

# Dynamics of Dark Fermentation Microbial Communities in the Light of Lactate and Butyrate Production

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## Research

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# Abstract

**Background:** This study focuses on the processes occurring during acidogenic step of anaerobic digestion, especially resulting from nutritional interactions between dark fermentation (DF) bacteria and lactic acid bacteria (LAB). Previously, we have confirmed that DF microbial communities fed on molasses are able to convert lactate and acetate to butyrate in batch experiments. The aims of the study were: (i) to recognize biodiversity of DF microbial communities able and unable to convert lactate and acetate to butyrate and (ii) to define the conditions for the transformation in static batch experiments.

**Results:** Sucrose stimulated bacterial growth, especially LAB. In the samples where the microbial communities fermented media containing carbohydrates the two main tendencies were observed: (i) a low pH ( $\text{pH} \leq 4$ ), lactate and ethanol as the main fermentation products, microbial communities dominated with *Lactobacillus*, *Bifidobacterium*, *Leuconostoc* and *Fructobacillus* was characterised by a low biodiversity; (ii) pH in the range 5.0-6.0, butyrate dominated among the fermentation products, the microbial communities composed mainly of *Clostridium* (especially *Clostridium sensu stricto* 12), *Lactobacillus*, *Bifidobacterium* and *Prevotella*. The biodiversity increased with the ability to convert acetate and lactate to butyrate. The microbial communities processing exclusively lactate and acetate showed the highest biodiversity and was dominated by *Clostridium* (especially *Clostridium sensu stricto* 12). LAB were reduced, other genera such as *Terrisporobacter*, *Lachnoclostridium*, *Paraclostridium* or *Sutterella* were found. Butyrate was the main metabolite and pH was 7. WGS analysis of the selected butyrate-producing microbial communities independently on the substrate, revealed *C. tyrobutyricum* as a dominant *Clostridium species*.

**Conclusions:** The batch tests revealed dynamics of metabolic activity and composition of DF microbial communities dependent on fermentation conditions. The results expand our knowledge on lactate to butyrate conversion by DF microbial communities. The relevant factor for conversion of lactate and acetate to butyrate in the presence of carbohydrates is pH in the range 5-6 and the balance between LAB (especially *Lactobacillus*), lactate and acetate producers (*Bifidobacterium*) and butyrate producers (mainly *Clostridium*) as well *Prevotella*. The pH below 4 and ethanol concentration might be the signalling factors responsible for metabolic shift of the dark fermentation microbial communities towards lactate fermentation.

## Background

Anaerobic digestion (AD) is a complex and multistep conversion of biomass to methane and carbon dioxide resulting from metabolic activity and nutritional interactions between many groups of microorganisms. It involves four main stages: hydrolysis of polymeric organic matter to monomers, acidogenesis, acetogenesis and methanogenesis.[1–3] This study focuses on the processes during acidogenesis when the products of hydrolysis are converted to non-gaseous short-chain fatty acids, alcohols, aldehydes and the gases, carbon dioxide and hydrogen[4]. The dominant end-products of the fermentation process determine the type of fermentation. A part of acidogenesis, hydrogen-yielding

fermentations (dark fermentation) are considered to be one of the most attractive alternative biological methods of hydrogen (biohydrogen) production. The main types of hydrogen-yielding fermentation under mesophilic conditions, especially from carbohydrates degradation, are acetic/butyric acid fermentation (*Clostridium*-type fermentation) and mixed-acid fermentation (*Enterobacteriaceae*-type fermentation) [3, 5, 6]. Hydrogen can be also produced during transformation of products other fermentation types. Fermentative biohydrogen production offers additional advantage of potentially using various waste streams from different industries as feedstock such as sugar beet industry. Optimization of biohydrogen yield during acidogenesis is challenging and requires better understanding of the microbial community dynamics in bioreactors and their metabolic substrate conversion along hydrogen-promoting pathways. In a multispecies microbial communities, nutrient utilization is a complex process and frequently involves competition and symbiotic cross-feeding (syntrophy) [7]. The former is when two or more groups of microorganisms compete for substrate that usually leads to a temporary increase in relative abundance of one interacting partner over the other. The latter is when the metabolic products yielded by one microbe constitute energy resource or nutrients supporting growth for another one. Therefore, the analysis of nutrient metabolism in fermentative processes should integrate the dynamics of microbial community composition with metabolic nutrient conversion.

Lactic acid bacteria (LAB) are ubiquitous in the environment, they accompany the plant biomass to anaerobic bioreactors, and constitute a relevant component of acidogenic microbial communities. It is commonly believed that development of LAB in bioreactors inhibit hydrogen production due to substrate competition and/or excretion of bacteriocins that inhibit growth of other bacteria. In the homolactic fermentation two molecules of pyruvate formed during glycolysis are converted to lactate; in heterolactic fermentation, one molecule of pyruvate is converted to lactate and the other to ethanol and carbon dioxide [8]. Substrate competition includes replacement of hydrogen fermentation by lactic acid or ethanol fermentation. Decrease in hydrogen production is observed with simultaneous increase of lactic acid and ethanol concentrations among nongaseous fermentation products [9–13].

On the other hand, cross-feeding of lactate involves the conversion of lactate and acetate to butyrate, hydrogen and carbon dioxide. It is a symbiotic nutritional interaction recognized between lactate- and acetate-producing bacteria and butyrate producers. This phenomena of metabolic interactions between different bacterial groups was described in the gut of many animals including in the human gut. The end product, butyrate, is a crucial molecule necessary in maintaining gut health, homeostasis, and serving as an energy source for the colonic epithelial cells [14–17]. Cross-feeding of lactate is also observed in dark fermentation bioreactors during fermentative conversion of organic substrates to biohydrogen both in mesophilic [18–23] and thermophilic conditions [24, 25].

The studies on fermentation of agave bagasse, tequila vinasse and wastewater from nixtamalization supplied data supporting the thesis that cross-feeding of lactate is significant in the microbial communities of dark fermentation bioreactors. The authors postulated that conversion of lactate and acetate to butyrate is the main pathway of biohydrogen production [19–23]. A specific succession of bacteria was observed in batch experiments. In the first stage, the substrate was processed to acetate

and lactate, which were transformed to butyrate and hydrogen in the second stage. The pH was an important factor ensuring balance and syntrophy between lactate- and butyrate- producers [19–23]. Studies on thermophilic dark fermentation of sugarcane vinasse also showed lactate as the primary substrate for biohydrogen production and relevance of pH in this process [24, 25]. Cross-feeding of lactate was also observed in reduced microbial communities composed of two components: butyrate-producing *Clostridium beijerinckii* and lactate-producer *Yokenella regensburgei* [26]. Furthermore, pure cultures of *Clostridium acetobutylicum* [27], *Butyribacterium methylotrophicum* [28], *Clostridium diolis* [29], *Clostridium butyricum* [18] and *Clostridium tyrobutyricum* [30, 31] anaerobically grown in media with acetate and lactate as exclusive carbon sources produced carbon dioxide, hydrogen and butyrate.

Our previous work demonstrated that dark fermentation microbial communities fed molasses under mesophilic conditions are able to convert lactate and acetate to butyrate in batch experiments [18]. Here we propose a logical continuation and extension of the previously published studies aimed at (i) recognition of biodiversity and dynamics of dark fermentation microbial communities able and unable to convert lactate and acetate to butyrate and (ii) definition of the conditions for the process of transformation. We examined batch cultures of dark fermentation microbial communities grown in media containing molasses or sucrose supplemented with lactate and acetate, or a mixture of lactate and acetate without added carbohydrates. The balance between lactic acid bacteria and the butyrate-producing clostridia and the pH values were shown to be the most relevant for the process of lactate and acetate conversion to butyrate. The putative main lactate producers and lactate and acetate utilizers were identified.

Since fermentation processes are ubiquitous in anaerobic environments, butyrate and lactate producers are found in anaerobic digesters and among the gut microbiota, the results obtained in this study should interest both the researchers dealing with studies on (i) AD and production of gaseous biofuels as well as (ii) the butyrate production by the gut bacteria.

## Methods

### ***Experimental set-up for the examination of lactate to butyrate transformation in batch experiments***

Tests on the transformation of lactate and acetate to butyrate were conducted in static batch experiments, analogous to those described previously [18], in 250-ml Erlenmeyer flasks for 18 days in a Vinyl Anaerobic Chamber (Coy Laboratory Products, Inc.) without shaking at 30°C. Five-milliliter samples of microbial community taken from the dark fermentation hydrogen producing packed bed reactor described previously was used as inoculum. The liquid growth medium (200 mL) was M9 after 10-fold dilution, without glucose, supplemented with 1% sucrose (Chempur Poland) or molasses at the concentration corresponding to 1% sucrose; sodium lactate (VWR Chemicals) 7.41 g/L; sodium acetate (Chempur Poland) 3.5 g/L; and 0.2% yeast extract (BD Biosciences USA). The following combination of nutrients were tested: molasses (Experiment M); molasses plus sodium lactate (Experiment ML); molasses plus sodium lactate and sodium acetate (Experiment MLA); sodium lactate and sodium acetate

(Experiment LA); sucrose plus sodium lactate and sodium acetate (Experiment SLA). All the variants were tested in three independent repetitions designated as A, B, and C. Molasses is a by-product of sugar processing from sugar beets. It contains 50% of sucrose. Other components are water, glucose, fructose, amino acids, mineral salts, betaine, B vitamins, glutamic acid, inositol, nitrogen compounds. In this study, molasses came from the Dobrzelin Sugar Factory, branch of the Polish Sugar Company "Polski Cukier". Starting pH of all media was 7.0. No additional means of pH control were used. Before inoculation, 4-5 sterile slag pieces were placed in each flask to be covered by bacterial biofilm. Bacterial growth in batch cultures was determined by OD<sub>600 nm</sub> measurements. After every 3 days of incubation, the Erlenmeyer flasks were shaken, the digestive liquids were removed and the respective fresh media for further growth were added. After every passage (on days 3, 6, 9, 12, 15 and 18) the digestive liquids were centrifuged (7,000 × g for 10 min), the supernatants analyzed and the pellets used for total microbial DNA isolation as described below. Composition of the selective media, lactate and acetate concentration were selected based on the data from previous studies [18, 29, 32, 33].

### ***Analytical methods***

The pH of the media and the digestive liquids was measured using a standard pH meter (ELMETRON model CP-502, Poland). Samples were centrifuged (7,000 × g for 10 min, 10°C) to remove microbial cells and debris, and concentrations of carbohydrates (sucrose, glucose and fructose), short-chain fatty acids, ethanol were determined. The carbohydrates and ethanol were analyzed using high performance liquid chromatography (HPLC) with refractometric detection (Waters HPLC system: Waters 2695 - Separations Module, Waters 2414 - Refractive Index Detector, a thermostat for column, and 300×6.5 mm Sugar Pak I column with guard column). The determination of carbohydrates was carried at 90°C, and ethanol at 70°C. The sample (10 µL) were injected onto the column and eluted for 20 min with an isocratic flow of 0.1 mM calcium disodium salt EDTA (0.5 mL/min). Short-chain fatty acids were analyzed by HPLC with photometric detection (Waters HPLC system as above, Waters 2996 – Photodiode Array Detector, and 300×7.8 mm Aminex HPX-87 H column with guard column at 30°C). The samples were eluted for 45 min with an isocratic flow (0.6 mL/min) of 4 mM sulphuric acid.

For the statistical analysis of bacterial growth (OD<sub>600nm</sub>), pH of the digestive liquids and the non-gaseous fermentation products, the STATISTICA (version 10.0) computer software (StatSoft, Inc.) was used. All variables were examined for normality and homogeneity of variance. Tukey's HSD (honestly significant difference) test was applied after ANOVA analysis to compare statistical significance among the variables in experiments. Statistical significance was considered at  $p \leq 0.05$ .

### ***Microbial DNA extraction***

The total DNA was isolated from the pellets obtained after centrifugation (see above) of 2 ml-samples of the digestive liquids taken after 3, 6, 9, 12, 15 and 18 days of the experiment. From each culture two samples (duplicates) were taken. DNA was extracted and purified using a DNeasy PowerSoil Pro Kit (Qiagen, Cat No. 47014) according to the manufacturer's protocol. Cell lysis was done using Vortex-Genie

2 equipped with a Vortex Adapter for 1.5–2 ml tubes (cat. no. 13000-V1-24). DNA was stored at –20 °C. The final samples of DNA extracted from the two replicates were pooled.

