PRODUCTION OF VOLATILE FATTY ACIDS FROM SLAUGHTERHOUSE BLOOD BY MIXED-CULTURE FERMENTATION

Jersson Plácido[1,2*], Yue Zhang[1]

1 Water and Environmental Engineering Group, Faculty of Engineering and the Environment, University of Southampton, Southampton, United Kingdom

2 Institute of Life Science, Medical School, Swansea University, Swansea, SA2 8PP, Wales, UK

ABSTRACT

The volatile fatty acids (VFA) production potential from animal blood and the factors affecting this process were investigated in this study. In order to simulate an industrial process different operation modes, batch, fed batch and semi-continuous, were also evaluated. Due to high ammonia concentration in fermentation broth, VFA concentration up to 100 g L^{-1} was achieved without addition of buffer and methanogen inhibitor. In general, acetic, n-butyric and iso-valeric acids were the most predominant species, although different operational conditions affected the VFA concentration, profile, production rate and yield. The microbial community analysis was conducted on the reactors with the best performance, revealing that 70-90% of the microbial population was from the *Clostridiales* order with a strong presence from the *Sporanaerobacter*

[*]Corresponding author.
Tel.: +44 (0)7809158696;
E-mail address: j.e.placidoescobar@swansea.ac.uk

genus. These results demonstrated the feasibility of a VFA-platform bio-refinery using high-protein wastes as substrate via mixed-culture fermentation under non-sterilised conditions.

KEYWORDS

Mixed-culture fermentation; volatile fatty acids; slaughterhouse blood; enzymatic hydrolysis, bio-refinery.

1. INTRODUCTION

The European Union produces more than 40 million tonnes of meat every year [1], with pig meat being the most important source (51%) followed by poultry (30 %), bovine (17%), and sheep and goat meat (2%) [1]. Meat production is associated with the generation of different types of solid (intestines, fat, bowels, etc.) and liquid (blood, manure, washing water, etc.) wastes [2]. Livestock blood from slaughterhouses is a protein rich waste. As most protein rich waste stream, its organic fraction is principally composed of proteins (94.4%), lipids (0.3%), and carbohydrates (5.3%) [3]. In order to transform blood into biogas via anaerobic digestion without suffering from ammonia toxicity, different strategies have been tested including inoculum acclimation, dilution and/or co-digestion [3-5]. VFA, however, could still be accumulated significantly even with those measures in place. For instance, Zhang and Banks [4] reported that the VFA reached up to 15 g L^{-1} when digesters were fed with organic fraction of municipal solids waste and sheep blood at a ratio of 80:20 on a volatile solids basis. The production of considerable quantities of VFA induced by blood addition to digesters has only been regarded as detrimental to the process

previously: it is often difficult to reduce these VFA accumulations and re-start methanogenesis. This observation, however, can be applied for production of VFA as renewable bulk chemicals.

Other investigations have proved that it is possible to produce organic acids using anaerobic processes by inhibiting the methane production and acids consumption [6-9]. However, all these substrates were carbohydrate based residues such as paper wastes, lignocellulosic material, municipal solid wastes, etc. Protein rich residues such as slaughterhouse blood have not been evaluated as substrates in the production of VFA yet, although this waste stream has several advantages for VFA production: the ammonia produced from acidogenesis can inhibit methanogenesis, provide buffering capacity to fermentation process and supply nutrients to microbial biomass. Therefore, in principle there is no external methanogen inhibitor, buffer and nutrients required when using protein rich materials as substrate, thus the operating cost is minimised at this aspect.

The aim of this study was to prove that significant VFA production can be achieved under non-sterilised mixed-culture conditions using slaughterhouse blood as substrate. The experimental methodology was initially looked at the batch kinetics of acid production from blood under conditions in which methanogenic activity is inhibited, to determine rates of production and the ratio of different acid products. This provided a first indication of process feasibility. As syntrophy and other complex interactions in the systems biology exert a strong influence on product formation and speciation, much of the research effort was thus spent on determining yields and productivities in semi-continuous trials to assess the effects of process manipulations

in the systems biology, with the aim of maximising the accumulation of recoverable intermediate acid products.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Substrate

The blood solution used in this study was prepared by mixing one part of dried blood (Tongmaster Seasonings, Lanarkshire, UK) with six parts of deionised water. This solution had a total solids (TS) content of 14.3% and volatile solids (VS) content of 13.5% on a fresh matter basis, lower than the real slaughterhouse blood which usually has a TS of 18~20% [3, 4, 10]. This lowered concentration was applied in this study to improve the rheology of reactor mixing in orbital incubator.

2.1.2. Inoculum

There were two types of inoculum used in this study: one un-acclimated and one acclimated inoculum. The un-acclimated inoculum was collected from an anaerobic digester treating biosolids (Millbrook Wastewater Treatment Works, Southampton, UK). It was kept at room temperature and sieved through a 1 mm mesh before inoculation to remove large particles. The acclimated inoculum was taken from the semi-continuous fermenter SC1 (section 2.2.3), after it had been running for 3 hydraulic retention times (HRT) (21 days).

2.1.3. Enzymatic pretreatment

In order to exam if enzymatic pretreatment could improve VFA production, a 25 g L^{-1} trypsin solution from porcine pancreas (Sigma-Aldrich) was selected to hydrolyse blood proteins. The hydrolysis process was carried out at an initial enzyme load of 1% (v/v) [11] to the blood solution for 48 h at 20 ^{o}C. Constant mixing at 150 rpm was applied during the hydrolysis.

2.2. Fermentation experiments

All experiments listed below were conducted in borosilicate glass reactors. Each of them had a working volume of 1 L, and was sealed with a rubber bung through which a stainless-steel metal tube was inserted and connected to the gas collection bags. To maintain anaerobic conditions, the headspace of the reactors was purged with nitrogen gas when starting the experiments, and when reactors were opened for sampling or for adding iodoform solution. The reactors were kept in an orbital incubator at 37 ^{o}C and 150 rpm during the course of the experiment.

2.2.1. Batch experiments

As shown in Table 1, three operational variables were tested for 41 days using four reactors (B1-B4): inoculum acclimation (IA), methanogen inhibitor (MI), and enzymatic hydrolysis (EH). The initial volumetric inoculum and blood loading (BL) of this experiment was 10% and 90%, respectively by filling 100 mL of inoculum and 900 mL of blood solution into each of the reactors. This resulted in a blood loading of 122 g VS L^{-1} at the beginning of the experiment.

2.2.2. Fed-batch experiment

This experiment tested the effect of enzymatic hydrolysis on VFA production in fed-batch mode using two reactors: FB1: No-EH and FB2: EH (Table 1). Un-acclimated inoculum was used for this experiment, and no methanogen inhibitor was added during the course of the test. The initial reactor working volume was 450 mL including 45 mL of inoculum and 405 mL of blood solution (FB1) and hydrolysed blood solution (FB2), indicating an initial blood load of 122 g VS L^{-1}. The experiment was run in batch mode for the first 7 days, and then the fed-batch operation was initiated. In fed-batch mode, 50 mL of blood or freshly hydrolysed blood were added to the reactors every 2.3 days without removing fermentation broth. The fed-batch mode was maintained until day 30 when the reactors reached their maximum volume of 1 L, and then the fermentation was continued in batch mode again until day 45.

2.2.3. Semi-continuous experiment

This experiment assessed the effect of initial inoculum loading and enzymatic hydrolysis on VFA production in semi-continuous process with a hydraulic retention time (HRT) of 7 days. As fed-batch experiment, un-acclimated inoculum was used for this experiment, and no methanogen inhibitor was added during the course of the test. The test was run for 9 HRT (63 days) using four reactors, SC1-SC4. As shown in Table 1, two initial blood loading levels were tested: 90% and 33% (v/v), and each were used to ferment both untreated blood and freshly hydrolysed blood solutions. In this experiment, different start-up procedures were adopted for different initial blood loading. The reactors (SC2 and SC4) with initial blood loading of 33% were operated in semi-continuous mode from the beginning: 670 mL inoculum and 330 mL of blood solution (135

g blood VS L^{-1}) were filled in the reactors on day 0. Then, after every 2.3 days, 330 mL of fermentation broth was replaced with 330 mL of blood solution, corresponding to an average organic loading rate of 19 g VS L^{-1} day^{-1}. The reactors (SC1 and SC3) with initial blood loading of 90% were started in batch mode during the first HRT (7 days) to avoid the wash-out effect: 100 mL inoculum and 900 mL of blood solution were filled in the reactors on day 0, and then after 7 days the reactors were started semi-continuous operation as described above.

2.3. Analytical methods

2.3.1. VFA concentration

Samples were centrifuged at 14,000 g (micro-centrifuge, various manufacturers) for 20 minutes and then the supernatant was used for analysis. Where dilution was necessary, deionised water was used. Formic acid was then added to give a concentration of 10% of the total volume for analysis. The acidified solution was transferred into vials and loaded onto the GC auto-sampler. VFA quantification was performed by a Shimadzu GC-2010 gas chromatograph (Shimadzu, Milton Keynes, UK), using a flame ionisation detector and a capillary column type SGE BP-21. The GC oven temperature was programmed to increase from 60 to 210^{o}C in 15 minutes with a final hold time of 5 minutes.

