The duo *Clostridium* and *Lactobacillus* linked to hydrogen production from a lignocellulosic substrate

Marisol Pérez-Rangel, José Eleazar Barboza-Corona, Marcelo Navarro-Díaz, Ana Elena Escalante and Idania Valdez-Vazquez

ABSTRACT

The study aimed to identify interspecies interactions within a native microbial community present in a hydrogen-producing bioreactor fed with two wheat straw cultivars. The relationships between the microbial community members were studied building a canonical correspondence analysis and corroborated through *in vitro* assays. The results showed that the bioreactor reached a stable hydrogen production of ca. 86 mL/kg·d in which the cultivar change did not affect the average performance. *Lactobacillus* and *Clostridium* dominated throughout the whole operation period where butyric acid was the main metabolite. A canonical correspondence analysis correlated positively *Lactobacillus* with hydrogen productivity and hydrogen-producing bacteria like *Clostridium* and Ruminococcaceae. Agar diffusion testing of isolated strains confirmed that *Lactobacillus* inhibited the growth of *Enterococcus*, but not of *Clostridium.* We suggest that the positive interaction between *Lactobacillus* and *Clostridium* is generated by a division of labor for degrading the lignocellulosic substrate in which *Lactobacillus* produces lactic acid from the sugar fermentation while *Clostridium* quickly uses this lactic acid to produce hydrogen and butyric acid. The significance of this work lies in the fact that different methodological approaches confirm a positive association in the duo *Lactobacillus–Clostridium* in a bioreactor with stable hydrogen production from a complex substrate.

Key words | agar diffusion method, antibacterial activity, bioenergy, complex substrate, microbial interactions

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HIGHLIGHTS

- Native microbiota of wheat straw reached a stable hydrogen production.
- Microbial structure was stable dominated by *Lactobacillus* and *Clostridium*.
- Statistical analysis and *in vitro* assays demonstrated positive interactions.
- Division of labor in lignocellulose consumption could promote positive interactions.

INTRODUCTION

Currently, one of the most important challenges in biological hydrogen production is the lack of long-term stability. To overcome this limitation, researchers have implemented the heat-treatment of inoculum, the removal of dissolved gases, the manipulation of operational conditions, and the application of other novel technologies achieving – at least for short periods – a stable hydrogen production in laboratory-scale reactors (Castelló *et al.* 2020). However, understanding the functions and interactions between the microorganisms involved in the process is mandatory to contribute a definitive solution to the stability problem.

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Thus, the first step is identifying those functional microorganisms that take part in hydrogen production. In this sense, Etchebehere *et al.* (2016) have reported three predominant groups of microorganisms in hydrogen production processes: high-yield producers (*Clostridium*, *Kosmotoga*, and *Enterobacter*), low-yield producers (*Veilonellaceae*), and competitors (lactic acid bacteria (LAB) such as *Lactobacillus*). The presence of these microorganisms in the community may vary accordingly to the inoculum, reactor configuration and operation, and concentration and type of substrate.

Members in a hydrogen-producing community can establish positive and negative interactions between them enhancing or reducing the system productivity. Traditionally, these microbial interactions refer to those relationships established with *Clostridium*. For instance, positive interactions include the cell aggregation that promotes biomass retention, pH regulation, oxygen consumption to generate anaerobic conditions, and degradation of complex substrates (Cabrol *et al.* 2017; Mazareli *et al.* 2020; Montoya *et al.* 2020). Conversely, the negative interactions previously described are hydrogen consumption, substrate competition, and growth inhibition due to the presence of metabolites or bacteriocins (antimicrobial peptides) (Gomes *et al.* 2016; Cabrol *et al.* 2017; Park *et al.* 2018). In this context, LAB are well known for their capability to produce high concentrations of lactic acid and bacteriocins, and therefore attributing them to lower productivity and stability in systems dominated by *Clostridium* (Gomes *et al.* 2016). However, LAB can establish positive interactions in hydrogen-producing communities through cell retention and lactic acid production as an alternative substrate for hydrogen production (Cabrol *et al.* 2017; García-Depraect *et al.* 2021). Thus, the past studies that have attributed positive or negative roles to LAB are not entirely convincing (Castelló *et al.* 2020).

