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Abstract

Nowadays mixed cultures are considered as a serious alternative to pure cultures in biotechnological processes. Mixed cultures can use various organic substrates and be operated under unsterile and continuous conditions. Although most of studies dealing with fermentative hydrogen production have focused on dominant species, sub-dominant bacteria can also have a significant effect despite their low abundance. The determination of their exact ecological role is essential for better understanding microbial metabolic networks in mixed cultures.

In this work, the contribution of sub-dominant bacteria to fermentative H_2 production was investigated using chemostats continuously fed with a glucose-based medium. Interestingly, *Clostridium pasteurianum* was dominant in six assays on seven at steady state, and only bacterial populations in low abundance differed. Acting as keystone species, these bacteria impacted substantially the microbial metabolic network of the overall ecosystem despite their low abundance. While *Bacillus* spp. and *Lactobacillus* spp. lowered the H_2 yields by diverting a part of the H_2 potential to lactate production, the presence of *E. coli* increased the H_2 yield by redirecting the metabolic network to acetate and butyrate hydrogen–producing pathways.

Keywords

Biohydrogen / Chemostat / Dark fermentation / Microbial interactions / Minority bacteria / Mixed cultures

1 Introduction

Hydrogen (H_2) is considered as one of the most interesting energy carriers in a global context of exploring new sources of renewable energy and reducing greenhouse gases. H_2 has a high energetic yield (122 $kJ.g^{-1}$), can be stored and its combustion produces only water. Nowadays, H_2 is mainly produced by chemical processes such as natural gas reforming or water electrolysis. These processes produce large amounts of H_2 but are high energy-consuming and release high amounts of CO_2 into the environment [1].

Hydrogen can also be produced in biological processes and becomes more environmental friendly. Especially, in anaerobic digestion bioprocesses, H_2 is a key metabolic intermediate which is produced by acetogenic bacteria during fermentation of organic compounds, and is then immediately consumed by microorganisms coupling the oxidation of H_2 to the reduction of more oxidized compounds [2]. Such H_2 consumption can be avoided by using appropriate operating conditions [3,4]. In addition, fermentative processes operated with mixed bacterial cultures can use a large range of renewable, complex, non-sterile and inexpensive substrates to produce H_2 [5–7]. Compared to pure cultures, these bioprocesses are more robust and susceptible to adapt themselves more easily to changes of environmental conditions [4,7]. Finally, such complex microbial communities are able to provide useful combinations of metabolic pathways for the degradation and the transformation of complex substrates [8].

In counterpart, maximal conversion yields of carbohydrates are limited to 2.5 mol $H_2.mol^{-1}$ hexose under experimental conditions [3], whereas the maximal theoretical yield is 4 mol $H_2.mol^{-1}$ hexose through the acetate pathway. Instability problems of these processes are also often encountered. Instability is mainly due to either a shift of microbial metabolic pathways in response to environmental conditions, or the emergence competitive non hydrogen-producing bacteria for carbohydrates or direct H_2-consuming bacteria [9].

To limit this instability and thus optimize bioprocesses, many operational parameters have already been tested, such as pH, temperature and hydraulic retention time [10,11]. It is recommended to

maintain pH lower than 6.5, and more generally between 5.5 and 6.0 [8,12,13]. Hydrogen production occurs at mesophilic (around 37°C), thermophilic (around 55°C) or even hyperthermophilic (60 – 80°C) conditions, although most of the studies found in the literature have been carried out under mesophilic conditions [12,14]. When reactors are operated in a continuous mode, especially with glucose as single substrate, a short hydraulic retention time ranging from 2 to 12 hours is recommended to avoid the presence of methanogenic *Archaea* which consumes efficiently H_2 and fermentation end-products to produce methane and carbon dioxide [3,10,15]. Another way to limit the presence of H_2-consuming microorganisms is to apply several pre-treatment methods of the inoculum such as heat-shock treatment, acidic and alkaline treatments and microaerobic treatment. These treatments, especially heat-shock, favour specifically the growth of spore-forming H_2-producing bacteria while H_2-consuming microorganisms are strongly inhibited, such as methanogens or homoacetogenic bacteria.

So far, most of literature studies have focused on dominant hydrogen-producing species such as *Clostridium* spp. Nevertheless, the presence of low abundant bacterial species are always observed in reactors operated under continuous mode, but their exact role is still unknown [3]. These experimental observations are not consistent with the competitive exclusion principle stating that microbial diversity should not be maintained in a chemostat at steady-state, unless the microbial populations are subject to product inhibition, cross-feeding or other direct microbial interactions [16–18]. Thus, low abundant bacteria should participate significantly to ecosystem functioning despite their low abundance, and therefore some of them may be considered as keystone species as defined by Smee [19]. The influence of these keystone species occurs through interactions between microorganisms. These interactions are often trophic: a microorganism is dependent on another microorganism for the degradation of specific substrates or products [17,20], or different microorganisms outcompete for the same substrate [21]. In amensalism cases, a microorganism has an adverse effect on other microorganisms, for example by producing antibiotics or toxic compounds [22]. Other physical or chemical, intra and extracellular mechanisms can also occur, including cellular communication between microorganisms, so called "quorum sensing" or through direct exchange of molecules or electrons *via* cytoplasmic connections, pilis or nanotubes [23,24].

The aim of this study was to determine the ecological role of low abundant bacteria in mixed cultures producing H_2 by dark fermentation. Seven inocula originated from anaerobic sludge, caecotrophs, fermented cassava, or a mixture of them were tested. Heat shock treatment was also used to reduce the microbial diversity in initial inoculum. Hydrogen-producing microbial communities were characterized after 40 hydraulic retention times at steady state in continuous chemostats operated under same conditions.

