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Derivation of volatile fatty acid from crop residues digestion using a rumen membrane bioreactor: a feasibility study

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Abstract

This study evaluates the feasibility of a novel rumen membrane bioreactor (rumen-MBR) to produce volatile fatty acids (VFA) from crop residues (i.e. lignocellulosic biomass). Rumen-MBR can provide a sustainable route for VFA production by mimicking the digestive system of ruminant animals. Rumen fluid was inoculated in a reactor coupled with ultrafiltration (UF) membrane and fed with maize silage and concentrate feed at 60:40% (w/w). Continuous VFA production was achieved at an average daily yield of 438 mg VFA/g substrate. The most abundant VFA species were acetic (40-80%) and propionic (10-40%) acids. The majority (73 ± 15%) of produced VFA was transferred through the UF membrane. Shifts in dominant rumen microbes were observed upon the transition from *in vivo* to *in vitro* environment and during reactor operation, however, stable VFA yield was maintained for 35 days, providing the first proof-of-concept of a viable rumen-MBR.

Keywords: volatile fatty acids, lignocellulosic biomass, anaerobic digestion, membrane bioreactor.

1. Introduction

Crop residues (i.e. lignocellulosic biomass) present an abundant, inexpensive, renewable and yet untapped carbon-based resource. It is estimated that 146 billion metric tons of lignocellulosic biomass are generated globally each year (Balat & Ayar, 2005). Lignocellulosic biomass can be converted into a variety of valuable products including biofuel, bio-based materials and chemicals; thus can serve as an alternative for fossil fuels in energy and chemical production (Sawatdeenarunat et al., 2015). Utilization of lignocellulosic biomass does not interfere with food production and also incur lower cost compared to other important biofuel feedstocks (Nanda et al., 2015). The major challenge hindering lignocellulosic biomass application is the recalcitrant structure consisting of cellulose encapsulated in a hemicellulose-pectin-lignin matrix (Sawatdeenarunat et al., 2015). Ruminant animals such as cows and sheep can readily digest lignocellulosic biomass thanks to their unique rumen's microbial community. Rumen fluid harbours bacteria with superior cellulolytic activities (e.g. *Ruminococcus flavefaciens* and *Ruminococcus albus*) and rhythmic coordination between different functional groups (Christopherson et al., 2014; Flint et al., 2008). Each cellulolytic bacterial strain as well as fungi genus target specific component(s) of lignocellulosic biomass and the combination of different strains ensure complete substrate hydrolysis. The growth of cellulolytic bacteria is promoted as fermenters and acetogens utilize hydrolysis products to generate VFA and hydrogen. The produced VFA (mainly include acetic, propionic and butyric acid) is continuously absorbed by the animal to produce energy (up to 80% of total energy provided to the host animal), while hydrogen is consumed by hydrogenotrophic methanogens during methanogenesis. Hydrogen removal by methanogens also favours the activity of fermentative and acetogenic bacteria (Nguyen et al., 2019b).

Exploiting rumen's microbial community is an attractive approach for VFA production from lignocellulosic biomass (Lazuka et al., 2015; Lueangwattanapong et al., 2020). Previous studies on this topic have demonstrated promising results with VFA yield of up to 377 mg/g substrate (Hu & Yu, 2005; Nguyen et al., 2019b; Njokweni et al., 2019). High VFA yield can lead to VFA accumulation and trigger pH drop that is detrimental to microorganism growth. Nguyen et al. (2019b) studied rumen digestion of four different lignocellulosic substrates and observed a rapid increase in VFA concentration to (12,000 mg/L after 2 days of incubation), followed by a pH drop and process inhibition. Thus, continuous removal of the produced VFA from an engineered rumen reactor is required to achieve long-term operation.

Membrane separation is a potential solution for continuous VFA recovery from the reactor. Membrane can provide effective solids/liquids separation, and has shown prominent capacity to separate fermentative products from fermentative broth (Aydin et al., 2018; Pan et al., 2018; Zacharof et al., 2016). Membrane can also completely prevent the washout of microbes from the reactor based on membrane pore size, which is beneficial for slow-growing rumen microbes. It is envisaged that the combination of rumen microbes with membrane separation in a reactor for continuous generation and removal of VFA can sustain stable operation. The transfer of VFA into a clean matrix (permeate) also facilitates subsequent purification as the final products. Thus, a new rumen membrane bioreactor (rumen-MBR) is proposed to alleviate microbe washout and VFA accumulation in the reactor.

This study aims to develop and evaluate the feasibility of a rumen-MBR to derive VFA from lignocellulosic biomass. The VFA yield was determined under similar conditions to the cow's natural rumen and VFA produced was continuously extracted using an ultrafiltration (UF) membrane module. The composition of the VFA produced and their transfer ratios through the UF membrane module were also investigated. Amplicon sequencing of the 16S rRNA marker gene on the Illumina Miseq platform was performed to elucidate how rumen's

microbial community changes during the rumen MBR continuous operation and subsequent impacts on VFA yield. Results from this study provide proof of concept of an engineered system to generate VFA from lignocellulosic biomass.

2. Materials and Methods

2.1. Preparation of substrate, rumen fluid and artificial saliva

The rumen MBR was fed with a mixture of maize silage and concentrate feed at 60:40% (w/w) representative of typical cow's diet. These substrates were obtained from The University of Sydney, Corstorphine (Camden Farm Dairy, Cobbitty, NSW 2570, Australia). Substrates were ground into powder using an electrical blender and stored in zip-locked bags at room temperature until use. The volatile solids (VS), chemical oxygen demand (COD), moisture, and ash content of maize silage were 87.8%, 1.022 kg/kg, 7.8 and 4.4%, respectively. The high moisture content in maize silage is similar to other lignocellulosic biomass. These substrates were characterized for moisture, volatile solids (VS), ash content and chemical oxygen demand (COD). The VS, COD, moisture, and ash content of concentrate feed were 81.2%, 1.325 kg/kg, 7.3 and 1.5%, respectively. The VS contents of both substrates were above 80%, indicating substantial levels of organic matters presented. The chemical composition of concentrate feed consists of non-fibre carbohydrates (52. 2%), crude fat (2.7%), crude protein (15.5%) and neutral detergent fibre (20.8%).