Based on the importance of the presence of *Clostridium* as a high-yield producer, and the undefined role of *Lactobacillus*, in this study, we focussed on the interactions that both microorganisms establish in a hydrogen-producing reactor fed with raw wheat straw (WS), a complex substrate. First, the hydrogen productivity from raw WS was evaluated in a semicontinuous reactor. Then, the microbial community was studied through the operation time and the data were used to identify statistical interactions between the members of the native microbial consortium and the hydrogen productivity. Finally, the information obtained was experimentally complemented using antagonistic assays between isolated bacteria from the hydrogen producer reactor.

METHODS

Hydrogen production

Substrate

Raw WS was used as substrate. Table 1 shows the different WS cultivars used through the operation time and their chemical composition determined according to the National Renewable Energy Laboratory (Sluiter *et al.* 2012), xylose and glucose concentrations were determined using a YSI 2900 biochemistry analyzer (YSI, Inc., Yellow Springs, OH, USA). Samples of 15 kg containing $9 \pm 5\%$ water were milled and sieved through 3.35 and 2.00 mm meshes (Endecotts, London, UK). The particles retained between these meshes were stored in opaque plastic containers at room temperature (25 °C and relative humidity of 55%) until further use.

Inoculum

The inoculum source was the native microbial community naturally present on the WS surface, previously reported as hydrogen producers (Valdez-Vazquez *et al.* 2017). Therefore, WS was used without sterilization and hydrated with distilled water for 24 h.

Bioreactor operation

The hydrogen productivity was evaluated in a 1-L semicontinuous reactor with a working mass of 660 g. The reactor was fed with hydrated WS of different cultivars and locations (Table 1) to identify possible changes in hydrogen productivity as a consequence of different microbiotas. The total solid content was adjusted to 10% using a non-sterile culture medium whose composition per liter was: 0.3 g urea, 2.4 g KH_2PO_4, and 0.5 g K_2HPO_4. During the feeding (every 4 days), 60% of digestate was replaced by hydrated wheat straw (HWS) and medium maintaining the total solids content and the initial pH was adjusted to 6.5 with a 1 N NaOH solution. The reactor was operated with an organic loading rate of 15 g-TS/kg.d and a retention time of 6.6 d, maintained at 37 °C with manual stirring twice a day. The feeding was carried out in aerobic conditions and the reactors were hermetically closed with air in the headspace. The reactor was operated in duplicate.

Table 1 | Wheat straw cultivars used as substrate and its chemical composition

Operation period (days)	Cultivar	Growth period	Collection site	Field location	Chemical composition (g/kg substrate)					
					TS*	VS†	Ash	Glucose	Xylose	Lignin
1–56	Cortazar S94	2016 autumn–winter	Irapuato, Guanajuato, Mexico	Latitude: 20°51′N Longitude: 101°16′W	947	899	25	327	195	141
60–80	Urbina S2007	2016 autumn–winter	La Barca, Jalisco, Mexico	Latitude: 20°17′N Longitude: 102°32′W	913	832	81	283	203	137

Note: *, total solids; †, volatile solids.