2.3.2. Gas volume and composition

The fermentation gas was collected by gas-impermeable sampling bags during the fermentation process. Its volume was measured using a weight-type water displacement gasometer and

expressed as the volume at standard temperature (0 °C) and temperature (1 atm) [12]. Its composition was quantified using a Varian Star 3800 CX gas chromatograph (Varian Ltd, Oxford, UK) with a thermal conductivity detector. The GC was fitted with a Hayesep C column and employed argon as the carrier gas.

2.3.3. Soluble protein concentration

Samples were centrifuged at 14,000 g (micro-centrifuge, various manufacturers) for 20 minutes. The supernatant was then added to the Bradford reactive (Bio-Rad Protein Assay) and mixed thoroughly. After 5 minutes the mixture was read at 595 nm using a UV-Visible spectrophotometer (Cecil 3000 series, Cecil Instruments). The concentration was calculated by comparing against a Bovine serum albumin standard (Bio-Rad).

2.3.4. Total Ammonia Nitrogen (TAN) concentration

A sample aliquot was distilled using a Büchi K-350 Distillation Unit (Büchi, UK) and aqueous boric acid with mixed indicator as the receiving solution. The distillate was then titrated manually with 0.25 N H_2SO_4 using a digital titration system (Schott Titroline, Gerhardt UK Ltd). TAN concentration is expressed in units of g N L^{-1}, which represents the total concentration of free ammonia and ammonium ion in the sample.

2.3.5. TS and VS content

Approximately 10 g of homogenous sample was transferred into a weighed crucible. Samples were weighed to an accuracy of 1 mg (Sartorius LC6215 balance, Sartorius AG, Gottingen Germany) and placed in an oven (LTE Scientific Ltd., Oldham UK / Heraeus Function Line series, UK) for drying overnight at 105 ± 1 ^{o}C. Samples were then weighed and transferred to a muffle furnace (Carbolite Furnace 201, Carbolite, UK) and heated to 550 ± 10 ^{o}C for two hours. After this ashing step, samples were weighted to an accuracy of 1 mg.

2.3.6. DNA extraction and Sequencing

Microbial community analysis were carried out for reactors SC3 and FB2, with the sample from SC3 taken at the end of the seventh HRT and FB2 sample taken at the end of the process. The samples were stored at -20°C before DNA extraction. DNA was extracted from 200 mg of each sample using a freeze-thaw method. Microbial community analyses were performed based on the V4 region of the 16S rRNA gene [13-15]. Amplification was carried out using the primers as shown in Table 2. Illumina barcode oligonucleotides were used to barcode individual amplicons to allow their identification after sequencing. Next generation sequencing (NGS) of all amplicons was completed using the illumina MiSeq system with MiSeq Reagent Kit v3. The downstream analysis for taxonomic assignment was completed in three main steps: 1. Reduction of the dataset size with the selection of operational taxonomic units (OTUs). 2. The most abundant sequence in each OTU cluster was selected as the representative sequence for that cluster for downstream analysis; and 3. Taxonomy was assigned to each representative sequence by comparing it against the Green Genes 16S database.

3. RESULTS

As the preliminary work, blood loading, methanogens inhibition using iodoform and enzymatic hydrolysis using trypsin were evaluated in batch configuration (see supplementary material). A blood loading of 1% or 5% (v/v) could not inhibit the methanogenic activity and the VFA accumulated at the beginning of the test was consumed by day 7 and 20, respectively. At higher loadings, a continuous accumulation of VFA was observed until it reached a plateau. At loading of 15-50%, VFA increased until day seven, whereas the VFA accumulation extended to day 13 for loading 75 and 90%. The initial VFA accumulation rate in a reactor without iodoform addition was usually lower than that of its counterpart with iodoform at the same loading, probably due to the simultaneous VFA consumption by methanogens when the ammonia and/or VFA concentration was still low. This is the reason that caused the lower final concentrations when iodoform was not present. The presence of enzymatic hydrolysis did not alter the final concentration of VFA. The initial VFA production rate, however, was improved considerably. The VFA profiles were also different in reactors fed with hydrolysed blood, although the total VFA concentrations were at the same level. In the reactor with EH pretreatment, acetic and n-butyric acids were dominant, whereas n-butyric acid out-competed acetic acid in No-EH reactor. A blood loading of 90% was therefore chosen for the following experiments.

3.1. Batch fermentation experiment

The reactors B1 (Figure 1a) and B2 (Figure 1b) presented a similar VFA production pattern as the preliminary experiments described in the supplementary material. Using acclimated inoculum, B3 (Figure 1c) and B4 (Figure 1d) achieved a VFA concentration close to 100 g L^{-1}, indicating the importance of inoculum selection. No difference was detected on the effect of

methanogen inhibitor when using acclimated inoculum, because the methanogens had been inhibited and washed out before the fermentation broth was taken to inoculate B3 and B4. B4 had the fastest initial VFA production rate, and reached 60% of its final VFA concentrations in three days, following by B3 (5 d), B2 (10 d), and B1 (13 d). The high VFA production rate in B3 and B4 was attributed by the use of acclimated inoculum (B3 and B4), as well as the enzymatic pre-hydrolysis for B4. In all reactors, n-butyric and acetic acids were the most produced acids followed by iso-valeric, iso-butyric, propionic, and valeric acids. B3 had the highest concentration of n-butyric (35 g L^{-1}) and iso-valeric acids (23 g L^{-1}) and B4 achieved the highest concentration of acetic (30 g L^{-1}) and iso-butyric acids (13 g L^{-1}). The reactors using acclimated inoculum, however, had much less propionic acid concentration (< 2 g L^{-1}) than that of B1 and B2 (> 6 g L^{-1}).

In all reactors, the VFA production was linked with protein consumption and TAN production (Figure 2). As VFA production rate was different in different reactors, protein consumption rate was also different and reflected the VFA production kinetics. TAN production occurred at the same time as the reduction in protein concentration and the increase in VFA concentration, which provided a sufficient buffering capacity for all reactors and maintained their pH between 6.7 and 8.2 throughout the process. The TAN content reached a level of 5~7 g N L^{-1} towards the end of experiment.

3.2. Fed-batch experiment

The effect of enzymatic hydrolysis was also tested in fed-batch experiment, FB1: No-EH and FB2: EH (Figure 3). Although the EH pretreatment only affected the initial VFA production rate in batch mode; in fed-batch mode, it also influenced the final VFA concentration. The EH reactor (FB2) achieved a higher concentration (100 g L^{-1}) than the No-EH reactor (FB1) (80 g L^{-1}). The constant addition of hydrolysed blood generated similar VFA concentrations as the batch reactors using acclimated inoculum (100 g L^{-1}) and greater than the batch reactors using un-acclimated inoculum (70 g L^{-1}).

The acid profile was different in these two reactors. Acetic acid was the predominant VFA specie (32 % on a mass basis) in FB1, followed by n-butyric (25 %) and iso-valeric (20 %) acid. Acetic and n-butyric acid had equal 30% share in FB2 when pre-hydrolysis applied, but the percentage of iso-valeric acid was still in the third place (21%). Both reactors had a same iso-butyric acid percentage of 12%. Propionic acid was the fifth dominant species, and accounted for 10% and 6% in FB1 and FB2, respectively. The VFA profile resulted from pre-hydrolysed blood was the same in both batch and fed-batch fermentation processes, however the operation mode seemed to affect the percentage of acetic and n-butyric acid when the substrate was not pre-treated.

A same correlation between protein consumption and TAN and VFA production was observed in fed-batch reactors (Figure 3c), as shown in batch reactors. FB2 consumed proteins faster than FB1 at initial days, and this trend was maintained for the rest of the experiment duration. The

30% higher TAN concentration in FB2 compared with that of FB1 also reflected upon their different VFA production level. Fed-batch fermentation also maintained a stable pH (6.9-7.4).

Similar to VFA and TAN production, the fermentation gas production also reflected blood consumption (Figure 3d). As in batch reactors, the principal gas produced from both fed-batch reactors was CO_2, followed by CH_4 and H_2. FB2 formed a greater amount of gas than FB1, along with its higher VFA production. There was clear CH_4 production from FB1 after feeding stopped, probably because the TAN level in that reactor was not sufficient high to inhibit methanogens. H_2 content was no more than 0.2 % after the start-up stage in both reactors.

