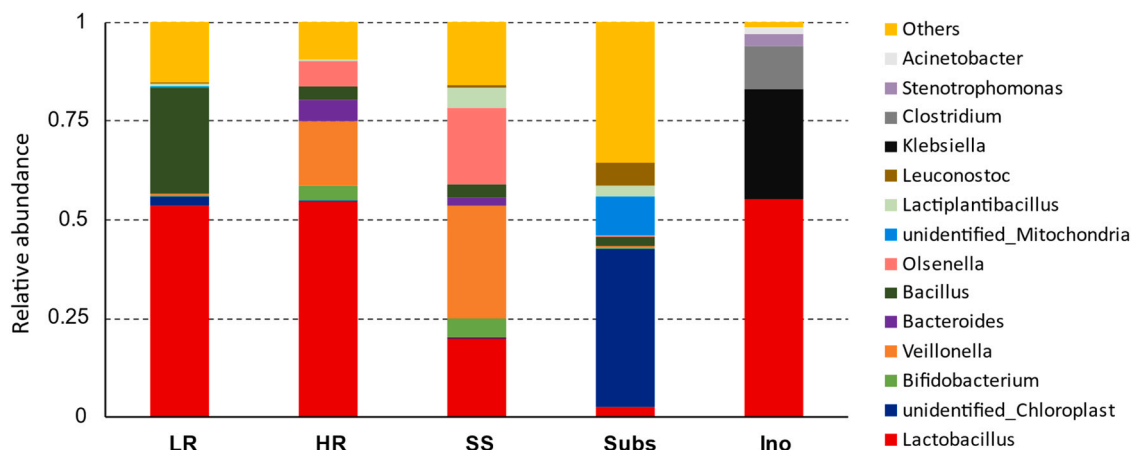


Fig. 5. Bar plot of the relative abundances of the main genera present during the steady states at optimal conditions. Genera with relative abundances < 1 % are included in the group “Others.” “LR” stands for lactate reactor; “HR” stands for H₂ reactor; “SS” stands for single-stage reactor; “Subs” stands for the natural microbiota present in the substrate; “Ino” stands for the inoculum microbiota, whose data were obtained from García-Depraect et al. (2022) [25].

implementation of two-stage LD-DF systems represents a promising strategy for optimizing bioH₂ production from FW. The rationale behind this two-stage concept is to mitigate H₂ inhibition issues associated with LAB overgrowth. Additionally, FW is rich in LAB and lactate, particularly when it undergoes pre-fermentation during storage, making this approach particularly relevant. Future studies are needed to optimize the process for its future viability, such as the implementation of FW storage as a replacement for the lactate production stage or the coupling with subsequent processes, like methanogenic stage or the production of other high-value compounds of interest in an integrative biorefinery scheme.

CRedit authorship contribution statement

Lois Regueira-Marcos: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Raúl Muñoz:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **García Depraect Octavio:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

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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Data Availability

Data will be made available on request.

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