Structure of the microbial community during the hydrogen production

Microbial diversity analysis was performed on samples from days 0 (hydrated WS1), 8, 20, 48, 60 (hydrated WS2), and 80. All these samples were stored at –80 °C until its processing. Cell pellets, a mixture of supernatant and unfermented WS, were recovered from wet samples and then genomic DNA was extracted using PowerSoil DNA extraction kit® (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. The DNA purity (A260/A280 ratio ~ 1.8) and concentration were verified in a NanoDrop Lite spectrophotometer (Thermo Scientific, USA). DNA concentration was normalized to 20 μg/μL using DNase/pyrogen-free water. DNA was sequenced based on the 16S rDNA gene using the set of primers 28F (GAGTTTGATCNTGGCTCAG) and 388R (TGCTGCCTCCCGTAGGAGT) through the Illumina MiSeq platform (Research and Testing Laboratories, LLC, Tx, USA). Data analysis of the bacterial communities DNA sequences were performed according to the standard method of the RTL Genomics NGS service. The full protocol can be reviewed at http://www.rtlgenomics.com/docs/Data_Analysis_Methodology.pdf. Briefly, the forward and reverse raw reads were merged using the PEAR Illumina paired-end read merger and prefix dereplication and clustering of the trimmed reads (sequences longer than 250 bp with a quality score higher than 30) were performed through the USEARCH algorithm. The classification of the large number of clusters into operational taxonomic units (OTUs) (with 4% of divergence) was carried out using the UPARSE OTU selection algorithm. For chimera checking on the selected OTUs we used the UCHIME chimera detection software executed in the *novo* mode. Lastly, taxonomy was assigned using the USEARCH global alignment algorithm against a database of high-quality sequences derived from the National Center for Biological Information (NCBI) database (Valdez-Vazquez *et al.* 2017). Metagenomic sequences were deposited in the GenBank database with the BioProjec PRJNA720769 (Biosamples: SAMN18676575, SAMN18676576, SAMN18676577, SAMN18676578, SAMN18676579, SAMN18676580).

Analytical and statistical methods

Volume, biogas composition, and organic acids were quantified as previously reported (Cardeña *et al.* 2018). Hydrogen production was reported at standard temperature and pressure. The statistical correlation between the hydrogen productivity, the main produced organic acids, and the structure of the microbial community were explored using canonical correspondence analysis (CCA). This analysis was performed with the OTU relative abundance and metabolites concentration values using XLSTAT software v2019.3.2 (Addinsoft Inc., NY, USA).

Calculations

The stability index of the hydrogen productivity was calculated according to the equation previously reported by Tenca *et al.* (2011):

$$\text{Stability} = 1 - \frac{\text{sd}(x)}{\bar{x}} \quad (1)$$

where x is the average of the hydrogen productivity during a known period, and sd is the standard deviation of the same period. In this regard, when the stability index is close to 1, the hydrogen productivity was constant during the period.

Isolation and molecular identification of native bacteria

The native bacteria were isolated from the digestate obtained from the hydrogen production reactor, taking the sample at 48 days of operation. Three groups of microorganisms were isolated: (i) facultative anaerobes; (ii) obligate

anaerobes; and (iii) LAB. For the isolation, 10 g of sample were diluted 1:1000 in peptone water (1% w/v). Then, 0.1 mL of the dilution was spread onto Petri dishes with a different medium: China lactose blue agar for facultative anaerobes, and Man, Rogosa, and Sharp agar for LAB. 0.1 mL of the dilution was deposited and spread with a loop into an inclined serological bottle containing reinforced clostridial agar for obligate anaerobes. Plates and bottles were incubated in an anaerobic chamber (Labconco Corporation, USA) at 37 °C for 48 h. Bacterial colonies with different morphology were streaked with a loop in plates to obtain axenic cultures.

Axenic cultures were molecularly identified by amplifying the 16S rRNA gene. Briefly, DNA was extracted following the phenol–chloroform method. The amplification of the 16S rDNA gene region was performed using the *Taq* DNA polymerase (Thermo Scientific, EP0402) and the primer set UBF/1942R (5′-AGAGTTTGATCCTGGCTAG-3′; 5′-GGTTACCTTGTTACGACTT-3′). The PCR was performed according to the procedure provided by the manufacturer. Amplicons were purified with the PCR Purification Kit (Jena Bioscience, PP201 L), and then sequenced at the National Laboratory of Genomics for Biodiversity (LANGEBIO Irapuato, Mexico). Sequences were compared with a database reported in the NCBI using the BLAST tool. Sequences were deposited into the GenBank database (accession numbers: MW600319, MW600320, MW600326, MW600327, MW600328, MW600329, MW624324, MW624326).