3.3. Semi-continuous experiment

There were 4 reactors used for semi-continuous experiment: SC1: BL90% and No-EH, SC2: BL33% and No-EH, SC3: BL90% and EH, and SC4: BL33% and EH. As shown in Figure 4, SC1 reactor had the highest concentration of VFA (Figure 4a) during the initial days of the fermentation (80 g L^{-1}), and its VFA production was stable during the first 4 HRTs. In SC1, acetic (25 g L^{-1}) and n-butyric acids (25 g L^{-1}) had the highest concentrations, followed by iso-valeric (17 g L^{-1}), iso-butyric (10 g L^{-1}), propionic (2 g L^{-1}), valeric (0.22 g L^{-1}) and hexanoic acids (0.05 g L^{-1}). Valeric and hexanoic acids were only detected during the batch phase and the following two HRTs. After the fourth HRT, the SC1 reactor showed a steady reduction in the production of n-butyric acid, although the production of the other acids was stable until the seventh HRT. During the last two HRTs, the production of these acids was also reduced in similar pattern as that of n-butyric acid. The SC2 reactor exhibited the second highest production

of VFA (70 g L^{-1}) after the SC1 reactor. SC2 formed all the VFAs during the first HRT (Figure 4b), however, when the reactor was more stable it only produced acetic (30%), propionic (5%), n-butyric (30%), iso-butyric (15%), and iso-valeric acids (20%). The total VFA production and its profile in SC2 were roughly stable during the entire course of the experiment. The SC3 reactor was the steadiest reactor (Figure 4c), but its VFA production (40 g L^{-1}) was lower than other reactors. The VFA profile in SC3 was similar to SC1 and SC2, with acetic (30%), propionic (5%), n-butyric (30%), iso-butyric (15%), and iso-valeric (20%) acids. No significant drop in VFA production was found in this reactor. SC4 reactor had a different and more dynamic behaviour compared with other reactors. This reactor exhibited a very high VFA peak of 85 g L^{-1} towards the end of the second HRT, and the VFA concentration declined to a stable level of around 50 g L^{-1} from the fourth HRT until the end of the fermentation. The acid composition was similar to the other reactors, acetic (30%), propionic (5%), n-butyric (30%), iso-butyric (15%), and iso-valeric (20%) acids; although at the initial HRTs a larger proportion of acetic acid was obtained.

SC1 exhibited a steady protein consumption (Figure 5a) when the reactor was having a stable production of VFA (the first 7 HRTs), after that, the protein concentration increased from approximately 2 g L^{-1} to 40 g L^{-1} towards the end of experiment. As a result, SC1 had a TAN concentration between 4 and 6 g L^{-1} during the first 7 HRTs but it decreased to 2.5 g L^{-1} after that (Figure 5a). The protein consumption in SC2 was stable during the majority of the fermentation process, although an increase in protein concentration was observed at the final HRT. This trend was also confirmed by the ammonia concentration and pH of this reactor. TAN had a concentration between 3.5 and 5 g L^{-1}, and the pH fluctuated between 6.6 and 7 in SC2

before the final HRT. In SC3 (Figure 5c), the soluble protein (60 g L^{-1}), was higher than that of the other reactors and its TAN concentration was lower (2.8-3.5 g L^{-1}), reflecting the less efficient VFA production. Low protein concentration (1 g L^{-1}) and high TAN concentration (5 g L^{-1}) were obtained in SC4 during the first two HRTs. After that, protein concentration increased and stabilised at a concentration around 55 g L^{-1}, and the TAN concentration decreased to values between 3 and 3.5 g L^{-1} in the meanwhile. The steady-state performance of SC4 was similar as that of reactor SC3, indicating that a negative effect was created by the combination of a semi-continuous operation and the enzymatic pretreatment. Along with its function of enzymatic hydrolysis for protein-rich substrate, trypsin also has potential to cleaving microbial cell surface proteins and affecting its functioning over time (Huang et al. 2010). It appeared that this antagonistic effect became evident after two weeks of operation, as shown in Figure 4. This did not affect batch fermentation because all VFA had already been produced over the initial two weeks of operation; however, this caused severe damage to long-term continuous system.

The SC1 gas phase (Figure 5b) mainly composed of CO_2 followed by H_2. CH_4 was not detected in this reactor after the semi-continuous mode started. The reactor SC2 (Figure 5b) produced smaller but constant amounts of CO_2 and H_2. Similar to reactor SC1, CO_2 was the gas with the greatest concentration and CH_4 was not found after the initial phase. SC3 achieved lower amounts of CO_2, CH_4, and H_2 (Figure 5d) compared with other reactors. This reactor generated all three gases during the entire course of experiment, and the principal gas was CO_2, followed by CH_4, and H_2. The initial gas production in reactor SC4 was the greatest among all reactors, but the gas production was reduced drastically later on. SC4 also produced all three gases throughout the fermentation process. It is worth noting that the CH_4 produced in SC3 and SC4

was negligible compared with the loss of VFA production potential, and therefore it is not the cause of the reduced VFA production in these two reactors.

Table 3 shows the daily VFA production yield calculated using equations in the supplementary material. SC1 and SC2 had the higher average daily VFA production rate and yield, but they were less stable. This evidenced the wide difference between their maximum and minimum production rates and their standard deviation, with propionic acid having the largest variation. SC1 and SC2 had daily average yields above 45%, whereas, SC3 and SC4 were below 40 %. The low yield of SC3 (30%) correlates with the results exhibited in Figure 4 and 5. In general, the VFA yield of semi-continuous reactors (30-50%) was lower than that of batch (55-60%) and fed-batch (60-70%) reactors, due to the shorter retention time of semi-continuous experiment.

3.4. Microbial community analysis

The microbial community analysis was performed for the reactors FB2 (EH) and SC3 (BL 90% and EH), where the reactor FB2 achieved the highest VFA yield, and the reactor SC3 was the most stable reactor in the semi-continuous operation. In both samples, 99.6% of the sequences were assigned to five phyla, Firmicutes, Proteobacteria, Synergistetes, Actinobacteria, and Bacteroidetes (Figure 6). The additional 0.4% of the sequences, however, was not allocated to any specific phylum. Among the five phyla identified, Firmicutes was the most dominant one with more than 99.5% abundance of their individual microbial community; this value indicates the strong participation of this phylum in the conversion of proteins to VFA in mixed-culture anaerobic fermentation. At the order level (Figure 6), both samples had more than 20 orders

present: 8 and 9 of them were from the Firmicutes phylum in FB2 and SC3, respectively. Six orders were identified in this phylum: Bacillales, Lactobacillales, Clostridiales, MBA08, Natranaerobiales, Thermoanaerobacterales and two (FB2) and three (SC3) unclassified orders. Where the unclassified orders were identified in upper taxa, two of them were from the clostridia and OPB450 class and another one with only Firmicutes phylum assignation. The major difference between both reactors occurred in the Clostridiales order. In both reactors, the order of Clostridiales obtained the greatest percentage (SC3: 98.6%, FB2: 71.8%), but the community structure of this order was different in these two reactors: FB2 has seven genus with more than 1% of sequences assigned, whereas, SC3 had only a subsection of them (*Sporanaerobacter sp.*, *Tepidimicrobium* sp., and *Clostridium sp*.). Among them, *Sporanaerobacter sp* was the genus that included the majority of the sequences in FB2 (49%) and SC3 (92%), and its high participation indicated that members from this genus were closely associated with the conversion of proteins to VFA. The second major difference between two reactors lied with the order of Thermoanaerobacterales. The abundance of this order in SC3 was just 0.53%, compared with 24.6% in FB2. Although Thermoanaerobacterales is the second largest order in FB2, 99.9% of its sequences were assigned to two unidentified families.

4. DISCUSSION

The VFA production using mixed-culture anaerobic fermentation of slaughterhouse blood was demonstrated feasible. The results obtained in this study using slaughterhouse blood as singular substrate achieved the highest VFA concentration up to 100 g L^{-1}. This VFA concentration is several times greater than the reported values (10~20 g L^{-1}) by previous studies using other types of waste streams. For instance, the VFA concentrations reached up to 20 g L^{-1} when using

370 lignocellulosic material such as paper wastes [6], and maize silage [16] as substrates. Waste
371 biomass such as food waste (Resulting VFA concentration: 20 g L^{-1}) [17], mixture of waste
372 activated sludge and henna plant biomass (11 g L^{-1}) [18], glycerol (16 g L^{-1}) [8], and cheese
373 whey (20 g L^{-1}) [16] have also been used for VFA production, with concentrations considerably
374 lower than what obtained in this study with slaughterhouse blood. The high VFA concentration
375 achieved in this work can be attributed to the sufficient buffering capacity in the fermenters
376 which is provided by the high concentration of TAN in the system [4]. Therefore, compared with
377 lignocellulosic substrates, one main advantage of using high nitrogen substrates such as
378 slaughterhouse blood is the simultaneous TAN and VFA production during acidogenesis. The
379 resulting high TAN concentration in the fermentation broth provides two important functions,
380 i.e. buffering capacity and methanogens inhibition, which are usually achieved by external input
381 when lignocellulosic materials are in use [6-9]. These additional requirements increase the
382 operating cost and the order of process complexity in terms of ancillary units and system control.

383

384 Compared with anaerobic digestion for biogas production, the selection of the substrate loading
385 (e.g. substrate to inoculum ratio for batch mode or organic loading rate for (semi-)continuous
386 mode) has less constrains with regard to fermentative VFA production. The substrate to
387 inoculum ratio is usually less than 1 on a VS basis for batch digestion [19] and less than 8 g VS
388 L^{-1} day^{-1} for continuous digestion operation [20]. This is because the conversion rates of
389 acidogenesis, acetogenesis and methanogenesis have to be well-controlled in that process to
390 prevent the accumulation of intermediate production such as VFA and the inhibition of
391 methanogens. This is, however, not the case for VFA production via fermentation. As can be
392 concluded from the preliminary experiment (supplementary material) and semi-continuous trial,

393 the higher blood loading (corresponding to higher substrate to inoculum ratio or higher organic
394 loading rate) provided higher VFA production rate and final VFA concentration, also inhibited to
395 a greater extent the methane production. The highest volumetric substrate to inoculum loading
396 used in this study was 9, corresponding to a ratio of 50 on a VS basis and a final VFA
397 concentration up to 100 g L^{-1}. The lower final VFA concentration (< 20 g L^{-1}) from other studies
398 using wastes such as food waste [17], maize silage, cheese whey, and microalgae biomass [16]
399 may be caused by the low substrate to inoculum ratio used (50% inoculum on a volume
400 basis)[16].

