Selecting for lactic acid producing and utilising bacteria in anaerobic enrichment cultures

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Abstract

Lactic acid producing bacteria are important in many fermentations, such as the production of biobased plastics. Insight in the competitive advantage of lactic acid bacteria over other fermentative bacteria in a mixed culture enables ecology-based process design and can aid the development of sustainable and energy-efficient bioprocesses. Here we demonstrate the enrichment of lactic acid bacteria in a controlled sequencing batch bioreactor environment using a glucose based medium supplemented with peptides and B vitamins. A mineral medium enrichment operated in parallel was dominated by Ethanoligenens species and fermented glucose to acetate, butyrate and hydrogen. The complex medium enrichment was populated by Lactococcus, Lactobacillus and Megasphaera species and showed a product spectrum of acetate, ethanol, propionate, butyrate and valerate. An intermediate peak of lactate was observed, showing the simultaneous production and consumption of lactate, which is of concern for lactic acid production purposes. This study underlines that the competitive advantage for lactic acid producing bacteria primarily lies in their ability to attain a high biomass specific uptake rate of glucose, which was two times higher for the complex medium enrichment when compared to the mineral medium enrichment. The competitive advantage of lactic acid production in rich media can be explained using a resource allocation theory for microbial growth processes.

Introduction

Lactic acid bacteria are key species in many fermentative processes (Axelsson and Ahrné, 2000), such as biogas production and food-related fermentations (Leroy and De Vuyst, 2004). They also are essential in promoting human health, *e.g.* a healthy human infant microbiome (Solís et al., 2010). In an industrial biotechnology setting, these microorganisms are applied in the production of lactic acid, which is used to preserve food and to produce the biobased and biodegradable plastic polylactic acid (Straathof, 2014). The lactic acid market is expected to reach 9.8 billion US dollars by 2025 which shows the economic significance of lactic acid.

Mixed culture biotechnology (Kleerebezem and van Loosdrecht, 2007) can aid to the development of more sustainable and energy-efficient bioprocesses. Such processes rely on "ecology-based design" of bioprocesses to perform a desired conversion, which contrasts with the traditional pure culture approach. Typically, enrichment cultures are used to function as a model system to develop such ecology-based bioprocesses. Compared to pure culture processes, ecology-based processes offer the advantage of (semi)-continuous bioprocessing and omit the need for sterilisation of the feedstock and equipment (Kleerebezem and van Loosdrecht, 2007). Examples of successful ecology-based bioprocesses are PHA production from VFAs (Johnson et al., 2009) or biological phosphorous removal (reviewed by Bunce et al., 2018). To create a stable ecology-based process, its design needs to be based on the competitive advantage of the concerned type of conversion. In

the case of lactic acid to be produced from carbohydrates, the ecological question is which environmental conditions provide lactic acid bacteria with a competitive advantage over other carbohydrate fermenting microorganisms?

Lactic acid bacteria tend to dominate in anaerobic, carbohydrate containing environments characterised by acidic pH and an abundant availability of compounds required for anabolism, such as in fermented milk, meats and vegetables (Axelsson and Ahrné, 2000). Most well-studied lactic acid bacteria are part of the *Bacilli* class, such as*Streptococcus* , *Lactococcus* , *Bacillus* and*Lactobacillus* species. Lactic acid bacteria have high maximal biomass specific growth rates (µmax), *e.g. Streptococcus salivarius* shows a µmax of 2.8 h-1 in a complex medium at 37°C, at neutral pH (Roger et al., 2011). This can be compared to µmax for*Escherichia coli* strain K12 of around 0.98 h-1 at similar conditions (Kim et al., 2007). Lactic acid bacteria seem to have a kinetic advantage over other species and have quite extraordinary growth rates while being anaerobic microorganisms.

Lactic acid bacteria only display fast growth when sufficient B vitamins and peptides are supplied to their medium environment. For example,*Lactococcus lactis* strains are auxotrophic for 14 of the 20 amino acids (Cocaign-Bousquet et al., 1995). A comparative genome study predicted that of the 46 *Lactobacillus* species analysed all are auxotrophic for biotin, folate, pantothenate and thiamine (Magnúsdóttir et al., 2015). Lactic acid bacteria grow poorly or do not grow at all in environments where such B vitamins or peptides are not available. We therefore suggest that auxotrophies are common among lactic acid bacteria, certainly under conditions of high growth rates.

Prototrophic fermentative microorganisms in general have lower µmax-values when compared to lactic acid bacteria. These organisms can be found in the genus of *Clostridium* and the families of *Enterobacteriaceae* and *Ruminococcaceae* . The extensively studied *Enterobacteriaceae* species *E. coli* is a prototroph, and is reported to have a µmax of 0.31 h-1 at 37 °C and a pH of 7 in a mineral medium with glucose (Hasona et al., 2004). *E. coli* here produced a mixture of acetate, ethanol and formate. We hypothesise that lactic acid bacteria will outcompete prototrophic fermenters by achieving a higher µmax in complex environments where there is an abundance of peptides and B vitamins.