### ***16S rRNA amplicon sequencing and data analysis***

The hypervariable V4 region of the 16S rRNA gene was amplified from each sample using barcoded reverse primers (806R) and common forward primer (515F). Both reverse and forward primers were extended with the sequencing primer pads, linkers, and Illumina adapters [34], and with MyFi™ Mix (Bioline Meridian, Cat No. BIO-25050). The PCR was performed on LightCycler 96 (Roche) in the final volume of 40 µL. Amplicons were quantified using Quant-It PicoGreen dsDNA Assay kit (ThermoFisher Scientific, Cat No. P7589), according to the manufacturer's protocol. Equal amount of amplified DNA (240 ng) from each sample were pooled and cleaned using UltraClean PCR Clean-Up Kit (MoBio, Cat No. 12500). Pooled amplicons were diluted and denatured with 0.1N NaOH. The library was sequenced at the Microbiome Core at the Steele Children's Research Center, University of Arizona, using MiSeq platform (Illumina) and custom primers [34]. Due to the limited sequence diversity among 16S rRNA amplicons, 5% of the PhiX Sequencing Control V3 (Illumina, Cat No. FC-110-3001) made from phiX174, was used to spike the library to increase diversity. The raw sequencing data were demultiplexed using the idemp script (<https://github.com/yhwu/idemp>). Filtering, dereplication, chimera identification, and merging of paired-end reads were performed with dada2 [35]. The amplicon sequence variants (ASVs) taxonomy was assigned using the Ribosomal Database Project (RDP) classifier [36] against SILVA database release 132 [37].

Taxonomic richness and evenness (Shannon and Simpson indices) were calculated and statistical significance within in each experiment was calculated using Kruskal-Wallis rank sum test followed by Dunn's multiple comparison test with Bonferroni correction (dunn.test R package).

Differences in microbial communities were evaluated using non-metric multidimensional scaling (NMDS) ordination analysis on Bray-Curtis distances followed by permutational multivariate analysis of variance (PERMANOVA) to analyze the contribution of different metadata variables to microbial communities composition dissimilarities. Also, to investigate and visualize the association between metadata variables and their effect on the species distribution pattern, redundancy analysis was used in *vegan* R package [38]. The obtained results were visualized with a *ggplot2* (ver 3.3.2) package [39] and with *heatplus* (ver. 3.11) R package [40].

The raw sequences generated in this study have been deposited in NCBI databases with the accession number PRJNA645198.

### ***Whole Metagenome Sequencing (WGS) and data analyses***

The libraries for WGS were constructed for the selected samples from the static batch experiments using QIASeq FX DNA Library Kit (QIAGEN) according to the manufacturer's protocol. Briefly, 50 ng of DNA from each sample (or pooled samples) was randomly fragmented with FX Enzyme Mix followed by the adapter

ligation step. Both i5 and i7 adapters contain unique 8 nucleotide barcodes. After removing free adapters from the reaction with AMPure XP magnetic beads, all individual libraries were amplified by PCR followed by the size selection with 2-step purification (the negative selection followed by the positive selection step) with AMPure XP magnetic beads. The quality and quantity of all libraries was determined with Agilent 4150 TapeStation DNA analyzer. The libraries were normalized and pooled, and the sequencing was performed on the Illumina NextSeq 500/550 platform using Illumina 400M HighOutput 300 cycles sequencing chemistry.

Adapter sequences were removed using Cutadapt v. 2.1 [41]. Reads shorter than 50 bp and low-quality bases were removed using Trimmomatic v. 0.38 [42]. The high-quality reads were *de novo* assembled using Megahit v. 1.1.4 [43]. After discarding assembled contigs shorter than 500 bp, protein-coding genes were predicted using Prodigal v. 2.6 [44]. Paired-end reads were mapped to the genes using BWA v. 0.7.16 [45].

The raw sequences generated in this study have been deposited in NCBI databases with the accession number PRJNA640235.

## Results

### *General characteristics of the microbial communities in static batch experiments*

To examine the capabilities of dark fermentation microbial communities to convert lactate and acetate to butyrate, five independent static batch experiments in three replicates were performed. Each one was inoculated with the same community derived from hydrogen-producing packed-bed reactors described previously [18, 46]. The experiments provided different carbon sources as shown in Table 1: molasses (Experiment M), molasses supplemented with lactate (Experiment ML), molasses supplemented with lactate and acetate (Experiment MLA), sucrose supplemented with lactate and acetate (Experiment SLA) and lactate and acetate (Experiment LA). The batch experiments were maintained for 18 days and passaged every 3 days.

Bacterial growths measured by  $OD_{600nm}$  of the digestive liquids after every passage are presented in Table 1. The results clearly show that sucrose stimulates bacterial growth. The densities were higher ( $OD_{600nm}$  after every three days  $\approx 2-3$ ) when bacteria grew on the media containing sucrose (either from molasses or used a pure additive; Experiments M, ML, MLA and SLA) compared to Experiment LA when lactate and acetate were provided as an exclusive carbon source ( $OD_{600nm}$  after every three days  $\approx 1$ ),  $0.001 < p < 0.005$  between LA group and any other group (Tukey's HSD test; Table 1, Additional file 1). Interestingly, in comparison to molasses and lactate alone (Experiment ML), addition of acetate in Experiment MLA increased bacterial growth on days 6, 9, and 12 ( $p < 0.05$ , Tukey's HSD test). Differences in bacterial growth were also found on day 9 between Experiments M and ML as well as between Experiments M and SLA ( $p < 0.05$ , Tukey's HSD test).

Biodiversity and microbial changes in all the experiments were analysed by sequencing of the 16S V4 amplicon profiling. Total of 119 samples were sequenced in one MiSeq run, and 7,431 ASVs were detected. After chimera identification and removal, 93.15% ASVs remained. 29 samples from an unrelated project were filtered out, and the remaining 90 samples were further analyzed. For detailed taxonomic assignments see Additional file 2. All negative controls for the V4 amplification by PCR (collection day 0 for each experiment) did not show any amplification and these controls were removed from analysis during the quality control steps due to insufficient number of reads. Alpha diversity analysis revealed that the microbial communities are moderately rich in taxa, and that communities grown in media supplemented with molasses only or molasses and lactate (Experiments M and ML) had the lowest diversity as compared to the inoculum alone or to other groups (Figure 1, Additional file 3). Taxonomic composition of each experimental microbial community (Figures 2 and 3) was analysed in the reference to its metabolites, i.e. non-gaseous fermentation products and pH of the digestive liquids (Figures 3 and 4, Table 2).

### ***Analysis of metabolites and microbial community composition after the initial 3 days of fermentation***

After the initial 3 days of fermentation, we found no statistically significant differences in the concentration of the analyzed non-gaseous fermentation products among the batch experiments where growth media contained sucrose (either as a component of molasses or pure sucrose; Experiments M, ML, MLA and SLA). The concentration of butyrate was low (<1 g/L; Table 2, Figure 4, Additional file 4). The main fermentation products were ethanol (5.5 – 6.7 g/L) and lactate. After the initial 3 days of fermentation with molasses only (Experiment M), lactate concentration was the lowest at 2.6 g/L. In the case of Experiments ML, MLA and SLA, the concentration of lactate in the digestive liquids (10.4 – 11.2 g/L) was the sum of that in the media and as a product of sucrose fermentation. Fermentation of sucrose (Experiments M, ML, MLA, and SLA) resulted in similar pH of the digestive liquids (4.0, 4.4, 4.6 and 4.6, respectively). Detailed data for each metabolite and time point are presented in Table 2 and Additional file 4.

After the initial 3 days of fermentation, communities grown on the media containing molasses (Experiments M, ML, and MLA) were composed primarily of *Lactobacillus* (38.3% ± 15.6, 34.7% ± 4.4, 38.3% ± 5.1, respectively), *Fructobacillus* (26.1% ± 17.6, 27.8% ± 12.0, 21.5% ± 5.8, respectively), *Bifidobacterium* (12.2% ± 3.0, 20.1% ± 7.8, 24.7% ± 1.4, respectively), *Leuconostoc* (20.7% ± 3.3, 11.7% ± 3.2, 10.6% ± 3.9, respectively), with a smaller proportion of *Clostridium sensu stricto 1* (0.6% ± 0.5, 3.3% ± 2.2, 6.7% ± 1.2, respectively). Compared to Experiments M, ML and MLA, microbial community grown with pure sucrose (Experiment SLA) showed lower contribution of *Bifidobacterium* (4.0% ± 3.4;  $p = 0.036$ ,  $p = 0.05$ , and  $p = 0.003$ , respectively), *Lactobacillus* (6.5% ± 5.8;  $p = 0.057$ ,  $p = 0.003$ ,  $p = 0.03$ , respectively), and *Leuconostoc* (2.6% ± 1.3;  $p = 0.005$ ,  $p = 0.025$ ,  $p = 0.062$ , respectively), and the community became dominated by *Clostridium sensu stricto 1* (51.1% ± 7.5;  $p = 0.007$ ,  $p = 0.005$ ,  $p = 0.008$ , respectively). However, at this stage of fermentation, this genus did not appear to correlate with butyrate production. The relative abundance of *Fructobacillus* (28.9% ± 3.2) in SLA community was

comparable to the communities in M, ML, and MLA ( $p = 0.81$ ,  $p = 0.89$ ,  $p = 0.14$ , respectively) (Figures 2 and 3, Additional file 2).

The dynamics of the fermentation process were followed over four additional passages until 18 days post-inoculation and showed considerable differences between experimental groups. These are discussed in detail in the following sections.

### ***Dynamics of fermentation processes with molasses only (Experiment M)***

When molasses were fermented without exogenous SFCAs, pH of the digestive liquids after six days dropped below 4 and remained in the 3.6 – 3.9 range (Table 2, Figure 4, Additional file 4). During the whole experiment, the main non-gaseous fermentation products were ethanol and lactate. Lactate was the only metabolite that significantly changed over time (ANOVA,  $p = 0.000015$ ), with a gradual increase from day 3 to day 18 (2.6, 3.9, 5.2, 6.3, 5.2 and 5.6 g/L, respectively). The results of the detailed statistical comparisons are presented in Table 2 and Additional file 1. Between 6-18 days, the concentrations of ethanol remained relatively stable at 5.6, 3.8, 3.1, 4.1 and 4.9 g/L. The concentrations of butyrate and acetate were low ( $\leq 1$  g/L) throughout the experiment (Table 2, Figure 4).

The overall biodiversity was low compared to the inoculum and showed changes over time, although without a clear trend (Figure 1). Microbial community was dominated by *Lactobacillus* (54.2%  $\pm$  7.8, 69.8%  $\pm$  5.3, 71.7%  $\pm$  11.6, 61.3%  $\pm$  11.9 and 72.6%  $\pm$  12.0, respectively after 6, 9, 12, 15 and 18 days), with *Bifidobacterium* as the second most abundant genus (11.3%  $\pm$  3.7, 15.7%  $\pm$  6.0, 19.5%  $\pm$  9.6, 10.1%  $\pm$  2.2 and 19.7%  $\pm$  7.9, respectively after 6, 9, 12, 15 and 18 days) (Figure 2). The *Leuconostoc* genus was a significant component of the microbial community on day 3 and 6 (20.7%  $\pm$  3.3 and 25.8%  $\pm$  9.7, respectively), but its relative abundance started to decline on day 9 and onward to eventually constitute a minor genus (10.1%  $\pm$  8.1 on day 6, 2.1%  $\pm$  1.5 on day 6, and < 1% on days 15 and 18). The relative abundance of the genera *Clostridia* sensu stricto was generally low (5-6%), among them *Clostridium* sensu stricto 12 dominated (4.8%  $\pm$  3.8%, 5.3%  $\pm$  2.8%, 3.8%  $\pm$  1.3%, 4.6%  $\pm$  0.5% and 3.5%  $\pm$  1.3%, respectively after 6, 9, 12, 15 and 18 days).