2 Material and methods

2.1 Inoculum sources

Three different sources of anaerobic inoculum were selected: (i) an anaerobic sludge (AS) was sampled from the outlet of a lab-scale methanogenic reactor fed with wine distillery wastewater (ii) cassava (Cas) was previously fermented in water and then homogenised with a sterile mixer, and (iii) cæcotrophs (Cæ) (*i.e.*, soft fæces of rabbits) were collected from the digestive system of rabbits and diluted in autoclaved physiological water solution (9 g $NaCl.L^{-1}$). For each inoculum, a heat treatment shock (90°C, 10 min) was additionally tested (named AS^{ht}, Cas^{ht} and $Cæ^{ht}$). Finally, a mixture of the three inoculum sources (Mix) was prepared in the same ratio of volatile solids (VS).

2.2 Feeding solution

A glucose solution of 10 $g.L^{-1}$ was used as sole carbon source in a feeding medium composed by the following nutrients (in $mg.L^{-1}$): K_2HPO_4, 500; NH_4Cl, 2 000; yeast extract, 200; HCl 37%, 55; $MgCl_2$, 55; $FeSO_4(NH_4)_2SO_4$, 7; $ZnCl_2$, 1; $MnCl_2$, 1.2; $CuSO_4$, 0.4; $CoSO_4$, 1.3; BO_3H_3, 0.1; $Mo_7O_{24}(NH_4)_6$, 1; $NiCl_2$, 0.05; Na_2SeO_3, 0.01; $CaCl_2$, 60.

The glucose and nutrient solutions were autoclaved separately at 121°C for 20 minutes. The feeding solution was prepared daily under sterile conditions in an Esco Labculture Class II Type A2 Biological Safety Cabinet, sparged with nitrogen gas and stored at 4°C prior to use.

2.3 Experimental set-up

Seven experiments were carried out using the different sources of inoculum (AS, Cas, Cae, AS^{ht}, Cas^{ht}, $Cæ^{ht}$ and Mix). Continuous stirred tank reactors, with a working volume of 1.5 L, were used. Before each experiment, reactors and sampling tubes were autoclaved at 121°C for 20 minutes. Each bioreactor was filled with the feeding solution, and was then inoculated with an inoculum to reach a final volatile solid content of 0.6 g $VS.L^{-1}$. pH was then adjusted at 5.5 using HCl, and the reactor was

flushed for 15 minutes with N_2 to achieve anaerobic conditions. Each assay started with a batch period of 24 h. Then, the reactors were operated in a continuous mode (24 h after inoculation). The hydraulic retention time (HRT) was fixed at 6 h for all experiments. The feed was stored at 4°C and was added continuously in the reactor using a peristaltic pump. A level sensor and a peristaltic pump were used to keep constant the liquid volume inside the reactor. Control of pH at 5.5, using NaOH 2 M, started with the continuous feeding of the reactor. The reactor was heated at 37°C using a silicon heating blanket wrapped around the vessel. The agitation speed was fixed at 300 rpm. Reactor was equipped with temperature and pH probes. The gas flow rate was monitored using an electronic gas volumeter. All continuous experiments lasted from 10 to 12 days, equivalent to more than 40-48 hydraulic retention times.

2.4 Chemical analysis

Biogas composition (H_2, CO_2, O_2, CH_4 and N_2 content) and volatile fatty acids (VFAs) were analyzed according to Aceves-Lara et al. [10]. Residual glucose as well as fermentation end-products (lactate, ethanol, acetone…) were quantified using high performance liquid chromatography (HPLC) coupled to a refractometer (Waters R410). The chromatograph was equipped with a HPX 87 column (Biorad) and the eluent corresponded to a H_2SO_4 solution (0.222 $\mu l.L^{-1}$) under isocratic elution at 0.4 $mL.min^{-1}$. The operating conditions were: temperature of column, 35°C; temperature of refractometer, 40°C.
The biomass concentration of reactor outlet was assessed by the determination of volatile suspended solids (VSS) by filtration at 0.2 µm and drying at 500°C overnight, according to Standard Methods [25].

2.5 DNA extraction and PCR amplification

Microbial cells were collected after centrifugation at 12,100 x g for 15 min of two milliliters of culture. Genomic DNA was extracted and purified using the Wizard Genomic DNA Purification kit (Promega). The amount and purity of DNA in the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan).

The 16S rRNA genes were amplified according to Quéméneur et al. [13] for further CE-SSCP analysis. Peaks were assigned by cloning and sequencing as previously described by Godon et al. [26].

2.6 CE-SSCP fingerprinting

CE-SSCP fingerprinting method was previously described by Quémeneur et al. [13]. CE-SSCP profiles were aligned with an internal standard to consider any electrophoretic variability. CE-SSCP profiles were then normalized using the StatFingerprints library [27] from R version 2.9.2 [28] and according to standard procedure [29]. On average, each peak of the CE-SSCP profile corresponded to the gene fragment of a unique 16S rDNA sequence, and thus represented a specific species within the microbial community. The area under a peak represented the relative abundance of the corresponding microbial species in the community.

2.7 Assignment of the CE-SSCP peaks

PCR products were purified using a PCR Purification Kit (Qiagen). The clone libraries were built using the TA Cloning kit (Invitrogen). After PCR amplification using plasmid-targeted primers T7 and P13, the PCR-CE-SSCP profiles of each PCR product, corresponding to an unique sequence and therefore an unique peak, were compared with the mixed profiles of the H_2-producing cultures for peak assignment. After assignment, the corresponding cloned inserts were sent for sequencing (MilleGen company, Toulouse, France). The nucleotide sequences, were aligned with reference sequences retrieved from the Genbank database using the CLUSTALW program [30], and were further refined manually using the BioEdit program [31]. The 16S rRNA gene sequences were deposited in the European Nucleotide Archive database and available in Genbank database under the accession numbers HE613257 to HE613267.

