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## Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium



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### HIGHLIGHTS

- A stable lignocellulolytic rumen-derived consortium was successfully obtained.
- The enriched consortium degraded 55.5% lignocellulose, producing mainly carboxylates.
- A three-phase behavior was observed for macrokinetic and enzymatic parameters.

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### ABSTRACT

A rumen-derived microbial consortium was enriched on raw wheat straw as sole carbon source in a sequential batch-reactor (SBR) process under strict mesophilic anaerobic conditions. After five cycles of enrichment the procedure enabled to select a stable and efficient lignocellulolytic microbial consortium, mainly constituted by members of *Firmicutes* and *Bacteroidetes* phyla. The enriched community, designed rumen-wheat straw-derived consortium (RWS) efficiently hydrolyzed lignocellulosic biomass, degrading 55.5% w/w of raw wheat straw over 15 days at 35 °C and accumulating carboxylates as main products. Cellulolytic and hemicellulolytic activities, mainly detected on the cell bound fraction, were produced in the earlier steps of degradation, their production being correlated with the maximal lignocellulose degradation rates. Overall, these results demonstrate the potential of RWS to convert unpretreated lignocellulosic substrates into useful chemicals.

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### 1. Introduction

Lignocellulose (LC) is the most abundant terrestrial reservoir of renewable carbon on Earth and thus its use as feedstock for industrial processes is considered to be a viable alternative to fossil resources. In the field of bioconversion, the production of ethanol, methane and H<sub>2</sub> from LC has been widely studied (Chang et al., 2010; Yan et al., 2012). Similarly, carboxylates (volatile fatty acids – VFAs) are also an interesting product of LC bioconversion, since these are intermediate building blocks for the production of polyhydroxyalkanoates, which are potentially useful as bioplastics

(Kleerebezem and van Loosdrecht, 2007). Nevertheless, whatever the target molecule, much progress remains to be achieved to overcome the recalcitrant nature of LC.

Lignocellulosic biomass is a composite material, with cellulose (30–55%), hemicelluloses (18–37%) and (10–30%) lignin as the main constituents. These macromolecules are organized in a complex, heterogeneous matrix that confers to LC hydrophobicity, strength and resistance to biotic and abiotic attack.

In Nature, the recycling of LC is performed by LC-utilizing microbial consortia, present in soils and guts of animals, which deploy complex arsenals of enzymes including, cellulases, hemicellulases and lignin-degrading enzymes to overcome the complexity of LC. Accordingly, microbial consortia constitute an interesting paradigm for LC bioconversion. In particular, the use of microbial consortia in controlled bioreactors is a promising alternative to the deployment of single microorganisms. In contrast to the latter, microbial consortia do not require sterile operating conditions,

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display a more diversified enzymatic arsenal, show high metabolic diversity and versatility and generally remain stable and robust in a broad range of conditions (Kleerebezem and van Loosdrecht, 2007). Moreover, microbial consortia are more advantageous than pure strains artificially mixed in the laboratory because they rely on natural symbiotic interactions and coupled metabolisms that provide the basis of their robustness and can prevent undesirable phenomena in bioreactors, such as feedback inhibition. Overall, assuming that the advantages of microbial consortia can be fully harnessed in artificial environments, these constitute an interesting biocatalytic alternative for the industrial bioconversion of LC into useful products.

In previous studies, microbial consortia operating under both aerobic and anaerobic conditions have been successfully enriched on different types of biomass (Gao et al., 2014; Guo et al., 2010; Jiménez et al., 2014; Reddy et al., 2011; Trifonova et al., 2008). The selection of microbial consortia has been mainly directed towards the production of proteins (Guo et al., 2010), or the biological pretreatment of LC and enhance methane production (Yan et al., 2012). Nevertheless, most of these studies used simplified cellulosic substrates (e.g. carboxymethylcellulose, Avicel, filter paper) or physico-chemically pretreated biomass (Eichorst et al., 2014; Yan et al., 2012), thus circumventing some of the difficulties linked to the complex structure of intact LC. Moreover, only a few studies have dealt with the enrichment of stable microbial consortia under strictly anaerobic conditions, thus the diversity and potential of microorganisms involved in LC decomposition in anaerobic environments remains poorly explored (Gao et al., 2014).

In this study, a natural bovine rumen consortium was enriched under strict anaerobic conditions, selecting for carboxylate production. Selection was performed by SBR cultivation, using raw wheat straw as the sole carbon source. Wheat straw was selected as substrate because it is considered one of the main LC feedstocks with about 34 Mt dry matter (DM) of extractable for being available in Europe for biorefinery purpose (Pajual and O'Donohue, 2013). Regarding the enrichment process, it is described herein in terms of biomass degradation, VFA production and also from the standpoint of the dynamic behavior of the microbial community. The major outcome of this study is the enrichment of a stable microbial community that displays good aptitude for the bioconversion of LC biomass into VFAs. Using the enriched consortium, the kinetic of LC deconstruction has been deeply studied, monitoring the physico-chemical composition of the substrate and characterizing the products. A particular effort was made to correlate enzyme deployment in both the liquid and solid fractions with biomass degradation to identify the rate limiting steps LC bioconversion process under strict anaerobic conditions.

## 2. Methods

### 2.1. Lignocellulosic substrate and inoculum

20 kg of straw from the winter wheat variety Koreli was harvested at an experimental farm (INRA, Boissy-le-Repos, France) in August 2011. After harvest, the straw was milled to 2 mm and stored at room temperature (20–25 °C).

The non-producing Holstein dairy cows used in this study were reared according to the national standards fixed by the legislation on animal care (Certificate of Authorization to Experiment on Living Animals, No. 004495, Ministry of Agricultures, France). In particular, the cows were fed a standard dairy cow ration composed of corn silage (64% DM), hay (6% DM) and concentrate (30% DM). They were fed *ad libitum* once a day in the morning and sampling was done just before feeding. For sampling, the

whole rumen content was collected just before feeding from two individuals fitted with rumen cannula. Rumen contents were taken from various parts of the rumen and manually homogenized. Subsamples (30 g) were immediately frozen in liquid nitrogen before storage at –80 °C.