The switch between lactate production on the one hand, and acetate and ethanol production on the other hand, has been reported for a single species under complex medium conditions. *Lactococcus lactis*(formerly known as *Streptococcus lactis*), switches its catabolism from lactate production to acetate, ethanol and formate or H2 production at lower dilution rates, *i.e.* lower growth rates (Thomas et al., 1979). Lactate is produced from pyruvate with one enzyme and delivers and acetate and ethanol with five enzymes. Lactate delivers 2 ATP by substrate level phosphorylation, while acetate and ethanol deliver 3 ATP. This switch is thought to be caused by resource allocation, which essentially describes that a cell has a certain amount of functional protein available, and shorter catabolic pathways can evoke a higher biomass specific substrate uptake rate, qsmax, (de Groot et al., 2018; Molenaar et al., 2009), often at the expense of less energy harvesting per unit of substrate.

Here, we tested the hypothesis that lactic acid producing enrichment cultures can be obtained by providing a complex medium and selecting on high growth rate. We compared two parallel anaerobic non-axenic or open mixed culture sequencing batch reactors (SBRs) operated under mesophilic (T = 30°) and slightly acidic conditions (pH = 5), with either mineral or complex cultivation media. The mineral medium was replicated from the work of Temudo (2008) and containing 4 g L-1 of glucose. The complex medium consisted of the mineral medium, and also 0.8 g L-1 of tryptone and 9 B vitamins. The cultures were characterised for their stoichiometric, kinetic and thermodynamic properties and the microbial community structure was analysed.

Material and Methods

Bioreactor enrichment

Both enrichments were performed in 3 L jacketed bioreactors with working volumes of 2 L. pH was maintained at 5.0 ± 0.1 using NaOH at 4 mol L-1 and HCl at 1 mol L-1. Temperature was maintained at 30°C ± 0.1. The

cultures were stirred constantly at 300 rpm. Anaerobic conditions were maintained by sparging the reactor with a flow of 576 mmol N2 h-1. The off-gas was cooled and dried at 5°C using a gas condenser. A hydraulic retention time (HRT) and solid retention time (SRT) of 12 h was maintained by removing 1 L of culture per cycle under continuous stirring and a cycle time set to 6 h.

The mineral cultivation medium was identical to the one used by Temudo et al. (2007), while the complex medium was supplemented by 9 B vitamins and peptides according to Table S1 in the supplementary information. The carbon source, peptides and B vitamins and the ammonium, phosphate and trace elements were fed separately from 12.5 concentrated stock solutions and diluted using N2-sparged demineralized water. Connected to the base pump was a pump supplying 3% (v:v) antifoam C (Sigma Aldrich, Germany), which ensured a flow of 3-5 mL h-1 or 14-17 mL cycle-1. The glucose solution was sterilised at 110°C for 20 min. For the complex medium, the peptides were sterilised separately at 110°C for 20 min and the B vitamins were added by filter sterilisation through 0.45 µm and 0.2 µm polyvinylidene fluoride filters.

The inoculum for all enrichments consisted of sludge taken from an anaerobic digester of the wastewater treatment plant (WWTP) Harnaschpolder, The Netherlands. The pH, temperature, and HRT and SRT of the digester in the WWTP were 7-7.2, 36-38@C, and 20 days, respectively. At the beginning of each experiment, the reactor was seeded with approximately 10 mL of 200 µm filtered inoculum (0.5% of the total volume). The reactors were gradually moving from 24-h and 12-h cycles in 3 days to the final desired 6-h cycles to maintain a HRT of 12 h. Steady state was assumed if during a period of at least 5 days little variation was detected in the product concentrations.

Analytical methods

Samples from the reactors were immediately filtered on 0.45 µm polyvinylidene fluoride membranes (Millipore, USA) and stored at -20°C until analysis. Volatile fatty acids (VFAs; formate to valerate), lactate, succinate and glucose were analysed using high performance liquid chromatograph (HPLC) method described previously (Rombouts et al., 2019). Ethanol was analysed using a gas chromatography (GC) method described previously (Rombouts et al., 2019). The off-gases were monitored on-line for H2 and CO2 by a connection to a NGA 2000 MLT 1 Multicomponent analyser (Rosemount, Shakopee, Minnesota, USA). Data acquisition (base, H2, CO2) was made using a BBI systems MFCS/win 2.1 (Sartorius, Göttingen, Germany).

Methane was measured manually using GC with a Varian CP 3800 (Varian Medical Systems, Palo Alto, California, USA) equipped with a MolSieve capillary column (1.2 m x 1 mm; 13 x 80/100 mesh, 50 °C) and a thermal conductivity detector (200 °C) with N2 as a carrier gas (2 mL min-1).

Biomass concentration was measured using a standard method which relies on centrifugation of 150 mL to separate the cells from the medium, drying these solids to obtain the total suspended solids (TSS) and burning these solids at 550 °C to determine the amount of volatile suspended solids (VSS) (APHA, 1998). This analysis was coupled to absorbance measurement at 660 nm to establish a correlation. Absorbance values were used to calculate the biomass concentration during the cycle analysis and batch experiments.