2.8 COD mass balance and statistical analysis

A COD mass balance of the glucose fermentation was calculated for all reactors at steady state to validate that all metabolic end-products were quantified. During H_2 fermentation, glucose was converted to VFAs, alcohols, hydrogen, carbon dioxide and biomass. In order to convert bacterial growth in COD or mmol flow, the chemical formula for biomass was assumed as $C_5H_7NO_2$ [32].

Significant differences between H_2 performances and metabolic network of the different chemostats were assessed by Student's t-tests and ANOVA using R software.

3 Results

3.1 Bacterial communities at steady state

At the end of the batch period (24 h after inoculation), a significant reduction of the microbial diversity was observed compared to the initial inoculum with the emergence of one dominant species and several minor species. Simplification of all ecosystems was observed during the continuous culture to reach a steady state after about 30 generations (hydraulic retention time, HRT). Throughout the steady state period representing at least 10 HRT, the microbial community remained stable. As an illustration of microbial community dynamics over experimental time, the behavior of the microbial community structure in the reactor inoculated with cassava (Cas) is presented in Figure 1. The behavior of all ecosystems inoculated with the seven different microbial inocula is presented as Supplementary Data (Figure S1).

[FIGURE 1]

The stable microbial community structures of the seven reactors at steady state are presented in Figure 2. All the CE-SSCP profiles were composed of one major *Clostridium* spp. peak (*peak 1* or *peak 2*) representing between 67 and 89 % of the community abundance, and several other minor peaks (from *peak 3* to *peak 11*) never exceeding 19% of the total abundance. Interestingly, except in the culture inoculated with the heat-treated anaerobic sludge (AS^{ht}), a single dominant bacterial species was found whatever the initial inoculum. Thus, only the nature, the number and the relative abundance of the low abundant bacterial populations differed from one ecosystem to another, at steady state.

The 16S rDNA sequence affiliations of the clones that match with the main different peaks are shown in Table 1. Sequence affiliations were always above 99% of similarity with sequences of known species. In the seven ecosystems, the most abundant peaks were assigned to known H_2-producing *Clostridium* spp., either *C. butyricum* (*peak 1*) in AS^{ht} culture, or *C. pasteurianum* (*peak 2*) in all other cultures. Among lower abundant bacterial populations, *peak 3* was detected in all chemostats, except in the culture inoculated with anaerobic sludge (AS). *Peak 3* was related to *Clostridium beijerinckii*.

Other minority bacteria corresponded mainly to lactate-producing bacteria: *peak 4* and *peak 5*, respectively affiliated to *Sporolactobacillus lævolacticus* and *Bacillus coagulans*, were detected in the reactor inoculated with AS^{ht}. *Bacillus racemilacticus* (*peak 6*) was detected in the reactors inoculated with AS and AS^{ht}. In the cultures generated from cassava (Cas), heat treated cassava (Cas^{th}) and heat-treated cæcotroph ($Cæ^{th}$), the sub-dominant populations were all related to *Lactobacillus* spp.: *L. paracasei*, *L. casei, L. nagelii* and *L. ghanensis* respectively for *peak 7*, *peak 8*, *peak 9* and *peak 10*. Finally, the only minority species (*peak 11*) neither belonging to *Clostridium* genus nor lactate-producing genera was affiliated to *Escherichia coli*, and was obtained in the reactor inoculated with untreated cæcotrophs (Cæ).

[TABLE 1]

[FIGURE 2]

3.2 Hydrogen production performances

Hydrogen productivities and yields were assessed at steady state for each culture, and are presented in Table 2. In this study, an HRT of 6 hours was applied and steady state was considered when H_2 production was stable for more than 2 days (*i.e.*, 8 HRT): the average values of H_2 productivity and yield were thus calculated over this period. At steady state, hydrogen productivity ranged from 5.47 to 9.07 mmol $H_2.L^{-1}.h^{-1}$ in AS^{ht} and AS cultures, respectively. Five different levels of H_2 production were observed ($p<0.05$, t-test). Hydrogen productivities were similar in Cæ and Cas cultures with 6.56 and 6.42 mmol $H_2.L^{-1}.h^{-1}$ ($p>0.05$, t-test), respectively. The Mix and Cas^{ht} cultures presented almost the same H_2 production rates of 8.06 and 8.04 mmol $H_2.L^{-1}.h^{-1}$, respectively. Finally, the $Cæ^{ht}$ culture presented an intermediate productivity of 7.45 mmol $H_2.L^{-1}.h^{-1}$.

The H_2 yield was calculated by considering both hydrogen productivity and glucose consumption at steady state. A minimum H_2 yield of 1.21 mol $H_2.mol^{-1}$ glucose_consumed was observed in AS^{ht} culture. A similar value was found in Cas culture with 1.41 mol $H_2.mol^{-1}$ glucose_consumed. The $Cæ^{ht}$ and Cas^{ht} cultures presented an intermediate H_2 yield of 1.72 and 1.79 mol $H_2.mol^{-1}$

glucose_consumed, respectively. Higher results were obtained for AS and Mix cultures with about 1.9 mol H_2.mol^{-1} glucose_consumed. Finally, the highest hydrogen yield was obtained in Cæ culture with 2.32 mol H_2.mol^{-1} glucose_consumed. This H_2 yield was significantly different from the yields of all other cultures ($p<0.05$, t-test). In Table 3, the H_2 production and yields obtained from different carbon sources using different inocula are reported and are mainly consistent with our results (Table 3).