### 2.2. Anaerobic reactor

Bioconversion was carried out in anaerobic SBR (2L BIOSTAT® A+, Sartorius, Germany) using a mineral medium containing wheat straw as the sole carbon source (20 g L<sup>-1</sup>). The mineral medium (MM) contained, per liter of distilled water: KH<sub>2</sub>PO<sub>4</sub>, 0.45 g; K<sub>2</sub>HPO<sub>4</sub>, 0.45 g; NH<sub>4</sub>Cl, 0.4 g; NaCl, 0.9 g; MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.15 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.09 g. MM was supplemented with 250 µL of V7 vitamin solution (Pfennig and Trüper, 1992), and 1 mL trace elements solution, containing per liter of distilled water: H<sub>3</sub>BO<sub>3</sub>, 300 mg; FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.1 g; CoCl<sub>2</sub>·6H<sub>2</sub>O, 190 mg; MnCl<sub>2</sub>·4H<sub>2</sub>O, 50 mg; ZnCl<sub>2</sub>, 42 mg; NiCl<sub>2</sub>·6H<sub>2</sub>O, 24 mg; NaMoO<sub>4</sub>·2H<sub>2</sub>O, 18 mg; CuCl<sub>2</sub>·2H<sub>2</sub>O, 2 mg; sterilized by filtration (0.2 µm).

Reactors were operated under mesophilic temperature (35 °C) and pH was regulated at 6.15 by the addition of 1 M NaOH. Anaerobic conditions were ensured by nitrogen flush at the beginning of the experiment. To avoid methanogenic conditions bromoethansulfonate (BES), a methanogenesis inhibitor, was added at the beginning of experiment (1 mM). During the experiment, if methane was detected in the biogas, BES was spiked until a maximum concentration of 10 mM.

To progressively enrich a LC-degrading microbial consortium, a first batch reactor was initiated by inoculating (2% w/v) fresh medium with raw cow rumen (pooled from the two cows, thawed overnight at 4 °C). This reactor was operated for 7 days before removing 10% v/v of its content, which was used as inoculum for the subsequent batch reactor. In this manner, SBR cultures were performed until VFA reached a stable concentration. Thereafter, 200 mL samples of the enriched consortium RWS were frozen under liquid nitrogen and stored at –80 °C. After thawing at 4 °C overnight, these frozen samples of the RWS consortium were used as inocula for further experiments. Deeply characterization of LC degradation by the enriched RWS consortium was performed in three biological replicate reactors incubated for fifteen days in the conditions described above. Reactors were carried out at three months intervals; they were inoculated with the same source of inoculum stored at –80 °C.

### 2.3. Chemical analyses

Total solids (TS) were measured using 10 mL samples that had first been centrifuged (7197 × g, 10 min), rinsed twice with distilled water and dried 24 h at 105 °C. The mineral fraction (MF) was estimated by mineralization of the samples at 500 °C for 2 h, and volatile solids (VS) were estimated from the difference between TS and MF. VS degradation was expressed as weight/weight percentages.

Wheat straw composition was determined using the sulfuric acid hydrolysis method described by de Souza et al. (2013) on 40 mg samples. Quantitative detection of monomeric sugars was achieved using high performance liquid chromatography (HPLC) on a Ultimate 3000 Dionex separation system equipped with a BioRad Aminex HPX 87H affinity column and a refractive index detector (Thermo Scientific) as described by Monlau et al. (2012).

Fourier transform infrared spectroscopy (FT-IR) analysis was performed on lyophilized samples using an attenuated total reflection (ATR) Nicolet 6700 FT-IR spectrometer (Thermo Fisher) equipped with a deuterated-triglycine sulfate (DTGS) detector. Spectra were recorded in the range 400–4000 cm<sup>-1</sup> with a 4 cm<sup>-1</sup> resolution. Each sample was recorded 5 times and analyses were performed using average spectra resulting from 32 individual

scans. For FT-IR spectral analysis, the peak ratio 1512:1375  $\text{cm}^{-1}$  was considered to be a lignin:holocellulose ratio (Monlau et al., 2012). The peaks 1430  $\text{cm}^{-1}$  and 898  $\text{cm}^{-1}$  were attributed to the amounts of crystalline and amorphous cellulose respectively (Monlau et al., 2012) and the ratio of these peaks was considered to be the lateral order index (LOI), which is the ratio of crystalline:amorphous cellulose.

VFA production was determined by gas chromatography (GC), using a Varian 3900 chromatograph as described by Cavaillé et al. (2013). The total organic carbon (TOC) content of the liquid fraction was measured using a TOC analyzer (TOC-V<sub>CSN</sub>, Shimadzu Co., Japan). Gas composition was analyzed using a chromatograph HP 5890 equipped with a conductivity detector and a HAYSEP D column.

All the macro-kinetic parameters are expressed as average values obtained in triplicate biological reactors.

#### 2.4. Enzyme activity assays

For enzyme activity measurement, triplicate reactor samples (5 mL) were removed at regular intervals. Samples were centrifuged at 7197 $\times$ g for 10 min at 4 °C thus yielding a supernatant and a solid pellet. It was assumed that the supernatant contains extracellular enzymes, while the pellet is representative of cell-bound enzymes. The latter was suspended in 6 mL of acetate buffer solution (50 mM, pH 6), placed on ice and sonicated four times for 20 s each using a sonication device (Bandelin Sonoplus HD 2070, MS73 probe) operating at 60 W.

For each reactor and each sampling time, end point enzymatic activities were measured in technical duplicates in both the extracellular and cell-bound fractions. Enzymatic activities were expressed as average values obtained on triplicate reactors.