Rumen fluid was collected from a 12-year old fistulated cow 2 h after feeding and used as the rumen microorganism's source. This rumen collection procedure was in accordance with The University of Sydney Animal Ethics Committee (Approved Protocol number 2015/835). The fistulated cow was housed at The University of Sydney, Corstorphine (Camden Farm Dairy, Cobbitty, NSW 2570, Australia). Rumen fluid was strained through two layers of cheesecloth to remove any coarse materials and then stored in insulated thermos bottles that had been pre-heated with warm water to maintain a temperature of approximately 39 ºC during

transportation to the laboratory and used immediately. The pH, total solids (TS), VS, and total COD of the rumen fluid were 6.95, 2.79%, 1.85%, and 27.5 g/L, respectively.

A modified version of the McDougall artificial saliva was used to control pH in the rumen MBR. McDougall saliva solution has been regularly used to control pH in the rumen simulation technique system (Ramos et al., 2018). The modified saliva contains (g/L) of Na_2HPO_4 1.845, $NaHCO_3$ 4.9, NaCl 0.235, KCl 0.285, $MgCl_2.6H_2O$ 0.0305 and $CaCl_2.2H_2O$ 0.0168.

2.2. Rumen membrane bioreactor

A 3 L Lambda Minifor fermenter (Lambda Pty Ltd, Czech Republic) was used as the rumen MBR (E-supplementary data). The reactor was equipped with two peristaltic pumps (i.e. saliva and permeate pump), an overhead mixer, a redox-temperature-pH probe, a temperature control unit and a submerged hollow fibre membrane module. The module was plotted using epoxy resin (Selleys Araldite Ultra Clear, Australia) comprising 20 PVDF fibres (Dupont, Australia) of 30 cm in length and 0.04 μm in pore size. The effective surface area of the membrane module was approximately 0.02 m^2.

Rumen fluid (1 L) was mixed with saliva (1 L), and 20 g of maize silage: concentrate (60:40 % w/w) to start up the rumen MBR. Pure N_2 gas was purged into the reactor until the oxygen redox potential dropped below -300 mV, indicating the anaerobic condition. The mixture of rumen microorganisms, saliva and substrates were continuously agitated at 150 rpm with one three-bladed propeller. The temperature control unit was set at 39 °C to simulate the rumen temperature. The feed and permeate pump were continuously operated to achieve a hydraulic retention time (HRT) of 57 h, corresponding to an operating flux of 2.07 L/m^2h. The operating flux was kept low to avoid significant membrane fouling in the rumen membrane reactor.

On a daily basis, 100 mL of reactor content was withdrawn from the reactor and 2 g of the substrate mixture in 100 mL of saliva was fed into the reactor to provide a loading rate (LR) of 1 g/L per day (equivalent to 1.14 kg COD/m^3 per day). The low LR was applied to avoid insufficient mixing of the substrate and microbial biomass as well as the possible overloading of the system. The solids retention time (SRT) in the rumen MBR was 480 h.

The rumen MBR was operated for 44 days. The pH and biogas volume were continuously recorded. VFA, TS, VS and soluble COD (sCOD) were measured twice a week.

2.3. Analytical method

Moisture, TS, VS and ash content were determined according to Standard Methods 1684. Briefly, one gram of substrate was transferred into a ceramic bowl and dried at 100 °C for 24 h. The ceramic bowl was then allowed to cool to room temperature in a desiccating glass chamber. The weight of ceramic bowl and material was recorded and used to calculate TS. Then the ceramic bowl was heated to 550 °C in a furnace for 15 min. The residual weight was recorded and used to calculate moisture, VS and ash content.

Total COD and sCOD concentration were measured by using digestion vials (Hach, Australia) and Hach DR3900 spectrophotometer program number 435 COD HR, following the US-EPA Standard Method 5220 D.

Samples (50 mL) of rumen MBR content and permeate were taken for quantification of VFA. Samples were centrifuged at 8,300 rpm for 5 minutes and the supernatants were filtered through 1.2 µm filter. The filtrates were mixed with metaphosphoric acid (25% w/v, 5:1 v/v) and centrifuged at 12,000 rpm for 2 minutes on a microcentrifuge (Sigma-Aldrich, Germany). The supernatant (1.2 mL) of was mixed with 0.2 mL crotonic acid solution (0.1 M), vortexed and allowed to stand at room temperature for 30 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes and the supernatants were transferred to a clean 1.5 mL auto-sampler vial. The VFA determination on Agilent technology 7820A gas-liquid

chromatography system, using a DB-FFAP column of dimensions 30 m × 0.32 mm × 1.00 mm, installed with a flame ionization detector set up at 250 °C, airflow 350 mL/min, H_2 fuel flow 30 mL/min, makeup flow (N_2) 30 mL/min Split Inlet heated to 225 °C, 9.526 PSI, Helium total flow 33 mL/min, septum purge flow 3 mL/min, split ratio 5:1, Split Flow 25 mL/min. The oven temperature was set to 150 °C and held for 1 min, then 5 °C per minute up to 195 °C and sustained for 3 min (Forwood et al., 2019). VFA concentration as mg/L, VFA yield, extent of acidification and transfer ratio of VFA through the UF membrane were calculated as described in the E-supplementary data.

2.4. DNA extraction and quality monitoring

Duplicate samples of the inoculum were collected at the beginning of the experiment duplicate samples of rumen MBR content were collected weekly for microbial community profiling. The inoculum/reactor content was mixed with 100% v/v ethanol (1:1 v/v) and stored at -20 °C prior to DNA extraction. Genomic DNA extraction was carried out using QIAamp DNA Stool Mini Kit (Qiagen) following the manual's instructions. The integrity, purity and concentration of the extracted DNA were evaluated by NanoDrop® spectrophotometer. DNA concentration of all samples was normalized to 20 ng/µl using DNase/Pyrogen-Free Water before sending to the sequencing facility.