[TABLE 2]

[TABLE 3]

3.3 Metabolic pathways

Figure 3 presents the COD mass balance of the different cultures at steady state. By considering the composition of reactor outlet in biomass, gas and liquid, the COD mass balance was identical to the inlet with a reasonable variability error of 10%. It confirmed that no major metabolite was missing during the analytical procedure.

[FIGURE 3]

Figure 4 presents the distribution of metabolic end-products for each reactor. All the cultures produced acetate, butyrate and lactate but in different proportions. Ethanol production was also detected in Cæ^{ht}, Cas and Cas^{ht} cultures with respectively 0.45, 0.86 and 0.86 mmol.L^{-1}.h^{-1}. The lactate production ranged from 0.75 to 3.44 mmol.L^{-1}.h^{-1} in Cæ and AS^{ht} cultures, respectively. The lowest acetate production was observed in AS^{ht} culture with 1.06 mmol.L^{-1}.h^{-1} and the lowest butyrate production was observed in Cæ culture with 1.59 mmol.L^{-1}.h^{-1}. Meanwhile, the highest production of acetate and butyrate was obtained in AS culture with 1.90 and 2.18 mmol.L^{-1}.h^{-1}, respectively. In addition, the Acetate/Butyrate ratio varied in all cultures (Table 2). It was lower than one for the AS^{ht}, AS and Cas^{ht} cultures (0.65, 0.84 and 0.86 respectively) and close to one for the Cas, Cæ^{ht} and Mix cultures.

Interestingly the Ac/But ratio of the Cæ culture was the only one greater than one, indicating a shift in microbial metabolic pathway in this culture.

Biomass concentration also differed significantly from one culture to another. The highest biomass concentration was observed in AS culture with 1 $mmol.L^{-1}.h^{-1}$, which is twice the concentration observed in AS^{ht} culture.

[FIGURE 4]

4 Discussion

4.1 Correlation between hydrogen production performances and metabolic network

In this study, a large range of H_2 yields and productivities were observed, but all were consistent with those previously reported in literature with mixed cultures [7-9; 12; 33]. Significant differences on H_2 production performances were observed and were directly related to the community structure of the microbial ecosystems present at steady state which also exhibited distinct metabolisms. Interestingly, community structures of the microbial ecosystems were not only influenced by the initial inoculum, but were also substantially different after batch incubation, with different proportions of sub-dominant bacteria. Therefore, initial conditions, i.e. inoculum diversity and microbial community structure, after the batch incubation had a high influence on the final microbial community found after 40 HRT.

In terms of reactor performances, the Cæ culture showed the highest H_2 yield with 2.32 mol H_2.mol^{-1} glucose_consumed, but the total amount of metabolites in Cæ culture was much lower than those observed with other inocula. Indeed, bacterial populations of Cæ culture degraded only 25% of inlet glucose suggesting a higher specificity and efficiency of the microbial pathways to produce hydrogen. The main end-products observed in Cæ culture were acetate (37%) and butyrate (34%), which are the two main metabolites linked to dark fermentative H_2 production pathways. According to stoichiometric reactions of dark fermentation, the formation of one mole of acetate or one mole of butyrate lead both to the release of two moles of H_2 (eq. 1 and 2). In contrast, there is no direct relationship between H_2 production and lactate or ethanol production (eq. 3 and 4). In Cæ culture, lactate represented only 13% of the end-product flow, and no ethanol production was observed. These results indicated that the degradation in the Cæ culture was more specific than with the other initial inoculum.

The other reactors produced also H_2 using the acetate and butyrate metabolic pathways, but showed lower H_2 yields. Two hypotheses could be formulated to explain this observation: Either a direct consumption of H_2 through homoacetogenesis (eq. 5) or a shift of microbial metabolisms towards non-H_2-production pathways, such as ethanol or lactate. In all reactors, the ratio of H_2 production and the

sum of acetate and butyrate remained almost constant around 2 mol H_2.mol^{-1} VFAs_produced which corresponded to the theoretical value. This suggested that there was no direct H_2 consumption due to homoacetogenesis, since acetate did not accumulate. The lower H_2 yield observed for the other reactors is mainly explained by less specific hydrogen-producing pathways and a higher production of other end-products, such as lactate and ethanol.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (4)$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 4H_2O \quad (5)$$

4.2 Role of dominant bacteria in hydrogen production

Interestingly, metabolites distribution as well as H_2 production was significantly different in all continuous cultures although the operating parameters were constant and similar. The only parameter that varied from one experiment to another was the bacterial community at steady state and, therefore, microbial community structure is the sole parameter that can explain such differences in metabolic network and H_2 performances. Consistently, when two cultures showed the same species distribution at steady state (Cas^{ht} and Mix), metabolic network and H_2 performances were similar.

In this study, whatever the inoculum source, the final microbial ecosystems were simplified with only one majority bacterial species and few low abundant minority species. At steady state, the main H_2-producing bacteria found in all the ecosystems selected belonged to *Clostridium* genus, which is in accordance with the literature where clostridia are the main dominant bacteria producing hydrogen

[12,38-39]. Furthermore, the type of *Clostridium* species had an effect on the overall H_2 production. Indeed, the lowest H_2 production was observed in AS^{ht} culture which was the only experiment with *Clostridium butyricum* as dominant species. This is explained by the preferential use of the butyrate pathway (acetate/butyrate ratio of 0.64) by *C. butyricum* as compared to *C. pasteurianum*. According to eq.1 and 2, the butyrate pathway leads to half of the amount of H_2 produced through acetate pathway. Such preferential usage of butyrate pathway of *C. butyricum* was already reported in previous studies with similar acetate/butyrate ratios ranging from 0.45 to 0.67 [40–42].

