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Research article

Anaerobic digestion of whey permeate: Impact of feedstock ratio and organic loading rate in batch and semi-continuous systems

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ABSTRACT

Anaerobic digestion (AD) plants have been facing significant challenges in maintaining a stable long-time operation when utilizing whey permeate as feedstock. In this study, we investigated the AD performance of whey permeate under batch and semi-continuous stirred tank reactor (s-CSTR) systems to optimize the process. Biochemical methane potential (BMP) tests were initially performed in batch reactors to assess whey permeate potential as AD substrate operating at different inoculum to substrate ratios (ISRs) and pH values under mesophilic temperatures. The kinetic parameters from the best AD performance under batch system were assessed to be used as guideline for s-CSTR operational set ups. The highest methane yield of 653.64 ± 12.16 $NL_{CH4}kg_{VS}^{-1}$ was observed at ISR 2, initial pH 7.5 at 37 °C incubation under batch system. However, when the kinetic parameters from this condition were applied to determine the organic loading rate (OLR) and hydraulic retention times (HRT) in the s-CSTRs, resulted in operational failure. The s-CSTRs were then operated at OLR 2.5 and 4 $g_{VS}L^{-1}d^{-1}$ with 30 d HRT for 150 d to assess the effect of different feeding regimes towards the overall AD performance. The CH_4 production rate declined for 3 HRTs in all reactors before stabilizing for the rest of the experiment. The decline of CH_4 rate was observed to be correlated with volatile solids degradation, volatile fatty acids and microbial composition. Initially, acetogenic bacteria (e.g., *Trichococcus* and *Sedimentibacter*) dominated the digestate which shifted to propionic acid producing bacteria (e.g., *Actinomyces* and *Acidipropionibacterium*) over the course of 150 d. A change in archaeal abundance was also observed where abundant *Methanosarcina* declined and finally substituted by *Methanobacterium*. The change in microbial population of whey permeate AD under s-CSTR system in our study, suggests a shift in methanogenesis pathway, which directly affects the AD performance.

1. Introduction

Anaerobic digestion (AD) has emerged as one of the technologies for renewable energy production by converting biomass into combustible biogas in the form of methane (CH_4) gas. AD-generated CH_4 is considered a promising source of renewable energy, especially considering its ability to reduce greenhouse gas emission in its system [1]. AD involves a sequential fermentative pathway,

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encompassing hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Through these processes, organic materials are degraded into volatile acids and concurrently transformed into CH_4. Alongside CH_4, AD yields slurry materials within the digester known as digestate. This digestate contains nutrients, such as nitrogen and minerals, that could be utilized to improve soil fertility for agricultural production [2]. As a result, AD could generate renewable energy, recover nutrients, and in parallel reduce organic waste volume. Moreover, AD has the ability to valorize a wide range of organic waste materials, such as wastewater sludge [3], agricultural residues [4,5], food waste [6,7] and other forms of disposed organic materials [8,9].

Whey permeate is a component of dairy wastewater derived through the membrane filtration of cheese whey [10,11]. The filtration process retains approximately 80% of the lactose present in milk, transferring it to the resulting whey permeate [12]. A study on whey permeate characterization showed its high levels of biochemical oxygen demand [13]. An extensive evaluation of the technological and economic viability across different whey processing methods emphasized that converting cheese whey into whey protein results in a considerable volume of whey permeate, requiring additional treatment due to its notable lactose content [14]. In 2020, the global production of whey protein reached 2.8 million tonnes, with estimates projecting a rise to 3.1 million tonnes by 2029 [15]. Anaerobic digestion thus offers a solution to support the growth of the dairy industry by enabling the conversion of whey permeate into renewable energy. In this scenario, the gap derived from wasted whey permeate can be closed and circular production practices can be established.

Currently, there are some valorization approaches to utilize whey permeate, such as into food, beverage, and animal feed products [16–18]. However, these approaches are limited in the capacity of whey permeate to be utilized as well as other factors such as a challenge in its organoleptic properties [19] and its poor nutritional values [20]. These limitations are not applicable in AD, where high amount of whey permeate can be potentially utilized depending on the volume of the AD reactor. However, achieving efficient AD operation for dairy wastewaters remains a challenge [21]. Several operational parameters, such as pH [22], temperature [23], inoculum to substrate ratio (ISR; [24]), reactor configuration [25–27], organic loading rate or OLR [28], and hydraulic retention time (HRT; [29]) have been investigated across various biogas-producing systems for their impact on AD efficiency. The pH reflects microbial enzyme activity and impacts on the kinetics of AD reactions [30]. Temperature plays a significant role in the growth rate and metabolism of the microorganisms involved, and the population dynamics within the digester. Inoculum to substrate ratio (ISR) indicates the ratio of volatile solids (VS) originating from the inoculum to the substrate within a batch reactor. A low ISR increases the probability of acidification and methanogenesis inhibition [31], whereas a high ISR diminishes the volume of substrate utilized in the digester and may lead to non-reproducible operation [32,33].