Cycle analysis and batch experiments

To characterise one cycle, product and biomass concentrations were measured in parallel to H2 and CO2 in the off-gas. Sampling and off-gas analysis were carried out as described above. The biomass concentration was determined spectrophotometrically at 660 nm (OD660) and this value was correlated to the three previous measurements of VSS.

A batch test with lactate and a batch test with H2, and CO2 was performed in the complex medium enrichment. This went by adding the peptides and B vitamins and peptides together with the N, P, S, trace elements, and either 11 mmol of lactate or 0.46% of H2 and 1.00% of CO2. Sampling was conducted as in a cycle measurement.

Microbial community analysis

Genomic DNA was extracted using the Ultra Clean Soil DNA extraction kit (QIAGEN, Hilden, Germany) following manufacturer's instructions, with the exception of heating the samples for 5 minutes at 65°C prior to bead beating. DNA extracts were checked on a 1% agarose gel. High molecular weight DNA was obtained (>10 kb) with a concentration of 10 ng μL-1 or higher. Extracted DNA was stored at -20°C until further use.

Analysis of the V3-V4 region of the 16S rRNA gene was conducted using amplicon sequencing. The extracted DNA was sent for amplification and sequencing at a commercial company (Novogene, China). Amplification was achieved using the universal primer set 341f (CCTAYGGGRBGCASCAG) / 806r (GGACTACNNGGGTATCTAA T) (Muyzer et al., 1993)(Caporaso et al., 2011). All polymerase chain reactions (PCR) were carried out in 30 μL reactions with 15 μL of Phusion® High_fidelity PCR Master Mix (New England Biolabs, USA), 0.2 μmol L-1 of forward and reverse primers, and 10 ng template DNA. Thermal cycling started with denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 60 s for 30 cycles, prior to ending with 72°C for 5 min. These pools of amplicons were sequenced using an Illumina HiSeq2500 platform. The sequencing datasets were cleaned and trimmed according to Jia *et al.* (2016) and processed with Qiime (Caporaso et al., 2010) using Uparse with a 97% stringency to yield operational taxonomic units (OTUs). OTUs were taxonomically classified using the Mothur classifier (Wang et al., 2007) with 0.8 confidence interval against the SILVA database 123 release of July 2015. The clean and trimmed sequences can be retrieved at NCBI using accession number SAMN11350619 - SAMN11350630. The inoculum was sequenced twice as a technical replicate, this data can be retrieved at NCBI (data not presented in this paper).

Cell fixation and fluorescence *in situ* hybridisation (FISH) were carried out as described by Johnson *et al.* (Johnson et al., 2009) using the probes listed in Table S2, except that hybridization was carried out overnight. Additionally, DAPI staining was used to stain all microbial cells by incubating the multi-wells microscopy slides of fixed cells with 10 μL of a solution of 10 mg DAPI mL-1 per well for 15 min. The samples were analysed using an epifluorescence microscope, Axioplan 2, (Zeiss, Oberkochen, Germany). Digital images were acquired using a Zeiss MRM camera together with Zeiss imaging software AxioVision version 4.7.

Parameter estimation of kinetics of the enrichment cultures using minimisation of residual error

To estimate the kinetic parameters of the enrichments and to derive the stoichiometry of the process a kinetic model was built. The uptake rate was modelled using Monod equations and no biomass decay or pH inhibition was considered. The saturation constant of the Monod equations for the different microorganisms was assumed to be 0.1 g/L. It is assumed that there is a microbial group degrading glucose and other degrading lactate (only in the complex medium enrichment). In this last case, the measured biomass was divided between glucose degraders (65%) and lactate degraders (35%), based on the derived stoichiometry and the ATP yield on the substrate corresponding to that stoichiometry. Parameter estimation was performed following the method proposed by González-Gil*et al.* (Gonzalez-Gil et al., 2018) and is further explained in Appendix II.

ATP yield estimation using the obtained parameters

The model estimates the distribution of substrate to select a set of catabolic pathways to obtain the lowest residual error with respect to the measured metabolic product distribution during a cycle analysis. These fractions are combined with the ATP yield per catabolic reaction to obtain the overall yield of ATP on substrate (YATP,S). This yield is than combined with the biomass yields on glucose (Yx,s) observed in time (n=3) to obtain the biomass yield on ATP harvested (YX,ATP). An argumentation which catabolic routes were selected for both enrichments can be found in Appendix III.

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A script was developed in Matlab version 2014 (MathWorks, Natick, Massachusetts, USA) to estimtate the growth rate over each SBR cycle. This script is based on determining the last moment of base dosage in a cycle, which indicates the end of the fermentation, and thus the growth. This method is explained in detail

in the supplementary information section published previously (Rombouts et al., 2019). The maximum biomass-specific substrate conversion rate (qsmax) is calculated using μmax and Yx,s using the Herbert-Pirt equation (Pirt, 1965) and neglecting maintenance.