In contrast, *Clostridium pasteurianum* led to a more equilibrated byproducts distribution (acetate/butyrate ratio between 0.87 and 1.08). Therefore, *C. pasteurianum* favoured H_2 production by using the acetate pathway compared to *C. butyricum*. This explains partly why H_2 yields were higher in the six other cultures when *C. pasteurianum* was the dominant species.

4.3 Sub-dominant bacteria as keystone species within the metabolic network

The presence of the same single dominant species in these ecosystems constituted a major driving force for H_2 production. Nevertheless, substantial differences were observed in metabolic pathways, although operating conditions remained the same. When considering the six ecosystems with *C. pasteurianum* as dominant species (AS, Cæ, $Cæ^{ht}$, Cas, Cas^{ht} and Mix), the significant differences in H_2 production and in end-products distribution were attributed to the persistence of different sub-dominant species after 40 HRT. Interestingly, each of these species never exceeded 19% of the total abundance. According to the competitive exclusion principle [43], which states that a simple unstructured environment containing a single resource can only support the growth of one competitor, these sub-dominant bacteria should have been washed out unless they interacted with their environment. Despite their low abundance, the sub-dominant bacteria had the highest influence on the overall ecosystem metabolism. In that sense, these sub-dominant bacteria may be viewed as true keystone species [19,44].

4.3.1 Role of competitive Clostridium spp.

Clostridium beijerinckii was one of the sub-dominant bacteria in the Cas, Cas^{ht}, Cæ, Cæ^{ht} and Mix cultures. A competitive (trophic) interaction with the dominant *Clostridium* was expected. No significant effect of *C. beijerinckii* was shown on H_2 production. However, this species is known to produce H_2 through both acetate and butyrate pathways. In some cases it may become a substrate competitor and produces propionate, butanol, lactate or ethanol [45]. In this study, the exact role of the different species of *Clostridium* genus cannot be distinguished. Whatever the experiment, the abundance of bacteria belonging to the *Clostridium* genus ranged from 79-97%. Therefore, the differences in H_2 production resulted from the microbial activity of only 3 to 21% of the bacteria that belong to other species.

4.3.2 Role of homolactic bacteria through either competition or amensalism.

Five cultures (AS, Cas^{ht}, Cæ^{ht}, Cas and Mix) contained lactic acid bacteria, although heat treatment should have drastically reduced the amount of lactic acid bacteria in the initial inoculum [46]. The presence of non-spore-forming lactic acid bacteria at steady state was likely due to low survival by heat resistance of these microorganisms in initial heat-treated inocula. In the five cultures, the lactate concentration was higher with negative effects on H_2 production. Karadag and Puhakka [47] also found high lactate production co-occurred with low H_2 production in presence of *Bacillus coagulans*. Indeed, homolactic bacteria are known to produce lactate with no associated H_2 production, according to eq. 3. [36]. These bacteria do not use H_2 directly but consume glucose which is then no longer available for H_2 production. These lactic bacteria outcompete with *Clostridium* spp. for glucose.

Ethanol production was observed in cultures Cas^{ht}, Cas and Cæ^{ht}. For *Clostridium* species, such ethanol production can be caused by a physiological stress [9]. Noike et al. [48] showed that *Lactobacillus* spp. can decrease H_2 production by secreting bacteriocins in the culture media. The presence of bacteriocins is stressfull for clostridia which leads to a switch in the metabolic pathway from H_2 production to solvent production (ethanol and lactate mainly) [9]. Bacteriocins can also affect

the presence of other bacteria in the ecosystem. In fact, the only culture (Cæ) where *E. coli* was found was the only one containing no lactic bacteria. As amensalistic interaction, similar observations were reported in the human gastrointestinal tract where lactic acid bacteria have an antimicrobial activity against coliforms like *E. coli* [49].

In addition, some homolactic bacteria might have a positive effect on H_2 production. Indeed, *Bacillus racemilacticus* was found as sub-dominant bacteria in AS culture where the highest biomass concentration was observed, and consequently the highest H_2 productivity. Such influence on biomass accumulation within the AS culture could be explained by the presence of such *Bacillus* sp. since lactic acid bacteria are known to produce exopolysaccharides (EPS) [50]. Fang et al. [34] showed that several *Bacillus* sp. produce EPS which can favour floc formation and thus increase biomass concentration. In our study, biomass accumulation in flocs would have led to higher H_2 production. In nature, EPS generated by lactic acid bacteria play a wide role in flocs and biofilm development for colonization of environmental niches, such as in fermentative human gastrointestinal tract. In addition, EPS protects the bacteria against hostile environmental conditions [51-52].

4.3.3 Escherichia coli, a keystone species driving Clostridium spp. metabolism.

The highest H_2 yield, i.e. 2.3 mol H_2.mol^{-1} glucose_consumed, was observed in the Cæ culture. In this culture, only two low abundant species were found: *Clostridium beijerinckii* and *Escherichia coli*.

The presence of *E. coli* in this continuous reactor was likely due to interaction with *Clostridium* sp., but the exact role of *E.coli* in terms of H_2 production remains to be elucidated.

