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Feasibility Study of Biohydrogen Production from Acid Cheese Whey via Lactate-Driven Dark Fermentation

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Abstract: The high loading of lactic acid bacteria (LAB) present in cheese whey still limits its use as hydrogen feedstock. This study aims to investigate the feasibility of producing hydrogen from acid cheese whey via lactate-driven dark fermentation (LD-DF). Mesophilic batch fermentations were performed with delipidated acid cheese whey at a fixed pH of 5.8 and driven by an acidogenic bacterial culture containing LAB and lactate-oxidizing hydrogen producers (LO-HPB). The results obtained indicated that it is technically feasible to produce hydrogen from undiluted cheese whey through lactate oxidation-mediated fermentation. It was elucidated that the acidogenic fermentation of cheese whey followed a two-step lactate-type fermentation, in which fermentable carbohydrates were first converted into lactate, and then lactate was metabolized into hydrogen with the co-production of butyrate. The hydrogen yield and the maximum volumetric hydrogen production rate achieved were 44.5 ± 2.9 NmL/g-COD_{fed} and 1.9 NL/L-d, respectively. Further microbial community analysis revealed that *Lactobacillus*, *Clostridium*, and *Klebsiella* were the dominant bacterial genera when the hydrogen production rate peaked. It was therefore suggested that the metabolic potential behind the association between LAB and LO-HPB was important in driving the two-step lactate-type fermentation. Overall, the LD-DF can be a strategic hydrogen-producing pathway to be implemented with cheese whey.

Keywords: bioenergy; cheese whey; dark fermentation; hydrogen; lactate

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1. Introduction

Cheese whey (CW) is the greenish-yellow liquid generated after the precipitation and removal of milk casein during cheese processing [1,2]. In the dairy industry, it is estimated that each kilogram of cheese produced generates about 10 L of CW [3,4]. Linking that figure with the fact that around 18 million tons of cheese are produced annually worldwide [5], it can be estimated that the global annual CW generation amounts to 160–200 million tons. The composition of CW varies mainly depending on the source of milk (e.g., sheep, goat, cow, or buffalo), quality of milk, type of cheese, and elaboration process [1,6]. Despite that intrinsic variation, CW typically has high chemical oxygen demand (COD; 50–80 g/L) and biochemical oxygen demand (BOD; 40–60 g/L) and retains about 55% of milk nutrients, including lactose, soluble proteins, lipids, and mineral salts [1,7,8]. Unfortunately, the

sustainable management of CW is still challenging for some small and medium dairy industries, which collectively dispose of large amounts of highly polluting CW into water bodies or drains without applying proper treatment [9]. This prohibited practice is of serious concern due to its social, environmental, and economic implications [4], but it also represents an opportunity for the development and deployment of sustainable and cost-effective (bio)technologies aimed at valorizing CW.

Due to its biodegradable nature and high organic load (mainly associated with milk carbohydrates), CW can be used as feedstock to produce biofuels or other value-added products within a biorefinery framework following the circular economy principles. In this context, the valorization approach of transforming CW to renewable hydrogen has gained interest in recent years [6,10,11]. Hydrogen is indeed used as a feedstock in various industries, such as the food industry, petroleum processing, electronic industry, and fertilizer industry, among others [12]. Hydrogen can also be used as a clean energy vector, as it has a high calorific value of 120 MJ/kg (2.75 times higher than hydrocarbon fuels) and produces only water vapor when combusted [13]. In this context, dark fermentation (DF) is one of the leading biological processes for producing biogenic hydrogen from a wide range of organic wastes and wastewater [14,15]. In the DF process, fermentable carbon sources are mainly transformed (under anoxic and dark conditions) into hydrogen, but other by-products such as short-chain organic acids (e.g., acetate, butyrate, propionate, lactate) can be produced concomitantly. Over the last two decades, several DF studies have been carried out with different substrates, including food and agricultural wastes, energy crops, lignocellulosic residues, and carbohydrate-rich wastewater [16,17]. The use of CW as a fermentative hydrogen feedstock has been studied by several researchers [18–22]. One of the main limitations for efficiently producing fermentative hydrogen from CW is indeed related to the high density of lactic acid bacteria (LAB) that it presents [23–28]. Considering that LAB is commonly the dominant spoilage bacteria in CW [29], it can be expected that the use of CW as feedstock increases the latent risk of LAB invasion during fermentation. LAB may outcompete hydrogen producers by different mechanisms, such as substrate competition, acidification of the broth, and bacteriostatic inhibition, due to their capacity to release bacteriocins into the broth (which are compounds with inhibitory activity against target bacteria) [30,31]. It is worthwhile to note that lactose is the main carbohydrate present in CW, and lactate is a key intermediate of lactose fermentation [27,32].