2.5. Amplicon sequencing and bioinformatics analysis

The universal primer set Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3') was used to amplify 16S rRNA V3 – V4 regions of both bacterial and archaeal communities (Takahashi et al., 2014). Paired-end amplicon sequencing (2 × 300 bp) was carried out on the Illumina MiSeq platform (Australian Genome Research Facility, Melbourne, Australia). Raw sequence data were generated with the Illumina *bcl2fastq* pipeline (version 2.20.0.422).

Raw reads were analysed according to Quantitative Insights into Microbial Ecology (QIIME) 2 (version 2019.10) pipeline (Bolyen et al., 2019). In brief, reads were denoised using DADA2 with the following parameters: trunc-len-f = 280, trucn-len-r = 280, trim-left-f = 17, trim left-r = 21, min-fold-parent-over-abundance = 8 and all other parameters as the default setting. Taxonomy assignment was performed against the SILVA database (release 132) (Glöckner et al., 2017) with a confidence of 0.7. Rarefaction curves of Observed amplicon sequence variants (ASVs) at a maximum depth of 70,000 showed that all samples approached a saturation plateau at about 68,500 (E-supplementary data), and this sampling depth was chosen for alpha diversity analysis.

3. Results and Discussion

3.1. Volatile fatty acids yield by the rumen-MBR

3.1.1. Total volatile fatty acids yield

The rumen MBR achieved a stable TVFA yield of 438 ± 87 mg VFA/g substrate from day 9. This stable TVFA yield was recorded for 35 days (Fig. 1), which is longer than stable operation periods reported in previous studies using rumen fluid as the inoculum. For example, stable performance was only reported for 8 – 21 days in studies using rumen simulation technique apparatus (RUSITEC) (Duarte et al., 2017a; Ramos et al., 2018). In another study by Jin et al. (2018), stable VFA production was only reported for 18 days at a loading rate of 1% solid content. These authors observed a drastic decrease in VFA production during the first 32 days of the experiment. The decrease in VFA yield was also observed in this study, but mainly during the first 9 days (Fig. 1). This decrease was due to the adaptation of rumen microbes after transferring to *in vitro* environment. The adaptation phase is evident by major changes in microbial diversity and composition as revealed by microbial community analysis (Section 3.4.2).

[FIGURE 1]

The rumen MBR achieved higher VFA yield than most previous studies using rumen fluid as the inoculum (Barnes & Keller, 2004; Duarte et al., 2017b; Hu & Yu, 2005; Jin et al., 2018; Ramos et al., 2018). VFA yield in these studies ranged from 130.4 to 446.2 mg VFA/g substrate. The higher yield in this study can be attributed to the different reactor configuration (membrane bioreactor) compared to other studies (RUSITEC fermentation vessels, continuous stirred tank reactor, sequencing batch reactor). The integration of UF membrane allows the achievement of a long SRT (480h) which provides slow-growing microbes with sufficient time to proliferate in the *in vitro* conditions. The membrane also prevents the washout of slow-growing microbes (as the pore size of the UF membrane used in this study was 0.04 µm) and maintains a high biomass concentration inside the rumen MBR, which can contribute to high productivity (Waszak & Gryta, 2016). Microbial washout cannot be completely prevented with solids/liquids separation methods used in previous studies such as coarse filtration or sedimentation in sequencing batch reactor. Moreover, membrane enables continuous VFA extraction from the reactor, avoiding VFA loss due to internal conversion reactions as well as process inhibition due to VFA accumulation. Continuous extraction of VFA, while they are being formed, can enhance the rate of VFA production (Arslan et al., 2017). It is noted that the yield from this study (438 mg VFA/g substrate) is slightly lower than the one reported by Barnes and Keller (2004). These authors observed a VFA yield of 446 mg VFA/g substrate from a sequencing batch reactor inoculated with rumen fluid and fed with fibrous-alpha cellulose (Barnes & Keller, 2004). The higher yield reported by Barnes and Keller (2004) can be attributed to the high degradability of their substrate.

3.1.2. Volatile fatty acids composition

The VFA composition or the rumen MBR closely resembles VFA composition in the rumen fluid inoculum (Fig. 2). Seven VFA species were detected in both the rumen inoculum and reactor content, with the two most abundant VFA species were acetic acid (59.8 ± 14.5 %)

234 and propionic (24.6 ± 12.2 %). These results were in agreement with VFA composition in
235 previous studies using rumen fluid as the inoculum and similar substrate type and ratio.
236 Ramos et al. (2018) found that acetic and propionic acid accounted for 52.2 and 27.1 % of
237 total VFA produced from RUSITEC apparatus fed with hay and concentrate (70: 30%, w/w).
238 In another study by Duarte et al. (2017a) using hay and concentrate at 66.7: 33.3% (w/w),
239 acetic and propionic accounted for 51.5 and 18.1 % of total VFA. A relatively low but stable
240 presence of branched-chain VFA (iso-butyric and iso-valeric acid) in the rumen MBR (0.5 –
241 0.9 mmol/L) was also observed, indicating the digestion of protein content in the substrate.
242 Branched-chain VFA has been shown to play an important role in the growth of ruminal
243 cellulolytic bacteria and their enzyme activities (Liu et al., 2018).
244 [FIGURE 2]
245 The predominance of acetic acid in the reactor content shows the potential of the rumen MBR
246 as a promising option for acetic acid production in the future. Acetic acid is the precursor for
247 synthesising a wide range of important compounds such as vinyl acetate monomer,
248 terephthalic acid, and ethanol. In 2018, the wholesale price of acetic acid was 400–800 €/ton
249 (Atasoy et al., 2018). The price of acetic acid is expected to grow at a predicted annual rate of
250 more than 4.3% over the 2019-2024 period due to strong demand (MordorIntelligence, 2018).
251 Conventional production routes including methanol carbonylation or oxidation of
252 hydrocarbons depend heavily on fossil fuels and emit approximately 3.3 ton $CO2_{eq.}$/ton acetic
253 acid produced (Atasoy et al., 2018). Acetic acid production from lignocellulosic biomass
254 using rumen MBR could be a more sustainable alternative for these production routes.