Xylanase and endoglucanase (CMCase) activities were measured using 1% w/v xylan beechwood (Sigma) and 1% w/v carboxymethyl cellulose (CMC) (Sigma) respectively dissolved in buffer solution. Activities were estimated by measuring the release of reducing sugar equivalents after incubation at 35 °C for 1 h (xylanase) or 4 h (CMCase). Reducing sugars were determined using the dinitrosalicylic acid (DNS) method, measuring absorbance of the sample at 570 nm using a UV/VIS spectrophotometer (Multiskan Ascent, Thermo Scientific). One unit of CMCase or xylanase activity (UA, unit of activity) was defined as the amount of enzyme that produces 1  $\mu\text{mol}$  of reducing sugars per minute. Exoglucanase (cellobiohydrolase) and  $\beta$ -glucosidase activities were assessed using fluorescent methylumbelliferyl (MUF) substrates (MUF cellobioside and MUF glucopyranoside, Sigma) and methylumbelliferone as standard. Incubation was carried out for 2 min in the same conditions as above. Measurements were made with a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). One unit of exoglucanase or  $\beta$ -glucosidase activity (UA) was defined as the amount of enzyme that produces 1  $\mu\text{mol}$  of methylumbelliferone per minute.

#### 2.5. Analysis of microbial diversity

For bacterial diversity analysis, samples (1.5 mL) were removed and immediately centrifuged (13,000 $\times$ g, 5 min, 4 °C). Subsequently, the supernatant was removed and the solid pellet was frozen in liquid nitrogen and stored at  $-80$  °C. Simultaneous DNA/RNA extraction was performed using the PowerMicrobiome RNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. Cell lysis was carried out using a FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA;  $2 \times 30$  s at 4  $\text{ms}^{-1}$ ). Subsequently, DNA purification was carried out using the AllPrep DNA/RNA mini kit (Qiagen), following the manufacturer's instructions.

Microbial diversity was analyzed using MiSeq Illumina sequencing performed by the GenoToul Genomics and Transcriptomics facility (Auzéville, France). A 460 bp fragment corresponding to the highly variable V3–V4 region of 16S rRNA gene was amplified from genomic DNA samples using bacterial universal primers F343 and R784 (Table 1). Primer sequences and PCR conditions are detailed in Table 1. The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions. Joined-pair reads (56,000 reads per sample, minimum 30,224) were pre-processed and filtered for chimeras using a Mothur pipeline (Schloss et al., 2009).

Operational taxonomic units (OTU; 97% similarity) were defined using a random subsample of 15,000 sequences per sample using Mothur default parameters, and OTUs that were less than 10 sequences across all samples were filtered. OTUs were taxonomically classified using Mothur and LTPs115 SSU database. Comparative analysis of samples was performed on a random subsample of 10,000 high quality sequences per sample. These produced saturated rarefaction curves (Supplementary data S. 1) and were sufficient to assess relationships among samples (Caporaso et al., 2011).

Community diversity was estimated using the Simpson and Shannon diversity index. The richness, at 97% similarity, was estimated using Chao and the abundance coverage estimator (ACE). The distance between communities present in different samples was estimated using the Bray–Curtis dissimilarity index and weighted Unifrac distances, calculated using the Phyloseq R package v1.6.1 (McMurdie and Holmes, 2013). These distances were used to ordinate samples with Hclust R function.

The sequencing data generated in this study were deposited in the NCBI Sequence Read Archive and are available under the project number PRJNA284586.

### 3. Results and discussion

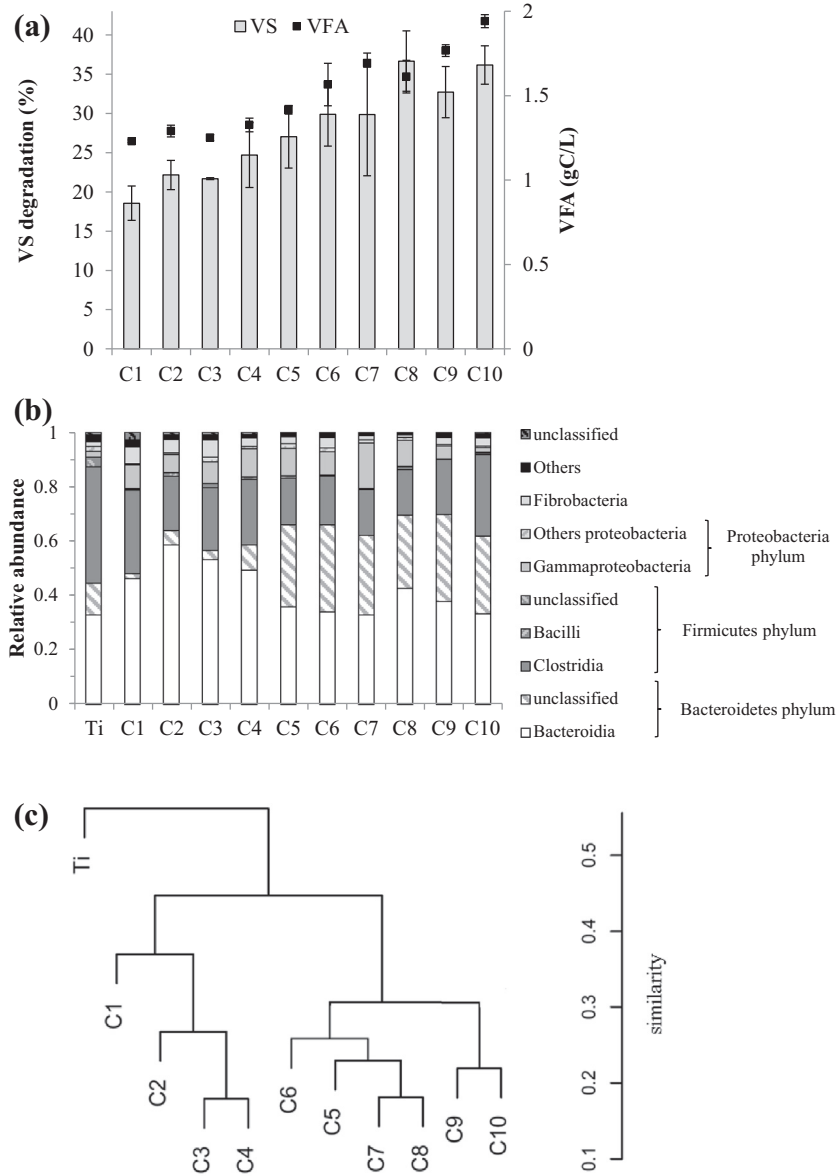
#### 3.1. Enrichment: macro-kinetic and diversity analyses

In this study, a sequential culture enrichment strategy was deployed to obtain a stable rumen-derived microbial community displaying good LC biomass hydrolysis capability. Likewise, the aim was to reduce the complexity of the initial microbial inoculum and to study the resultant LC-degrading, VFA-producing microbial community.