There is evidence that has been disclosed recently arguing that lactate-driven dark fermentation (LD-DF) can be a smart approach to cope with the excessive thriving of LAB and associated hydrogen production inhibition [33]. This fermentative hydrogen-producing route lies in harnessing the interactions existing between LAB and certain types of hydrogen-producing bacteria (HPB) that are metabolically capable of oxidizing lactate and releasing hydrogen gas (hereafter referred to as lactate-oxidizing hydrogen-producing bacteria, LO-HPB) [33,34]. LAB drive the transformation of fermentable carbohydrates mainly into lactate, yet sometimes it is accompanied by small amounts of acetate or ethanol, depending on whether the dominant fermentation pathway is homo- or heterofermentative. Likewise, LO-HPB are responsible for producing hydrogen, butyrate, and carbon dioxide (CO_2) by taking up lactate, commonly hand-in-hand with acetate or even ethanol, which are both utilized as acceptor donors [35]. The cross-feeding of lactate that has been observed in dark fermenters resembles the lactate cross-feeding that occurs in the human intestinal microbiota. Previous in vitro experiments have revealed that the rate of lactate utilization by butyrogenic bacteria can exceed two times that of lactate production by LAB such as *Bifidobacterium* spp. [36]. In line with this, the LD-DF pathway has been observed with the use of synthetic wastewater mimicking cheese whey [10,37]. In another study, De Gioannis et al. [38] suggested the presence of LD-DF using real CW, while Asunis et al. [6] reported that the autochthonous microflora of freeze-thaw CW was able to perform the oxidation of lactate with concomitant hydrogen production. Recently, Muñoz-Páez et al. [39] found that the LD-DF pathway played a significant role in the continuous hydrogen production from real, diluted CW rich in lactate (13.8 g/L). Based on the aforementioned information,

it can be argued that despite the utmost importance of the LD-DF pathway to produce hydrogen efficiently from real, lactate-rich CW enriched in LAB, only a limited number of reports on this field have been disclosed. In the present study, it is hypothesized that the use of a highly specialized bacterial consortium with proven LD-DF activity will ensure the LD-DF of CW [40–43]. Hence, the aim of this study was to investigate the feasibility of hydrogen production from real CW via LD-DF by a specialized mixed culture. An overarching approach, including typical process characterization and microbial community analysis, was employed.

2. Materials and Methods

2.1. Feedstock

CW was collected from a cow-made cheese producer in Lagos de Moreno, Jalisco, Mexico. A representative sample of 50 L was collected and kept at 4 °C until use. To remove fats and suspended solids, CW was centrifuged at 4000 rpm for 20 min. with a centrifuge Rotina 380 (Hettich, Tuttlingen, Germany), and then decanted and sieved through an 850 µm mesh strainer. The CW used was not sterilized, it therefore contained autochthonous bacteria such as LAB that might have an impact on the process. The physicochemical composition of the raw and preconditioned CW is presented in Table 1.

Table 1. Physicochemical characteristics of the acid CW used in this study.

Parameter	Raw CW	Preconditioned CW
pH	5.4 ± 0.02	5.4 ± 0.04
Total alkalinity (g $CaCO_3$/L)	3.8 ± 0.1	1.0 ± 0.02
Total COD (g/L)	118.1 ± 1.3	83.3 ± 0.5
Soluble COD (g/L)	95.4 ± 0.64	66.3 ± 0.2
Total nitrogen (g/L)	1.7 ± 0.3	0.4 ± 0.09
Ammonia nitrogen (mg/L)	ND	67.4 ± 0.3
Protein (g/L)	5.4 ± 0.03	3.6 ± 0.03
Total phosphorous (g/L)	2.9 ± 0.2	0.3 ± 0.02
Total sugars (g/L)	51.8 ± 1.3	44.9 ± 1.1
Lipids (g/L)	6.0 ± 0.01	0.1 ± 0.01
Total solids (g/L)	67.5 ± 0.8	52.2 ± 0.6
Volatile solids (g/L)	62.0 ± 0.1	46.8 ± 0.3
Lactate (g/L)	1.0 ± 0.1	2.9 ± 0.1
Formate (g/L)	1.3 ± 0.4	0.4 ± 0.04
Acetate (g/L)	1.0 ± 0.01	3.1 ± 0.02

ND: No determined.