255 **3.2. Conversion of the substrate's organic fraction by the rumen-MBR**

256 The digested substrate has a lower VS/TS ratio compared to the original substrate mixture
257 (92.2%), indicating that the organic fraction in the substrate has been utilized by the rumen
258 MBR (Fig. 3). The VS removal efficiency of the reactor was in the range of 28.8 – 49.6 %

after day 9, and changes in VS content corresponded with changes in sCOD concentration (E-supplementary data), indicating that insoluble substrates were converted to soluble products. These changes determine the extent of acidification and dictate the amount of VFA produced during the process (Atasoy et al., 2018). Thus, the stable VS removal efficiency and sCOD concentration observed explained for the stable VFA production of the rumen MBR.

[FIGURE 3]

The VFA/sCOD ratio shows how much soluble substance is converted into VFA and is commonly used as an indicator of the extent of acidification. The average extent of acidification in the rumen MBR was 36.4 ± 10.3 % (Fig. 3). This value is comparable with the extent of acidification of 40.6% in the rumen fluid inoculum (shown as day 0 in Fig. 3), indicating the ability of the rumen MBR to simulate natural rumen. Due to the recalcitrant structure of lignocellulosic biomass, a higher extent of acidification would be hard to achieve, unless optimal operating conditions or substrate/inoculum pre-treatment are applied. For example, Wang et al. (2017) acclimated their inoculum with oilseed rape straw's leachate for 5 months before utilization and observed a 60% extent of acidification during anaerobic digestion of the substrate. A similar extent of acidification was recorded by Kullavanijaya and Chavalparit (2019) when they performed leachate recirculation to enhance hydrolysis and acidogenesis rates of Napier grass in an anaerobic leach bed reactor.

3.3. VFA transfer to the permeate

[FIGURE 4]

The UF membrane module (0.04 μm nominal pore size) achieved an average transfer ratio of 73 ± 15% for TVFA (n = 8) (Fig. 4). This is the first study to report the continuous transfer of TVFA directly from a rumen reactor using a UF membrane. The transfer ratio observed in this study was comparable to that of an electrodialysis system (75%) used by Pan et al. (2018) to extract acetic, propionic and butyric acid from fermentation broth. Higher transfer

ratio (up to 99%) have been reported in other studies using different extraction methods (e.g. nanofiltration, membrane contractor, solvent extraction) (Atasoy et al., 2018). Nevertheless, it is worth mentioning that in these studies the VFA recovery step was separated from VFA production and/or pre-treatment of the broth were performed before recovery (Aydin et al., 2018; Pan et al., 2018; Zacharof et al., 2016). Overall, the ability of the UF membrane to allow continuous and effective VFA transfer from the rumen MBR contributes to the system practical feasibility.

The specific transfer ratio for individual VFA was 43% for butyric acid, 58% for propionic acid and more than 77% for acetic, iso-butyric, valeric, iso-valeric and caproic acid (Fig. 4). These numbers are comparable with previous studies on membrane filtration for fermentation products recovery. Alexandri et al. (2018) reported that 78.5 % lactic acid could be recovered (21.5% loss) by MF module from the fermentation broth of crust bread hydrolysate, while Tessier et al. (2005) showed a transfer rate of 81 – 91% of benzylpenicillin from the fermentation broth of cheese whey liquor using UF membranes. Specific VFA transfer ratio could be enhanced through optimization of filtration conditions (e.g. initial flux/pressure applied, cross-flow velocity, pH).

The UF membrane module was able to operate continuously during the experimental period, which can be explained by the low operating filtration flux applied to the membrane (~2 L/m^2h). This flux was relatively low compared to the membrane's typical filtrate flux range (~60 L/m^2h) as specified by the manufacturer. However, membrane fouling might become a challenging issue for large scale operation of rumen MBR with higher applied pressure/permeate flux and higher loading rates, as fouling can significantly escalate the cost of the process. Tessier et al. (2005) observed significant fouling (indicated by up to 74% decrease of permeate flux) after 22 – 34 minutes of UF filtration of cheese whey liquor fermentation broth (initial flux of 65 L/m^2h). Therefore, the effect of substrate loading rate

and membrane fouling on VFA production from lignocellulosic substrates using the rumen MBR needs to be investigated in future studies.

3.4. Rumen microbes and their fates during rumen-MBR operation

3.4.1. Rumen microbes' prominent capacity for lignocellulosic biomass degradation

Rumen fluid inoculum in this study harbours various cellulose-degrader and in total, they accounted for 22.2% of the microbial community (E-supplementary data). Cellulolytic bacteria in this study's inoculum target different component of lignocellulosic biomass. For example, some bacteria digest both cellulose and hemicellulose (xylan), e.g *Ruminococcaceae* (Flint et al., 2008), some attack hemicellulose and pectin, e.g. *Prevotella* and *Butyrivibrio* (Krause et al., 2003; Palevich et al., 2019), while some only degrade cellulose, e.g. *Fibrobacter* (Ransom-Jones et al., 2012). This is because each bacterial strain can only produce specific enzyme(s) targeting specific substrate(s). The synergy between multiple cellulolytic bacteria in rumen fluid is the key to efficient degradation of the lignocellulosic substrate.