Microorganisms belonging to the genera *Escherichia* can carry out a mixed acid fermentation and produce lactate, acetate, succinate, ethanol, formate, CO_2 and H_2 [53]. Thus *E. coli* potentially produces H_2 using the formate pathway [53-54]. Nevertheless, this is not coherent with the results obtained for the H_2 productivity since the quantity of acetate and butyrate produced in the Cæ culture was highly correlated to H_2 production. Indeed, the ratio between H_2 production and acetate/butyrate concentration was equal to 1.9, slightly lower than theoretical ratio. This suggests that H_2 was exclusively produced by acetate and butyrate pathways, but not by formate pathway.

Some studies use the association between *E. coli* and *Clostridium* to maintain an anaerobic environment by oxygen depletion and thus increase the H_2 yield [55]. But for all our experiments the redox potential was highly negative (-330 mV), suggesting that this commensalistic interaction did not occur in Cæ culture.

Another hypothesis, even though very speculative, can be formulated in order to explain such high H_2 yield in Cæ culture: Recently, Dubey and Ben-Yehuda [24] showed that some bacteria, of which *E. coli* belongs, could form nanowires with Gram+ bacteria and interact through direct electrons transfer or metabolites exchanges. That type of interaction could also be considered between *Clostridium* spp. and *E coli*. This interaction could disturb the metabolism of *Clostridium* spp. and redirect it to the acetate pathway under unknown cellular mechanisms. It might explain why the Cæ culture was the only one to reach an acetate/butyrate ratio higher than one. This type of interaction would also explain why *E. coli* was not washed out after 40 hydraulic retention times, according to the competitive exclusion principle, by attachment to *Clostridium* sp. cells, after division. However, further investigations are needed to clarify the exact interaction existing between *E.coli* and *Clostridium sp.*

5 Conclusions

In this study, H_2 production by dark fermentation in mixed cultures was clearly influenced by the structure of the bacterial community. Even though dominant bacteria played a role in H_2 production, the metabolic network of the ecosystem seemed to be driven by low-abundant sub-dominant bacteria. These bacteria in low abundance influenced H_2 production from a benefic or a negative way. Particularly, *E. coli* seemed to have a positive effect on H_2 production, by redirecting the global metabolism of the ecosystem towards the acetate pathway and by increasing the H_2 yield. In contrast, the homolactic bacteria had a negative impact by using a part of the carbohydrates for lactate production which is not linked to H_2 production. These bacteria are also known for their ability to produce bacteriocins which could stress clostridia or prevent the implantation of other bacteria, such as *E.coli*. In this case, the influence of lactic acid bacteria on the overall ecosystem is high even at low abundance. In conclusion, it was shown that bacteria in low abundance can be considered as keystone species controlling the metabolism of the global microbial ecosystem in fermentative mixed cultures.

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Table and Figure captions

Table 1: Phylogenetic affiliation of bacterial 16S rDNA cloned from samples of the different cultures at steady state. The number of each clone indicates the peak of the CE-SSCP profile, to which the clone was assigned. The relative abundance of each species in the ecosystem was calculated from the area under each peak. The different cultures at steady state were: heat-treated anaerobic sludge (AS^{ht}), anaerobic sludge (AS), heat-treated cassava (Cas^{th}), cassava (Cas), heat-treated cæcotroph ($Cæ^{th}$), cæcotroph (Cæ), and mixture of the three non heat-treated inocula (Mix).

Table 2: H_2 production performances at steady state for the seven selected ecosystems: heat-treated anaerobic sludge (AS^{ht}), anaerobic sludge (AS), heat treated cassava (Cas^{th}), cassava (Cas), heat-treated cæcotroph ($Cæ^{th}$), cæcotroph (Cæ) and mixture of the three non heat-treated inocula (Mix). Statistical differences are indicated by letters ($p<0.05$, t-tests).

Table 3: Hydrogen productivity and yields observed in the literature for mixed cultures in continuous reactor.

Figure 1: Dynamics of the microbial community structure: example of the culture inoculated with cassava (Cas). The first CE-SSCP profile corresponds to the structure of the inoculum, followed by the profile of the community structure after a batch period of 24 hours. The last two profiles correspond to the microbial community structure at steady state after 32 and 40 hydraulic retention times (HRT), respectively. The CE-SSCP profiles were aligned on the basis of the common ROX internal standard, and areas normalized. The X- and Y-axes represent the relative peak migration distance and the relative peak intensity, respectively. The dynamics of all cultures are given in Supporting Information (Figure S1).

Figure 2: CE-SSCP profiles of the seven hydrogen-producing ecosystems at steady state: heat-treated anaerobic sludge (AS^{ht}), anaerobic sludge (AS), heat-treated cassava (Cas^{th}), cassava (Cas), heat-treated cæcotroph ($Cæ^{th}$), cæcotroph (Cæ), and mixture of the three non heat-treated inocula (Mix).

The CE-SSCP profiles were aligned on the basis of the common ROX internal standard, and areas normalized. The X- and Y-axes represent the relative peak migration distance and the relative peak intensity, respectively. A representative profile of the ecosystem at steady state is presented for each culture. Phylogenetic affiliation of the clone sequences corresponding to the numbered peaks is given in Table 1.

Figure 3: COD mass balance of the different cultures at steady state: heat-treated anaerobic sludge (AS^{ht}), anaerobic sludge (AS), heat-treated cassava (Cas^{th}), cassava (Cas), heat-treated cæcotroph ($Cæ^{th}$), cæcotroph (Cæ), and mixture of the three non heat-treated inocula (Mix).