2.2. Inoculum

The microbial consortium coded as PTA-124566 by the American Type Culture Collection (ATCC) was used as biocatalyst, as it has a proven metabolic capacity to perform LD-DF [40–43]. The inoculum is composed of LAB such as *Lactobacillus*, acetic acid bacteria such as *Acetobacter*, and LO-HPB such as *Clostridium* [40]. Prior to use, the inoculum was reactivated for 24 h in a 500-mL Erlenmeyer flask with a working volume of 0.2 L, at 35 °C, 100 rpm, and under anoxic conditions. The operational pH was not controlled during fermentation but was initially adjusted to 6.5 using a 10 M NaOH solution. The inoculum size was 10% *v*/*v*, whereas the mineral growth medium used had the following constituents (in g/L): lactose, 10; NH_4Cl, 2.4; K_2HPO_4, 2.4; $MgSO_4 \cdot 7H_2O$, 1.5; KH_2PO_4, 0.6; $CaCl_2 \cdot 2H_2O$, 0.15; and $FeSO_4 \cdot 7H_2O$, 0.05.

2.3. Experimental Setup and Fermentation Conditions

Triplicate batch fermentation tests were performed in a 3 L stirred tank reactor (Applikon Biotechnology, Schiedam, The Netherlands) with a 2 L working volume. The reactor was equipped with the Applikon ez-Control system (Applikon, Schiedam, The Netherlands), which allowed for the monitoring and control of agitation speed, pH, and temperature.

A schematic diagram of the system is presented in Figure 1. The reactor was filled with 90% v/v of the preconditioned CW and 10% v/v of the activated inoculum. No gas flushing was employed as a measure to achieve anoxic conditions. The fermentations were carried out at 35 ± 1 °C, a fixed pH of 5.8, and 150 rpm. The pH was kept constant by automatically adding either a 3 M NaOH or 5 M HCl solution. The amount of biogas produced was measured using the µFlow biogas meter (Bioprocess Control™, Lund, Sweden). Headspace gas samples were taken once a day to analyze the acidogenic off-gas composition. Additionally, liquid samples were also drawn once a day to analyze COD, total reducing sugars, volatile solids (VS), and organic acids. The fermentation was stopped when the amount of hydrogen gas produced during two consecutive days was <1% of the total cumulative volume of hydrogen. The volumetric hydrogen production rate (VHPR) expressed as NL H_2/L-d was calculated according to Equation (1), where R_{max} is the maximum hydrogen production rate estimated from modeling (in NL H_2/d), and V is the working volume (in L). As shown in Equations (2) and (3), hydrogen production yield (YH_2) was calculated in terms of either the amount of VS (VS_{fed}, g/L) or COD (COD_{fed}, g/L) added; VH_2 stands for the cumulative hydrogen production (NmL H_2). Finally, the cumulative hydrogen production recorded was also kinetically modeled with the modified Gompertz model (Equation (4)), where H is the cumulative hydrogen (mL); P is the hydrogen production potential (mL); R_m is the maximum hydrogen production rate (NmL/h); e is ca. 2.71828; λ is the lag phase time (h), and t is the cultivation time (h).

$$\mathrm{VHPR} = \frac{Rmax}{\mathrm{V}} \quad (1)$$

$$\mathrm{YH_2} = \frac{VH_2}{\mathrm{VS_{fed}}*\mathrm{V}} \quad (2)$$

$$\mathrm{YH_2} = \frac{VH_2}{COD_{fed}*\mathrm{V}} \quad (3)$$

$$\mathrm{H(t)} = P\cdot\exp\left\{-\exp\left[\frac{R_m\cdot e}{P}(\lambda-\mathrm{t})+1\right]\right\} \quad (4)$$