### ***Dynamics of fermentation processes with molasses supplemented with lactate (ML)***

After the addition of lactate, pH of the digestive liquids after the first passage (days 6-18) remained in the range 4.4–4.6. The concentrations of ethanol decreased steadily (ANOVA,  $p = 0.004$ ) from 6.7 g/L on day 3 to 3.4, 2.7 and 2.8 g/L on days 9, 15 and 18, respectively. Concentration of lactate varied over time (ANOVA,  $p = 0.006$ ). It peaked on day 6 at 12 g/L and decreased to 6.3 and 6.8 g/L on days 15 and 18, respectively. The concentration of butyrate gradually increased (ANOVA,  $p = 0.006$ ) from 0.03 g/L on day 3 to 3.2 g/L on days 15 and 18. The concentration of acetate remained low and steady ( $\leq 1$  g/L) throughout the experiment (Table 2, Figure 2, Additional files 1 and 4).

Supplementation of molasses with lactate as a source of carbon overall did not change the richness of the bacterial community, which remained similar to that in Experiment M, with molasses as a sole source

of carbon. After inoculation, we observed a transient drop of richness until day 12 followed by the restored number ASVs to the original level on day 15 followed by a not statistically significant decline on day 18 (Figure 1). Similar to the Experiment M, the microbial communities were dominated with *Lactobacillus* (49.9% ± 5.1, 67.7% ± 15.9, 74.5% ± 9.7, 55.1% ± 8.4 and 56.7% ± 7.1, after 6, 9, 12, 15 and 18 days, respectively) and *Bifidobacterium* (11.4% ± 0.8, 13.9% ± 5.6, 15.7% ± 6.0, 22.8% ± 2.3 and 24.7% ± 6.2, after 6, 9, 12, 15 and 18 days, respectively). The relative contribution of the *Leuconostoc* genus decreased over time (12.5% ± 6.3%, 7.4% ± 7.0% and <1%, on day 6, 9 days and on and beyond day 12;  $p = 0.016$ , Kruskal-Wallis test). The *Fructobacillus* genus followed a similar pattern (13.6% ± 12.8%, 4.7% ± 5.7% and <1%, on day 6, 9 days and on and beyond day 12;  $p = 0.015$ , Kruskal-Wallis test). Relative abundance of *Clostridium sensu stricto 12* genus showed a slight decline from day 3 to 6, followed by recovery and modest expansion (11.9% ± 13.8%, 6.1% ± 2.7%, 8.5% ± 4.1%, 15.2% ± 5.4% and 13.4% ± 3.1%, on day 6, 9, 12, 15 and 18, respectively;  $p = 0.06$ , Kruskal-Wallis test). Corresponding with increased butyrate synthesis on days 15 and 18, the microbial community showed relative expansion of *Lactobacillus*, *Clostridium sensu stricto 12* and *Bifidobacterium* genera (Figure 2). Among minor genera, contribution of *Caproiciproducens* increased to 1.3% ± 0.9% and 3.2% ± 1.2%, after 15 and 18 days, respectively ( $p = 0.011$ , Kruskal-Wallis test).

### ***Dynamics of fermentation processes with molasses or sucrose supplemented with lactate (MLA and SLA)***

In the MLA and SLA Experiments, the media contained molasses as a source or sucrose or pure sucrose, both supplemented with lactate and acetate. These two experiments are described together due to similar tendencies observed, which reflects the dominant effect of lactate/acetate supplementation over the source of sucrose (Figures 3 and 4, Table 2, Additional files 1 and 4). In the MLA Experiment, the pH of the digestive liquids changed from 4.6 on day 3 to 5.4 and 5.5 on days 6 and 9 ( $p = 0.0002$ ,  $p = 0.0002$ , respectively; Tukey's HSD test). In the SLA Experiment, the pH changed from 4.6 on day 3 to 6.2 and 5.8 on days 6 and 9 ( $p = 0.002$ ,  $p = 0.02$ , respectively; Tukey's HSD test). In the MLA Experiment, the pH increase was associated with increased butyrate concentration, from 0.2 g/L on day 3 to 7.1 and 6.5 g/L on days 6 and 9 ( $p = 0.002$ ,  $p = 0.004$ , respectively; Tukey's HSD test). In the SLA Experiment, the concentration of butyrate increased from 0.9 g/L on day 3 to 7.0 g/L and 8.0 g/L on days 6 and 9 ( $p = 0.0004$ ,  $p = 0.0003$ , respectively; Tukey's HSD test). During longer fermentation (days 12, 15 and 18), butyrate remained an abundant fermentation product and pH was maintained at ca. 5. On day 6 and onward, ethanol concentration decreased and it became a minor metabolite compared to samples collected on day 3 in either MLA or SLA Experiment ( $p = 0.0002$ , Tukey's HSD test).

The biodiversity measured by richness index in both MLA and SLA Experiments increased over time (Figure 1). The microbial communities (Figures 2 and 3) associated with the highest butyrate production in Experiments MLA and SLA, were dominated by *Bifidobacterium* (16-30% for both Experiments), *Clostridium sensu stricto 12* (20-30% for both Experiments), *Lactobacillus* (40-50% for Experiment MLA and 20-30% for Experiment SLA), *Prevotella* (up to 6% in Experiment MLA and above 15% in Experiment SLA on day 12 and 18), and *Caproiciproducens*. In the MLA and SLA Experiments, we detected a higher

contribution of *Prevotella* in comparison to the other culture conditions. In the MLA Experiment, the contribution of the *Leuconostoc* and *Fructobacillus* genera decreased over time, from  $6.6\% \pm 2.2$  and  $5.3\% \pm 0.4$  after 6 days, respectively, to below 1% from day 9 onward. In the SLA Experiment, *Leuconostoc* was a minor genus whereas *Fructobacillus* also decreased in time ( $23.2\% \pm 2.5$ ,  $18.0\% \pm 4.7$ ,  $5.1\% \pm 1.8$ ,  $1.2\% \pm 0.3$  and  $2.1\% \pm 2.9$ ) on days 6, 9, 12, 15 and 18, respectively ( $p=0.007$  and  $p=0.009$  for *Leuconostoc* and *Fructobacillus*, respectively; Kruskal-Wallis test). In both experiments, we observed an increasing contribution of *Caproiciproducens* genus (MLA:  $<1\%$ ,  $2.1\% \pm 1.8\%$ ,  $5.4\% \pm 0.6\%$ ,  $4.6\% \pm 1.8\%$ , on days 9, 12, 15, 18, respectively; SLA:  $<1\%$ ,  $3.4\% \pm 2.4\%$ ,  $7.9\% \pm 5.2\%$ ,  $2.3\% \pm 2.0\%$ ,  $5.0\% \pm 1.6\%$ , on days 6, 9, 12, 15 and 18, respectively;  $p=0.02$  for either genus, Kruskal-Wallis test).

Clustering analysis of each experiment revealed that in some individual experimental replicates differed from the other counterparts and were more similar to those from other experiments. For example, the replicate B after the 3<sup>rd</sup> passage (9 days) from the Experiment MLA grouped with the samples collected after the 3<sup>rd</sup> passage (9 days) from the Experiment ML. Other examples are replicates from the Experiment SLA after the 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> passages, respectively, 12, 15 and 18 days (Figure 3). We had no logical explanation and thus no reason to exclude these replicates from analysis.

### ***Dynamics of fermentation of lactate and acetate as the main carbon sources (LA)***

A distinct scenario occurred for Experiment LA when the source of carbon was limited to lactate and acetate (Table 2 and Additional files 1 and 4). The pH of the digestive liquids was maintained at approximately 7, and the lowest reached value of 6.6 was observed after the first three days. Since the second passage (after 6 days), lactate was efficiently utilized (90-100%) and the dominant fermentation product, butyrate, was maintained at similar level during the whole experiment at around 4 g/L (Table 2). Acetate was detected as the second component (1 – 2 g/L) of the digestive liquids, whereas propionate (0.2 – 0.4 g/L) and ethanol (0.05 – 0.2 g/L) were detected as minor products.

The initial richness of the LA community was higher compared to other experiments condition and did not significantly change over time (Figure 1). Taxonomic analysis to some extent reflected this result (Figure 2). The genera *Clostridium* sensu stricto constituted on average 45-50%. *Clostridium* sensu stricto 12 increased over time ( $0.5\% \pm 0.3\%$ ,  $5.7\% \pm 4.9\%$ ,  $28.4\% \pm 12.6\%$ ,  $28.2\% \pm 2.3\%$ ,  $29.8\% \pm 8.1\%$  and  $34.2\% \pm 1.4\%$ , respectively after 3, 6, 9, 12, 15 and 18 days, Kruskal-Wallis test  $p = 0.034$ ) whereas *Clostridium* sensu stricto 1 decreased ( $41.9\% \pm 0.6\%$ ,  $21.9\% \pm 0.8\%$ ,  $14.0\% \pm 3.0\%$ ,  $8.9\% \pm 2.3\%$ ,  $7.9\% \pm 3.1\%$  and  $6.0\% \pm 1.4\%$ , respectively after 3, 6, 9, 12, 15 and 18 days, Kruskal-Wallis test  $p = 0.012$ ) in time. *Clostridium* sensu stricto 1 was a dominant genus after the first 3 days when conversion of lactate to butyrate was on the lowest level (Figures 3 and 4, Table 2). Relative abundance of *Clostridium* sensu stricto 13 was also high, but maintained at a similar level over time ( $6.8\% \pm 1.5\%$ ,  $13.9\% \pm 3.5\%$ ,  $7.2\% \pm 1.5\%$ ,  $7.9\% \pm 1.6\%$ ,  $6.1\% \pm 2.1\%$  and  $6.3\% \pm 1.1\%$ , respectively after 3, 6, 9, 12, 15 and 18 days). Compared to the other experiments, *Lactobacillus* ( $9.2\% \pm 4.7\%$ ,  $3.1\% \pm 2.4\%$ ,  $2.1\% \pm 2.0\%$ , respectively, after 3, 6, 9 days and  $<1\%$  since the 12<sup>th</sup> day), *Fructobacillus* ( $1.0\% \pm 0.2\%$  after 3 days and  $<1\%$  since the 6<sup>th</sup> day), *Bifidobacterium* ( $3.5\% \pm 0.4\%$  after 3 days and  $<1\%$  since the 6<sup>th</sup> day) and *Leuconostoc* ( $1.6\% \pm 0.4\%$

after 3 days and and <1% since the 6<sup>th</sup> day) were significantly reduced. Contribution of the following genera increased: *Terrisporobacter* (5-10%), *Sutterella* (5-10%), *Paraclostridium* (up to 10%), *Lachnoclostridium* (up to 10%), *Escherichia* (up to 5%) and *Dialister* (4-6%).

### **Summary of the static batch experiments and redundancy analysis**

Detailed statistical comparison of the pH and metabolite (ethanol, butyrate, propionate, and lactate) formation between Experiments M, ML, MLA, LA and SLA are depicted in Additional file 1. For simplicity, in this section we focus on the results from day 6 and 9. The pH values were significantly different among all experiments ( $0.001 > p > 0.0002$ , Tukey's HSD test; Additional file 1) with the lowest pH recorded in Experiment M (molasses only; Table 2). Butyrate synthesis in Experiments M and ML was significantly lower than in Experiments MLA, SLA and LA ( $0.02 > p > 0.0004$ , Tukey's HSD test; Additional file 1, Table 2). A reverse tendency was observed for ethanol production which was higher in Experiments M and ML compared to MLA, SLA and LA ( $0.05 > p > 0.0005$ , Tukey's HSD test). Detected lactate concentrations in the digestive liquids from Experiments ML, MLA, SLA and LA, where lactate was added to the media, clearly show more efficient utilization of lactate in Experiments MLA, SLA and LA as compared to Experiment ML ( $0.04 > p > 0.0004$ , Tukey's HSD test; Additional files 1 and 4, Table 2).