401

402 Enzymatic hydrolysis appeared to have a positive effect on batch and fed-batch operation and the
403 hydrolysed substrate was degraded to VFA at faster rate and to a greater extent, and especially
404 facilitated acetic acid production. Its negative effect, however, was detected in the semi-
405 continuous reactors (SC3 and SC4): the VFA concentration drop correlated with the trypsin
406 concentration. In batch operation, trypsin was only added in the initial load and its activity
407 decreased through the fermentation as results of trypsin denaturation, microbial trypsin
408 inhibitors, or microbial proteases [21, 22]. In contrast, in the semi-continuous reactors the
409 continuing addition of hydrolysed blood with trypsin into reactors generated a negative effect by
410 degrading proteins of microorganisms [23, 24]. In the fed-batch reactors, the addition rate of
411 hydrolysed blood with trypsin was much lower than that of the semi-continuous reactors;
412 therefore, its activity reduction rate was probably higher than the amount of trypsin addition
413 avoiding the negative effects observed in the semi-continuous reactors. Therefore for (semi)-
414 continuous operation, the other pre-treatment methods should be used to facilitate the rate and

extent of VFA production, such as heat-alkaline pre-treatment [25] and ultraviolet pre-treatment [26].

The microbial community analysis proved the importance of the Firmicutes phylum and its Clostridiales order in the transformation of proteins into VFA at high concentrations, consistent with the results of Bacteria community composition analysis of digester using pig blood as substrate [5]. The differences between the fed-batch and semi-continuous reactor community may be explained by the duplication rate of relevant microorganisms. In fed-batch operation, the duplication rate is not a selection mechanism because the medium is not removed from the reactor; therefore, microbes with low duplication rate but adaptability to the reactor conditions can flourish. In contrast, the semi-continuous reactor requires microorganisms with a doubling time much shorter than the HRT, otherwise they are just washed out of the reactor over time.

In terms of operation mode comparison, the batch fermenters with acclimated inoculum and the fed-batch fermenters achieved the highest concentration of VFA (100 g L^{-1}) at an initial blood loading of 122 g blood VS L^{-1}, indicating a conversion rate of 0.82 g VFA g^{-1} VS. This high level of VFA concentration was expected mainly because the duration of the experiments (45 days) allowed the conversion process to proceed to a greater extent than the semi-continuous mode. The reduced concentration of VFA produced in semi-continuous fermenters versus fed-batch was caused by the much shorter hydraulic retention time (7 days). The best-performed semi-continuous reactor (SC2) achieved a total VFA production of 444 g of VFA L^{-1} over the initial 45 days of its operation, which corresponding to a conversion of 0.51 g VFA g^{-1} blood VS.

The production capacity of the semi-continuous operation was therefore considerably longer than that of the batch and fed-batch reactors. It is important to point out, that in batch processes 80% of the total VFA production is achieved by day 10. If the batch fermentation is performed only during the initial 10 days a larger VFA production can be achieved. Batch processes can be improved by reducing the fermentation time, although this VFA increment is achieved at the expense of lower blood conversion rate. Similarly, to improve semi-continuous production, it is necessary to evaluate if longer HRT can result in a more efficient process in the semi-continuous reactors.

The result obtained in this study demonstrates a fermentation process capable to produce a VFA concentration much higher than the feasibility threshold for an industrial process. The MixAlco process is a technology that transforms any biodegradable material in to alcohol based principally on VFA production. The MixAlco process needs a minimum VFA concentration of 22 g L^{-1} to achieve the low cost for VFA fermentation and purification processes [9, 27]. The VFA production achieved in this research by the transformation of slaughterhouse blood is 2~5 times higher than that threshold level. The high VFA production achieved has also stimulated new research on alternative feasible industrial downstream processing, and the preliminary study on the initial steps of VFA recovery was performed using esterification and a membrane solvent based extraction [28]. Both techniques achieved similar recovery comparable to other purification processes [29-31], and each of them had specific features to be preferably integrated with different operation mode(s) demonstrated in this research. Batch and fed-batch processes were more suitable for esterification recovery because untransformed blood proteins was remained in fermentation froth was much less compared with semi-continuous fermentation.

460 The advantage of this approach is the possibility of producing VFA methyl esters and
461 ammonium sulphate simultaneously, with the first an important product for the scents and
462 fragrance industry, and the second a well-known fertiliser. The principal drawback of
463 esterification recovery is that it requires a high VFA concentration, (above 80%). In contrast, the
464 solvent based membrane system is a better fit for the continuous fermentation process because it
465 selectively recovers butyric and iso-valeric acids (>80%) in favour of acetic acid (5 %), reduces
466 the product-induced inhibition effect on longer-chain VFA, and allows the utilisation of acetic
467 acid in recycled stream. Additionally, this is a method is a less expensive option compared with
468 water removal processes such as distillation. The issue of this method is the use of organic
469 solvents (octanol); although adding a stripper membrane step into the recovery process can
470 recover and reuse these solvents. In the future research, the economic feasibility of the process
471 needs to be confirmed by using process-modelling software.

472

473 **5. CONCLUSIONS**

474 Anaerobic mixed-culture fermentation is a suitable technology to transform slaughterhouse blood
475 into high concentrations of VFA (45-100 g L^{-1}). In this process, the VFA produced were mainly
476 acetic, n-butyric, iso-valeric, iso-butyric and propionic acids. Blood loading and inoculum
477 acclimation were the main factors that influenced the VFA concentration. Enzymatic hydrolysis
478 improved the initial production rate; however, it did not affect the final VFA concentration and
479 was inhibitory in the semi-continuous reactors. Iodoform increased the VFA production in the
480 low blood loading and modified the VFA profile in the high blood loadings. The batch and semi-
481 continuous reactor generated good results in terms of total VFA production and yield, and the
482 selection of operation mode should be based on the analysis of the economic and logistic

parameters and the downstream recovery unit of the system. These results pave a way for the development of future cost-effective processes to produce VFA from slaughterhouse blood and to increase the economic and environmental feasibility of a VFA bio-refinery from high protein wastes.

6. CONFLICTS OF INTEREST

There are no conflicts to declare

7. ACKNOWLEDGEMENTS

The authors thank the UK Biotechnology and Biological Sciences Research Council (BBSRC) and the Anaerobic Digestion network (ADNet) for funding this project (POC2014016). Dr. Jersson Plácido would like to thank the financial support provided by the European Regional Development Fund / Welsh Government funded BEACON research program (Swansea University). Data supporting this study are openly available from the University of Southampton repository at http://doi.org/10.5258/SOTON/xxxxxx.

8. REFERENCES

1. Marquer P, Rabade T, Forti R (2014) Meat production statistics. Eurostat. http://ec.europa.eu/eurostat/statistics-explained/index.php/Meat_production_statistics#Further_Eurostat_information 2015.

2. Marcos A, Al-Kassir A, Mohamad AA, Cuadros F, López-Rodríguez F (2010) Combustible gas production (methane) and biodegradation of solid and liquid mixtures of meat industry wastes. Appl Energy 87:1729-1735. doi: http://dx.doi.org/10.1016/j.apenergy.2009.09.037.

3. Hejnfelt A, Angelidaki I (2009) Anaerobic digestion of slaughterhouse by-products. Biomass Bioenergy 33:1046-1054. doi: http://dx.doi.org/10.1016/j.biombioe.2009.03.004.

4. Zhang Y, Banks CJ (2012) Co-digestion of the mechanically recovered organic fraction of municipal solid waste with slaughterhouse wastes. Biochem Eng J 68:129-137. doi: http://dx.doi.org/10.1016/j.bej.2012.07.017.

5. Kovács E, Wirth R, Maróti G, Bagi Z, Rákhely G, Kovács KL (2013) Biogas production from protein-rich biomass: fed-batch anaerobic fermentation of casein and of pig blood and associated changes in microbial community composition 8:e77265

6. Forrest AK, Hollister EB, Gentry TJ, Wilkinson HH, Holtzapple MT (2012) Comparison of mixed-acid fermentations inoculated with six different mixed cultures. Bioresour Technol 118:343-349. doi: http://dx.doi.org/10.1016/j.biortech.2012.05.043.

7. Fu Z, Holtzapple MT (2010) Consolidated bioprocessing of sugarcane bagasse and chicken manure to ammonium carboxylates by a mixed culture of marine microorganisms. Bioresour Technol 101:2825-2836. doi: 10.1016/j.biortech.2009.11.104.

8. Forrest AK, Sierra R, Holtzapple MT (2010) Effect of biodiesel glycerol type and fermentor configuration on mixed-acid fermentations. Bioresour Technol 101:9185-9189. doi: http://dx.doi.org/10.1016/j.biortech.2010.07.041.

9. Domke SB, Aiello-Mazzarri C, Holtzapple MT (2004) Mixed acid fermentation of paper fines and industrial biosludge. Bioresour Technol 91:41-51. doi: http://dx.doi.org/10.1016/S0960-8524(03)00156-1.