The most dominant cellulolytic taxa in the inoculum were *Ruminococcaceae* (relative abundance of 13.3%) and *Prevotella* (relative abundance of 7.5%), which have been commonly detected in the rumen of various ruminants and other foregut fermenters across the globe (Henderson et al., 2015). Members of *Ruminococcaceae* have been reported to have superior substrate attachment ability compared to other taxa species, comprehensive collections of glucosidase enzymes with high catalytic activity and versatility, as well as novel cell surface-anchored cellulose-binding protein which enhances their degradation capacities (Christopherson et al., 2014; Flint et al., 2008). Meanwhile, *Prevotella* strains produce a wide range of enzymes (Krause et al., 2003) that are specialized for hemicellulose and pectin degradation, and this taxon work alongside *Ruminococcaceae* to fully break down lignocellulosic substrates. *Butyrivibrio*, *Fibrobacter*, and *Pseudobutyrivibrio* also contribute

to the cellulolytic activity of the rumen inoculum (Palevich et al., 2019; Ransom-Jones et al., 2012), but to a lesser extent than *Ruminococcaceae* and *Prevotella* due to their low relative abundances (< 0.5%).

Other dominant taxa detected in the rumen fluid inoculum are non-cellulolytic microbes that function synergistically with cellulolytic bacteria (E-supplementary data). These microbes including fermenters (*Olsenella*, unassigned *Clostridiales*, *Veillonellaceae* UCG-001, *Corynebacteriaceae*), acetogens (other *Lachnospiraceae*, Succiniclasticum and *Acetitomaculum*) and hydrogenotrophic methanogens (*Methanobrevibacter*, *Methanosphaera* and unassigned *Methanobacteriaceae*) quickly utilize products of cellulolytic bacteria to prevent the feedback inhibition (Nguyen et al., 2019b). Together, the microbial composition and synergistic interactions between different functional groups in the rumen fluid make it an ideal inoculum source for an engineered system degrading lignocellulosic biomass. This is in agreement with results from a previous study where the superior ability of rumen fluid to degrade lignocellulosic substrates compared to another inoculum (i.e. anaerobic sludge) has been pinpointed to its unique microbial community (Nguyen et al., 2019b).

3.4.2. Shifts in microbial composition during rumen-MBR operation

Most dominant taxa in the inoculum showed decreases in their relative abundances during the start-up period of the rumen MBR (the first 9 days) (Fig. 5A). Changes in the microbial community are expected since the *in vitro* environment cannot perfectly simulate the natural environment. This decrease was also accompanied by a reduced diversity level (Table 3), which was also reported in previous studies (Cieplik et al., 2018; Han et al., 2019). Specifically, decreases of 19.3% in community richness (Observed ASVs) and 10.5% in evenness (Shannon index) compared to the inoculum was observed in the rumen MBR during the start-up period. Together these changes explained for the decrease in VFA yield observed during the first 9 days of the experiment (Section 3.1.1).

[FIGURE 5]

[TABLE 3]

Further decrease in the relative abundance of inoculum's dominant taxa was recorded during the remaining period of the experiment (Fig. 5A). These data reflect the experiment selection pressure on the community, where taxa that cannot adapt to the new environment will be eliminated or replaced by other taxa. This was confirmed by sharp decreases in microbial diversity indices (Table 3). The decrease of dominant ruminal taxa can also be attributed to the dilution of the original rumen fluid inoculum. Rumen fluid contains specific nutrients that are essential for the growth of many ruminal microbes, such as hemin for *P. ruminicola*, 3-phenylpropanoic acid for *R. albus*, and mercaptoethanesulfonic acid for *Methanobrevibacter ruminantium* (Stack & Cotta, 1986). This is the reason why clarified rumen fluid is often supplemented to cultivation media of ruminal bacteria. Meanwhile, in the rumen MBR the inoculum liquid was continuously diluted by the artificial saliva and thus negatively affected the abundance of inoculum's indigenous taxa.

The rumen MBR ability to maintain stable performance despite the changes in microbial composition can be attributed to the microbial community functional redundancy. Functional redundancy means multiple taxa can perform the same ecological function (Nguyen et al., 2019a). Indeed, novel cellulolytic bacteria such as *Bacteroides* and *Ruminofilibacter* emerged in the rumen MBR (Fig. 5B) to compensate for the reduction of *Ruminococcaceae* and *Prevotella*. Multiple bacteria belong to the order *Bacteroidales* and *Synergistaceae* were also enriched to take over the role in fermentation and acetogenesis steps. Microbial adaptation to the experimental conditions was also indicated by the significant growth of proteolytic bacteria (*Proteiniphilum* and *Proteiniclasticum*) that can utilize the protein content of the concentrate feed. In contrast, there was only a low presence of methanogens in the rumen MBR after the start-up period (Fig. 5B). Methanogens are more susceptible to environmental

changes than bacteria and their slow-growing rate make it harder for them to recover after disturbance (Nguyen et al., 2019a). The decrease in methanogens abundances explains the negligible methane production observed during the experiment.

Other taxa with promoted growth during the operation of the rumen MBR are also commonly found in the rumen of cow and yak, however, they have unclear functions. These taxa includes *Christensenellaceae* R-7 group (4.4 ± 1.1%), *Rikenellaceae* RC9 gut group (3.0 ± 1.7%), uncultured *Bacteroidales* bacterium Bact_22 (1.7 ± 2.0%), uncultured *Pedosphaeraceae* (0.9 ± 0.8%) and uncultured *Tannerellaceae* (0.7 ± 1.5%) (De Mulder et al., 2016; Schären et al., 2018). Their high relative abundances and the consistency in their presence in the rumen as previously reported suggest that they play key roles in the rumen digestion of process.

4. Conclusion

This study demonstrates the proof-of-concept of a rumen MBR for continuous VFA production from lignocellulosic biomass with an average daily yield of 438 mg/g substrate. The produced VFA contain mostly acetic and propionic acids (over 80% in combined molar fraction). These low molecular weight organic acids can replace petroleum-based raw chemicals in the future. Membrane separation offers a promising solution for VFA removal from the reactor with an average transfer ratio of 73±15%. Shifts in the microbial composition of the rumen MBR during the stable operation were observed, indicating the role of functional redundancy to support VFA yield.