To integrate the targeted metabolomic data with the analyses of sample biodiversity, we performed redundancy analysis (RDA), a direct gradient analysis technique which summarises linear relationships between components of response variables that are "redundant" with (i.e. "explained" by) a set of explanatory variables. The results of RDA analysis and the correlation between the fermentation products, pH of the digestive liquids and the dominant bacterial genera in respective experiment are presented in Figure 5. The following positive correlations were observed: *Clostridium sensu\_stricto\_12* with butyrate and pH (Experiments ML, MLA, SLA); *Fructobacillus* and *Leuconostoc* with lactate and ethanol (Experiments ML); *Fructobacillus*, *Leuconostoc* and *Clostridium sensu\_stricto\_1* with ethanol (Experiment MLA); *Clostridium sensu\_stricto\_1* with lactate and ethanol (Experiment SLA) or with lactate and acetate (Experiment LA); *Bifidobacterium* with acetate (Experiments M and ML) or acetate and lactate (Experiment MLA); *Fructobacillus* with ethanol and pH; *Lactobacillus* and lactate (Experiment M); collection day with butyrate and pH (Experiment LA). It is noteworthy that *Lactobacillus* correlated with lactate only in Experiment M.

As a synthesis of our observations, two main scenarios for microbial communities fermenting sucrose-containing media (Experiments M, ML, MLA, SLA) can be proposed: (i) Low pH of the digestive liquids ( $\leq 4$ ) is associated with lactate and ethanol as the main non-gaseous fermentation products. Under such condition, the production of butyrate is very low. Microbial communities are dominated with LAB (especially *Lactobacillus*) and lactate- and acetate-producer *Bifidobacterium*. Contribution of *Clostridium* is very low. This scenario is best illustrated by Experiment M and to some extent by Experiment ML (till the 12<sup>th</sup> day). (ii) In the second scenario, illustrated by Experiments MLA and SLA, butyrate dominates among the non-gaseous fermentation products and the pH of the fermentation process is in the range 5-

6. Lactate and ethanol are the minor products. The *Clostridium* genus constitutes at least 25% of the microbial community.

Samples collected late (on days 15 and 18) in Experiment ML indicate an intermediate state between both scenarios. In these conditions, lactate is still the dominant fermentation product, concentration of ethanol decreases, while butyrate production increases and pH of the digestive liquids reaches 4.5-4.6. This corresponds with a higher contribution of *Clostridium* in the microbial communities. In all scenarios, propionate remains a minor product during the experiments, a decreasing contribution of *Fructobacillus* is observed over time, and *Lactobacillus* remains to be an abundant genus. Butyrate formation is related to pH increase, higher contribution of *Clostridia* (e.g. *Clostridium sensu stricto* 12) in the microbial community and increase of biodiversity that is the especially prominent in Experiment LA.

### ***Carbon balance in the selected static batch experiments***

We have previously described an approximate balance of carbon during the fermentation of lactate and acetate to butyrate by *Clostridium butyricum* and proposed a model of lactate/acetate conversion to butyrate [18]. To illustrate metabolic transformations in the batch experiments performed in this study, the approximate millimolar balance of carbon for the selected data from Experiments LA and SLA (as shown in Figure 4) was calculated and presented in Table 3. The selection criterion was butyrate concentration in the digestive liquids, low on day 3 and high on day 6 in both experiments. The carbon balances are based on the concentrations of sucrose (Experiment SLA only), acetate, lactate, propionate, butyrate and ethanol in the media and the digestive liquids. The calculations take into account (i) concentrations of the remaining non-fermented sucrose in the digestive liquids (~3 millimoles of carbon) which were subtracted from the initial amount of sucrose in the media; (ii) the concentrations of the yeast extract-derived butyrate (18 millimoles of carbon) and propionate (3 millimoles of carbon) in the media that were subtracted from the butyrate and propionate detected in the fermentation products.

### ***Metagenomic analysis of the selected microbial communities***

For better understanding of the dynamics of the microbial communities and explanation of the observed differences in their metabolic activity, we selected samples from the static batch experiments designated as MLA-3-AC, MLA-9-AC, LA-3-BC, LA-18-AB (summarized in Table 4) and subjected them to shotgun metagenomics analysis. MLA-3-AC is derived from the MLA Experiment (pooled replicates A and C) after the first passage (day 3), when the main non-gaseous fermentation products were lactate and ethanol, concentration of butyrate was very low (Figure 4, Additional file 4). Sample MLA-9AC is also derived from the MLA Experiment (pooled replicates A and C) but after the third passage (day 9), when lactate was efficiently utilized and the main fermentation product was butyrate (Figure 4, Additional file 4). Sample LA-3-BC comes from the LA Experiment (pooled replicates B and C) after first passage (day 3) when lactate was partially metabolized (Figure 4, Additional file 4). Sample LA-18-AB comes from the LA Experiment (pooled replicates A and B) after the sixth passage (day 18) when lactate was efficiently utilized and the main fermentation product was butyrate (Figure 4, Additional file 4). A total of 34,545,964 to 78,144,622 reads per sample was obtained. Taxonomic composition of the microbial communities on

the level of phylum, class, family and genus are presented in Additional file 5. For detailed taxonomic assignments see Additional file 6. Metagenomic analysis confirmed the results obtained by 16S rRNA sequencing. The goal of this analysis was to identify species potentially responsible for sucrose, acetate and lactate utilization. However, due to limitations of the approach we chose, we limited the data interpretation to two aspects. Since the MLA community produces initially (on day 3) large quantity of lactate, the first goal was to identify the putative main lactate producers from sucrose (molasses) fermentation. The species more highly represented in MLA3 vs LA3 communities (>2-fold higher in MLA3, > 0.02% abundance in MLA3) were selected and 72 species that may be involved in fermentation of sucrose to lactate were identified (Figure 6, Additional file 7). They were the *Lactobacillus*, *Leuconostoc*, *Bifidobacterium*, *Weissella*, *Enterococcus*, *Gardnerella*, *Pediococcus*, *Oenococcus*, *Peptoaerobacter* species. The top species were *Lactobacillus uvarum* (7-fold higher in MLA3,  $\square$ =11.3%), *L. brevis* (7-fold higher in MLA3,  $\square$ =1.7%), *Leuconostoc fallax* (22-fold higher in MLA3,  $\square$ =8.9%), *L. mesenteroides* (5-fold higher in MLA3,  $\square$ =5.8%), *Bifidobacterium crudilactis* (8-fold higher in MLA3,  $\square$ =10.5%) and *B. subtilis* (10-fold higher in MLA3,  $\square$ =6.4%).

The second aspect of the analysis was a comparison of LA\_3 vs. LA\_18 and MLA\_3 vs. MLA\_9 microbial communities to find lactate and acetate utilizers and butyrate producers (Figure 6, Additional file 7). The species more highly represented in MLA9 vs MLA3 communities (>2-fold higher in MLA9, > 0.02% abundance in MLA9) were selected and 52 species were identified (Figure 6, Additional file 7), They were mostly the *Clostridium*, *Prevotella*, as well as *Lactobacillus*, *Dakarella* and *Bacillus* species. The top species was *C. tyrobutyricum* (64.5-fold higher in MLA9,  $\square$ =11.3%). Interestingly, in comparison to MLA-3-AC in the sample MLA-9-AC a decreased contribution of *Leuconostoc* (below 1%) was observed whereas *Lactobacillus* (*L. uvarum* 12.4 %, *L. brevis* 4.4 %) and *Bifidobacterium* (*B. crudilactis* 3.3 % and *B. subtilis* 12.9%) were still top species.

The species more highly represented in LA18 vs LA3 communities (>2-fold higher in LA18, > 0.02% abundance in LA18) were selected and 48 species were identified (Figure 6, Additional file 7), They were mostly the *Clostridium*, *Terrisporobacter* as well as *Romboutsia*, *Shigella*, *Aerocolum*, *Gottschalkia*, *Klebsiella* and *Lactococcus* species. The top species were *Clostridium tyrobutyricum* (106-fold higher in LA18,  $\square$ =28.6%) and *Terrisporobacter glycolicus* (7.2-fold higher in LA18,  $\square$ =4.1%) suggesting that these species contributed to butyrate synthesis. Interestingly, In the LA-3-BC sample (low butyrate formation) the top species were *Clostridium sulfidigenes* (7%) and *Clostridium beijerinckii* (4%). The former maintained at the level 4.8% whereas the latter dropped to 0.3% in the LA-18-AB sample.

Finally, the common species between MLA9 and LA18 communities were found. All of them were the *Clostridium* species (the most abundant *C. tyrobutyricum* and minor *C. coskatii*, *C. kluyveri*, *C. ljungdahlii*, *C. ragsdalei*, *C. arbusti*, *C. estertheticum*, *Clostridium* sp. DMHC 10, *C. pasteurianum*, *C. carboxidivorans* and *C. acetobutylicum*) (Figure 6, Additional file 7). There are putatively the most involved in butyrate formation independent on the growth medium (MLA or LA).

## Discussion

This study describes dynamics of the metabolic activity and the structure of microbial communities sampled from hydrogen-producing dark fermentation bioreactor and tested in static batch experiments. The results contribute to better understanding of the dynamics and plasticity of dark fermentation microbial communities that are key factors responsible for the stability and instability of hydrogen production process. This study continues and expands our previous research [18] on lactate and acetate conversion to butyrate by the bacteria of dark fermentation. Here we provide new data on (i) the conditions that favour and unfavour transformation of lactate and acetate to butyrate by dark fermentation microbial communities and (ii) the key players of the microbial communities determining ability to the conversion process. The study involved five independent static batch experiments performed under anaerobic conditions differing with the media subjected to fermentation. The media contained molasses as a source of sucrose or pure sucrose with addition of lactate and acetate, or exclusively a mixture of lactate and acetate. Molasses has been used in our studies for hydrogen and methane production in a two-stage process [46–48]. Sucrose is an attractive substrate for glycolytic fermentations. Lactate and acetate are substrates for butyrate, hydrogen and carbon dioxide formation. Previously we have shown that dark fermentation microbial communities are unable to grow on lactate only-containing media [18].

The experiments in this study focused on the analysis of non-gaseous fermentation products and the examination of the microbial communities by 16S rDNA profiling complemented by metagenomics analysis of the selected samples from Experiments MLA and LA (Additional files 5 and 6). Changes in the microbial communities, selection of the specific groups of bacteria during the batch experiments point to the significance of substrates and metabolic activity of bacteria for the community structure and plasticity. It is noteworthy that the differences were also observed between replicates of the same experiments. This variability revealed in the batch experiments may explain unstable operation of dark fermentation bioreactors observed in many studies[9]. It may also illustrate metabolic microniches that can be formed in the bioreactors.

In the Experiments M, ML, MLA and SLA when the media contained a source of sucrose, stimulation of bacterial growth, especially of lactic acid bacteria, was observed compared to the Experiment LA where the only substrates were lactate and acetate.

The first three days of all batch experiments regardless of the substrate were characterised by a high concentration of lactate and a low concentration of butyrate. Microbial communities processing molasses-containing media (Experiments M, ML, MLA) consisted mainly of lactate-, ethanol- and acetate-producers. The metagenomic analysis of the MLA microbial community on day 3 revealed that the top species were *Lactobacillus uvarum*, *L. brevis*, *Bifidobacterium subtilis*, *B. crudilactis*, *Leuconostoc fallax* and *L.mesenteroides*. This period (day 3) resembles the first stage of hydrogen production from tequila vinasse and nixtamalization wastewater in batch co-fermentation experiments done by Garcia-Depraect et al. [20]. In their studies the majority of fermentable carbohydrates were metabolized to lactate by *Lactobacillus*, *Sporolactobacillus*, *Streptococcus* and acetate by *Acetobacter* that dominated in the microbial communities. Lactate and acetate were further used for production of butyrate and hydrogen

by hydrogen producing bacteria [19, 20]. In our Experiments SLA and LA (the latter with no carbohydrates in the medium), after the first three days the most abundant genus was *Clostridium sensu stricto* 1, unlikely to convert lactate to butyrate.

Our observations concerning microbial communities selected in the Experiment M confirm commonly recognized fact about replacement of clostridial type fermentation by lactic acid fermentation in hydrogen-producing bioreactors and support the thesis about negative role of lactic acid bacteria in the dark fermentation microbial communities [9, 49]. Analysis of the non-gaseous fermentation products in digestive liquids from the Experiment M revealed that besides lactic acid, the dominant product was also ethanol, both products of heterolactic fermentation. Butyric acids was a minor product and a drop in pH to < 4.0 was observed. Noteworthy was a high concentration of ethanol that together with a low pH (< 4.0) might be a relevant factor responsible for metabolic shift of microbial community towards lactic acid fermentation and maintenance of its stable metabolic activity. Previously, we have shown butyrate formation by the dark fermentation microbial communities grown on the medium containing only molasses [18]. However, compared to this study, the previous media contained a higher concentration of  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ , that increased their buffering capacity and helped to maintain pH at 5.