10. Bah CS, Bekhit AEA, Carne A, McConnell MA (2013) Slaughterhouse blood: an emerging source of bioactive compounds 12:314-331

11. Sigma-Aldrich (2013) Product Information: Trypsin solution from porcine pancreas. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/1/t4549pis.pdf. Accessed March/28 2018.

12. Walker M, Zhang Y, Heaven S, Banks C (2009) Potential errors in the quantitative evaluation of biogas production in anaerobic digestion processes. Bioresour Technol 100:6339-6346

13. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A 108 Suppl 1:4516-4522. doi: 10.1073/pnas.1000080107 [doi].

14. Apprill A, McNally S, Parsons R, Webe L (2015) Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton

15. Parada AE, Needham DM, Fuhrman JA (2015) Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environ Microbiol

16. Jankowska E, Chwialkowska J, Stodolny M, Oleskowicz-Popiel P (2017) Volatile fatty acids production during mixed culture fermentation–The impact of substrate complexity and pH. Chem Eng J 326:901-910

17. Stein UH, Wimmer B, Ortner M, Fuchs W, Bochmann G (2017) Maximizing the production of butyric acid from food waste as a precursor for ABE-fermentation. Sci Total Environ 598:993-1000

18. Huang J, Zhou R, Chen J, Han W, Chen Y, Wen Y, Tang J (2016) Volatile fatty acids produced by co-fermentation of waste activated sludge and henna plant biomass. Bioresour Technol 211:80-86

19. Raposo F, Fernández- Cegrí V, De la Rubia M, Borja R, Béline F, Cavinato C, Demirer G, Fernández B, Fernández- Polanco M, Frigon J (2011) Biochemical methane potential (BMP) of solid organic substrates: evaluation of anaerobic biodegradability using data from an international interlaboratory study 86:1088-1098

20. Liu C, Wang W, Anwar N, Ma Z, Liu G, Zhang R (2017) Effect of organic loading rate on anaerobic digestion of food waste under mesophilic and thermophilic conditions. Energy Fuels 31:2976-2984

21. Murthy S, Kostman J, Dinoso VP (1980) Effect of pH, substrate, and temperature on tryptic activity of duodenal samples. Dig Dis Sci 25:289-294

22. Grenier D (1991) Production of trypsin and chymotrypsin inhibitors by oral bacterial isolates. J Periodont Res 26:381-387

23. Goldberg AL (1972) Correlation between rates of degradation of bacterial proteins in vivo and their sensitivity to proteases. Proc Natl Acad Sci U S A 69:2640-2644

24. Sekirov I, Russell SL, Antunes LC, Finlay BB (2010) Gut microbiota in health and disease. Physiol Rev 90:859-904. doi: 10.1152/physrev.00045.2009 [doi].

25. Wang X, Li Y, Liu J, Ren N, Qu J (2016) Augmentation of protein-derived acetic acid production by heat-alkaline-induced changes in protein structure and conformation. Water Res 88:595-603

26. Xiao N, Chen Y, Ren H (2013) Altering protein conformation to improve fermentative hydrogen production from protein wastewater. Water Res 47:5700-5707. doi: http://dx.doi.org/10.1016/j.watres.2013.06.047.

27. Holtzapple MT, Davison RR, Ross MK, Aldrett-Lee S, Nagwani M, Lee C, Lee C, Adelson S, Kaar W, Gaskin D (1999) Biomass conversion to mixed alcohol fuels using the MixAlco process. Appl Biochem Biotechnol 79:609-631

28. Plácido J, Zhang Y (2017) Evaluation of Esterification and Membrane Based Solvent Extraction as Methods for the Recovery of Short Chain Volatile Fatty Acids from Slaughterhouse Blood Anaerobic Mixed Fermentation:1-11

29. Lopez AM, Hestekin JA (2013) Separation of organic acids from water using ionic liquid assisted electrodialysis 116:162-169. doi: http://dx.doi.org/10.1016/j.seppur.2013.05.028.

30. Ormerod D, Sledsens B, Vercammen G, Van Gool D, Linsen T, Buekenhoudt A, Bongers B (2013) Demonstration of purification of a pharmaceutical intermediate via organic solvent nanofiltration in the presence of acid 115:158-162. doi: http://dx.doi.org/10.1016/j.seppur.2013.05.007.

31. Lubsungneon J, Srisuno S, Rodtong S, Boontawan A (2014) Nanofiltration coupled with vapor permeation-assisted esterification as an effective purification step for fermentation-derived succinic acid. J Membr Sci 459:132-142. doi: http://dx.doi.org/10.1016/j.memsci.2014.02.006.

Table 1. Factors evaluated in Batch, Fed-batch and Semi-continuous experiments

1-liter reactor experiment in batch mode[1]			
Reactor	Factors evaluated		
	Inoculum Acclimation	Enzymatic hydrolysis	Methanogen inhibitor
B1	No	No	No
B2	No	No	Yes
B3	Yes	No	No
B4	Yes	Yes	No
Fed-batch experiment[1,2]			
Reactor	Factors evaluated		
	Enzymatic hydrolysis		
FB1	No		
FB2	Yes		
Semi-continuous experiment[2,3]			
Reactor	Factors evaluated		
	Inoculum Loading	Enzymatic Hydrolysis	
SC1:	10%	No	
SC2	67%	No	
SC3	10%	Yes	
SC4	67%	Yes	

1- The initial blood load was 122 g VS L^{-1} for batch and fed-batch experiments.

2-The fed-batch and semi-continuous experiments did not have methanogen inhibitor.

3- The initial blood load was 122 g VS L^{-1} for reactor SC1 and SC3 when it was run in batch mode for the first 7 days, and then their organic loading rate was 19 g VS L^{-1} day^{-1} in semi-continuous mode. Reactors SC2 and SC4 were run in semi-continuous mode from day 1 and their organic loading rate was 19 g VS L^{-1} day^{-1} as well.

Table 2. Primers'sequence used in the pyrosequencing analysis

Nex_16S_515 F[1]	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-3'
Nex_16S_806 R[1]	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT-3'

1-Primer sequences are underlined, with illumina Nextera Transposase adapters attached

Table 3. Daily VFA production rates and yields of the semi-continuous experiment.

Volatile Fatty Acids	Production rate (g $L^{-1} \cdot d^{-1}$)											
	SC1			SC2			SC3			SC4		
	Average	Max.	Min.	Average	Max.	Min.	Average	Max.	Min.	Average	Max.	Min.
Acetic acid	2.8±1.4	5.0	0.1	3.0±0.8	4.7	1.1	1.6±0.5	2.8	0.9	2.6±1.3	5.8	1.2
Propionic acid	0.2±0.4	1.2	-0.3	0.4±0.5	1.6	0.01	0.2±0.2	0.8	0.02	0.4±0.4	1.4	0.1
Iso-butyric acid	1.3±0.5	1.7	0.1	1.2±0.4	2.0	0.034	0.7±0.2	1.2	0.4	1.0±0.5	1.9	0.5
n-Butyric acid	2.4±1.2	5.2	0.3	2.4±1.1	4.6	-0.7	1.8±0.6	3.5	0.5	2.1±0.9	4.7	0.6
Iso-valeric acid	2.1±0.9	3.4	-0.2	1.9±0.8	3.6	-0.4	1.2±0.3	1.9	0.5	1.5±0.7	3.3	0.5
Total VFA	8.8±3.5	12.9	0.8	9.0±2.8	12.7	0.5	5574±1659	8.7	3.0	7.6±3.5	16.5	4.0
Volatile Fatty Acids	Yield (g^{-1} VFA g^{-1} VS)											
	SC1			SC2			SC3			SC4		
	Average	Max.	Min.	Average	Max.	Min.	Average	Max.	Min.	Average	Max.	Min.
Acetic acid	0.149±0.073	0.337	0.009	0.164±0.050	0.279	0.049	0.086±0.018	0.125	0.056	0.138±0.063	0.260	0.061
Propionic acid	0.010±0.016	0.052	0.000	0.021±0.023	0.072	0.001	0.012±0.012	0.048	0.001	0.024±0.021	0.077	0.004
Iso-butyric acid	0.067±0.024	0.103	0.005	0.067±0.026	0.133	0.002	0.039±0.009	0.060	0.021	0.051±0.024	0.122	0.021
n-Butyric acid	0.126±0.058	0.234	0.014	0.127±0.062	0.222	0.000	0.094±0.031	0.157	0.022	0.111±0.056	0.314	0.025
Iso-valeric acid	0.111±0.047	0.195	0.000	0.107±0.052	0.242	0.000	0.063±0.015	0.087	0.023	0.080±0.040	0.188	0.023
Total VFA	0.464±0.178	0.690	0.037	0.488±0.175	0.844	0.024	0.294±0.075	0.473	0.136	0.404±0.185	0.887	0.181

Figure 1. VFA profiles over the course of the 1-L batch experiment: a) reactor B1; b) reactor B2; c) reactor B3; d) reactor B4. The embedded images correspond to the VFA composition for each reactor.

Figure 2. Soluble protein and TAN concentration in 1-L batch experiment: a) B1 and B2; B) B3 and B4.