Figure 4: Metabolites and hydrogen produced by the different cultures at steady state. The Y-Axes have been scaled to represent the difference between the H_2 productivity and the associated metabolites (theoretically if H_2 is produced by acetate and butyrate pathways: H_2 Productivity = 2 (Ac + Bu)). The different cultures at steady state were: heat-treated anaerobic sludge (AS^{ht}), anaerobic sludge (AS), heat-treated cassava (Cas^{th}), cassava (Cas), heat-treated cæcotroph ($Cæ^{th}$), cæcotroph (Cæ), and mixture of the three non heat-treated inocula (Mix).

Table1_YR.doc

Clone number	Closely related to	Sequence similarity (%)	Relative abundance of species in each ecosystem (%)						
			AS^{ht}	AS	Cas^{ht}	Cas	$Cæ^{ht}$	Cæ	Mix
1	*Clostridium butyricum*	100	74.0	-	-	-	-	-	-
2	*Clostridium pasteurianum*	100	-	89.1	67.7	66.8	74.0	78.8	73.2
3	*Clostridium beijerinckii*	99	15.7	-	18.3	12.6	15.8	18.8	16.8
4	*Sporolactobacillus laevolacticus*	99	3.5	-	-	-	-	-	-
5	*Bacillus coagulans*	99	1.8	-	-	-	-	-	-
6	*Bacillus racemilacticus*	100	5.0	10.9	-	-	-	-	-
7	*Lactobacillus paracasei*	99	-	-	14.0	13.6	-	-	10.0
8	*Lactobacillus casei*	100	-	-	-	7.0	-	-	-
9	*Lactobacillus nagelii*	99	-	-	-	-	7.3	-	-
10	*Lactobacillus ghanensis*	99	-	-	-	-	2.9	-	-
11	*Escherichia coli*	100	-	-	-	-	-	2.4	-

Table2_YR.doc

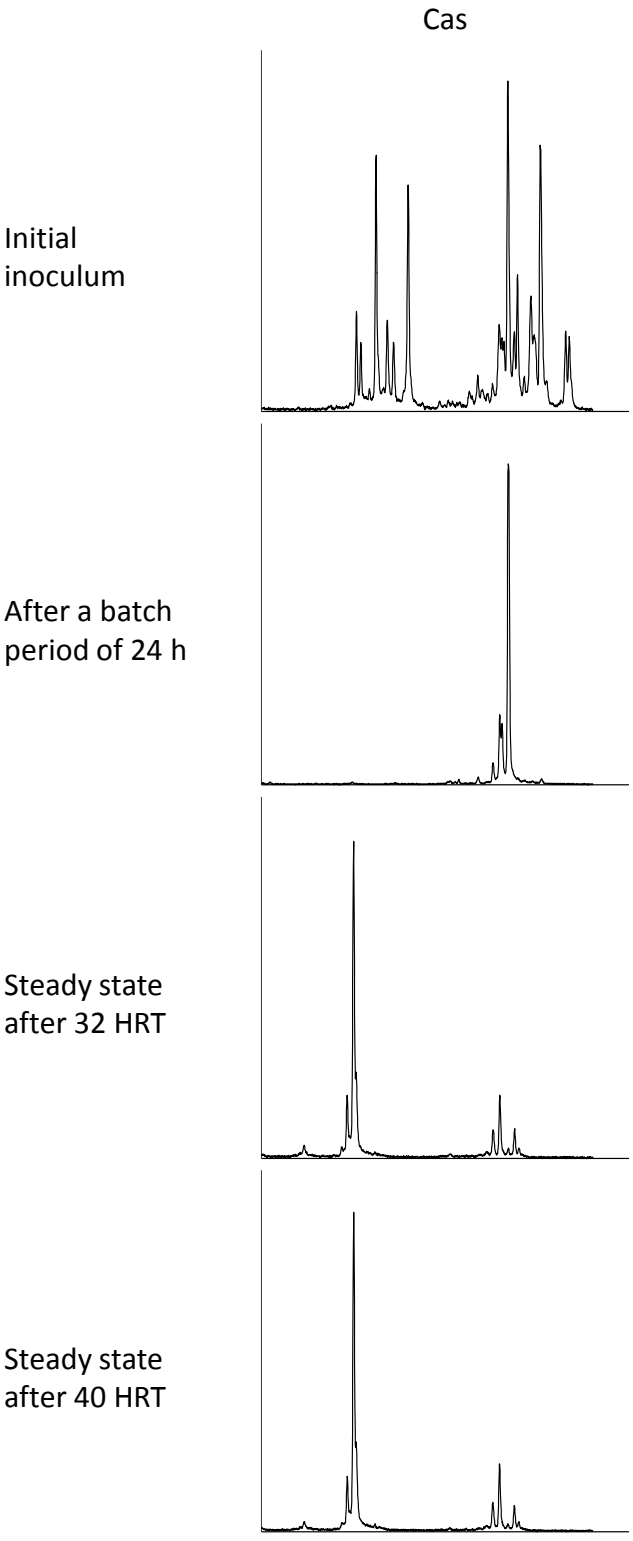
	AS^{ht}	AS	Cas^{ht}	Cas	Cæ^{ht}	Cæ	Mix
H_2 productivity (mmol.L^{-1}.h^{-1})	5.47 ^{a}	9.07 ^{e}	8.04 ^{d}	6.42 ^{b}	7.45 ^{c}	6.56 ^{b}	8.06 ^{d}
Y (mole H_2.mole^{-1}Glc $_{consumed}$)	1.21 ^{a}	1.92 ^{c}	1.79 $^{b, c}$	1.41 ^{a}	1.72 ^{b}	2.32 ^{d}	1.87 ^{c}
acetate / butyrate ratio	0.64 ^{a}	0.87 ^{b}	0.88 ^{b}	0.99 ^{d}	0.99 $^{c, d}$	1.08 ^{e}	0.94 ^{c}