Presence of external lactate or lactate and acetate in the medium was a stimulating factor for the growth of butyrate producers (Experiments ML, MLA and SLA). Lactate and acetate are also fermentation products. Our results clearly show that the balance between lactic acid bacteria and butyrate producers is key for the conversion of lactate and acetate to butyrate. Dark fermentation microbial communities that the most effectively converted lactate and acetate to butyrate (Experiments SLA and MLA) were composed of *Clostridium sensu stricto* 12, *Lactobacillus*, *Fructobacillus*, *Bifidobacterium* and *Prevotella*. Summary of the batch experiments where lactate and acetate were transformed to butyrate clearly shows a significant consumption of lactate (or its low concentration) among the non-gaseous products despite the presence of lactic acid bacteria in the microbial communities. It is in accordance with the previous studies where the effluents from hydrogen producing bioreactors and the microbial communities were examined [46, 47, 50]. It can also explain a lack or a weak correlation between *Lactobacillus* and lactate in Experiments ML, MLA and SLA. Interestingly, Esquivel-Elizondo and co-workers [51] considered *Lactobacillaceae* as a putative butyrate-producers.

It is noteworthy that *Clostridium sensu stricto* 12 was an abundant taxon in all butyrate producing microbial communities. Metagenomic analysis of the selected samples from Experiments MLA (day 9) and LA (day 18) revealed a significant contribution of *Clostridium tyrobutyricum* in the microbial communities. *C. tyrobutyricum* is a recognized hydrogen- and butyrate-producing bacterium via conversion of lactate and acetate [30, 31].

Previously, we have postulated that pH may be a critical factor responsible for a balance of dark fermentation microbial communities. In our experiments, pH was established and maintained intrinsically in the flasks with no additional pH adjustments. Lactate and acetate were transformed to butyrate at pH  $\approx 7$  when the substrate did not contain carbohydrates and or 5–6 when the substrate contained

molasses or pure sucrose. Other studies reported pH in the range of 5.5–6.5 as optimal for hydrogen production and butyrate formation from lactate and acetate [13, 21, 24, 29–31, 50].

Interestingly, Garcia-Depraect et al. [21] reported that increase of pH above 6.5 caused domination of *Blautia* and *Propionicum* genera in the microbial community processing tequila vinase and nixtamalization wastewater, and a metabolic shift leading to propionate production. However, in our study, low concentrations of propionate were detected within the non-gaseous fermentation products in all the samples. Propionate-type fermentation characteristic of e.g. *Clostridium propionicum* [32] was thus seemed irrelevant.

The results of our research are generally consistent with those of other groups. On one hand they confirm that lactic acid bacteria compete with dark fermentation bacteria and inhibit their growth [9–13]. However, the role of ethanol as the promoting factor is novel. Ethanol and a low pH are thought to provide unfavourable conditions for butyrate producers and conversion of lactate and acetate to butyrate. On the other hand our results strongly support thesis that conversion of lactate and acetate to butyrate is a common process in dark fermentation bioreactors. Furthermore, it is believed that this metabolic pathway is the main route of hydrogen production during acidic fermentation of carbohydrates-rich substrates [19–25]. Further investigation should concentrate on search for quorum-sensing mechanisms regulating hydrogen producing microbial communities with regard to pH and ethanol contribution. The regulation seems to be more complex than maintaining lactate and acetate transformation and likely includes mutual metabolic stimulation of bacteria.

Although during the batch test in this study the hydrogen production was not measured, we calculated the balances of carbon for selected time points. The balances of carbon performed for Experiment SLA differ dependently on the contribution of butyrate formation. The X value (meaning bacterial biomass and other fermentation products including fermentation gases) was higher when transformation of lactate and acetate to butyrate was observed. Since the bacterial biomass was similar in every experiment the differences included fermentation gases and eventually other non-analysed products. Balance of carbon for the transformation of lactate and acetate to butyrate in the Experiment LA was similar to that for pure culture of *Clostridium butyricum* [18] with regard to milimoles of butyrate. Formation of butyrate was not limited by propionate synthesis. A lower concentration of ethanol within the non-gaseous fermentation products in comparison to the previous study [18] may have resulted from a lower concentration of acetate in the medium and/or activity of other bacteria within the community. It should be noted that acetate is a substrate and an intermediate on the pathway of lactate to butyrate transformation [15, 27, 28].

It is noteworthy that the biodiversity of microbial communities measured by the taxonomic richness and evenness increased with the capability to transform lactate into butyrate. This capacity was the lowest when the microbial communities were dominated by lactic acid bacteria and the most abundant fermentation products were lactate and ethanol. Additionally, the highest biodiversity was observed in microbial communities grown in the presence of lactate and acetate (Experiment LA) and was related to

the presence of taxa not found in other Experiments. These include *Terrisporobacter*, *Lachnoclostridium*, *Paraclostridium* or *Sutterella*. *Paraclostridium* strain CR4 was isolated from sugar cane bagasse involved in hydrogen and butyric acid production [52]. Other genera were found in and were isolated from human intestine microbiome [53–56].

Since lactic acid and butyric acids fermentations are ubiquitous and some analogies can be found between anaerobic digesters and the mammalian gut, our results are also relevant in the context of human microbiomes analyses. Interestingly, studies on the microbiomes from the patients with autism spectrum disorder revealed an increased number of *Terrisporobacter*, *Lachnoclostridium* [55]. *Lachnoclostridium* was identified as a novel bacterial marker for the non-invasive diagnosis of colorectal adenoma [56]. It was also found that most of the gut butyrate-producing bacteria were significantly decreased in patients with non-small-cell lung cancer compared to healthy adults [57]. Also diarrhoea is often associated with the accumulation of lactate in the hindgut in the case of intestinal disorders such as short-bowel syndrome, inflammatory bowel disease, ulcerative colitis, dyspepsia, antibiotic-associated diarrhoea [58].

## Conclusions

The batch tests revealed dynamics of metabolic activity and relevant differences in composition of dark fermentation microbial communities dependent on fermentation conditions. These results expand our knowledge on lactate to butyrate conversion by dark fermentation microbial communities and are relevant for understanding the processes inside hydrogen-producing bioreactors. The microbial communities unable to butyrate formation are dominated by lactic acid bacteria. The main fermentation products are lactate and ethanol, the drop of pH to  $< 4.0$  is observed. Further investigations should concentrate on the role of pH and ethanol in the changes of microbial communities structure and metabolic shifts towards lactate fermentation.

With the ability to convert lactate and acetate to butyrate, biodiversity of microbial communities increase. The process of conversion proceeds at  $\text{pH} \approx 5-6$  when the media contain carbohydrates. The most relevant for lactate to butyrate conversion is the balance between lactic acid bacteria (mainly *Lactobacillus*) and butyrate producers (especially *Clostridium sensu stricto* 12, also *Prevotella*). Mutual metabolic stimulation of bacteria is likely and should not be discounted. In the absence of carbohydrates, the process of conversion lactate and acetate to butyrate proceeds at  $\text{pH} \sim 7$  and the most abundant bacteria belong to the clostridial species. The increased contribution of *Terrisporobacter*, *Lachnoclostridium*, *Paraclostridium* or *Sutterella* is also observed. Metagenomic analysis revealed *C. tyrobutyricum* as the most abundant *Clostridium* species in the butyrate producing microbial communities fermenting media with and without carbohydrates.

## List Of Abbreviations

AD – anaerobic digestion

DF – dark fermentation

Experiment M – experiment with molasses

Experiment ML – experiment with molasses plus sodium lactate

Experiment MLA – experiment with molasses plus sodium lactate and sodium acetate

Experiment SLA – experiment with sucrose plus sodium lactate and sodium acetate

Experiment LA – experiment with sodium lactate and sodium acetate

HPLC – high performance liquid chromatography

LAB – lactic acid bacteria

NMDS – non-metric multidimensional scaling analysis

OD – optical density

SD – standard deviation

SFCA – short-chain fatty acids

Tukey's HSD – Tukey's honestly significant difference test

WGS – whole genome sequencing

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable

### **Availability of data and material**

All data generated or analysed during this study are included in this published article [and its supplementary information files]. The raw DNA sequences generated in this study have been deposited in NCBI databases with the accession numbers PRJNA645198 (16SrRNA) and PRJNA640235 (WGS).

### **Competing interests**

The authors declare that they have no competing interests.

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## Authors' contributions

ASi, AD, AC planned the work, conceived and designed the experiments. AD, AC performed the batch experiments and isolated microbial DNA. DL performed DNA sequencing (16SrRNA and WGS), analysed the sequence data and contributed to writing the manuscript. ASa performed analyses of short chain fatty acids and ethanol. ASi, AD, DL, PK analyzed the results. AD contributed to writing the manuscript. AB, YC, FY analyzed the metagenomics data. PK revised the manuscript and contributed to writing the manuscript. ASi wrote the paper. MKB revised the paper. All authors read and approved the final manuscript.

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## Tables

Table 1

Bacterial growth measured by OD<sub>600nm</sub> of the digestive liquids after every passage. The data show a mean from three replicates with ± SD. Tukey's HSD test was applied after ANOVA variance analysis to compare statistical significance, for detailed comparisons see Additional file 1.

Experiment	M	ML	MLA	LA	SLA
Day	Molasses	molasses + lactate	molasses + lactate + acetate	lactate + acetate	sucrose + lactate + acetate
3	2.8 ± 0.7	3.1 ± 0.3	3.4 ± 0.1	0.8 ± 0.05 <sup>b</sup>	3.2 ± 0.1
6	3.2 ± 0.4	2.4 ± 0.5 <sup>a</sup>	3.6 ± 0.1 <sup>a</sup>	0.9 ± 0.1 <sup>b</sup>	3.2 ± 0.03
9	3.3 ± 0.4 <sup>c</sup>	2.3 ± 0.2 <sup>a, c, d</sup>	3.4 ± 0.3 <sup>a</sup>	1.4 ± 0.2 <sup>b</sup>	3.3 ± 0.1 <sup>d</sup>
12	2.1 ± 0.1	2.3 ± 0.5 <sup>a</sup>	3.4 ± 0.05 <sup>a</sup>	1.2 ± 0.3 <sup>b</sup>	2.7 ± 0.4
15	2.8 ± 0.7	3.3 ± 0.01	3.4 ± 0.1	1.1 ± 0.1 <sup>b</sup>	3.3 ± 0.1
18	3.3 ± 0.2	3.2 ± 0.1	3.2 ± 0.2	1.3 ± 0.2 <sup>b</sup>	3.1 ± 0.1
<sup>a</sup> $p < 0.05$ (Experiment ML vs Experiment MLA; Tukey's HSD test)					
<sup>b</sup> $0.001 < p < 0.005$ (Experiment LA vs any other group; Tukey's HSD test)					
<sup>c</sup> $p < 0.05$ (Experiment M vs Experiment ML; Tukey's HSD test)					
<sup>d</sup> $p < 0.05$ (Experiment ML vs Experiment SLA; Tukey's HSD test).					

Table 2

Characteristics of the digestive liquids. The data show a mean from three replicates with  $\pm$  SD, for each the analyses were performed in duplicate. Tukey's HSD test was applied after ANOVA variance analysis to compare statistical significance, for detailed comparisons see Additional file 4.