Gratifyingly, after only 10 SBR subculture cycles a stable consortium (RWS) was obtained that is characterized by its ability to release up to 35% VS, accumulating up to  $1.94 \pm 0.04$  gC VFA  $\text{L}^{-1}$  over a 7-day period when operating at 35 °C (Fig. 1a). Amplicon sequencing of the 16S rDNA gene revealed that important changes in the microbial community had accompanied the acquisition of higher VFA production and LC biomass degradation capability (Fig. 1b). The majority of these changes occurred in the initial enrichment phase, specifically by the end of the second SBR cycle. These radical changes can almost certainly be attributed to the transition from the natural environment of the rumen to the artificial conditions of the bioreactor and thus mainly correspond to community acclimation. Compared to the rumen inoculum, the community richness, estimated by the ACE and Chao methods, decreased strongly from 282.9 and 287.4, respectively, to 241.5 and 255.1 after the first SBR cycle and to 203.7 and 205.6 at the second SBR cycle. Similarly, determination of the Simpson diversity and Shannon index revealed a loss in diversity, which was particularly marked during the two firsts SBR cycles and then stable along the subsequent enrichment cycles (Table 2). Comparison of the Simpson diversity across the whole experiment, from the rumen-derived inoculum to the final SBR cycle (C10), revealed that

**Table 1**  
PCR primers and conditions.

PCR1	F343	5'-CTTTCCTACACGACGCTCTTCCGATCTTACGGRAGGCAGCAG-3'
	R784	5'-GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGTATCTAATCCT-3'
	Cycles	94 °C/60 s – 30 × (94 °C/60 s – 65 °C/60 s – 72 °C/60 s) – 72 °C/10 min
PCR2	FP2	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3'
	RP2	5'-CAAGCAGAAGACGGCATACGAGAT- <b>index</b> -GTGACTGGAGTTCAGACGTGT-3'
	Cycles	94 °C/60 s – 12 × (94 °C/60 s – 65 °C/60 s – 72 °C/60 s) – 72 °C/10 min



**Fig. 1.** VS degradation and VFA production (a), relative community composition (b) and community clustering (c) through the enrichment procedure. Error bars in VS degradation are standard deviations of two technical replicates.

**Table 2**  
Variation of richness (ACE and Chao) and diversity index (Simpson and Shannon) through the enrichment process.

SBR cycle	Ti	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Shannon index	4.45	3.71	3.25	3.51	3.47	3.04	3.11	3.16	3.17	3.08	3.38
Simpson index	36.8	21.1	12.6	16.1	14.9	7.7	7.8	8.9	9.8	8.2	11.7
ACE richness	282.9	241.5	203.7	219.7	195.5	191.4	199.0	192.4	195.5	191.7	186.5
Chao richness	287.4	255	205.6	224.7	194.2	191.3	201.3	193	217.8	185.5	187.3

Ti: initial time. Cn: cycle number.

it had dropped from 36.8 to 11.7. Similarly, the Shannon index decreased from 4.45 to 3.38. Hierarchical Unifrac clustering of the initial community presented in the inoculum and those generated at the end of each SBR cycle revealed that two community groups cluster together (Fig. 1c). The first cluster encompasses the communities present during the first four SBR cycles, whereas the second one contains the communities from SBR cycles 5 to 10. Correlation of these results with the higher degradation of LC biomass and VFA production (described below) suggests that the microbial community evolved towards a structure that better expresses the wheat straw degradation and VFA production functions within the conditions defined by the experiment. Significantly, the communities that grouped in the second cluster are rather similar, reflecting the relative stability of the community's structure after five cycles of SBR enrichment. Similar rapid stabilization of enriched microbial communities has already been reported by Jiménez et al. (2014) and Trifonova et al. (2008), with stable microbial communities being obtained after six transfers on wheat straw and five transfers on grass fiber respectively. Overall, this rapid stabilization underlines the stringency of the enrichment conditions and suggests that the use of LC biomass as a sole carbon source is a good way to select a LC-degrading microbial community.

The analysis of the community's composition during the successive SBR cycles showed a dominance of OTUs related to the phylum *Bacteroidetes* (44–70%), followed by *Firmicutes* (17–46%) and *Proteobacteria* (2–18%), which constituted more than 85% of the total community (Fig. 1b). The relative abundance of the *Bacteroidetes* phylum increased during the first five SBR subcultures (from 44% to 66%); and stabilized between the fifth and the last cycle of enrichment (Fig. 1b). Within the *Bacteroidetes* phylum, it is noteworthy that the *Bacteroidia* class diminished during the enrichment process to the benefit of an unclassified class, composed of only one OTU. This latter was related to the unclassified *Bacteroidetes* clone P5\_J07 (EU381760, 100% identity) that has already been identified in the ruminal microflora (Kong et al., 2010). This OTU increased from a relative abundance of 3% in the inoculum to 28% in the SBR cycles 5–10. A second dominant OTU that was enriched during the SBR subculture process (from 1% to 14%) was closely related to *Bacteroides graminisolvens* strain JCM 15093 (*Bacteroidetes* phylum, 100% identity), a known xylanolytic anaerobic bacterium able to produce acetate, propionate and succinate (Nishiyama et al., 2009). *Prevotella* genus was well represented with several OTUs including *Prevotella ruminicola* and *Prevotella brevis*, known ruminal bacteria able to produce succinate that can be further decarboxylated into propionate (Ramsak et al., 2000); OTUs related to unclassified *Prevotella* species were also present. The *Clostridia* class (*Firmicutes* phylum), highly abundant in the rumen inoculum (43%), decreased after the first cycle of SBR enrichment, but then stabilized at a relative abundance of 20% in subsequent SBR cycles. This class included *Butyrivibrio* species, able to transform xylan and produce butyrate (Krause et al., 2003); *Ruminococcus albus*, a ruminal cellulose degrader and acetate producer (Christopherson et al., 2014); various *Clostridium* species, reported as displaying cellulase and/or hemicellulase activities; *Eubacterium cellulosolvens*, a cellulose degrader known to produce mainly butyrate, succinate and valerate (Prins et al., 1972), as well as numerous unclassified *Clostridiales*. Therefore, it appears that members of RWS consortium are particularly well-equipped to degrade LC biomass and produce VFA. These observations are consistent with those of previous studies that have identified *Firmicutes* and *Bacteroidetes* as dominant phyla in LC-degrading ecosystems and are known to participate in the hydrolytic and acidogenic steps in carboxylate production systems (Hollister et al., 2011). Moreover, the enrichment of members of