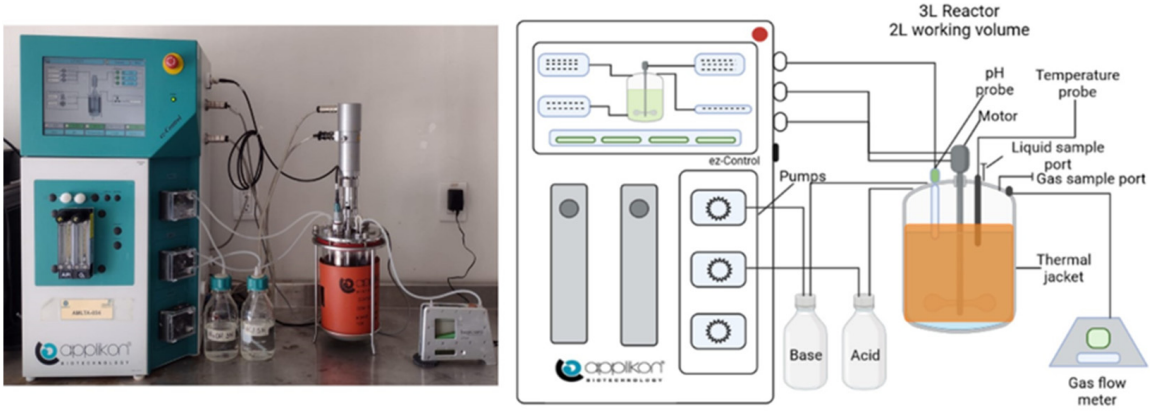

Figure 1. Schematic diagram of the hydrogen-producing reactor used in the experiments.

2.4. Analytical Methods

The determination of pH, solids, soluble proteins, COD, total nitrogen, phosphorus, alkalinity, lipids, and ammoniacal nitrogen was carried out according to standard methods [44]. Total reducing sugars were measured by the Miller method (DNS; 3,5-dinitrosalicylic acid method) [45]. The soluble metabolites (formate, acetate, propionate, iso-butyrate, butyrate, and lactate) were determined by high-performance liquid chromatography (HPLC) using the Varian Prostar system 230 equipped with a Varian 325 UV-Vis

detector at 210 nm and a Biorad Aminex HPX-87H column. The column temperature was kept at 55 °C, while the mobile phase was 5 mM sulfuric acid at a constant flow rate of 0.5 mL/min. Gas composition was analyzed by gas chromatography using a Perkin-Elmer Clarus 580 gas chromatograph (Perkin Elmer, Inc., Waltham, MA, USA) equipped with a thermal conductivity detector and a HayeSep D column (3 m × 3.2 mm, 100/120 mesh; Perkin Elmer, USA). The temperatures of the injection port, oven, and detector were 75, 30, and 120 °C, respectively. Nitrogen was used as a carrier gas with a flow rate of 30 mL/min.

2.5. Microbial Community Analysis

The microbial community structure was determined by duplicate samples drawn from the 2nd day of fermentation, corresponding to the acceleration phase in regard to hydrogen production. Genomic DNA was extracted with the MoBio PowerSoil DNA Extraction Kit (Mo Bio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. The quantification and evaluation of the purity of the DNA were determined with a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The isolated DNA samples were submitted to RTL Genomics (RTL, Lubbock, TX, USA) for 16S rRNA amplicon-based sequencing using the Illumina MiSeq platform. The universal primers 28F (GAGTTTGATCNTGGCTCAG) and 388R (TGCTGCCTCCCGTAGGAGT) were utilized [41]. Finally, the bioinformatic analysis was performed according to RTL's protocol [46].

3. Results and Discussion

3.1. Undiluted CW Can Support Hydrogen Production via LD-DF

As shown in Figure 2, the total amount of hydrogen produced after 5 days of fermentation was 3332.18 ± 220.95 NmL/L. The associated YH_2 values were 80.0 ± 5.3 NmL H_2/g VS_{fed} and 44.5 ± 2.9 NmL H_2/g COD_{fed}, whereas the maximum VHPR achieved was 1.9 ± 0.5 NL H_2/L-d. A similar hydrogen production outcome was reported by [10], who reported a peak VHPR of 2.0 NL H_2/L-d (average values of 1.0–1.3 NL H_2/L-d) during the continuous DF of synthetic CW by mixed culture at pH 4.5–5.0, 35 °C, and 24 h hydraulic retention time. Recently, Muñoz-Páez et al. [39] reported a transient VHPR of up to 7.1 NL H_2/L-d (average 4.1 NL H_2/L-d) when applying an automatic control strategy to the DF of raw acid diluted CW. That comparative analysis indicates that the hydrogen production herein obtained can be optimized further. It is therefore necessary to evaluate the continuous LD-DF of CW in future works. From Figure 2, it is also possible to observe that the time evolution of cumulative hydrogen production was appropriately described by the modified Gompertz model (R^2 value of 0.9959; Table 2). It was observed a short latency phase of 4.2 h, suggesting that organic matter was readily available for fermentation. This behavior can be verified with the relatively rapid degradation trend of total reducing sugars (Figure 2). More concretely, a marked decrease in fermentable sugars was observed within the first day of fermentation and was almost depleted (0.07 ± 0.01 g/L) by day 2, achieving an average removal of 99.6 ± 0.1%.