Carbon and COD balancing

During steady state, carbon and electron (as chemical oxygen demand, COD) balances were defined using the elemental metabolite matrix given in Table S3 multiplied by the in- and outgoing rates in the reactor. COD balances were set up for the cycle analyses by dividing the amount of COD present in the metabolic compounds measured at a time in the cycle by the measured COD at the start of the cycle.

Results

A complex medium promotes fermentation to VFAs and ethanol with little hydrogen while a mineral medium promotes acetate-butyrate-hydrogen formation

Two anaerobic SBRs were operated with either a mineral or complex medium. The enrichments displayed distinct fermentation patterns after 20 SRTs. Initially, the mineral-medium enrichment showed a 1:2:1 acetate:propionate:butyrate product spectrum with little ethanol and no lactate (Figure S1). This shifted after 10 SRTs to primarily acetate and butyrate, with a small amount of lactate and ethanol (Figure 1). Hydrogen was the major gaseous product in the mineral medium enrichment (13%±1% of incoming COD). Up to 96% ± 3% of the incoming COD could be recovered for this enrichment, which indicates that a minor by-product might have been missed. Succinate, valerate or formate concentrations were below the detection limit of 50 μM.

The complex medium enrichment showed a more dynamic product spectrum development. Initially lactate and acetate were the dominant products (Figure S1). After 3 SRTs, the product spectrum shifted to acetate, propionate, butyrate, valerate, and lactate. After 31 SRTs, 0.19 Cmol ethanol Cmol-1 sugar was produced, and only a minor amount of hydrogen were detected in the off-gas (1%±0% of the incoming COD) (Figure 1). This product spectrum was more diverse than for the mineral medium enrichment. The carbon balance (based on glucose as the only substrate) displayed a recovery of 113% ± 2%, which most likely is caused by the uptake of tryptone for biomass production. Taking into account that tryptone uptake is equivalent to the nitrogen requirements for biomass production, the carbon recovery would be 100% ± 2%.

The mineral medium enrichment showed a 25% higher biomass yield on glucose then the complex medium enrichment culture. The μmax values for the cultures were derived with a cut-off at 20 SRTs (Figure S2). The μmax in the complex medium enrichment was 58% higher than the mineral medium enrichment, while the maximal biomass specific substrate uptake rate (qsmax) was even 94% higher (Table 1).

Cycle analysis reveals potential storage of glucose when using a mineral medium and an intermediate lactate peak when using a complex medium

The product and substrate concentrations during a representative cycle in the SBR are shown in Figure 2 for both enrichments. In the enrichment culture on mineral medium, glucose was converted to mainly acetate and butyrate with minor amounts of ethanol and lactate (Figure 2A). The formation of fermentation products proceeds after glucose depletion. The COD recovery during the cycle showed that during the glucose consumption phase the COD of consumed glucose is not fully recovered in the measured products (Figure S7A), while in the subsequent period in the cycle the product concentration increased and finally a full recovery of consumed COD is observed. This indicates formation of an intermediate product which is probably a storage product, most likely a polymer of glucose.

Supplementing peptides and B vitamins led to significant presence of lactic acid bacteria and more microbial diversity

Clostridium and *Ethanoligenens* were the dominant OTUs in the mineral medium enrichment culture (Figure 3). FISH analysis showed a very different result and demonstrated dominance in biovolume of *Ruminococcaceae* using the Rums278 probe, to which the genus of *Ethanoligenens* belongs. Only a minor biovolume of *Clostridium*

was detected using the Chis150 probe.*Lactobacillus* was present as a very minor population (Figure S4) in the mineral medium enrichment. The discrepancy between sequencing results and FISH evaluation shows that complementary observations of the microbial community structure are needed when analysing a microbial community, referred to as the "full cycle rRNA analysis" by Amann*et al.* (Schleifer et al., 1995).

Lactococcus and *Lactobacillus* were dominant OTUs in the complex medium enrichment (Figure 3). Their dominance was confirmed by FISH analysis using the Lactococcus4 (*Lactococcus*) and Lacto722 (*Lactobacillus*) probes. The presence of *Megasphaera* and minor presence of *Clostridium* was also confirmed using the Mega-X (*Megasphaera*) and the Chis150 (*Clostridium*) probes.

The diversity of the obtained microbial community structures using 16S rRNA amplicon sequencing can be calculated using the Shannon index for the observed genera with a presence $> 3\%$ of the total OTU's. For the mineral medium the Shannon index was 0.94 (sample at 32 SRTs) and for the complex medium this was 1.34 (sample taken at 41 SRTs). Thus, more microbial diversity was observed in the complex medium enrichment compared to the mineral medium enrichment on the basis of the number of genera found (Figure 3).

Discussion

Supplementation of peptides and B vitamins leads to dominance of lactic acid bacteria and high qsmax through resource allocation

In this work we demonstrate that lactic acid bacteria outcompete prototrophic type fermenters (*e.g.* , *Clostridium* species) when nutritive conditions were favourable, *i.e.* , with sufficient amount of amino acids and B vitamins in an SBR cultivation mode. Kim and colleagues (Kim et al., 2016) have shown that lactic acid bacteria can be enriched in a continuous-flow stirred tank reactor (CSTR) process. They operated the CSTR anaerobically, at pH 5.0 and thermophilic (50 °C) conditions with a SRT of 12 h, with glucose and yeast extract as fermentable organic substrates. Yeast extract is a well-known source of peptides, amino acids, B vitamins and carbohydrates. In cabbage fermentations lactic acid bacteria are known to be the dominant organism (Plengvidhya et al., 2007), while fermentable substrates with low protein content, such as starch, *Clostridium* species are the dominant organism (Lin et al., 2008).