Figure 3. Fed-batch experiment: a) VFA profile of reactor FB1; the embeded image corresponds to the VFA composition. b) VFA profile of reactor FB2; the embeded image corresponds to the VFA composition. c) Protein consumption and TAN concentration. d) Cumulative gas production and pH.

Figure 4. VFA profiles of the semi-continuous experiment: a) reactor SC1; b) reactor SC2; c) reactor SC3; d) reactor SC4.

Figure 5. Soluble protein, TAN, gas produciton and pH of semi-continuous experiment: a) Soluble protein and TAN concentrations of reactors SC1 and SC2. b) Gas production and pH of reactors SC1 and SC2; gas produciton data represent the CO2, H2 and CH4 produced during each of SC2 feeding cycle. c) Soluble protein and TAN concentrations of reactors SC3 and SC4. d) Gas produciton and pH of reactors SC3 adn SC4; gas production data represent the CO2, H2 and CH4 produced during each of SC4 feeding cycle.

Figure 6. Percentage of sequences assigned to order; the embedded plot is the sequences assigned to the genus level for the Clostridiales order: a) reactor SC3; b) reactor FB2.

Figure 1

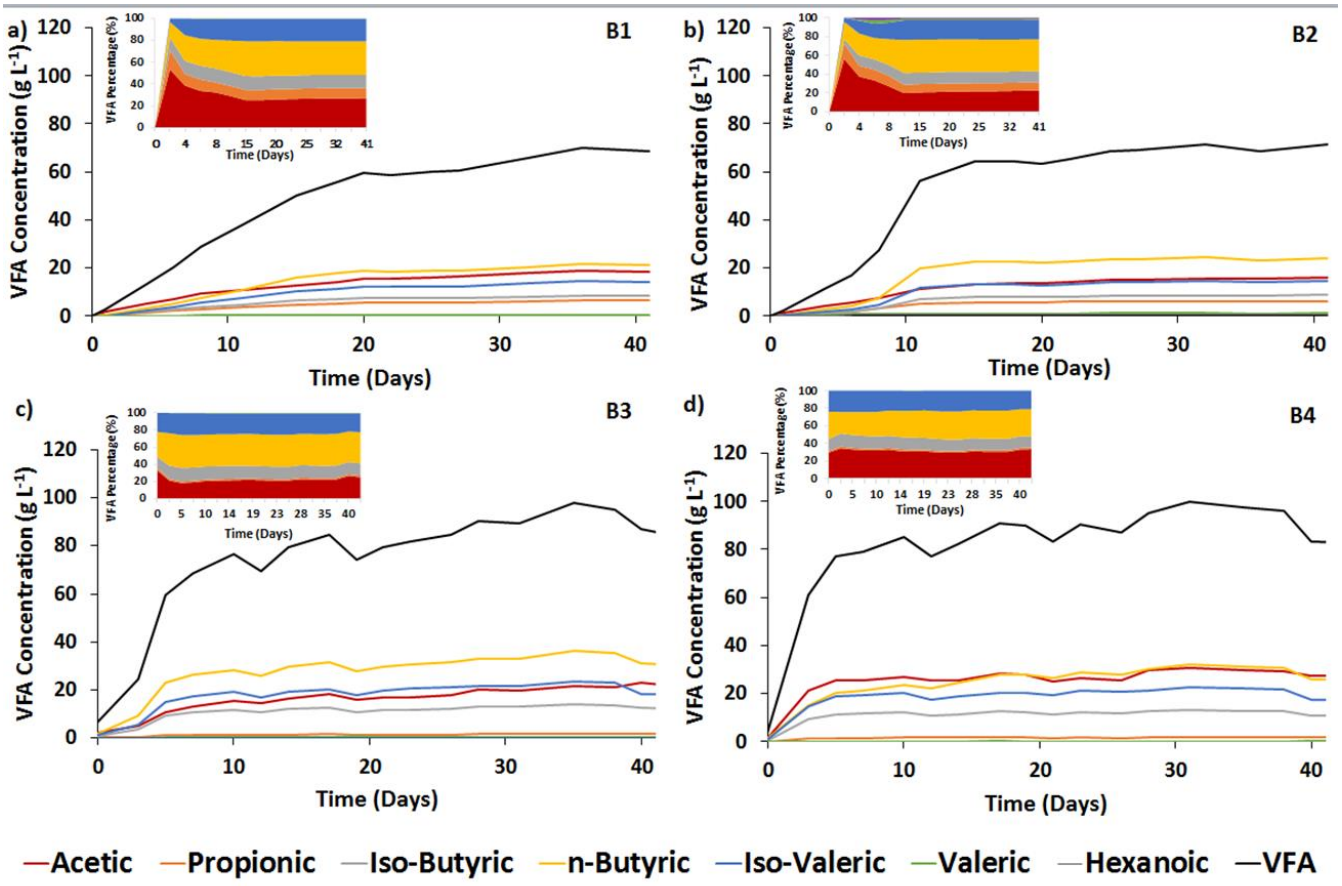

a)
B1
b)
B2
c)
B3
d)
B4
VFA Concentration (g L-1)
Time (Days)
VFA Percentage (%)
Acetic
Propionic
Iso-Butyric
n-Butyric
Iso-Valeric
Valeric
Hexanoic
VFA

Figure 2

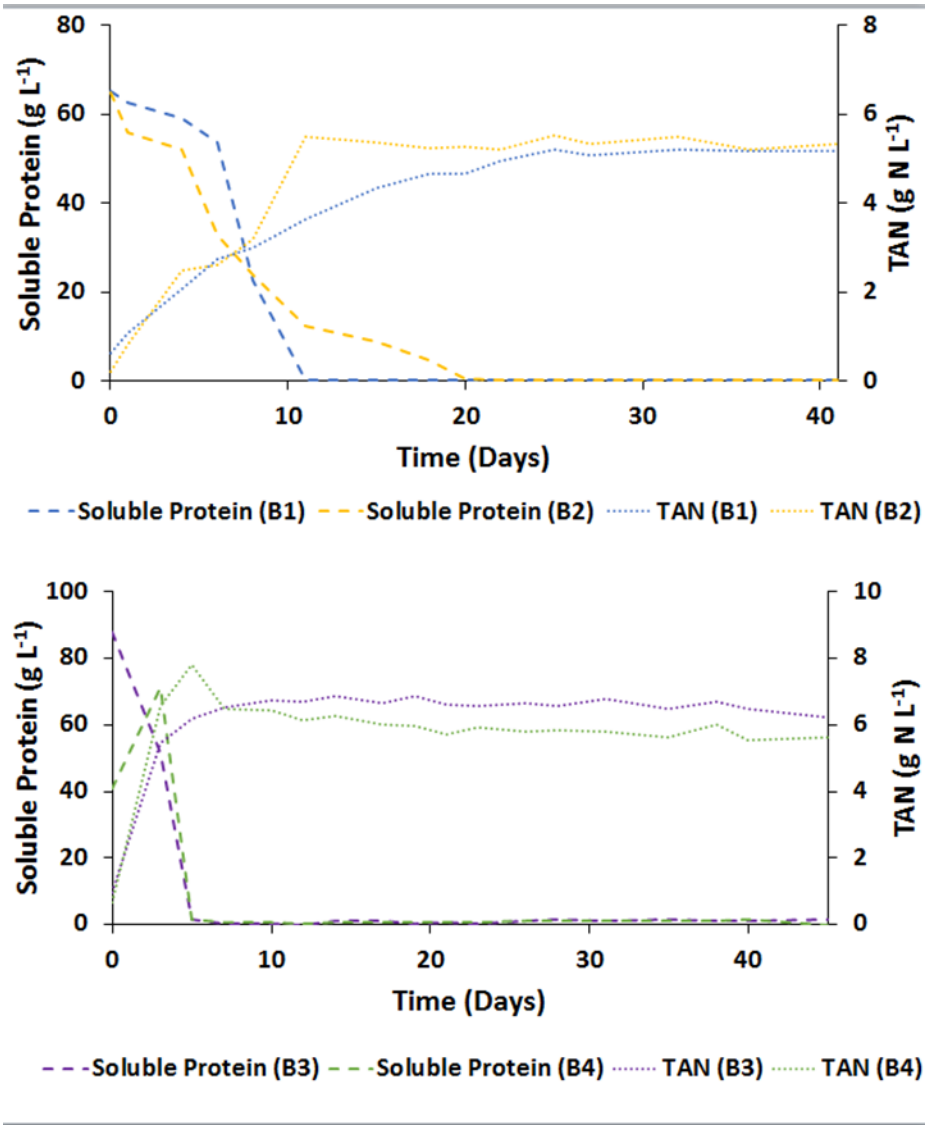

Soluble Protein (g L-1)
TAN (g N L-1)
Time (Days)
Soluble Protein (B1)
Soluble Protein (B2)
TAN (B1)
TAN (B2)
Soluble Protein (B3)
Soluble Protein (B4)
TAN (B3)
TAN (B4)