Antimicrobial activity

Supernatants from native strain cultures were used to evaluate their inhibitory activity. Briefly, the facultative anaerobes and LAB were grown in 250 mL flasks with 100 mL of culture medium. Obligate anaerobes were grown in 150 mL serum bottles with 110 mL of culture medium at 37 °C, 300 rpm for 24 h. Supernatants were centrifuged at 6,000 × *g* for 15 min at 4 °C and stored at −20 °C until further use.

The inhibitory activity of native strains was evaluated through the agar well diffusion assay using the cell-free supernatants (Barboza-Corona *et al.* 2007). The native strains were grown in trypticase soy broth for 16 h and were incubated at 37 °C. Next, 140 µL of the bacterial culture were deposited in 20 mL of soft trypticase soy agar, mixed to obtain a uniform cell content, and then poured into Petri dishes. 7-mm wells were made inside the plates and filled with 70 µL of supernatants. Sterile culture medium was used as the negative control. Plates were maintained to 4 °C for 12 h to carry out the supernatants diffusion, and after that, incubated at 37 °C for 18 h. Diameters of clearing zones were measured to determine the inhibition area.

RESULTS AND DISCUSSION

Hydrogen productivity from raw WS was studied in a semi-continuous reactor operated for 80 days. The hydrogen production started during the first 4 days of operation, reaching hydrogen productivity of 325 ± 43 mL/kg·d; nevertheless, in the next 12 days, the hydrogen productivity declined to 125 mL/kg·d. The hydrogen productivity reached a period with the highest stability between 36 and 52 days of operation with average productivity of 80 mL/kg·d and a stability index of 0.90 (Figure 1(a)). When the feeding was changed from cultivar Cortazar S94 (WS1) to cultivar Urbina S2007 (WS2) at 60 days of operation, the hydrogen productivity showed a new peak of 132 ± 19 mL/ kg·d. Then, in the last 16 days of operation, the reactor recovered the steady-state performance with a stability index of 0.86, where no significant differences were observed in productivity among WS cultivars ($p > 0.5$; an average of 91 ± 26 mL/kg·d with cultivar Cortazar S94 and 80 ± 34 mL/kg·d with cultivar Urbina S2007). In the steady state, butyric > acetic > iso-valeric > propionic acids were the main fermentation end-products (Figure 1(b)), maintaining the pH range between 4.58 and 5.16. These results seem to indicate that large-scale bioreactors could be fed with different WS cultivars within location and year without affecting the performance, however, more research is needed to confirm this observation.

Native microbiota in hydrated WS (HWS1 and HWS2, fed between the operation days 1–56 and 60–80, respectively) was composed of typical epiphytic genera such as *Leuconostoc*, *Enterobacter*, *Erwinia*, *Pantoea*, and *Rahnella* (Figure 1(c)). Then, the environmental conditions imposed by the hydrogen-producing bioreactor such as anaerobiosis, an acid pH, and availability of substrate promoted the stable coexistence of *Lactobacillus*, *Clostridium,* and *Azotobacter* throughout the whole operation time in despite the differences in the microbial composition of feeding (Figure 1(d)). A canonical correspondence analysis (CCA) correlated the hydrogen productivity, microbial diversity, and the main fermentation end-products explaining 93.56% of the total variability of such variables (Figure 2). The analysis corroborated a positive relationship