*Bacteroidetes* has been previously observed in experiments where strict anaerobic conditions have been applied (Gao et al., 2014).

During the enrichment process the hydrolytic capability of the microbial community was estimated by measuring the residual VS content and metabolite production. This revealed that biomass degradation almost doubled between the first SBR cycle and the final enrichment step, increasing from 20% to 35% VS (Fig. 1a). This increase in biomass degradation was particularly marked during the first four SBR cycles (from  $18.5 \pm 2.2\%$  VS to  $24.7 \pm 4.1\%$  VS), after which progress was steadier (between  $24.7 \pm 4.1\%$  VS and  $36.1 \pm 2.4\%$  VS). Similar behavior was observed on VFA accumulation, increasing from  $1.23 \pm 0.01$  to  $1.56 \pm 0.12$  gCL<sup>-1</sup> between the first and sixth cycles (Fig. 1a). Significantly, stabilization of the community's LC biomass-degrading function correlates quite well with the evolution of the community's composition, since from the fifth SBR cycle the community grouped together with the final one (Fig. 1c). This result clearly underlines the benefit of the enrichment procedure and the fact that only ten successive cultures were necessary to procure a functionally- and microbiologically-stable consortium. Moreover, from the fifth to the tenth cycle of enrichment, the LC degradation ability and community composition were stable and reproducible after each cycle of culture, showing the robustness and reproducibility of the selected microbial community. These observations strongly suggest that the LC biomass degradation function, concomitant to VFA production, is linked to the presence of specific species. In this respect it is useful to recall that during the enrichment process the microbial community became progressively enriched in OTUs related to the *Bacteroidetes* phylum and the *Clostridia* class; some of them known for their capacity to produce VFA (see Supplementary data S. 2).

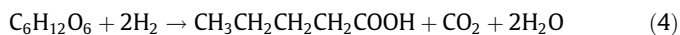
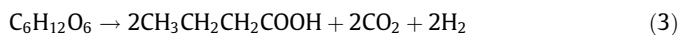
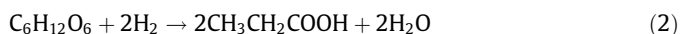
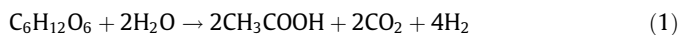
Previous studies focused on the production of stable consortia presenting high LC biomass degradation capability, already demonstrated how progressive enrichment of inocula (e.g. compost, soil, and sediment) can be used to procure microbiologically-stable LC biomass-degrading communities, but did not always evidence increases in the potency of the LC biomass-degrading function (Gao et al., 2014; Guo et al., 2010). However, working with a bovine rumen inoculum (1% v/v), Chang et al. (2010) did obtain results that are consistent with those described here. However, in this latter work a much longer enrichment process (18 cycles) was necessary to obtain a stable community, composed mainly of *Clostridia*. Such community was able to release 27% w/w of hemicellulose and 2% w/w of cellulose after 8 days of cultivation on milled (1 mm) *Napiergrass* (1.5% w/v); whereas in the present study degradation was much higher (35.5% w/w in 7 days) after only 10 cycles of enrichment. Similarly, the aerobic enrichment of a compost-derived communities on switchgrass and corn stover procured very fast increases in LC biomass-degrading function, with degradation increasing from 8% to 34% (switchgrass) and from 19% to 23% (corn stover) in only 4 cycles (Reddy et al., 2011). Remarkably, in this study it was shown that the main changes occurred between the first and second enrichment cycles, since thereafter both the function and the community structure remained quite stable. Taken together with results obtained in the present study, it is clear that enrichment can be an efficient method to select for LC biomass-degrading function with strong selection occurring after only a relatively small number of subculture steps. However, the efficiency of the process is almost certainly determined by the biological potential of the initial inoculum, the culture conditions and the specific composition of the biomass.

To the authors' knowledge, this study represents the first work demonstrating anaerobic enrichment of rumen-derived inoculum on raw wheat straw as an efficient tool to increase substrate degradation capability and generate a stable and robust consortium.