Table 2. Summary of the kinetic parameters obtained from the modified Gompertz model and other process performance indicators of the dark fermentation process of acid CW.

Parameter	Value
λ (h)	4.2 ± 1.8
Hmax (NmL H_2/L)	3313.2 ± 207.4
Rmax (NmL H_2/L-h)	77.9 ± 20.5
R^2	0.9959
VHPR (NL H_2/L-d)	1.9 ± 0.5
YH_2 (NmL H_2/g VS_{fed}-L)	80.0 ± 5.3
YH_2 (NmL H_2/g COD_{fed}-L)	44.5 ± 2.9
COD removal (%)	24.6 ± 3.9
VS removal (%)	48.7 ± 3.4

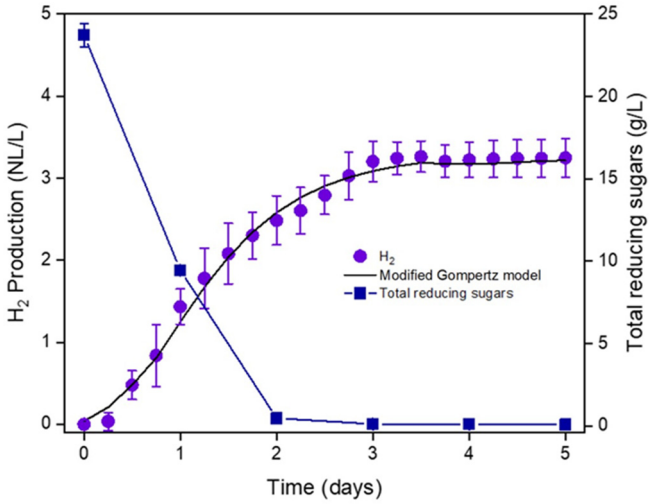

Figure 2. Time course of cumulative hydrogen production and total reducing sugars concentration.

Regarding metabolic by-products (Figure 3), an increase in the concentration of lactate, acetate, formate, and butyrate was observed in the first 2 days of fermentation, from 5.1, 3.3, 0.6, and 1.1 g/L to 15.0, 6.1, 3.5, and 4.5 g/L, respectively. Notably, the major organic acid detected was lactate, yet it was further depleted. The increase in lactate was consistent with the decrease in the concentration of reducing sugars (Figure 2). The titer of butyrate in the broth significantly increased during hydrogen production, achieving a concentration of 12.4 ± 1.7 g/L at the end of fermentation. The two-step LD-DF herein observed was also reported by [47], who studied the hydrogen production by DF with raw CW without the addition of inoculum at different operating pH values (from 5 to 7.5). As discussed before, the LD-DF pathway has been observed with other types of substrates such as bagasse [48], tequila vinasse [40], and synthetic CW [37]. The two-step LD-DF can be attributed to the interaction between LAB and LO-HPB, in which LAB degrades sugars to lactate, which in turn serves as a substrate for LO-HPB for hydrogen production. It is important to note that the cross-feeding of lactate can occur in a parallel way rather than a sequential one, thus explaining the hydrogen production observed during the first day of fermentation. It should be also considered that fermentation of carbohydrates to hydrogen might have also occurred, although to some minor extent based on the amounts of hydrogen recorded along with the concentration of lactate measured. According to the net amount of acetate and butyrate recorded at the end of the process, the experimental amount of hydrogen produced, and the theoretical hydrogen production dictated by Equations (5) and (6), the hydrogen amount produced by LD-DF would explain 75% of the total hydrogen produced; the remaining amount could be derived from carbohydrates. Contrarily, the hydrogen-producing pathway described by Equation (5) led to a theoretical hydrogen production 77% higher than the experimental one. Estimated amounts of hydrogen production via acetic-type fermentation far exceeded those recorded experimentally. In future research work, it would be interesting to perform a comparative study of the hydrogen production from CW through the LD-DF process and the direct fermentation of carbohydrates. Finally, the removal efficiency of COD and VS was determined to be 24.6 and 49%, respectively. According to the COD mass balance, at the end of the process, the soluble by-products represented 79.8 ± 6.8% of the inlet total COD, whereas the production of hydrogen accounted for only 3.1 ± 0.2%, reinforcing the need for further optimization. Electron flow diverted towards biomass production was considered to be 10.0% of the initial COD, entailing an average recovery of 93% (Table 3).