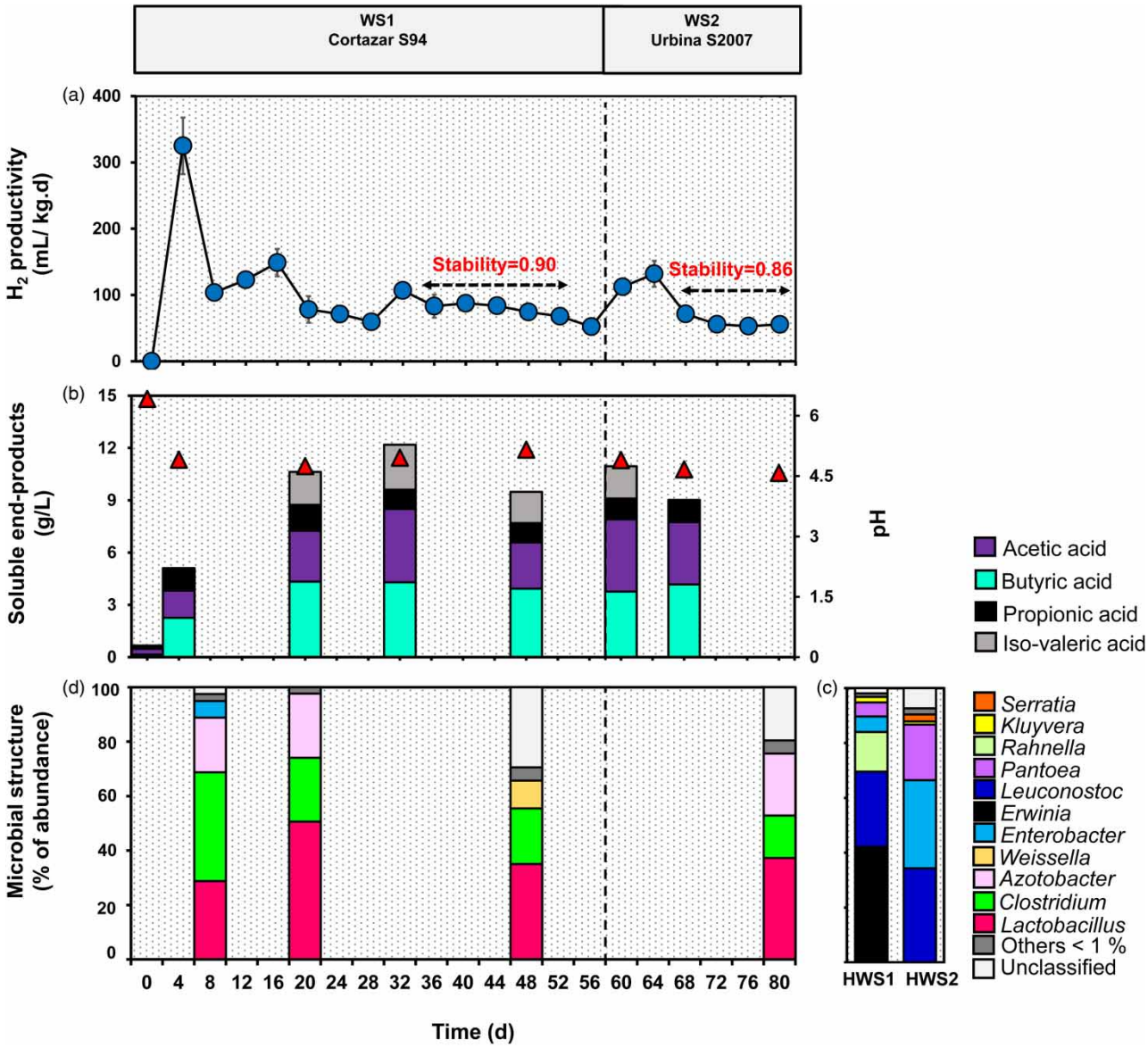

Figure 1 | Performance of the hydrogen-producing bioreactor fed with two wheat straw cultivars (Cortazar S94 from day 0 to day 60 and Urbina S2007 from day 60 to day 80). (a) Hydrogen productivity, (b) Main fermentation end-products, (c) Structure of the native microbial community in the substrate. HSW1, hydrated wheat straw cultivar Cortazar S94; HSW2, hydrated wheat straw cultivar Urbina S2007, and (d) Structure of native microbial community during hydrogen production.