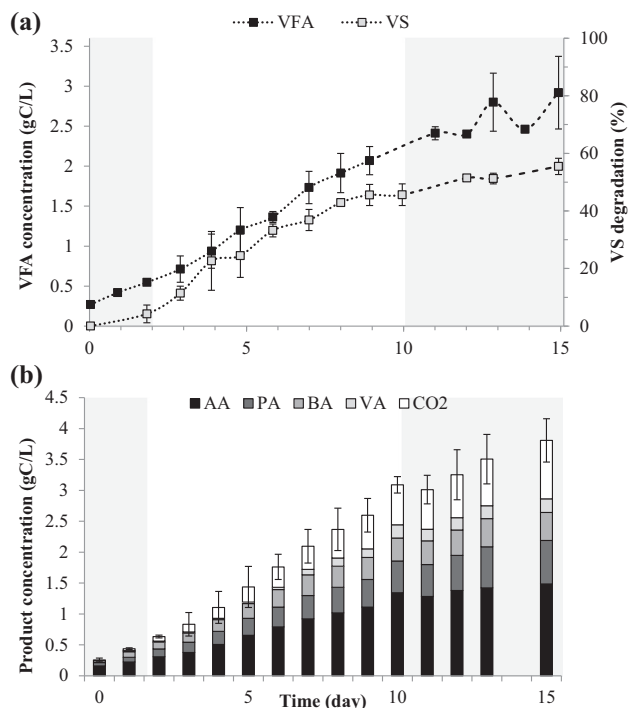
### 3.2. Characterization of wheat straw degradation by consortium RWS

#### 3.2.1. Wheat straw degradation and VFA production by RWS

The LC degradation kinetics of the enriched microbial consortium RWS, obtained after ten SBR subcultures, was studied in detail using the same culture conditions as those used for enrichment. The inoculation rate was 10% v/v and incubation was pursued over a 15-day period. After 7 days, % VS degradation was  $36.8 \pm 3.6$  (Fig. 2a), reaching  $55.5 \pm 2.8$  after 15 days. Simultaneously, VFA production reached  $2.92 \pm 0.45$  gC L<sup>-1</sup>. It is important to note that, although the reactors were not performed simultaneously and that the same source of inoculum was stored for different periods (as detailed in Methods), similar LC degradation and VFA production rates were observed in all replicate reactors. Taken together with the remarkable stability of the consortium observed over SBR cycles 5–10, it is clear that RWS is highly robust. The study of the kinetics of VS release and VFA production revealed a 3-phase dynamic behavior. VS degradation was rather low at the beginning of the experiment and then strongly increased from days 2 to 10. Consistently, before day 2, average VFA productivity was  $0.155 \pm 0.027$  gC-VFA L<sup>-1</sup> day<sup>-1</sup>, increasing thereafter to  $0.224 \pm 0.008$  gC-VFA L<sup>-1</sup> day<sup>-1</sup>. However, from day 10 onwards, both VS degradation and VFA productivity declined ( $0.110 \pm 0.100$  gC-VFA L<sup>-1</sup> day<sup>-1</sup>). TOC measurements (data not shown) performed on the supernatants showed that no other metabolites (other than VFA) were present. The VFA molar ratio (expressed as %) for acetic (AA), propionic (PA), butyric (BA) and valeric (VA) acid was  $65.6 \pm 3.9\%$ ,  $20.5 \pm 3.4\%$ ,  $10.0 \pm 2.9\%$  and  $3.8 \pm 3.1\%$  respectively, with this composition remaining rather constant during the 15-day period (Fig. 2b). This VFA profile is quite typical for acidogenic systems at neutral and slightly acidic pH (Hu and Yu, 2005). Similarly, the CO<sub>2</sub>:VFA ratio also remained constant ( $0.33 \pm 0.04$  gC-CO<sub>2</sub>/gC-VFA), with CO<sub>2</sub> production representing approximately 25% (gC/gC) of all products. Using the theoretical stoichiometry of the bioconversion of glucose to C<sub>2</sub>–C<sub>4</sub> VFA (AA, PA, BA) in ruminant digestive systems (Eqs. (1–3)), and assuming that valeric acid production results from the condensation of acetyl-CoA and propionyl-CoA with oxidation of reduced cofactors (Eq. (4)), the yields of AA, PA, BA and VA can be calculated as 0.66, 0.82, 0.48 and 0.56 g g<sup>-1</sup> respectively (Hu and Yu, 2005; Nozière et al., 2010). Moreover, calculation of the theoretical stoichiometry of VFA production from C<sub>6</sub> degraded sugars based on the wheat straw composition determined in this study reveals a yield of 0.65 g g<sup>-1</sup> (or 0.74 g eq AA g<sup>-1</sup>). However, the experimentally-measured VFA yield was 0.49 g g<sup>-1</sup> (or 0.61 g eq AA g<sup>-1</sup>), which represents 75% of the theoretical yield. This high VFA yield was achievable because of anaerobic conditions, since unlike aerobic conditions these limit carbon loss in the form of CO<sub>2</sub> and microbial biomass (Feng et al., 2011). It is noteworthy that the yield of VFA observed in this experiment is quite consistent with that observed in previous studies (0.6–0.7 g eq AA g<sup>-1</sup> VS) in which rumen inocula were used to treat corn stover (Datta, 1981; Hu and Yu, 2005).



Using the same equations, the theoretical CO<sub>2</sub> yield is 0.35 g g<sup>-1</sup>, while that measured in the present experiments was  $0.26 \pm 0.03$  g g<sup>-1</sup> biomass-derived sugars. The discrepancy between the theoretical and experimentally-measured yields is probably the result of the utilization of a part of the carbon for microbial



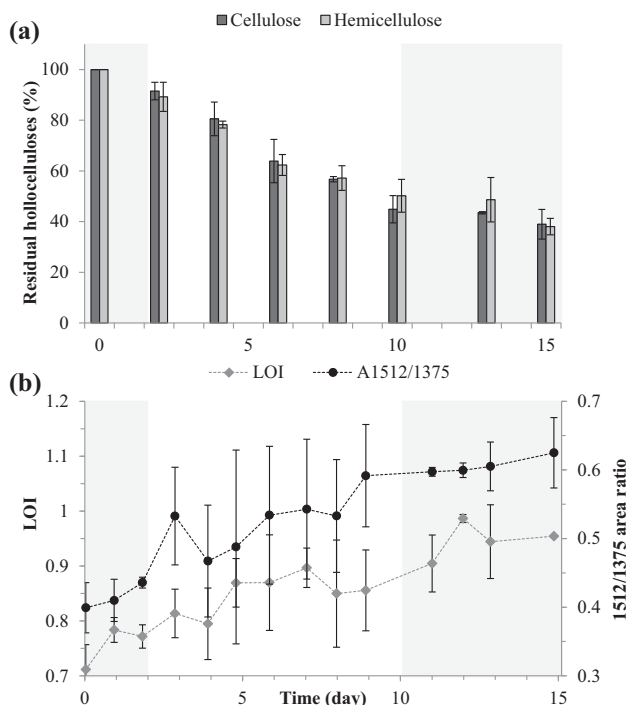
**Fig. 2.** Profile of VS degradation and VFA production (a) and products composition of RWS cultivated on wheat straw (b). Error bars are standard deviations of three biological replicates.

growth and maintenance, but could also result from CO<sub>2</sub> consumption via homoacetogenic reactions. However, although increased quantity of DNA clearly indicated that microbial growth did occur during the experiment (data not shown), it was not possible to estimate quantitative microbial growth using standard methods, because of interference from the solid wheat straw fraction.