Letters indicated the statistical confidence for grouping ($p < 0.05$, t-tests)

Table3_YR.doc

Inoculum	Substrate	Productivity (mmol $H_2.L^{-1}.h^{-1}$)	H_2 Yield (mol H_2.mol $hexose^{-1}$)	Reference
Different types heat treated or not	Glucose	5.5 – 9.1	1.2 – 2.3	This study
Sewage sludge	Glucose	0.3 – 15	0.6 – 1.7	[33]
Sludge of hydrogen reactor	Glucose	4.3	2.1	[34]
Agricultural soil heat treated	Glucose	3.6 – 96.7	1.7 – 2.8	[35]
Sludge from an anaerobic digester heat treated	Sucrose	14.2	1.2	[36]
Sludge from an anaerobic digester	Sucrose	-	0.1 – 1.7	[37]

Rafrafi_et_al_Figure1.pdf



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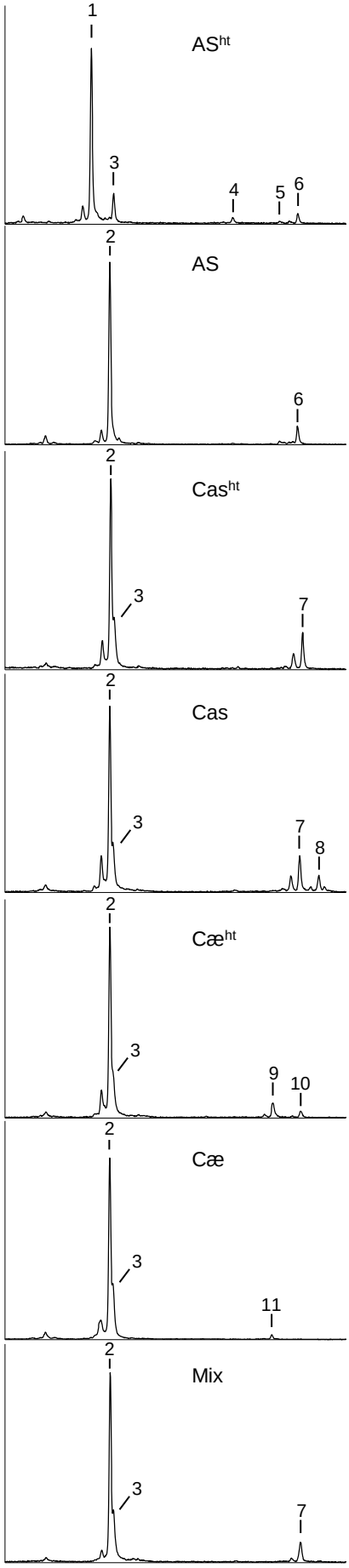

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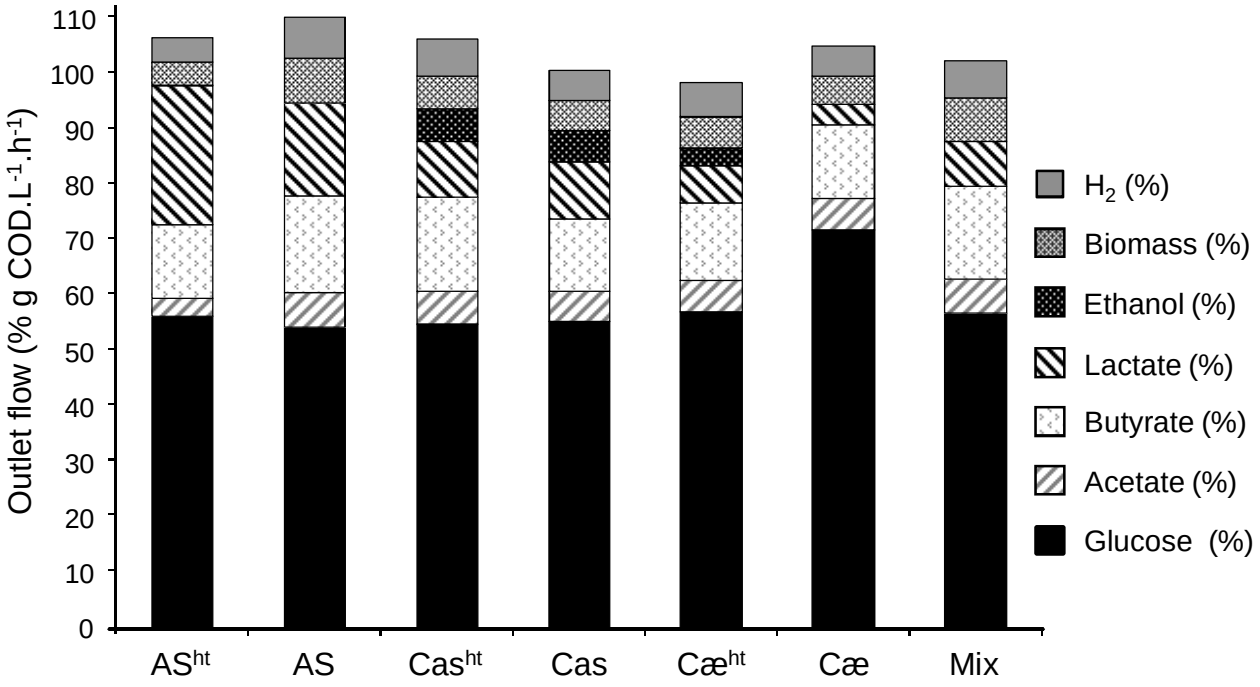

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