Lactic acid bacteria are well known to be auxotrophic for amino acids (Kitay and Snell, 1950), while their auxotrophy for B vitamins is more ambiguous. Some lactic acid bacteria might actually be producers of B vitamins (LeBlanc et al., 2011). Studies with lactic acid bacteria on synthetic medium have demonstrated the specific compounds needed for growth (Novak et al., 1997), up to individual amino acids (Cocaign-Bousquet et al., 1995). The effect of decreasing medium complexity has been illustrated by Olmos-Dichara *et.al*(Olmos-Dichara et al., 1997). When the "richness" of the growth medium was decreased, the qsmax remained stable, while the growth yield decreased. This shows that the medium complexity directly influences the bioenergetics of *L. casei* , resulting in a lower biomass production when peptides and/or B vitamins are insufficiently supplied in the medium.

Lactic acid bacteria have a kinetic advantage leading to their dominance in enrichments at high growth rates and complex media. The biomass yield of the complex medium enrichment culture was 20% lower than for the enrichment culture on a mineral medium. The maximal substrate uptake rate was almost double for the community enriched on a complex medium versus mineral medium (Table 1). Lactic acid production is clearly a metabolic strategy of high flux but low efficiency. This is supported by the observation that lactic acid bacteria switch to acetate and ethanol production when substrate conversion rates decrease, *i.e.* lower growth rates (De Vries et al., 1970). Acetate/ethanol production generates 3 instead of 2 moles ATP for lactate fermentation on glucose. This can be placed well in the context of resource allocation theories, given a certain protein budget (Bachmann et al., 2017). Less biosynthetic enzymes needed for amino acids and B vitamin synthesis lead to a smaller anabolic proteome. A smaller anabolic proteome can imply a bigger catabolic proteome, as demonstrated when comparing the proteome from *E.coli* grown in a mineral and complex medium (Li et al., 2014). Lactate catabolism requires one enzyme from pyruvate, while acetate/ethanol production requires at least 5 enzymes. Furthermore, at increasing growth rates, ribosome

and RNA polymerase content is higher (Bosdriesz et al., 2015). Lactic acid bacteria are assumed to have optimally distributed their metabolic enzyme levels (Teusink et al., 2011), enabling a high overall metabolic flux.

Mineral medium enriched for an acetate-butyrate type fermentation, potential glucose storage and the class of*Clostridia*

The product formation spectrum from the mineral medium enrichment culture was evaluated to identify the most dominant catabolic route. It was found that our flux-based model fitted best when 42% of the glucose was converted through the acetate-butyrate pathway involving electron bifurcation (Table S5). The microbial community was populated by two genera from the class of *Clostridia* : an *Ethanoligenens*population and *Clostridium* population. The *Ethanoligenens*population showed dominance, as *Ruminococcaceae* were dominant while *Clostridium* was a minority which was shown with fluorescent*in situ* hybridisation (Figure 3F and 3G). *Ethanoligenens harbinense* is a strictly anaerobic species known to produce ethanol, acetate and butyrate from carbohydrates (Tang et al., 2012; Xing et al., 2006). Cluster 12 (*sensu strcito* XII) of *Clostridium* was identified (Figure 3A and 3F). *Clostridium pasteurianum* is a well-studied species in this cluster (SILVA release 138), and is known for acetate-butyrate production involving electron bifurcation (Buckel and Thauer, 2013). This organism has also been found in a fermentative granular enrichment culture which stored poly-glucose (Tamis et al., 2015). In the mineral medium enrichment, effectively 20% of the glucose was potentially metabolised via a carbon storage pool (Figure S6A). The storage response in the mineral medium enrichment causes uncoupling of substrate uptake and growth. The community thereby maximises its substrate uptake rate (competitive advantage) while growing at a more balanced growth rate over the SBR cycle.

Complex medium enriched for production of VFAs through lactic acid formation and consumption and is linked with lactic acid bacteria and *Megasphaera*

Evaluating the pathways for the complex medium enrichment showed a best fit when glucose was catabolised through the heterofermentative (69%) and homofermentative (31%) pathway (Table S5). Lactate was subsequently fermented into propionate, butyrate, valerate, H2 and CO2. The secondary lactate fermentation was confirmed in a batch experiment with the enrichment culture and replacing glucose with lactate (Figure S5). The microbial community analysis revealed a dominance of *Lactobacillus* , *Lactococcus* and*Megasphaera* (Figure 3B, 3C and 3E).