$$\text{Glucose} \rightarrow \text{Butyrate} + 2CO_2 + 2H_2 + \text{ATP} \quad (5)$$

$$\text{Lactate} + 0.4\ \text{Acetate} \rightarrow 0.7\ \text{Butyrate} + CO_2 + 0.6H_2 \quad (6)$$

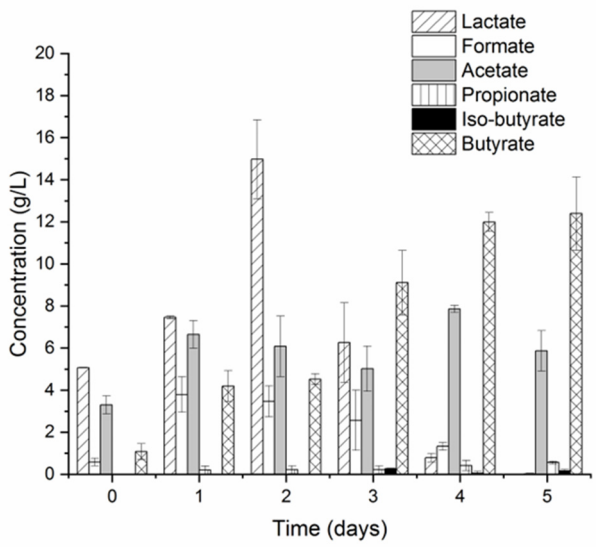

Figure 3. Time course of organic acids during the LD-DF of acid, undiluted, delipidated CW.

Table 3. COD balance for the batch fermentative hydrogen production from acid CW.

Parameter	Value
SCOD effluent (%)	79.8 ± 6.8
Hydrogen (%)	3.1 ± 0.2
Biomass (%)	10.0 ± 0.0
Recovered fraction (%)	92.9 ± 6.6
Non-recovered fraction (%)	7.1 ± 6.6

SCOD: Soluble chemical oxygen demand (COD).

3.2. Microbial Communities and Metabolic Pathways

Table 4 shows the groups of bacteria found in this study during the acceleration of the hydrogen production phase (day 2). The bacterial genus with the highest relative abundance was *Lactobacillus* (86.6 ± 4.2%), followed by *Clostridium* (12.3 ± 3.7%), and *Klebsiella* (1.0 ± 0.5%). It is known that the genus *Lactobacillus* belongs to LAB along with *Lactococcus*, *Streptococcus*, *Bacillus*, and *Enterococcus* [49]. These bacteria produce lactate through a series of reactions. First, it is necessary that lactose, the dominant type of sugar in CW, be hydrolyzed by β-galactosidases (produced by LAB) into glucose and galactose; subsequently, these monosaccharides are converted into lactate [6]. According to the predominant metabolism, it is possible to classify the LAB group into homofermentative and heterofermentative [31,47,49,50]. Homofermentative species produce lactate as the only final product, while heterofermentative ones, besides lactate, can produce acetate, CO_2, and ethanol. In the homofermentative lactate fermentation pathway, glucose is transformed into pyruvate via the glycolysis pathway (also known as the Embden-Meyerhof-Parnas pathway). Subsequently, pyruvate is reduced to lactate by reductive power in the form of NADH (Equation (7)) [31,50]. Some homolactic species are *L. acidophilus*, *L. amylophilus*, *L. bulgarius*, *L. helveticus*, and *L. salivarius* [50].

$$\text{Glucose} \rightarrow 2\ \text{Lactate} + 2\text{ATP} \quad (7)$$

Table 4. Microbial community structure present in the LD-DF of CW during the exponential hydrogen production phase.