E-supplementary data of this work can be found in online version of the paper.

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Tables Captions

Table 1. Changes in apha diversity indices of the rumen MBR microbial community. The percentage decrease (%) calculation was normalized against the values from the inoculum. The error bar represents the standard deviation of 4 samples for the start-up phase and 9 samples for stable operation phase.

Figures Captions

Figure 1. Total volatile fatty acids yield in the rumen membrane bioreactor and permeate during the experimental period.

Figure 2. Percentages of individual volatile fatty acids (VFA) of total VFA (based on molar fraction) in the original inoculum (day 0) and rumen membrane bioreactor content (from day 2) during the experimental period. Branched-chain VFA includes iso-butyric and iso-valeric acid.

Figure 3. Volatile solids/total solids (VS/TS) ratio, volatile fatty acids/soluble chemical oxygen demand (VFA/sCOD) ratio and VS removal by the rumen membrane bioreactor as a function of time. Samples were collected in a time series and one sample was collected per time.

Figure 4. Transfer ratio for total volatile fatty acids and individual volatile fatty acids species. The error bar represents the standard deviation of at least 2 measurements.

Figure 5. Changes in the relative abundance of dominant taxa from the inoculum during operation of the rumen membrane bioreactor (A) and functional redundancy in the rumen membrane bioreactor indicated by the emergence of novel functional taxa (B). The error bar represents the standard deviation of 4 samples for the start-up phase and 10 samples for stable operation phase.

603 **List of Tables**

604 **Table 1**

Index	Inoculum	Start-up phase (day 0 – 9)	% decrease	Stable operation (day 10 – 44)	% decrease
Observed ASVs	1905	1537.3 ± 220.2	19.3	692.1 ± 107.4	63.7
Shannon index	9.3	8.4 ± 0.5	10.5	5.5 ± 0.4	41.0

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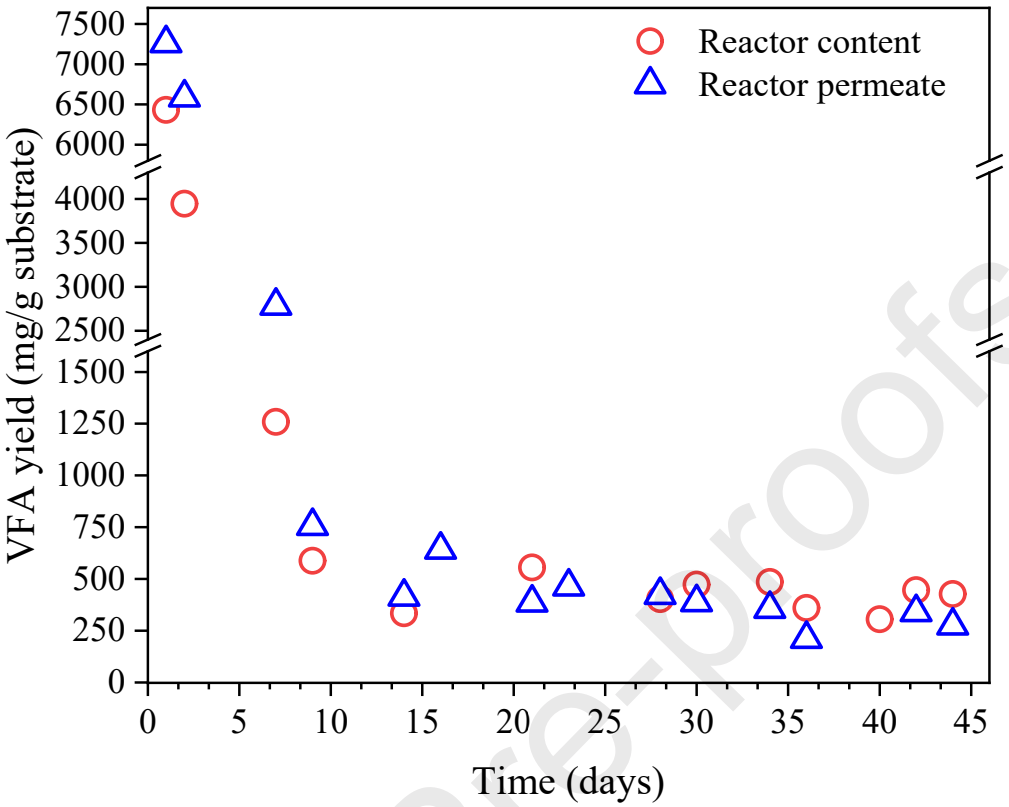
606 **List of Figures**