Figure 3

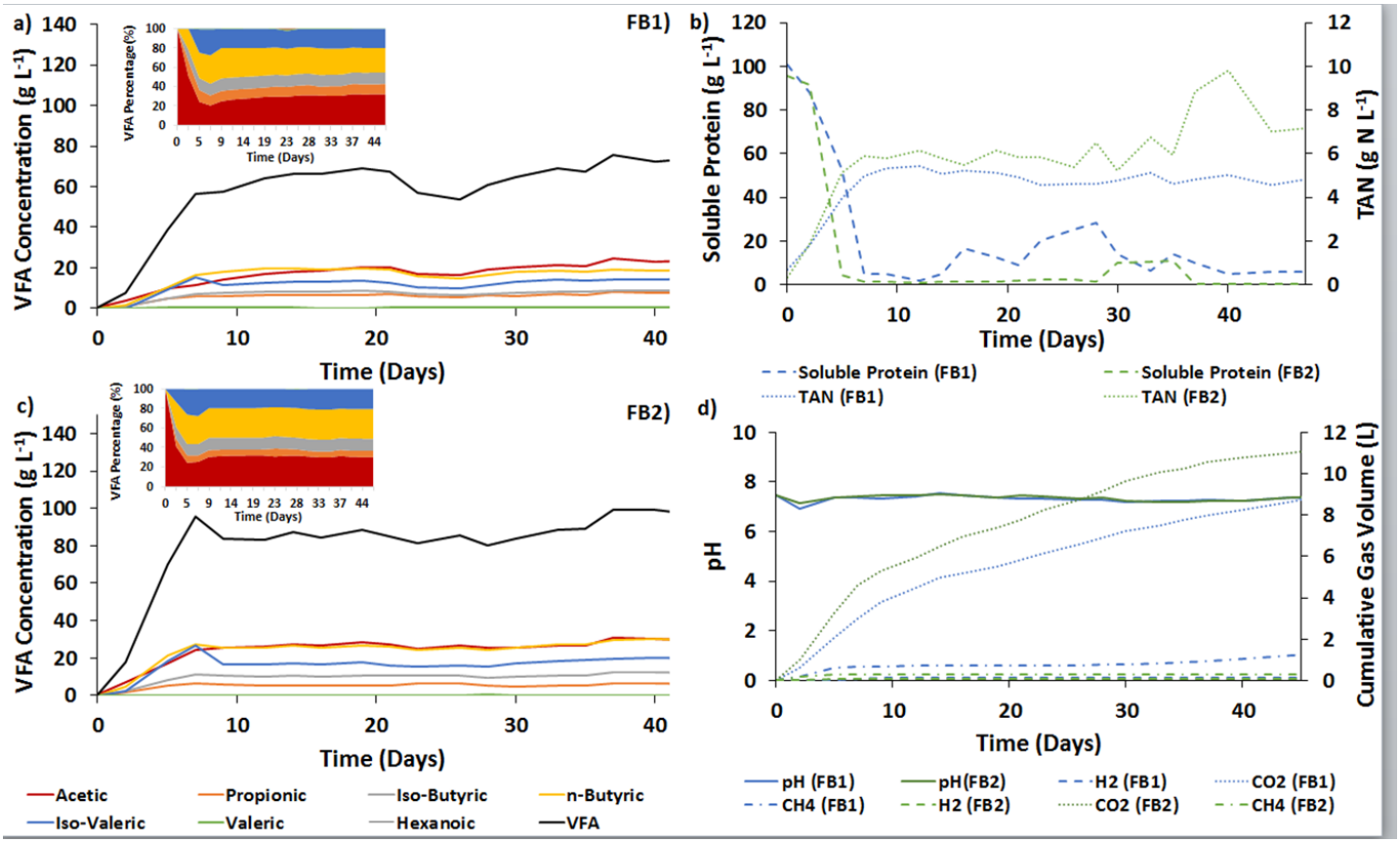

a)
FB1)
VFA Concentration (g L-1)
VFA Percentage (%)
Time (Days)
b)
Soluble Protein (g L-1)
TAN (g N L-1)
Time (Days)
Soluble Protein (FB1)
Soluble Protein (FB2)
TAN (FB1)
TAN (FB2)
c)
FB2)
VFA Concentration (g L-1)
VFA Percentage (%)
Time (Days)
d)
pH
Cumulative Gas Volume (L)
Time (Days)
pH (FB1)
pH(FB2)
H2 (FB1)
CO2 (FB1)
CH4 (FB1)
H2 (FB2)
CO2 (FB2)
CH4 (FB2)
Acetic
Propionic
Iso-Butyric
n-Butyric
Iso-Valeric
Valeric
Hexanoic
VFA

Figure 4

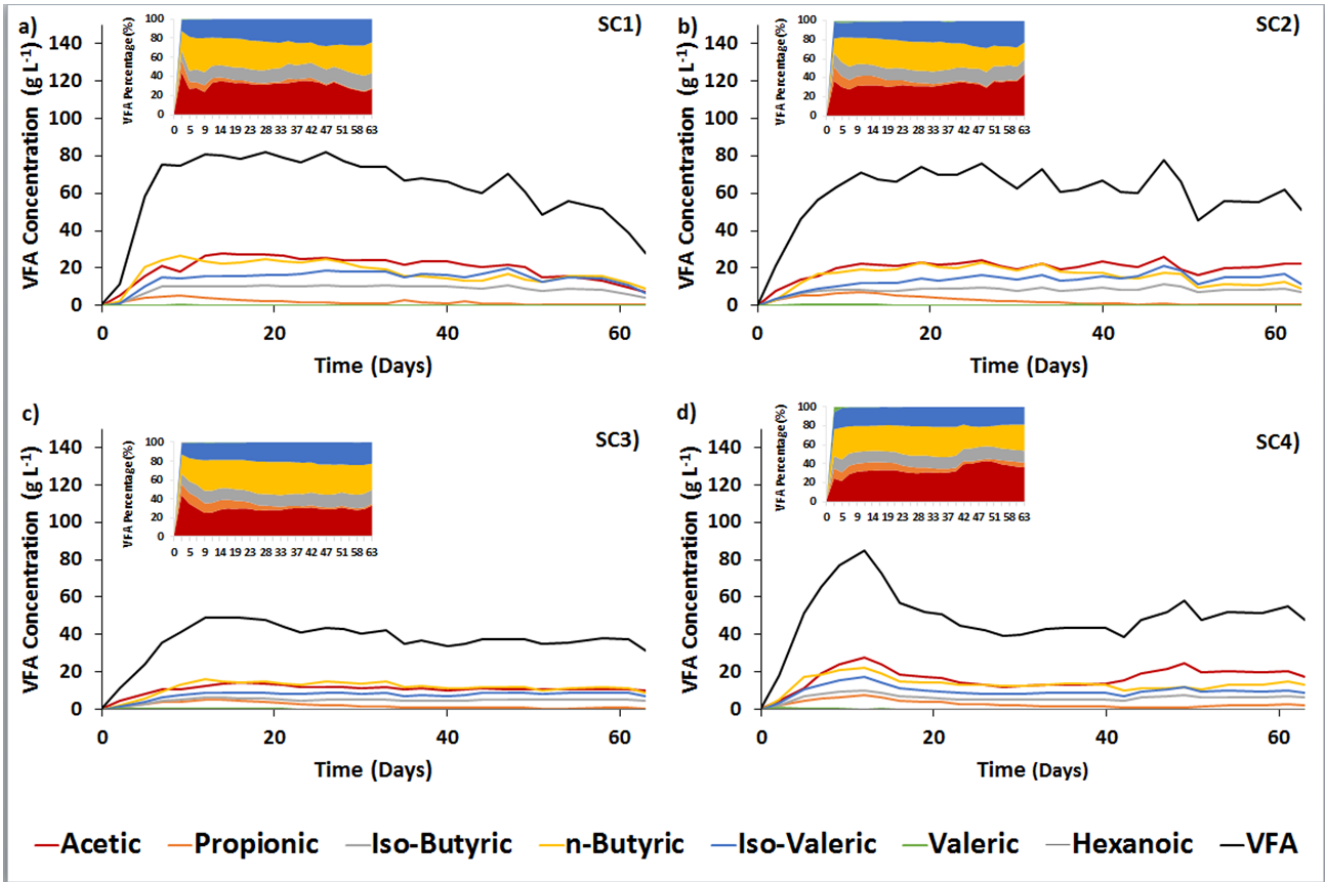

a)
SC1)
b)
SC2)
c)
SC3)
d)
SC4)
VFA Concentration (g L-1)
Time (Days)
VFA Percentage (%)
Acetic
Propionic
Iso-Butyric
n-Butyric
Iso-Valeric
Valeric
Hexanoic
VFA