between hydrogen productivity, the production of butyric and acetic acids with the presence of *Clostridium, Lactobacillus,* and *Azotobacter*. *Azotobacter* is an obligate aerobe characterized by their production of laccases for the oxidative depolymerization of lignin (Herter *et al.* 2011). Thus, this genus could be related to the generation of anaerobic conditions needed for the obligate anaerobes and the degradation of lignin present in the substrate. Focus on *Clostridium* and *Lactobacillus*, of special interest in this study, a complex substrate as WS may promote positive interactions between them due to the division of metabolic labor. Two previous studies using complex substrates (banana and coffee wastes) also found the coexistence of *Clostridium* and *Lactobacillus* during the period most productive (Mazareli *et al.* 2020; Montoya *et al.* 2020). Complex substrates influence the ecology of microbial communities differently than simple substrates (Lindemann 2020). Members in a microbial community can divide metabolic labor in exchange for other required metabolites. Thus, previous studies demonstrated that *Lactobacillus* and *Clostridium* are hydrolytic microorganisms when they are grown on lignocellulosic substrates (Valdez-Vazquez *et al.*

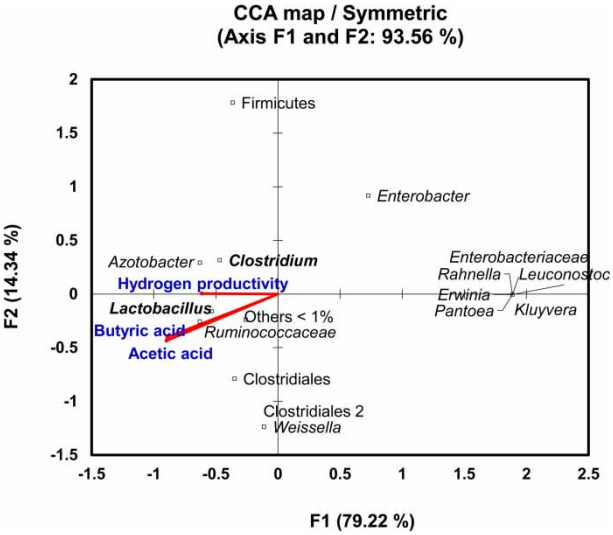

Figure 2 | Canonical correspondence analysis for hydrogen productivity, fermentation end-products, and structure of native microbial community.

2017; Mohamad Zabidi *et al.* 2020). Members of *Lactobacillus* can ferment carbohydrates present in hydrolysates of alfalfa fibers and sugarcane bagasse producing lactic acid and trace of acetic acid (Sreenath *et al.* 2001; Wischral *et al.* 2019) while *Clostridium* can use these two acids to produce hydrogen with butyric acid as the main fermentation end-product (García-Depraect *et al.* 2021). The hypothesis of a division of labor between native *Lactobacillus* and *Clostridium* during the self-fermentation of WS is supported by the abundance of *Lactobacillus* but the absence of lactic acid as well as the dominance of butyric acid in the fermentation end-products.

The type of microbial interactions established between the native *Clostridium* and *Lactobacillus* was studied using the agar well diffusion method. First, eight native bacteria were isolated from day 48 of operation. The isolated strains were grouped as hydrogen producer facultative anaerobe (*Escherichia coli* A), hydrogen producer obligate anaerobe (*Clostridium beijerinckii* B), hydrogen producer LAB (*Enterococcus faecium* F, *Enterococcus gallinarum* G), and non-hydrogen producer lactic acid bacteria (*Lactobacillus plantarum* LB1, *Pediococcus acidilactici* LB4, *Lactobacillus dextrinicus* LB5, *Lactobacillus pentosus* LB7). The cell-free supernatants of the eight isolated strains were used to evaluate their capability to inhibit their own growth. Only 16.0% of the combinations showed inhibition between the strains (Table 2). The results were similar to the CCA, where it identified a positive relationship between the dominant members in the microbial community (Figure 2). The native LAB generated the greatest inhibitory activity. *L. plantarum* LB1, *L. pentosus* LB7, and *P. acidilactici* LB4 inhibited the growth of hydrogen producer LAB such *E. faecium* F and *E. gallinarum* G. A reduced antimicrobial activity was detected by hydrogen producer bacteria. *E. faecium* F inhibited to *E. gallinarum* G, and *C. beijerinckii* B inhibited to *P. acidilactici* LB4.