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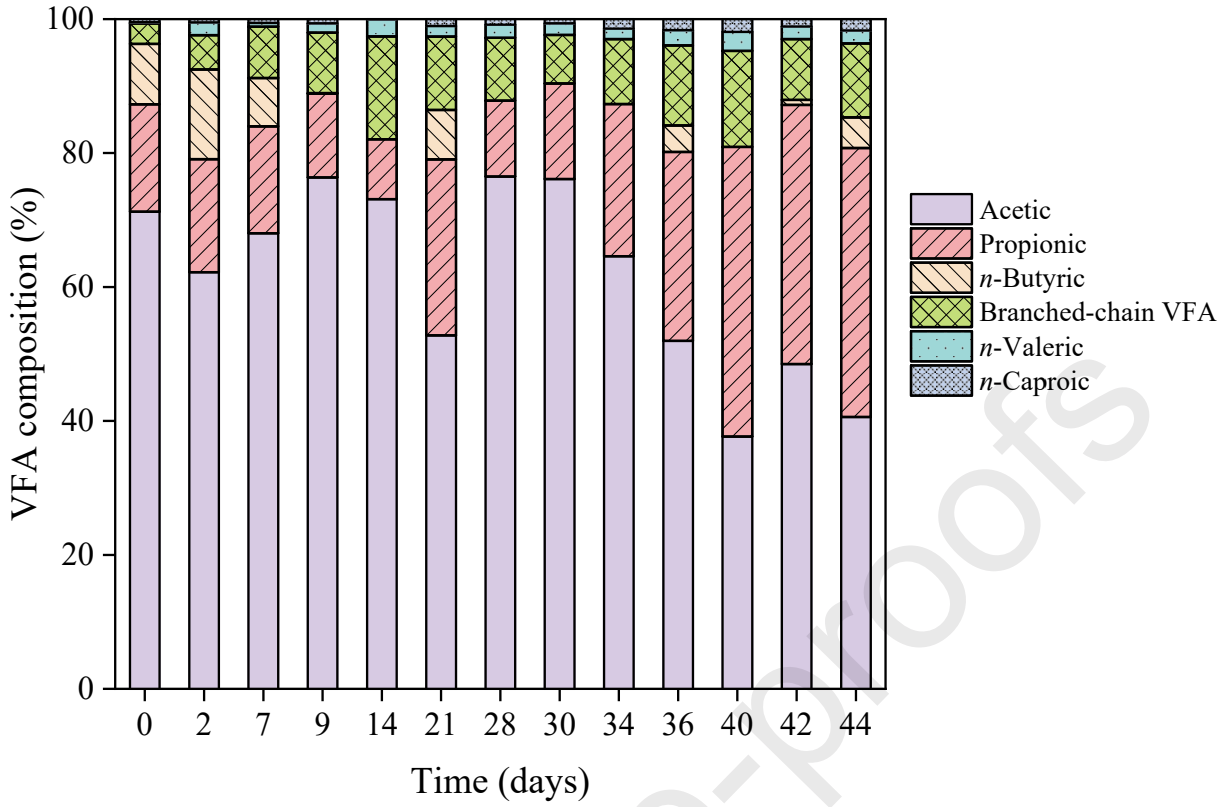

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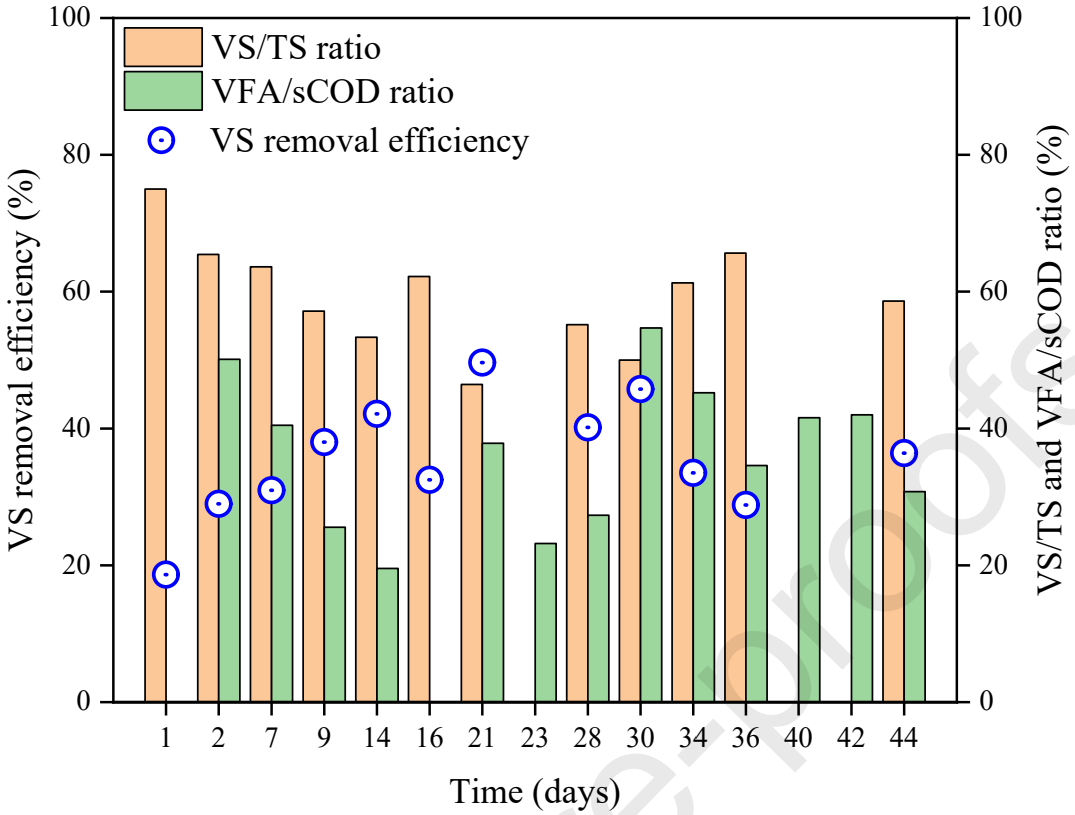