Figure 5

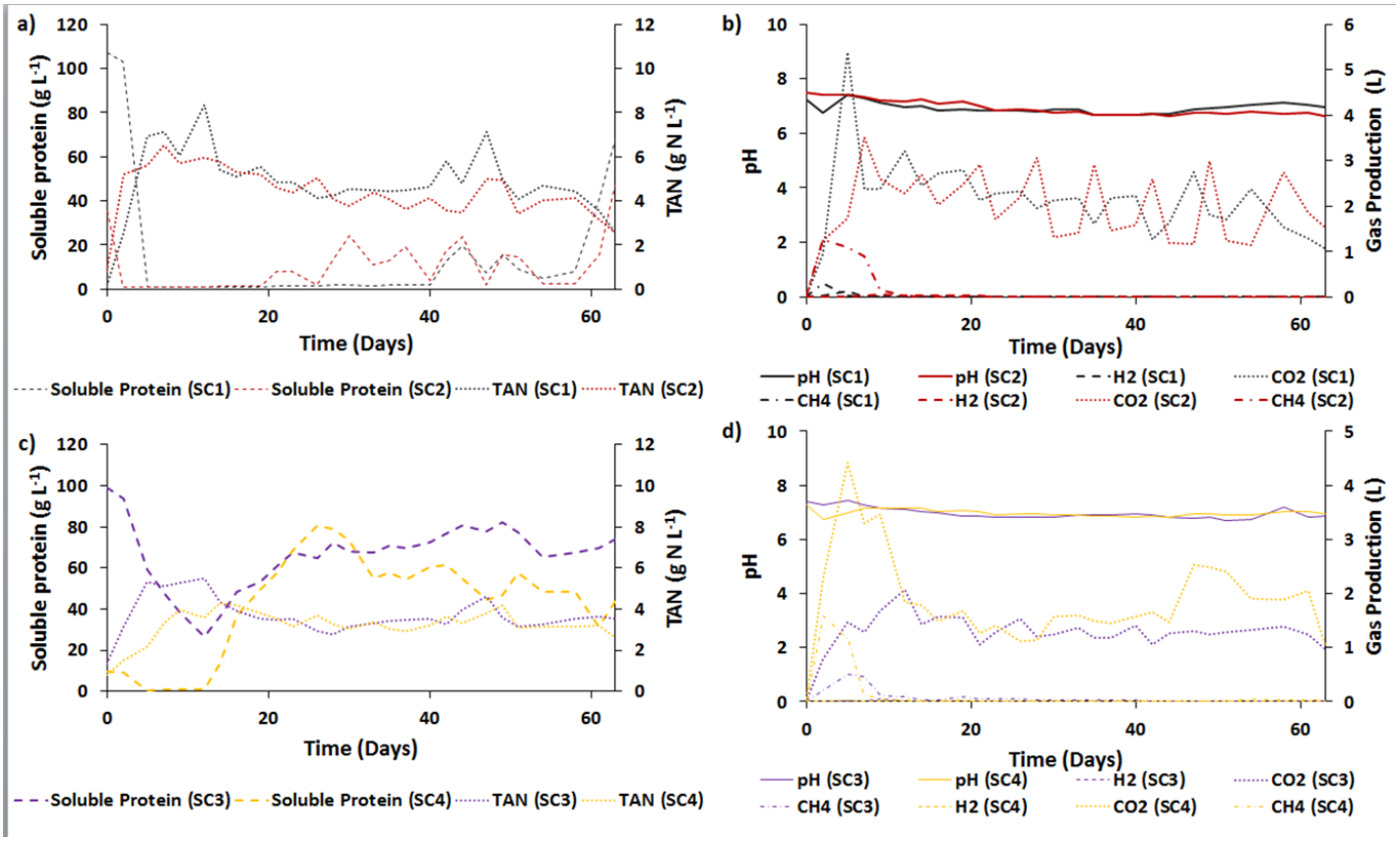
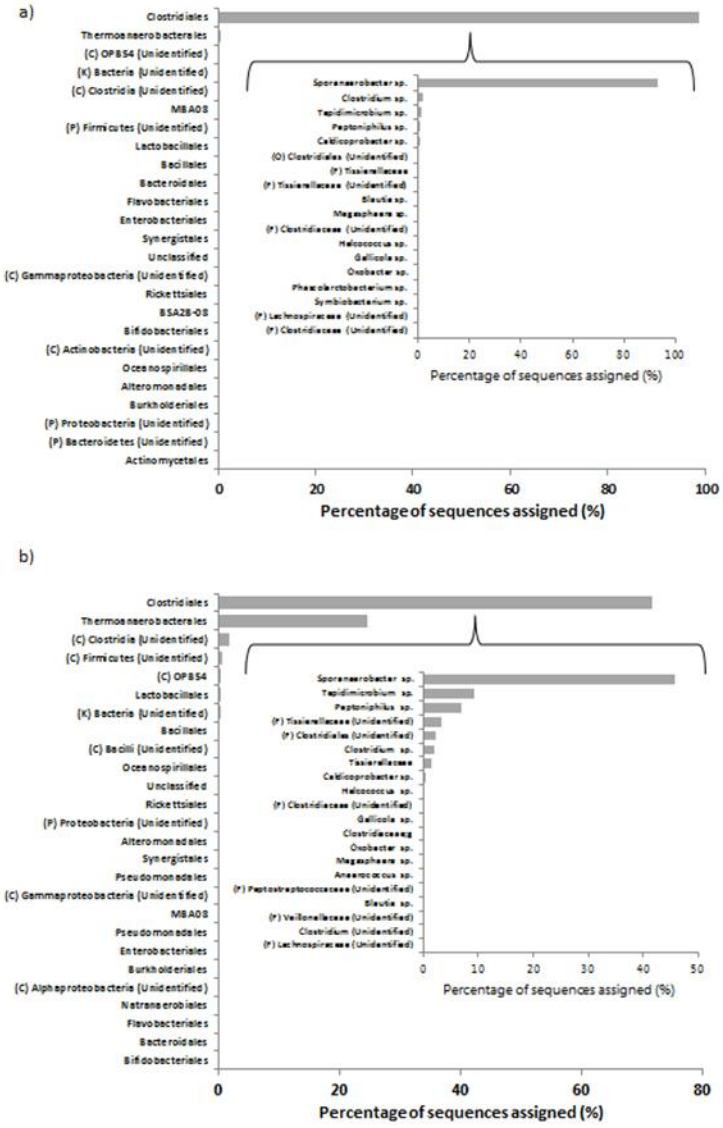

a)
Soluble protein (g L-1)
TAN (g N L-1)
Time (Days)
Soluble Protein (SC1)
Soluble Protein (SC2)
TAN (SC1)
TAN (SC2)
b)
pH
Gas Production (L)
Time (Days)
pH (SC1)
pH (SC2)
H2 (SC1)
CO2 (SC1)
CH4 (SC1)
H2 (SC2)
CO2 (SC2)
CH4 (SC2)
c)
Soluble protein (g L-1)
TAN (g N L-1)
Time (Days)
Soluble Protein (SC3)
Soluble Protein (SC4)
TAN (SC3)
TAN (SC4)
d)
pH
Gas Production (L)
Time (Days)
pH (SC3)
pH (SC4)
H2 (SC3)
CO2 (SC3)
CH4 (SC3)
H2 (SC4)
CO2 (SC4)
CH4 (SC4)

Figure 6


a)
Clostridiales
Thermoanaerobacterales
(C) OPB54 (Unidentified)
(K) Bacteria (Unidentified)
(C) Clostridia (Unidentified)
MBA08
(P) Firmicutes (Unidentified)
Lactobacillales
Bacillales
Bacteroidales
Flavobacteriales
Enterobacteriales
Synergistales
Unclassified
(C) Gammaproteobacteria (Unidentified)
Rickettsiales
BSA28-08
Bifidobacteriales
(C) Actinobacteria (Unidentified)
Oceanospirillales
Alteromonadales
Burkholderiales
(P) Proteobacteria (Unidentified)
(P) Bacteroidetes (Unidentified)
Actinomycetales
0
20
40
60
80
100
Percentage of sequences assigned (%)
Sporanaerobacter sp.
Clostridium sp.
Tepidimicrobium sp.
Peptoniphilus sp.
Caldicoprobacter sp.
(O) Clostridiales (Unidentified)
(F) Tissierellaceae
(F) Tissierellaceae (Unidentified)
Blautia sp.
Megasphaera sp.
(F) Clostridiaceae (Unidentified)
Helcococcus sp.
Gallicola sp.
Oxobacter sp.
Phascolarctobacterium sp.
Symbiobacterium sp.
(F) Lachnospiraceae (Unidentified)
(F) Clostridiaceae (Unidentified)
0
20
40
60
80
100
Percentage of sequences assigned (%)
b)
Clostridiales
Thermoanaerobacterales
(C) Clostridia (Unidentified)
(C) Firmicutes (Unidentified)
(C) OPB54
Lactobacillales
(K) Bacteria (Unidentified)
Bacillales
(C) Bacilli (Unidentified)
Oceanospirillales
Unclassified
Rickettsiales
(P) Proteobacteria (Unidentified)
Alteromonadales
Synergistales
Pseudomonadales
(C) Gammaproteobacteria (Unidentified)
MBA08
Pseudomonadales
Enterobacteriales
Burkholderiales
(C) Alphaproteobacteria (Unidentified)
Natranaerobiales
Flavobacteriales
Bacteroidales
Bifidobacteriales
0
20
40
60
80
Percentage of sequences assigned (%)
Sporanaerobacter sp.
Tepidimicrobium sp.
Peptoniphilus sp.
(F) Tissierellaceae (Unidentified)
(F) Clostridiales (Unidentified)
Clostridium sp.
Tissierellaceae
Caldicoprobacter sp.
Helcococcus sp.
(F) Clostridiaceae (Unidentified)
Gallicola sp.
Clostridiaceae g
Oxobacter sp.
Megasphaera sp.
Anaerococcus sp.
(F) Peptostreptococcaceae (Unidentified)
Blautia sp.
(F) Veillonellaceae (Unidentified)
Clostridium (Unidentified)
(F) Lachnospiraceae (Unidentified)
0
10
20
30
40
50
Percentage of sequences assigned (%)