Lactococcus species are known homofermentative lactic acid bacteria, while *Lactobacillus* and *Leuconostoc* species can also be heterofermentative (Madigan and Martinko, 2006).*Megasphaera elsdenii* is known to produce acetate, propionate, butyrate and valerate from lactate (Prabhu et al., 2012).*Megasphaera* is known to convert lactate in the intestinal tract of cows, pigs and humans (Duncan et al., 2004) and is linked to lactate-mediated medium-chain length carboxylate production microbiomes (Spirito et al., 2014). *Megasphaera elsdenii* interestingly prefers lactate uptake over glucose uptake, taking up limited amounts of glucose when lactate is present (Marounek et al., 1989), which argues for the *Megasphaera* species having a mainly lactate consuming role in the community.

Bioenergetics of complex and mineral-type fermentation: supplementation might lead to a more efficient metabolism

The lower growth yield in the complex medium (Table 1) is counterintuitive since these bacteria grow on a complex medium and do not need to produce the amino acids themselves. To compare the impact of the supplementation of peptides and B vitamins on the metabolism, we calculated the Yx,ATP for both enrichments from the catabolic ATP yields estimated (Table 1). For the mineral enrichment a 11% lower Yx,ATP value was estimated, but is not statistically significant (one-tailed t-test gives p=0.45).. Amino acid degradation (Stickland fermentation) was omitted from this bioenergetic evaluation, as the metabolic evaluation of 20 different amino acid utilising pathways would seriously complicate the bioenergetic evaluation. Incorporating amino acid degradation might lower the YX,ATP for the complex medium enrichment, bringing it closer to the value of the mineral medium enrichment.

Prototrophic fermenters such as *Escherichia coli* and auxotrophic fermenters such as *Lactococcus lactis* have similar protein and RNA content (Table S6). Stouthamer estimated that the supplementation of amino acids induces a 0.7% decrease in Yx,ATP (Stouthamer, 1973). The biomass yield of *E. coli* fermenting glucose in complex medium is 13% higher than when fermenting in mineral medium (Lawford and Rousseau, 1995). According to Stouthamer (1973, Table 5) the synthesis of amino acids consumes a relatively low amount of ATP, while uptake of amino acids or ammonium consumes ATP, making both environment bioenergetically equivalent. The polymerisation process for proteins consumes most ATP, about 55% of the available ATP (Stouthamer, 1973). We expect that the biosynthesis of B vitamins requires a relatively small ATP-flux, as B vitamins are present in trace amounts in bacterial biomass (<10-5 in g g-1) (Waller and Lichstein, 1965).

The difference in anabolic efficiency we estimated is much less than the difference observed in μmax, indicating that the complex medium promotes high substrate uptake rates rather than high biomass yields. Functional protein is a valuable resource for a cell. Not only minimising the fermentative pathway length, but also minimising biosynthetic enzymes for amino acid and B vitamin production makes that more cellular protein can be allocated to increase the growth rate of the cell. This is in essence the "trick" that lactic acid bacteria use to dominate anaerobic environments where carbohydrates and peptides are available.

The impact of feedstock protein content on fermentative product spectrum

The consequence of these different ecological types of fermentations are important for understanding applications of mixed-culture fermentation, especially for non-aseptic or "open" bioprocesses aimed to produce economically interesting compounds. The difference in hydrogen production we observed here (Figure 1) has been reflected in a meta-study comparing different feedstocks for the production of hydrogen: food and municipal waste streams generate 32-42% less hydrogen than industrial and (pre-treated) agricultural residue waste streams (Moscoviz et al., 2018). Food waste typically contains more than 10% (w:w) of protein (Paritosh et al., 2017), while agricultural residues contain low amounts of protein, *e.g.* , wheat straw contains 0.6% (w:v) (Kaparaju et al., 2009). This leads to food waste fermentations being dominated by lactic acid bacteria and the secondary lactate fermentation producing no or small amounts of hydrogen gas. In contrast, fermentations of (pre-treated) agricultural residues are dominated by acetate/butyrate producing bacteria, such as*Clostridium* species, resulting in significant amounts of hydrogen produced. This difference in performance is a direct consequence of the ecology of these two different fermentative microbial groups. Lactic acid bacteria seem to dominate environments abundant in carbohydrates and peptides where selection occurs on a maximal growth rate or maximal substrate uptake rate. The consequence of striving for a high growth rate is that the organisms have to optimise their proteome in preference for high growth rate enabling proteins, making them auxotrophic for e.g. amino acids and vitamins.

Using this ecological concept, feedstocks with readily fermentable carbohydrates and a sufficient protein content are a good target to directly produce lactic acid. Protein-poor feedstocks on the other hand are a good target to produce VFAs and hydrogen (Figure 5). In this study, we have obtained an enrichment producing only 0.11 Cmol per Cmol of lactate at the end of the batch (Figure 1). If lactic acid production from low value feedstocks is the desired bioprocess, lactate consumption has to be managed effectively. Lactate consumption can be managed by creating a selective environment which does not select for lactate consuming organisms such as *Megasphaera* , by using a different pH or solid retention time for example.