#### 3.2.2. Effect of RWS activity on the physicochemical composition of wheat straw

The residual LC biomass was characterized using biochemical (hydrolysis and HPLC) and physical (FT-IR) methods. The monitoring of cellulose and hemicellulose content, performed at several points during the experiment, revealed that these plant polysaccharides were simultaneously degraded, with their hydrolysis following the same kinetic profile and displaying similar residual percentages (Fig. 3a). This behavior was also similar to the VS degradation kinetics, displaying a high degradation between days 2 and 10, and a relative stability beyond day 10. Thus, the RWS consortium did not preferentially degrade one or the other of these fractions. At the end of the 15-day incubation, more than 60% w/w of holocellulose was degraded. In contrast, the determination of lignin content revealed that this macromolecule was not degraded (data not shown).

Monitoring wheat straw degradation by RWS using FT-IR analysis revealed an increase in both the ratio of the 1512:1375 cm<sup>-1</sup> peaks (representing the lignin:holocellulose ratio) and the LOI, representing the ratio of crystalline:amorphous cellulose (Fig. 3b). Although these measurements are associated with quite large standard deviations, the time-dependent profile nevertheless correlates well with the 3-phase dynamic behavior of VS loss (Fig. 2a) and the evolution of the two ratios is opposite to that of the residual holocellulose measurements (Fig. 3a). Therefore, accounting for the fact that lignin was not degraded, the increase of 1512:1375 cm<sup>-1</sup> ratio is consistent with a decrease of the holocellulose. Moreover, the increase of the LOI ratio indicates that the RWS consortium preferentially degraded the amorphous cellulose



**Fig. 3.** Substrate characterization: residual percentages of cellulose and hemicellulose calculated as percentage from their respective initial concentrations (a) and lateral order index (LOI) and 1512:1375  $\text{cm}^{-1}$  area ratio (b). Error bars are standard deviations of three biological replicates.

fraction, leaving a residual cellulose fraction enriched in crystalline cellulose.

So far, only a few studies involving the use of enriched microbial consortia have provided detailed insight into how the LC biomass is degraded during the process. Using a compost-derived consortium (XCD-2) enriched on alkali-pretreated lignocellulosic substrates, Guo et al. (2010) showed that LC biomass degradation mainly involved the destruction of the hemicellulose fraction (up to 89% w/w), with cellulose degradation being only about 12% w/w. This is in sharp contrast to the present study, but the fact that a rumen-derived consortium and untreated raw LC biomass were used may well have orientated the microbial enrichment process, leading to a more efficient functional community. Indeed, alkaline treatment leads to saponification of ester bonds that link hemicellulose to lignin and decorating side-chain groups (e.g. acetyl and feruloyl moieties) to the main xylan chain. The overall effect is to increase the solubility and accessibility of hemicellulose and thus provide a prime target for microbial attack (Hendriks and Zeeman, 2009).

The finding that RWS preferentially degrades the amorphous fraction of cellulose is logical, since it is known that the crystalline fraction is quite resistant to the action of microbial enzymes (Lynd et al., 2002). The absence of lignin degradation is consistent with the fact that anaerobic bacteria are not widely associated with lignin degradation, this function being much more frequently associated with aerobic fungi, which produce oxidative enzymes such as peroxidases and laccases (Ten Have and Teunissen, 2001). Moreover, studies on bacterial consortia incubated on unpretreated LC substrate (switchgrass) under aerobic conditions, where lignin modification by peroxidase and laccase oxidation-reactions likely occurs, showed that the physicochemical composition of lignin remain unchanged in the residual biomass after two weeks of incubation (Eichorst et al., 2014). It is clear that native lignin in raw biomass is resistant to bacterial attack. Nevertheless, despite the fact that lignin was not degraded by RWS, the consortium was able

to efficiently degrade holocellulose, demonstrating that lignin modification is not a prerequisite feature of biomass deconstruction by microbial consortia under the applied experimental conditions. The ability of RWS to concomitantly degrade hemicellulose and cellulose fractions of raw LC substrate to produce VFA is a clear advantage that may have led to the higher LC degradation observed in the present study.