Experiment	Collection day	pH	Acetic acid	Lactic acid	Propionic acid	Butyric acid	Ethanol
			g/L				
Experiment M	3	4.0 $\pm$ 0.0	0.8 $\pm$ 0,2	2.6 $\pm$ 0.4 <sup>a</sup>	0.4 $\pm$ 0.1	0.3 $\pm$ 0.5	5.5 $\pm$ 1.9
	6	3.9 $\pm$ 0.1 #	0.8 $\pm$ 0,1	3.9 $\pm$ 0.4 <sup>b</sup>	0.5 $\pm$ 0.1	1.0 $\pm$ 0.1 \$	5.6 $\pm$ 1.5 @
	9	3.7 $\pm$ 0.1 ##	1.0 $\pm$ 0.2	5.2 $\pm$ 0.3 <sup>a</sup>	0.4 $\pm$ 0.1	0.9 $\pm$ 0.6 \$\$	3.8 $\pm$ 2.1 @@
	12	3.6 $\pm$ 0,03	1.2 $\pm$ 0.3	6.3 $\pm$ 0.7 <sup>a,b</sup>	0.5 $\pm$ 0.1	0.5 $\pm$ 0.3	3.1 $\pm$ 0.6
	15	3.7 $\pm$ 0.03	1.0 $\pm$ 0.2	5.2 $\pm$ 0.7 <sup>a</sup>	0.3 $\pm$ 0.03	0.4 $\pm$ 0.2	4.1 $\pm$ 0.4
	18	3.6 $\pm$ 0.03	1.0 $\pm$ 0.1	5.6 $\pm$ 0.2 <sup>a,b</sup>	0.5 $\pm$ 0.1	0.4 $\pm$ 0.2	4.9 $\pm$ 0.5
Experiment ML	3	4.4 $\pm$ 0.01	1.0 $\pm$ 0.2	10.4 $\pm$ 1.5	0.5 $\pm$ 0.1	0.03 $\pm$ 0.01 <sup>d</sup>	6.7 $\pm$ 1.1 <sup>e</sup>
	6	4.4 $\pm$ 0.2 #	0.7 $\pm$ 0.4	12.0 $\pm$ 2.1 <sup>c,&amp;</sup>	0.6 $\pm$ 0.1	1.1 $\pm$ 1.7 \$	5.2 $\pm$ 0.7 @
	9	4.5 $\pm$ 0.1 ##	0.8 $\pm$ 0.1	10.0 $\pm$ 0.5 <sup>&amp;&amp;</sup>	0.5 $\pm$ 0.1	1.1 $\pm$ 1.0 \$\$	3.4 $\pm$ 1.1 <sup>e</sup> , @@
	12	4.5 $\pm$ 0.03	0.7 $\pm$ 0.2	8.3 $\pm$ 2.5	0.7 $\pm$ 0.1	1.9 $\pm$ 0.6	4.2 $\pm$ 1.5
	15	4.6 $\pm$ 0.04	0.7 $\pm$ 0.2	6.3 $\pm$ 0.6 <sup>c</sup>	0.6 $\pm$ 0.1	3.2 $\pm$ 0.8 <sup>d</sup>	2.7 $\pm$ 0.6 <sup>e</sup>
	18	4.6 $\pm$ 0.04	1.1 $\pm$ 0.3	6.8 $\pm$ 1.2 <sup>c</sup>	0.7 $\pm$ 0.1	3.2 $\pm$ 0.7 <sup>d</sup>	2.8 $\pm$ 1.1 <sup>e</sup>
Experiment MLA	3	4.6 $\pm$ 0.01 <sup>f</sup>	3.8 $\pm$ 0.4	11.2 $\pm$ 0.9	0.5 $\pm$ 0.1	0.2 $\pm$ 0.03 <sup>g</sup>	5.9 $\pm$ 0.6 <sup>h</sup>
	6	5.4 $\pm$ 0.1 <sup>f,#</sup>	1.4 $\pm$ 0.1	2.5 $\pm$ 0.8 <sup>&amp;</sup>	0.6 $\pm$ 0.01	7.1 $\pm$ 0.5 <sup>g,\$</sup>	0.6 $\pm$ 0.04 <sup>h,@</sup>
	9	5.5 $\pm$ 0.01 <sup>f,##</sup>	2.7 $\pm$ 1.4	4.4 $\pm$ 6.5 <sup>&amp;&amp;</sup>	0.6 $\pm$ 0.02	6.5 $\pm$ 3.5 <sup>g,\$\$</sup>	0.9 $\pm$ 0.7 <sup>h</sup> , @@

	12	4.7 ± 0.2	3.1 ± 0.9	5.2 ± 1.8	0.6 ± 0.1	4.5 ± 0.9	1.8 ± 0.8 <sup>h</sup>
	15	5.1 ± 0.1	1.4 ± 0.6	2.9 ± 2.3	0.4 ± 0.1	5.2 ± 1.6	0.6 ± 0.1 <sup>h</sup>
	18	4.6 ± 0.03	6.3 ± 1.8	8.8 ± 2.2	0.5 ± 0.1	2.2 ± 0.4	1.0 ± 0.1 <sup>h</sup>
Experiment LA	3	6.6 ± 0.1	2.1 ± 0.5	3.4 ± 0.2 <sup>i</sup>	0.1 ± 0.04	1.5 ± 0.7 <sup>j</sup>	0.1 ± 0.03
	6	7.0 ± 0.1 <sup>#</sup>	1.7 ± 0.5	0.2 ± 0.2 <sup>l, &amp;</sup>	0.2 ± 0.1	3.5 ± 0.8 <sup>j, \$</sup>	0.1 ± 0.1 <sup>@</sup>
	9	7.2 ± 0.1 <sup>##</sup>	1.7 ± 0.1	0.02 ± 0.01 <sup>l, &amp;&amp;</sup>	0.2 ± 0.1	4.2 ± 0.2 <sup>j, \$\$</sup>	0.04 ± 0.04 <sup>@@</sup>
	12	7.2 ± 0.05	1.1 ± 0.4	0.5 ± 0.3 <sup>i</sup>	0.3 ± 0.1	3.8 ± 0.7 <sup>j</sup>	0.2 ± 0.03
	15	7.3 ± 0.03	2.1 ± 0.2	0.2 ± 0.2 <sup>i</sup>	0.2 ± 0.0	4.0 ± 0.1 <sup>j</sup>	0.1 ± 0.04
	18	7.3 ± 0.1	1.7 ± 1.0	0.2 ± 0.15 <sup>i</sup>	0.4 ± 0.04	3.8 ± 0.5 <sup>j</sup>	0.1 ± 0.03
Experiment SLA	3	4.6 ± 0.03 <sup>k</sup>	3.4 ± 0.1	10.4 ± 0.7	0.02 ± 0.01	0.9 ± 0.2 <sup>l</sup>	5.8 ± 1.0 <sup>m</sup>
	6	6.2 ± 0.04 <sup>k, #</sup>	0.3 ± 0.1	0.7 ± 1.1 <sup>&amp;</sup>	0.4 ± 0.2	7.0 ± 1.3 <sup>l, \$</sup>	1.1 ± 0.2 <sup>m, @</sup>
	9	5.8 ± 0.03 <sup>k, ##</sup>	2.2 ± 0.7	0.02 ± 0.01 <sup>&amp;&amp;</sup>	0.5 ± 0.2	8.0 ± 0.6 <sup>l, \$\$</sup>	0.4 ± 0.1 <sup>m, @@</sup>
	12	4.7 ± 0.5	3.7 ± 0.7	4.4 ± 3.9	0.2 ± 0.1	4.4 ± 3.6	0.7 ± 0.1 <sup>m</sup>
	15	4.8 ± 0.5	3.1 ± 2.1	6.7 ± 6.9	0.3 ± 0.2	4.7 ± 3.2	0.8 ± 0.8 <sup>m</sup>
	18	5.1 ± 0.6	4.7 ± 2.5	5.9 ± 5.1	0.3 ± 0.1	5.4 ± 4.1	0.3 ± 0.1 <sup>m</sup>

Results of the Tukey's HSD test:

<sup>a</sup> day 3 vs day 9,  $p = 0.0005$ ; day 3 vs day 12,  $p = 0.0002$ ; day 3 vs day 15,  $p = 0.0005$ ; day 3 vs day 18,  $p = 0.0003$

<sup>b</sup> day 6 vs day 12,  $p = 0.0009$ ; day 6 vs day 18,  $p = 0.02$

<sup>c</sup> day 6 vs day 15,  $p = 0.01$ ; day 6 vs day 18,  $p = 0.02$

<sup>d</sup> day 3 vs day 15, $p = 0.01$ ; day3 vs day 18, $p = 0.01$
<sup>e</sup> day 3 vs day 9, $p = 0.02$ ; day 3 vs day 15 $p = 0.006$ ; day 3 vs day 18, $p = 0.007$
<sup>f</sup> day 3 vs day 6, $p = 0.0002$ ; day vs day 9, $p = 0.0002$
<sup>g</sup> day 3 vs day 6, $p = 0.002$ ; day vs day 9, $p = 0.004$
<sup>h</sup> day 3 vs days 6, 9, 12, 15 and 18, $p = 0.0002$
<sup>i</sup> day 3 vs day 6, $p = 0.0002$ ; day 3 vs day 9, $p = 0.0002$ ; day 3 vs day 12, $p = 0.0002$ ; day 3 vs day 15, $p = 0.0002$ ; day 3 vs day 18, $p = 0.0002$
<sup>j</sup> day 3 vs day 6, $p = 0.009$ ; day 3 vs day 9, $p = 0.001$ ; day 3 vs day 12, $p = 0.003$ ; day 3 vs day 15, $p = 0.002$ ; day 3 vs day 18, $p = 0.003$
<sup>k</sup> day 3 vs day 6, $p = 0.002$ ; day vs day 9, $p = 0.02$
<sup>l</sup> day 3 vs day 6, $p = 0.0004$ ; day vs day 9, $p = 0.0003$
<sup>m</sup> day 3 vs days 6, 9, 12, 15 and 18, $p = 0.0002$
<sup>#</sup> pH after 6 days: Exp. 1/M vs Exp. 2/ML, $p = 0.001$ ; Exp. 1/M vs Exp 3/MLA, 4/LA, 6/SLA, $p = 0.0002$ ; Exp. 2/ML vs Exp. 3/MLA, Exp. 4/LA, Exp. 6/SLA, $p = 0.0002$ ; Exp. 3/MLA vs Exp. 4/LA, Exp. 6/SLA, $p = 0.0002$ ; Exp. 4/LA vs Exp. 6/SLA, $p = 0.0002$
<sup>##</sup> pH after 9 days: Exp. 1/M vs Exp. 2/ML, Exp 3/MLA, 4/LA, 6/SLA, $p = 0.0002$ ; Exp. 2/ML vs Exp. 3/MLA, Exp. 4/LA, Exp. 6/SLA, $p = 0.0002$ ; Exp. 3/MLA vs Exp. 4/LA, $p = 0.0002$ ; Exp. 3/MLA vs Exp. 6/SLA, $p = 0.0008$ ; Exp. 4/LA vs Exp. 6/SLA, $p = 0.0002$
<sup>\$</sup> butyrate concentration after 6 days: Exp. 1/M vs Exp. 3/MLA, Exp. 6/SLA, $p = 0.0004$ ; Exp. 2/ML vs Exp. 3/MLA, Exp. 6/SLA, $p = 0.0004$ ; Exp. 3/MLA vs Exp. 4/LA, $p = 0.01$ ; Exp. 4/LA vs Exp. 6/SLA, $p = 0.01$
<sup>\$\$</sup> butyrate concentration after 9 days: Exp. 1/M vs Exp. 3/MLA, $p = 0.01$ ; Exp. 1/ML vs Exp. 6/SLA, $p = 0.003$ ; Exp. 2/ML vs Exp. 3/MLA, $p = 0.02$ ; Exp. 2/ML vs Exp. 6/SLA, $p = 0.003$
<sup>&amp;</sup> lactate concentration after 6 days: Exp. 2/ML vs Exp. 3/SLA, Exp. 4/LA, Exp. 6/SLA, $p = 0.0003$
<sup>&amp;&amp;</sup> lactate concentration after 9 days: Exp. 2/ML vs Exp. 4/LA, Exp. 6/SLA, $p = 0.03$
<sup>@</sup> ethanol concentration after 6 days: Exp. 1/M vs Exp. 3/MLA, Exp. 4/LA, $p = 0.0002$ ; Exp. 1/M vs Exp. 6/SLA, $p = 0.0003$ ; Exp. 2/ML vs Exp. 3/MLA, $p = 0.0003$ ; Exp. 2/ML vs Exp. 4/LA, $p = 0.0002$ ; Exp. 2/ML vs Exp. 6/SLA, $p = 0.0005$
<sup>@@</sup> ethanol concentration after 9 days: Exp. 1/M vs Exp. 4/LA, Exp. 6/SLA, $p = 0.02$ ; Exp. 2/ML vs Exp. 4/LA, $p = 0.03$ ; Exp. 2/ML vs Exp. 6/SLA, $p = 0.05$

Table 3

The approximate balance of carbon for the selected data from experiments LA and SLA based on the concentration of the media components and fermentation products (mean from three replicates shown).