Here we used enrichment culture to better understand the ecological niche of lactic acid producing bacteria which showed:

1. Lactic acid bacteria outcompete prototrophic type fermentative bacteria on high biomass specific substrate uptake rate and growth rate.
2. This behaviour can be explained in line with the resource allocation hypothesis for protein allocation: LAB can dedicate a higher share of their proteome to catabolism, ribosomes and RNA polymerases and therefore are able to attain a significantly higher substrate uptake rate and growth rate.
3. The anabolic efficiency of the microbial community enriched on complex medium is higher but not significantly, and only accounts for a minor possible increase in μmax

4. Intermediately formed lactic acid is fermented to acetate, propionate, butyrate, valerate, H2 and CO2, resulting in a different fermentation product spectrum when lactate is an intermediate fermentation product.
5. A relatively high protein content of a feedstock can enhance the competitiveness of lactic acid bacteria, leading to lower hydrogen yield and the possibility of producing lactic acid by enrichment cultures.

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Conflict of intertest

The authors declare that they have no conflict of interest

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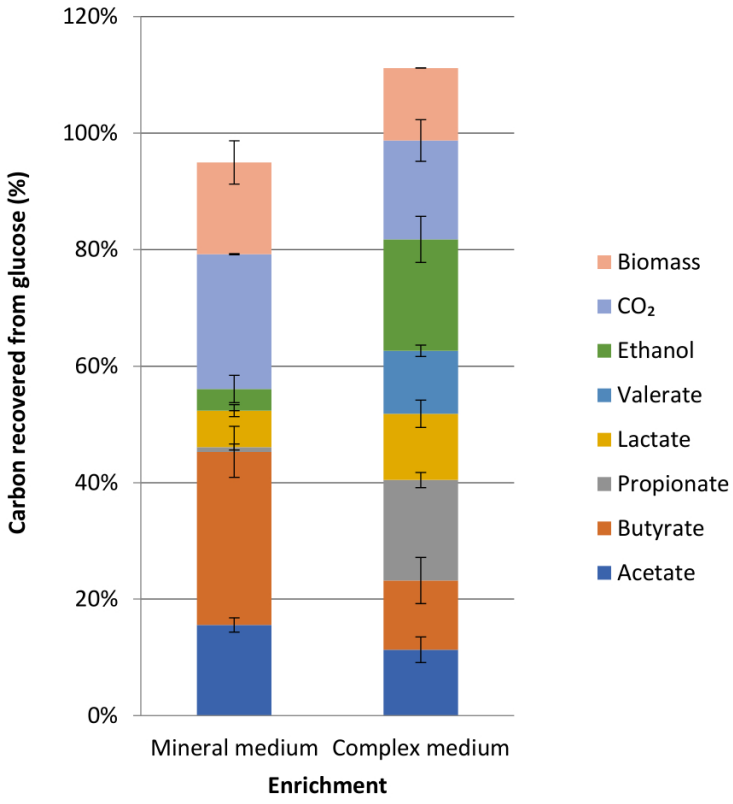
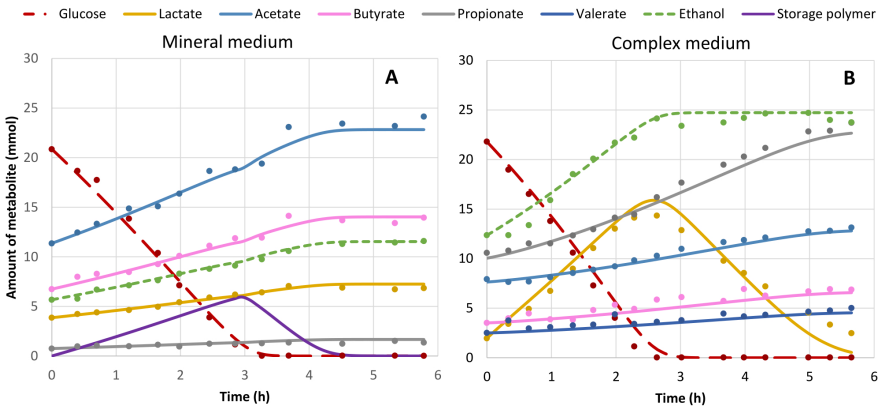
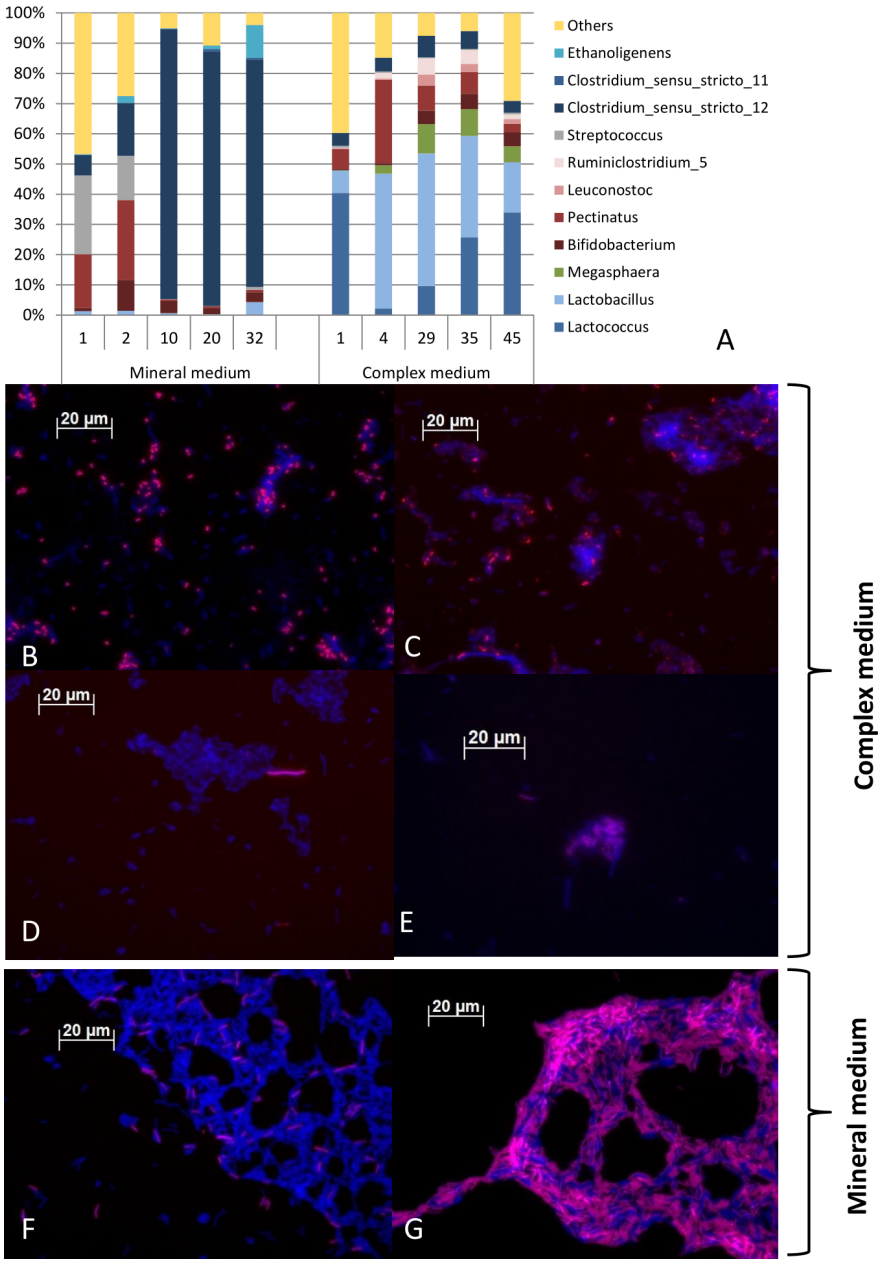
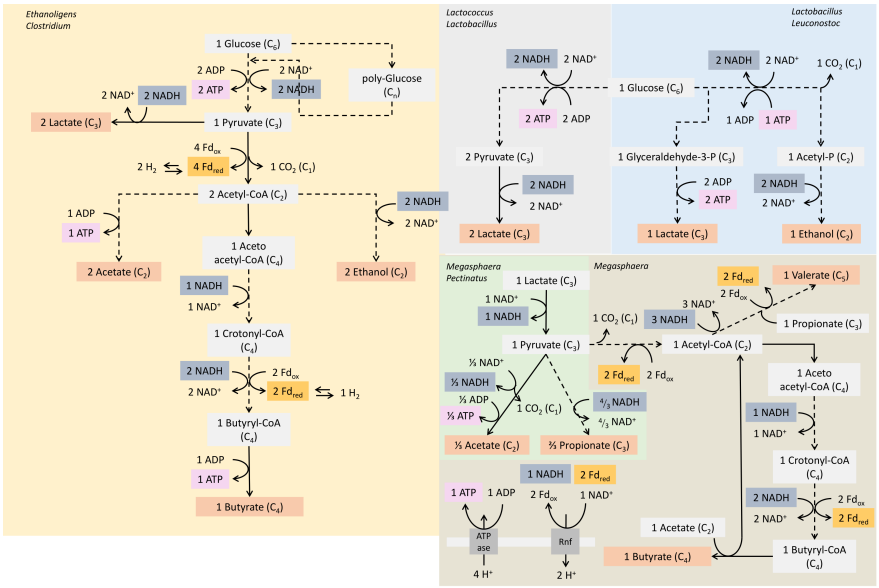
`Appendix.docx` available at https://authorea.com/users/285548/articles/409184-selecting-for-lactic-acid-producing-and-utilising-bacteria-in-anaerobic-enrichment-cultures



Enrichment	C-recovery (%)	COD-recovery (%)	H_2 yield (mol $Cmol^{-1}$)
Mineral	95% ± 3%	96% ± 3%	0.28 ± 0.00
Complex	113% ± 2%	118% ± 6%	0.02 ± 0.00







Food waste

Lactic acid bacteria
e.g. *Lactococcus*

Lactic acid

Paper mill effluent

Acetate-butyrate producing bacteria
e.g. *Clostridium*

VFAs
(butyrate, acetate, *etc*)