### 3.2.3. Enzymatic activity profiles along wheat straw degradation by RWS

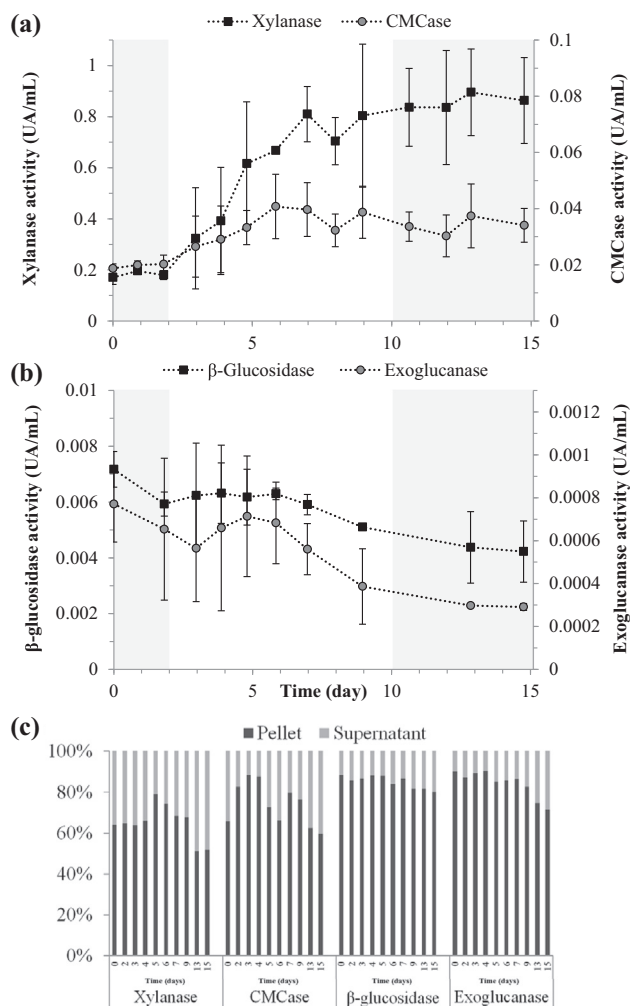
The enzyme activity (xylanase and CMCase) profiles expressed by RWS during the 15-day experiment displayed an initial lag phase that covered the first 2 days (Fig. 4a). Afterwards, from day 2 to 7 xylanase and CMCase activities increased 4- and 2.5-fold respectively, reaching maximum levels of  $0.81 \pm 0.11 \text{ UA mL}^{-1}$  and  $0.04 \pm 0.01 \text{ UA mL}^{-1}$ . Towards the latter part of the experiment CMCase and xylanase activities stabilized. In sharp contrast, exoglucanase and  $\beta$ -glucosidase activities decreased throughout the 15-day period, even if there was a slight increase in exoglucanase activity between days 5 and 6 (Fig. 4b). Taken together, these results imply that an endocellulase-like activity (i.e. CMCase) played a more important role in LC biomass degradation by RWS than an exocellulase activity. This is consistent with the fact that amorphous cellulose was the main target for RWS, since it is known that endocellulases mainly act on amorphous regions, whereas exocellulases preferentially act on crystalline cellulose (Lynd et al., 2002).

The localization of the different enzyme activities that were produced by RWS during the experiment revealed that the different enzyme activities were mainly localized in the insoluble pellet fraction (Fig. 4c). This might imply that different members of the RWS community produced cell-bound enzymes that are perhaps part of cellulosome-like complexes described by Lamed et al. (1983) and observed by Gao et al. (2014). Indeed, several *Clostridia* members known for having a cellulosome complex, such as *R. albus*, were detected in RWS (Christopherson et al., 2014). Nevertheless, the fact that both microbial biomass and wheat straw were present in the pellet fraction, it is impossible to completely affirm that all of the enzymes were actually cell-bound. This is because some free, extracellular enzymes could have remained associated with the solid biomass perhaps forming enzyme-substrate complexes involving high affinity carbohydrate binding domains (Himmel et al., 2010).

The integration of the different parameters (VS release, substrate characterization, VFA production, enzyme activities) that describe the degradation of wheat straw by RWS provides an overview of the whole experiment. An initial phase that covers the first two days was characterized by stable, low-level enzyme activity and VFA production and very little biomass degradation. During this phase VFA production was no doubt sustained by the degradation of readily-accessible biomass that could be easily dealt by only small amounts of enzyme. A second phase characterized by high enzyme activities, VFA production and biomass degradation was the result of the use of amorphous cellulose and hemicellulose, with this phase coming to end when the ability of the enzymes to furnish metabolizable sugars from the recalcitrant and more crystalline structure of the remaining substrate became overwhelmingly rate-limiting. Thereafter, despite maintenance of enzyme levels, substrate hydrolysis is low, a fact reflected by lowered VS degradation and VFA production. Overall, the present results clearly highlight the fact that enzyme hydrolysis and the increase of wheat straw recalcitrance throughout the experiment were the rate-limiting steps regulating of the wheat straw to VFA bioconversion process.

Among the studies of microbial consortia acting on LC biomass, only a few have so far simultaneously studied the dynamics of





**Fig. 4.** Enzymatic activity profiles of RWS trough wheat straw transformation: xylanase and CMCase activities (a);  $\beta$ -glucosidase and cellobiohydrolase activities (b) and pellet:supernatant repartition of the enzymatic activities measured (c). Error bars are standard deviations of three biological replicates.

enzyme production and substrate modification, and even when enzyme activities have been studied, the cell-bound fraction has often been neglected (Hui et al., 2013). In the case of the xylanolytic consortium XDC-2, preferential degradation of the hemicellulose fraction was correlated with strong extracellular xylanase activity, with only a minor fraction of enzyme activity being cell-bound (Guo et al., 2010). However, it should be recalled that this experiment was subject to micro-aerobic conditions, unlike the conditions that prevailed in the present study. In a previous study performed under strict anaerobic conditions, cellulosome-like systems were detected and associated with single *Clostridium*-related isolates arising from the consortium SQD-1.1 (Gao et al., 2014). In this respect it is noteworthy that the consortium RWS was also characterized by numerous OTUs belonging to *Clostridia*, a class that is well-known for its cellulosomes. In view of the performance of RWS, it is obvious that further investigation of the enzyme array produced by RWS could be highly profitable route of investigation. Also, studies of RWS at metatranscriptomic and metaproteomic level are required to ultimately confirm the microorganisms and enzymes acting along wheat straw degradation.

#### 4. Conclusions

This work reveals original insight into the dynamics and function of a microbial community acting on raw LC biomass and

operating in non-sterile, strictly anaerobic conditions. The RWS enriched consortium transformed 55.5% w/w of the lignocellulose into VFA as main products. RWS is mainly constituted by members of *Bacteroidetes* and *Firmicutes* phyla and produced the key cellulolytic and hemicellulolytic enzymes, mainly of cell-bound type, to simultaneously degrade cellulose and hemicellulose fractions of raw biomass. The holistic analysis of substrate structure and composition, VFA and enzymes production underlines the potency of non-sterile microbial enrichment for the development of high-performance biomass-degrading consortia.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2015.07.084>.

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