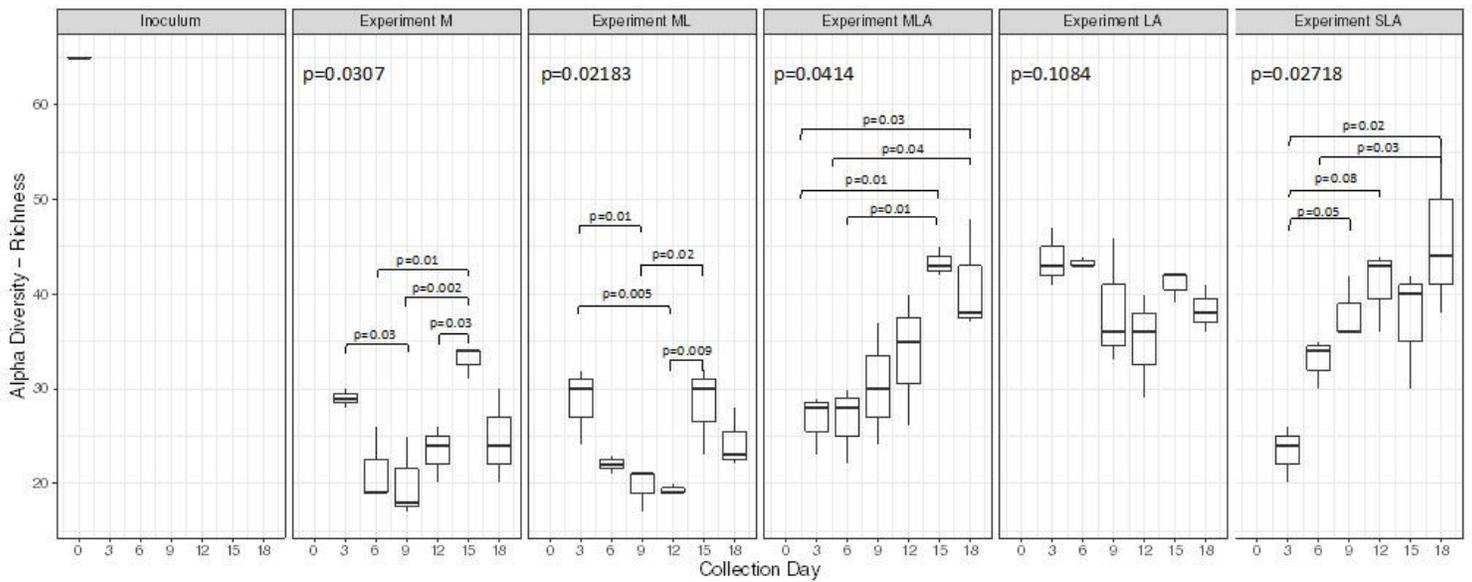
	<b>Experiment</b>	<b>Medium</b>		<b>Fermentation products</b>
Balance 1	Experiment LA after the first passage (3 days)	200 lactate + 85 acetate	→	112 lactate + 49 butyrate + 69 acetate + 3 propionate + 4 ethanol + X (27 millimoles of carbon)**
Balance 2	Experiment LA after the second passage (6 days)	200 lactate + 85 acetate	→	8 lactate + 140 butyrate + 56 acetate + 4 propionate + 4 ethanol + X (73 millimoles of carbon)**
Balance 3	Experiment SLA after the first passage (3 days)	200 lactate + 85 acetate + 347 sucrose	→	345 lactate + 22 butyrate + 113 acetate + 250 ethanol*
Balance 4	Experiment SLA after the second passage (6 days)	200 lactate + 85 acetate + 343 sucrose	→	22 lactate + 318 butyrate + 8 acetate + 14 propionate + 46 ethanol + X (220 millimoles of carbon)**
* The excess of millimoles of carbon in the fermentation products likely comes from the inoculum				
** X are estimated bacterial biomass and other products including carbon dioxide				

Table 4

Summary of the selected samples used for WGS

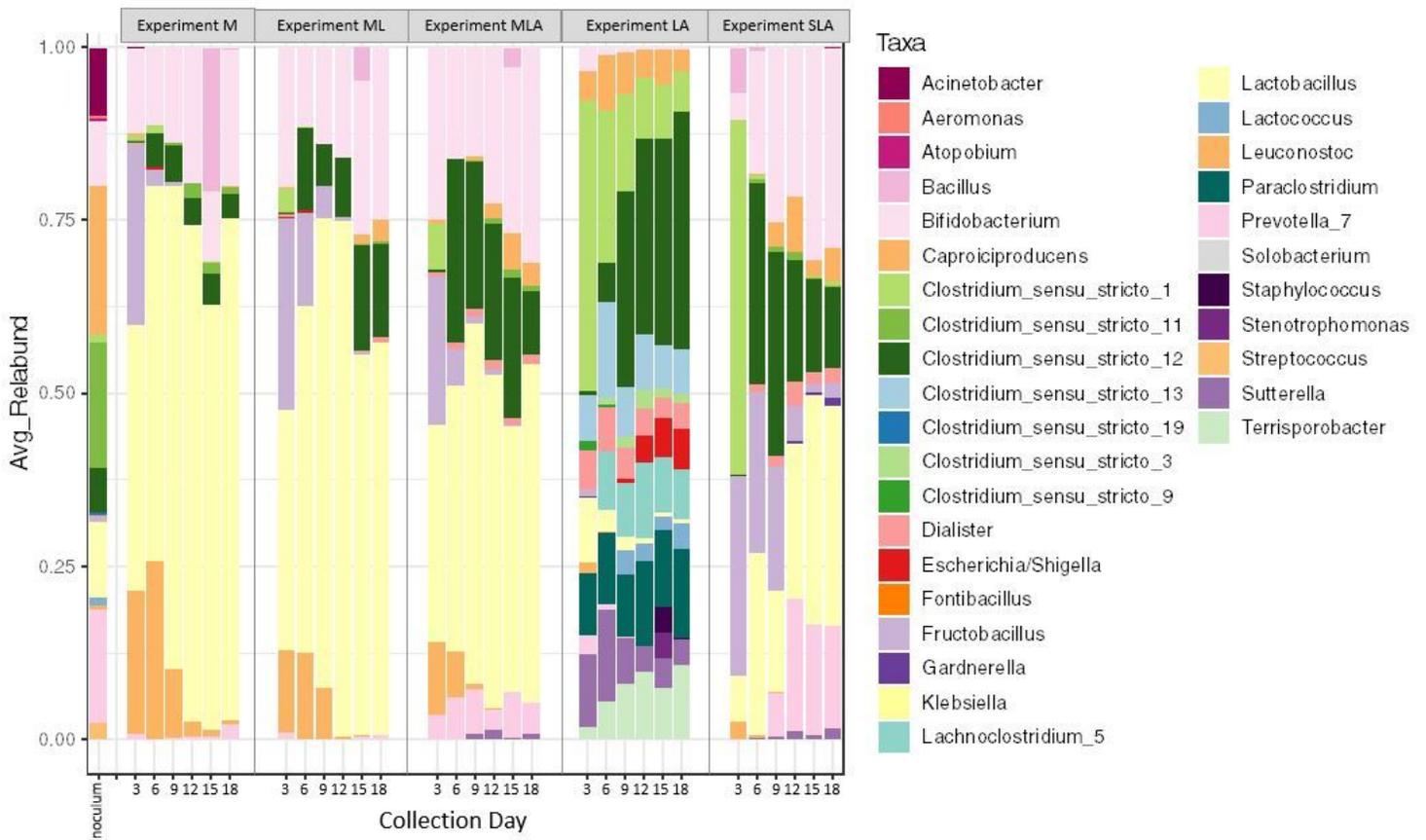
<b>Sample ID</b>	<b>Experiment</b>	<b>Day of passage/ replicates</b>	<b>Sample name in Fig. 3</b>	<b>Fermentation substrate /products description</b>
MLA-3-AC	MLA	3 / pooled A + C	MLA 3 A and MLA 3 C	The main non-gaseous fermentation products of molasses were lactate and ethanol, concentration of butyrate was very low
MLA-9-AC	MLA	9 / pooled A + C	MLA 9 A and MLA 9 C	The main fermentation product of molasses supplemented with acetate and lactate was butyrate; lactate as a substrate was efficiently utilized
LA-3-BC	LA	3 / pooled B + C	LA 3 B and LA 3 C	Lactate as a substrate was partially metabolized
LA-18-AB	LA	18 / pooled A + B	LA 18 A and LA 18 B	The main fermentation product of the medium containing exclusively lactate and acetate was butyrate; lactate as a substrate was efficiently utilized

## Figures



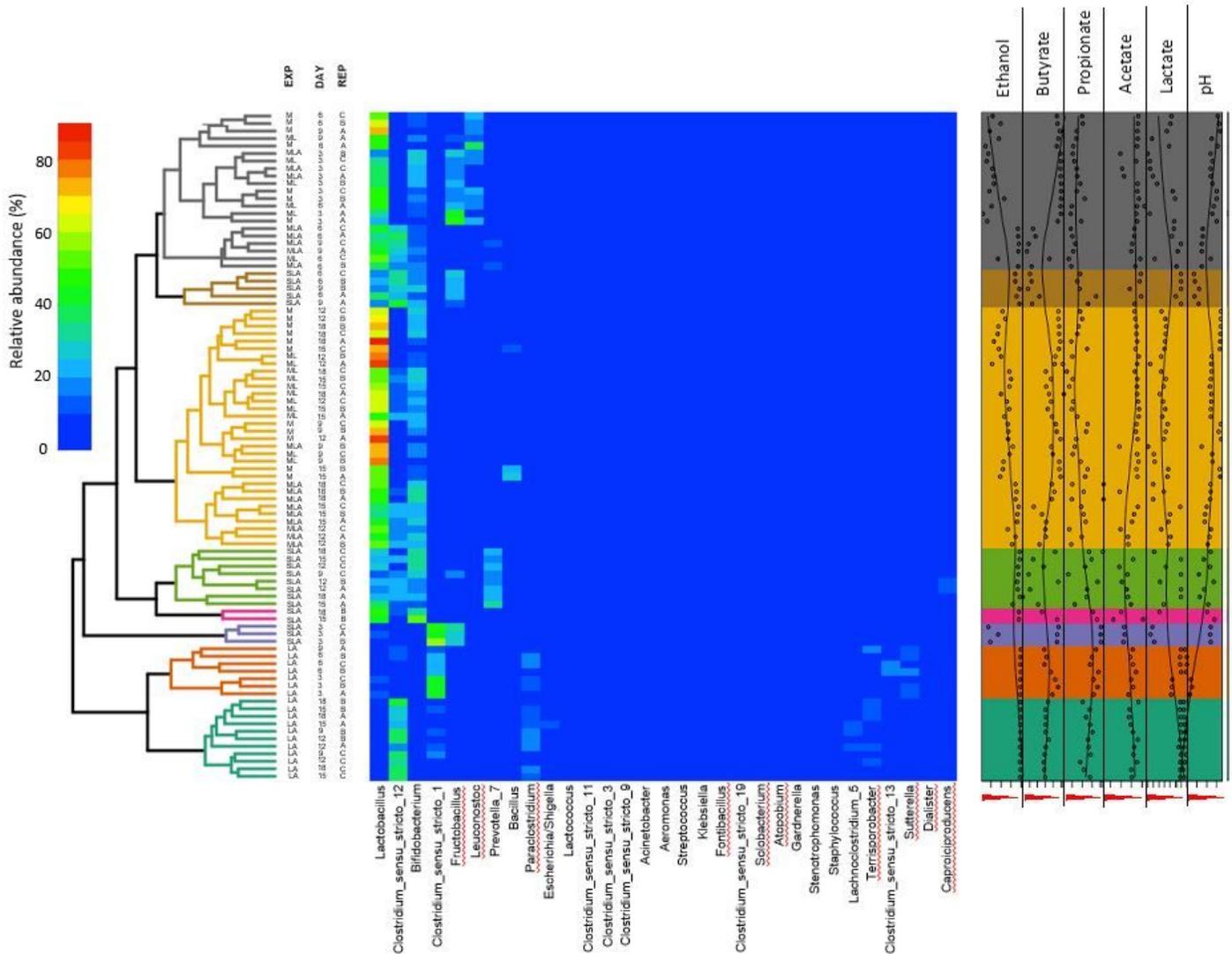
**Figure 1**

Alpha Diversity (Richness) of the microbial communities selected in time in the static batch experiments for each collection day, except day 0, which is an inoculation day. The lower and upper hinges represent the first and third quartiles respectively. The whiskers extends to the largest and lowest values. The middle line represents the median value. Dots represent individual samples. For statistical analysis Kruskal-Wallis rank sum test followed by Dunn's multiple comparison test were used.