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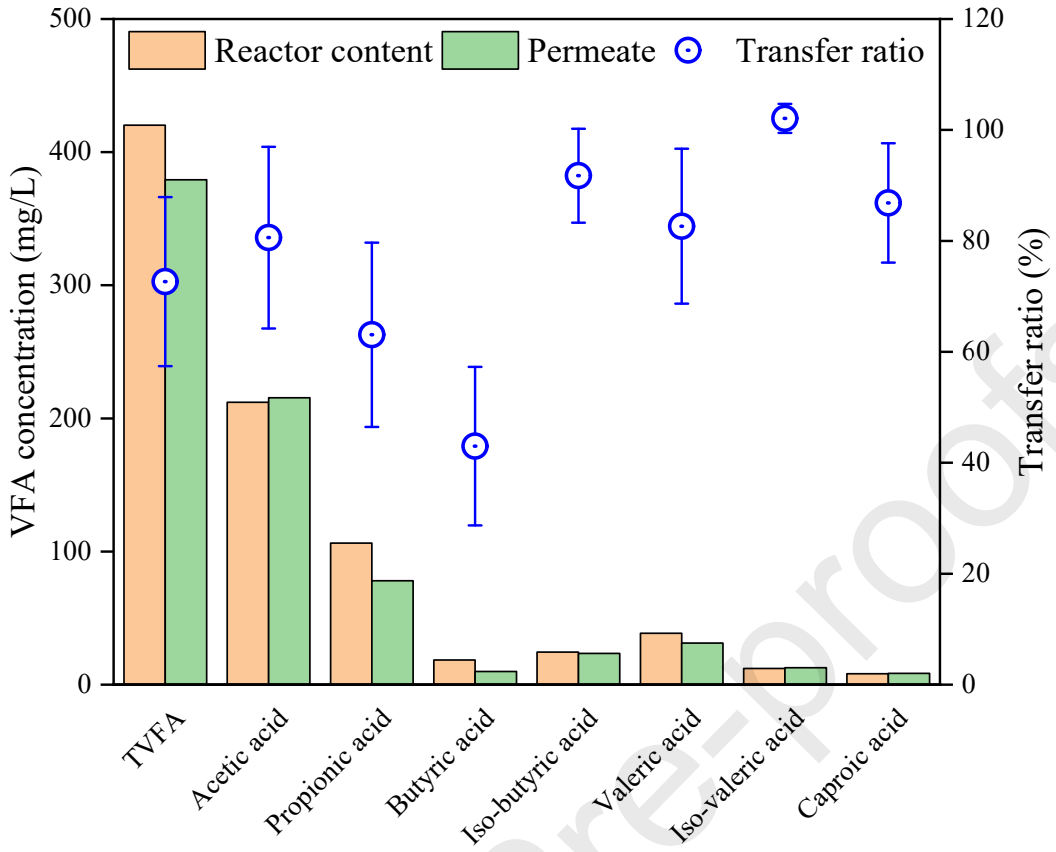

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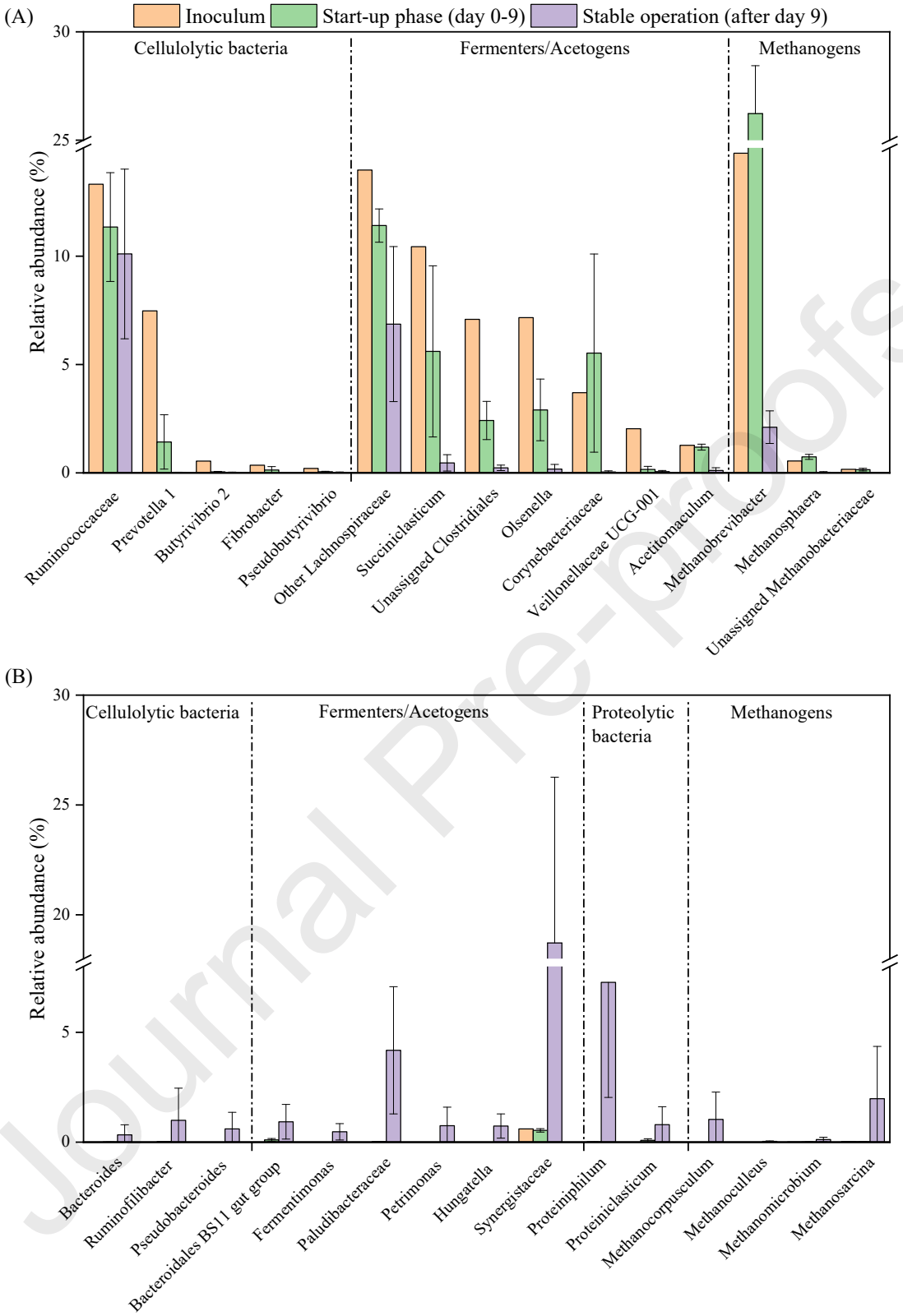

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616 **Figure 5**

617 **Author contribution**

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