The growth of some microorganisms can be limited by the presence of compounds such as organic acids, hydrogen peroxide, solvents, diacetyl, and protein compounds (bacteriocins and encrypted peptides), produced during the metabolism of other microorganisms (Piard & Desmazeaud 1991; Ghanbari *et al.* 2013). When the organic acids are present in high concentrations in the medium, they can limit certain microorganism growth through cellular damage modifying the lipidic permeability in the plasmatic membrane (Cherrington *et al.* 1991). In some cases, the microorganisms can release antimicrobial protein compounds during the fermentation. These compounds are mainly antimicrobial peptides such as encrypted peptides, non-ribosomal peptides, and ribosomal peptides such as the bacteriocins (Cotter *et al.* 2005; Hayes *et al.* 2007; Hur *et al.* 2012). LAB are widely known for their bacteriocins production.

The obtained results suggested that the inhibition growth between the native isolated strains could be due to organic acids due to the pH change from an initial value of 6.50 to final values between 3.17 and 4.27 in the supernatants and bacteriocins present in the study's supernatant. In this sense, the LAB have been identified as potential inhibitors of the hydrogen production process due to their production of lactic acid and bacteriocins (Gomes *et al.* 2016). Previous studies have discussed the role of the LAB on the stability of hydrogen production and reported their positive and negative effects (Castelló *et al.* 2020). This study presents evidence of positive effects of native *Lactobacillus* on the hydrogen production process from a raw lignocellulosic substrate, and their presence was correlated with the highest hydrogen productivity. The positive effect could be related to synergism with *Clostridium* (the most efficient hydrogen producer) via a cross-feeding of lactate and acetate. The amount of lactic acid present in the supernatants during the reactor operation time supports this hypothesis, i.e., *Lactobacillus* was one of the most abundant members. In addition, it is supported by the reduced inhibitory interactions identified in the antagonistic assays. Also, our results showed that interactions might be species or strain specific. These patterns might be difficult to observe when the microbial community is studied using broader taxonomic levels.

Table 2 | Antagonistic activity of isolated strains against fermentative and lactic acid bacteria

Supernatants from native strains (diameter in mm)

Native strains	*Escherichia coli* A	*Enterococcus faecium* F	*Enterococcus gallinarum* G	*Clostridium beijerinckii* B	*Lactobacillus plantarum* LB1	*Pediococcus acidilactici* LB4	*Lactobacillus dextrinicus* LB5	*Lactobacillus pentosus* LB7
Escherichia coli A	–	–	–	–	–	–	–	–
Enterococcus faecium F	–	–	–	–	22 ± 4	14 ± 1	–	23 ± 2
Enterococcus gallinarum G	–	16 ± 2	–	–	28 ± 5	26 ± 10	–	26 ± 11
Lactobacillus plantarum LB1	–	–	–	–	–	12 ± 1	–	–
Pediococcus acidilactici LB4	–	–	–	34 ± 2	–	–	–	–
Lactobacillus dextrinicus LB5	–	–	–	–	–	–	–	–
Lactobacillus pentosus LB7	–	–	–	–	–	-	–	–

CONCLUSIONS

The self-fermentation of WS by their native microbiota was stable despite changes in the WS cultivars. A *Lactobacillus–Clostridium* coexistence lasted the whole operation period and correlated positively with hydrogen production. An *in vitro* method confirmed that *Lactobacillus* was not antagonistic to *Clostridium* and only 16.0% of microbial interactions were negative.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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