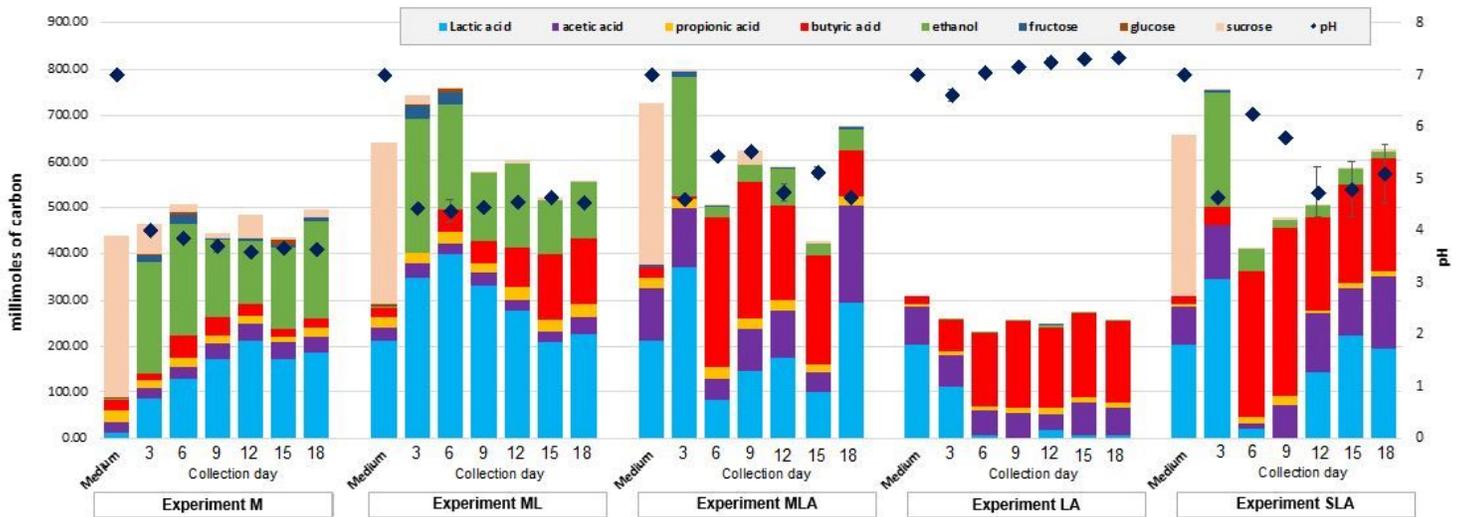
**Figure 2**

Taxonomic composition (genus level) of the microbial communities selected in batch experiment based on hypervariable V4 region of the 16S rRNA gene, sequenced on MiSeq platform (Illumina). The taxonomy was assigned using RDP classifier against SILVA database. All taxa with Relative Abundance lower than 0.1% were removed.



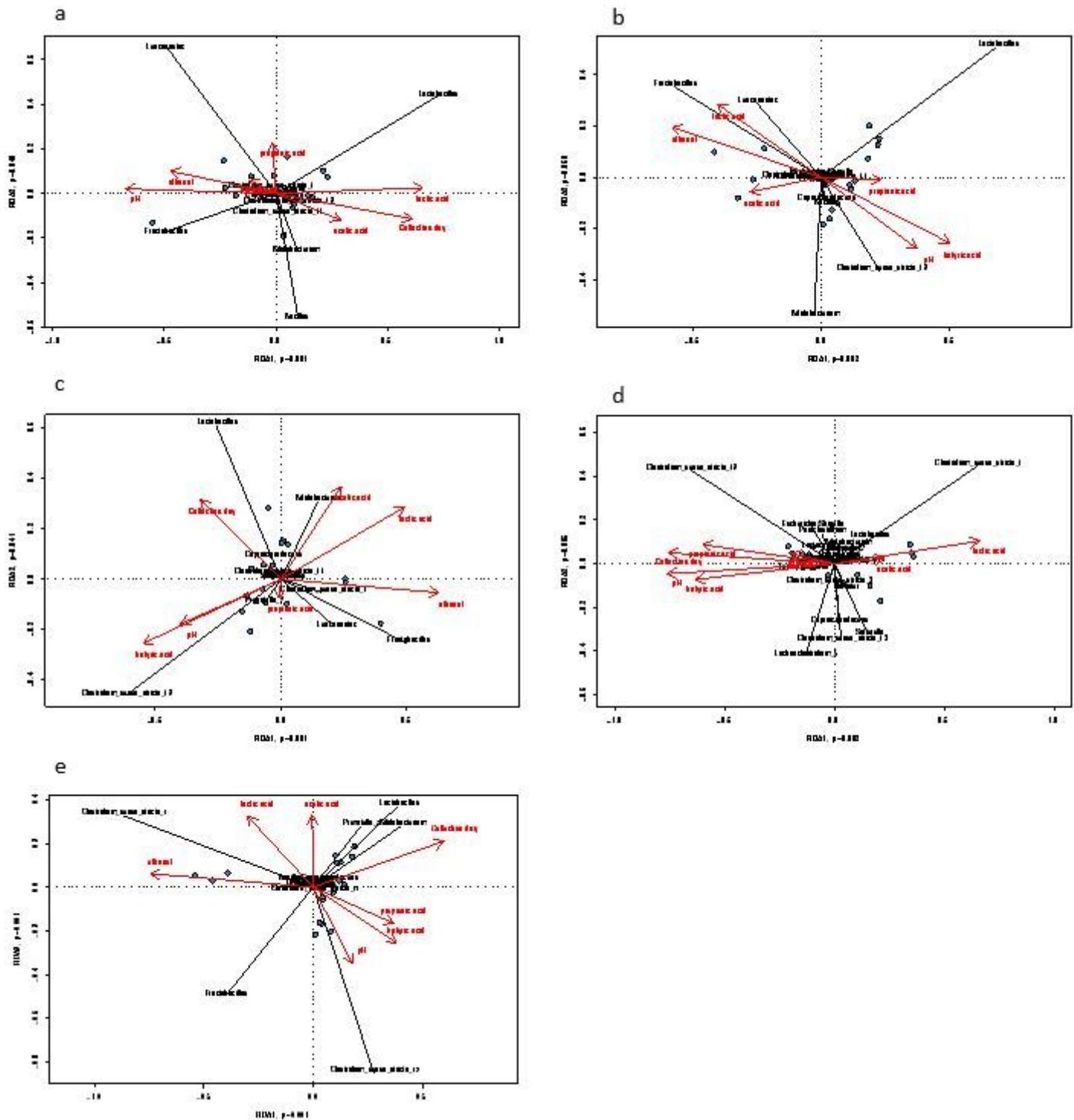
**Figure 3**

Heatmap showing the relative abundance of genera in the individual Experiments for all timepoints and annotation with measured metabolites and pH. The heatmap was generated in R (Heatplus package, annHeatmap2 function) using the relative abundance of the observed genera. For clarity, the “Inoculum” sample and all genera with summarized relative abundance lower than 0.1% were removed. Rows were clustered using average linkage hierarchical clustering based on the Bray–Curtis dissimilarity matrix of the dataset (‘vegdist’ from the vegan package).



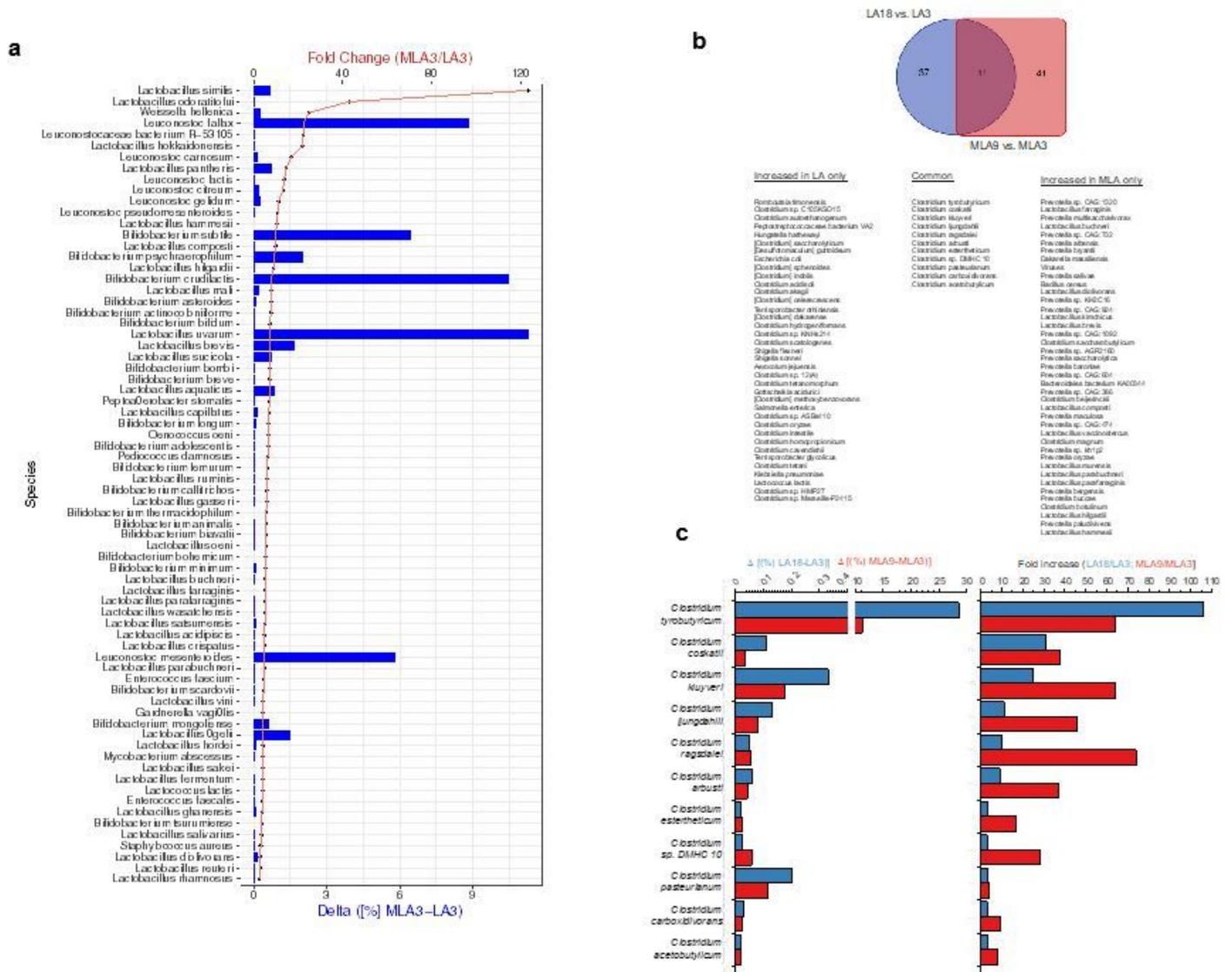
**Figure 4**

Non-gaseous fermentation products expressed in millimoles of carbon (bars, left axis) and pH (diamonds, right axis) of the digestive liquids from batch experiments presented in time for each experiment. The values are a mean from 3 replicates, for each the analyses were performed in duplicate. The composition of fermentation products were analyzed using high performance liquid chromatography. The pH values are a mean from 3 replicates  $\pm$  SD. For detailed data see Additional file 4.



**Figure 5**

The correlations between the non-gaseous fermentation products, pH of the digestive liquids and the dominant bacterial taxa presented as Redundancy Analysis (RDA) for the batch experiments: a. Experiment M, b. Experiment ML, c. Experiment MLA, d. Experiment LA, e. Experiment SLA .



## Supplementary Files

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