Phylum	Class	Family	Genus	Relative Abundance
Firmicutes	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	86.6 ± 4.2%
	Clostridiales	*Clostridiaceae*	*Clostridium*	12.3 ± 3.7
Proteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella*	1.0 ± 0.5

The application of molecular analyses deserves attention in future studies to elucidate the temporal dynamics in the microbial community structure during the CW LD-DF. Such insights could be of paramount importance to lay the foundation for the design of innovative continuous processes allowing enhanced and more stable hydrogen productivity.

On the other hand, in the heterofermentative lactate fermentation pathway, the first step is the degradation of glucose via the pentose phosphate pathway to produce glyceraldehyde 3-phosphate (G3P), acetyl-phosphate, and CO_2 [49,50]. G3P is assimilated via glycolysis to be transformed into lactate, while acetyl-phosphate is transformed into acetate and/or ethanol (Equations (8) and (9)). Therefore, after glycolysis, one pyruvate molecule is transformed into lactate, and the second pyruvate molecule is transformed into acetate and/or ethanol [31]. Some microorganisms that belong to the heterofermentative species are *L. brevis*, *L. fermentum*, *L. parabuchneri*, and *L. reuteri* [50].

$$\text{Glucose} \rightarrow \text{Lactate} + CO_2 + \text{Ethanol} + \text{ATP} \quad (8)$$

$$\text{Glucose} \rightarrow \text{Lactate} + CO_2 + \text{Acetate} + 2\text{ATP} + 2\text{NADH} \quad (9)$$

Since the production of lactate and acetate was observed between days 1 and 2 of fermentation (Figure 3), it is assumed that the pathway involved in the first stage of fermentation was the heterolactic pathway. On the contrary, Asunis et al. [47] investigated the self-fermentation of raw CW and found the homolactic pathway to be the dominant one since no metabolic by-products other than lactate were found. That discrepancy might be associated with the microorganisms involved; in this study, a mixed inoculum mainly composed of LAB and LO-HPB was used, whereas Asunis and co-workers [47] employed the autochthonous microbial communities of the substrate. On the other hand, LO-HPB can participate in the formation of organic compounds, as well as hydrogen and CO_2. Two types of fermentation are known depending on the compounds produced: (1) *Clostridia*-type fermentation and (2) *Enterobacteria*-type fermentation [31,51]. Glycolysis (Embden Meyerhof pathway) is the first step in both fermentations to produce pyruvate and generate NADH [31]. In *Clostridia*-type fermentation, strictly anaerobic microorganisms convert pyruvate into acetyl-CoA and CO_2 via the ferredoxin oxidoreductase (PFOR) pathway, where ferredoxin (Fd) is the coenzyme that acts as an electron acceptor. In *Enterobacteria*-type fermentation, facultative anaerobes convert pyruvate to acetyl-CoA via the pyruvate formate lyase (PFL) pathway, and hydrogen is then produced by formate hydrogen lyase [51–53]. Bacteria belonging to the genus *Klebsiella* are known to produce hydrogen from lactose and glucose [54]. Alternatively, it has been seen in LD-DF that some microorganisms belonging to the genera *Clostridium* and *Lactobacillus* can metabolize lactate to transform it into hydrogen and other organic compounds. For example, *Clostridium neopropionicum* (Equation (10)) through the acrylate pathway consumes lactate and produces propionate, CO_2, and H_2 [55], and *Lactobacillus bifermentans* (Equation (11)) use lactate to produce acetate, ethanol, CO_2, and H_2 [56]. Other microorganisms consume lactate together with acetate to produce butyrate, CO_2, and H_2 (Equation (6)) such as *C. acetobutyricum* P262 [57]. Therefore, primary and secondary lactate fermentation performed by LAB and LO-HPB, respectively, can coexist during the process.

$$\text{Lactate} \rightarrow 0.5\ \text{Propionate} + CO_2 + H_2 + 0.5\ H_2O \quad (10)$$

$$\text{Lactate} \rightarrow 0.5\ \text{Acetate} + 0.5\ \text{Ethanol} + CO_2 + H_2 \quad (11)$$

Therefore, it is herein suggested that there is a syntrophic relationship between LAB (*Lactobacillus*) and LO-HPB (*Clostridium*) during the fermentative hydrogen production from CW. Both genera of microorganisms have the pyruvate oxidation route in common. A schematic representation of the LD-DF process is depicted in Figure 4, which shows the lactate cross-feeding interactions between LAB and LO-HPB; a third bacterial group, namely acetic acid bacteria, was also included. The problems in hydrogen production caused by excessive growth of LAB (competition for substrate, antimicrobial release, acidification of the medium) are avoided because LAB and LO-HPB can not only coexist under certain conditions but also form an alliance [6,47,58]. Additionally, it has been observed that the balance between the groups of bacteria is dependent on the temperature and pH of the medium in the production of hydrogen and organic acids [58]. Therefore, it can be deduced that at the point of highest lactate production, there was an interaction between the microbial communities present (*Lactobacillus*, *Clostridium*, and *Klebsiella*) because, at that point, the highest lactate concentration coincided with the highest removal of total reducing sugars coupled with the production of hydrogen.

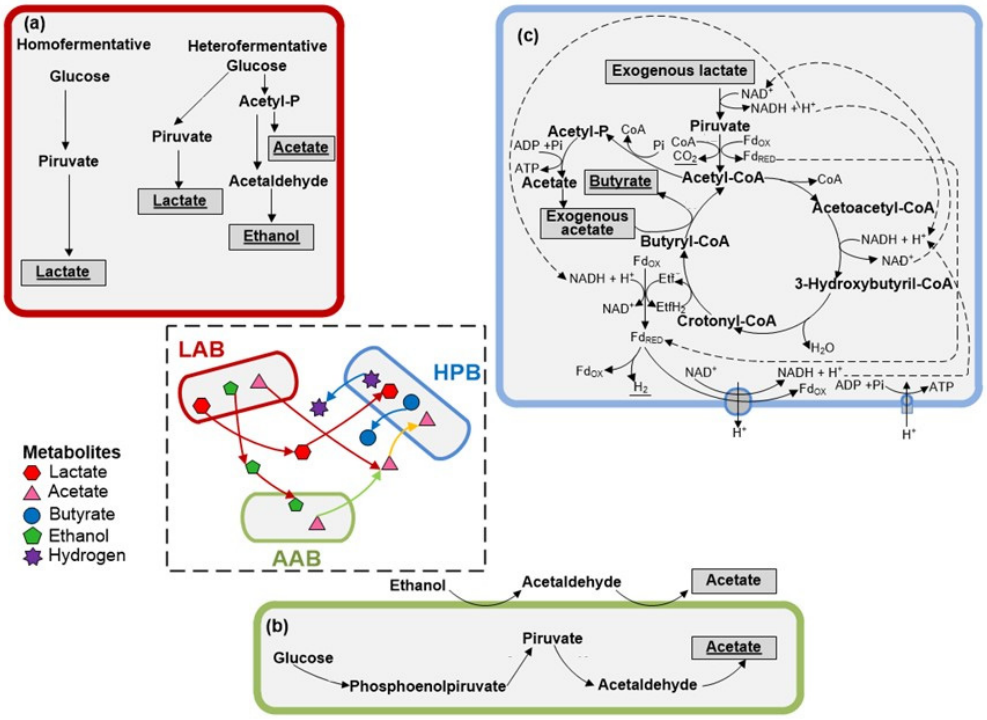

Figure 4. Schematic representation of (**a**) homo- and hetero-lactic fermentation by lactic acid bacteria (LAB), (**b**) the oxidation of glucose and ethanol by acetic acid bacteria (AAB), and (**c**) the production of hydrogen from the oxidation of lactate by hydrogen-producing bacteria (HPB). Putative lactate- and acetate-based cross-feeding interactions between LAB, AAB, and HPB are denoted inside the dotted rectangle. Adapted from [57–61].

4. Conclusions

The feasibility of producing hydrogen from acidic, undiluted, delipidated CW through the LD-DF pathway by a specialized mixed culture was investigated. It was observed that the use of the biocatalyst allowed the production of 3.3 L of hydrogen per liter of CW, with an associated hydrogen productivity of 1.9 L H_2/L-d. Based on the metabolic

analysis and the time evolution observed for carbohydrates and hydrogen production, it was elucidated that the LD-DF was the major metabolic pathway involved, explaining about 75% of the total amount of hydrogen produced. Carbohydrates were determined to be still important for co-producing hydrogen but also for allowing primary lactate-type fermentation to produce lactate. The microbial community detected, which was found to be consistent between the replicates, endorsed the process. *Lactobacillus*, *Clostridium*, and *Klebsiella* were found to be the core microbial groups. In conclusion, CW is a good feedstock for hydrogen production via LD-DF, which can be a key metabolic pathway to overcome hydrogen inhibition issues associated with the overwhelming growth of LAB.

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