In semi-continuous stirred tank reactor (s-CSTR) systems, both OLR and HRT directly impact AD efficiency and stability. Organic loading rate signifies the quantity of feedstock introduced into the AD reactor, whereas HRT refers to the duration of interaction between the substrate and anaerobic microbial flora during the digestion process. Studies have shown that the optimum HRT and OLR levels can enhance the processing efficiency and stability of the AD. However, at certain thresholds, it may hinder biogas production or even result in system collapse due to the changes in organic matter accessibility [34,35] and microbial washout [36].

Earlier research of whey permeate AD has focused on the determination and evaluation of kinetic models [37,38]. While these studies provided insight into kinetic parameters of the AD based on experimental data, they overlooked the AD performance under a continuous system. Furthermore, we have evidence from contact with industry stakeholders that anaerobic digestion plants of why permeate face significant challenges in maintaining a stable operation. Other strategies, such as co-digestion [39,40], offer a potential for whey permeate AD optimization, but they have also faced practical challenges, due to the variability of co-substrate characteristics [41]. Our study aims to optimize the AD performance of whey permeate, by initially assesses its potential as AD feedstock under batch system at different ISRS, initial pH levels and incubation temperatures. The kinetic parameters from the best AD performance observed under batch system were then used as operational set ups for the subsequent continuous AD using s-CSTR system. The effect of different ISRs, initial pH levels and incubation temperatures on CH_4 production were assessed under batch reactor, while different OLRs were subjected at s-CSTR. Furthermore, metagenomics analysis was conducted to examine the microbial diversity and dynamics within the s-CSTR reactor.

2. Materials and methods

2.1. Inoculum and substrate characterization

Whey permeate powder, used as substrate in this study, was obtained from Volac Ltd. (Hertfordshire, UK). The inoculum used in this study was biogas effluent from a full-scale plant that was previously fed with energy crops (i.e., mainly maize and small amounts of rye and beet pulp) supplied by Future Biogas Ltd (Guildford, UK). Prior to experiments, the inoculum was incubated at 30 ± 2 °C for 5 d and then sieved with 2 mm mesh to eliminate the non-degraded organic fraction, aiming to minimize endogenous CH_4 production from the inoculum [31].

The total solids (TS) for both substrate and inoculum were determined by drying the samples at 105 °C overnight. Subsequently, the dried sample were heated at 550 °C for 6 h to determine the volatile solids (VS). Both TS and VS analyses were following standard methods for water and wastewater analysis [42]. The pH was measured using a pH meter equipped with microelectrode (Mettler Toledo™ FiveEasy™ Plus FP20 pH/mV Meters). Total organic carbon (TOC) and nitrogen analyses were conducted using Leco CHN628 instrument (LECO corp., USA). Lactose, protein and fat content of whey permeate were analyzed using a Lactoscope FTIR Diary Analyzer (Delta Instruments, Netherlands).

2.2. Batch anaerobic digestion

2.2.1. Operational conditions for batch experiments

The batch experiment used in this study were 150 mL borosilicate glass flask with the working volume set at 70 mL. The inoculum weight was fixed at 42 $g_{VS}.L^{-1}$ before mixed with substrate to make up the final inoculum to substrate ratios (ISR) of 0.5, 1 and 2 by VS weight basis. Deionized water was then added in appropriate quantities to achieve the desired working volume. The pH was adjusted by adding 1 N NaOH until the target pH values of 7, 7.5, and 8 were achieved. All flasks were sealed with rubber stoppers and crimped with aluminum caps. Subsequently, all flasks were flushed with nitrogen (N_2) gas to remove oxygen and ensure anaerobic conditions. The reactors were then incubated at 20, 30, and 37 °C.

Methane production was measured daily using the liquid displacement method, where 1 N NaOH solution was used as CO_2 scrubber. Blank trials (flasks containing only inoculum and water) were prepared for all different pH levels and temperatures to measure the endogenous CH_4 coming only from the inoculum. The experiment concluded when daily CH_4 production was less than 1% of the accumulated volume of CH_4 (<1% BMP) for three consecutive days. In order to determine the CH_4 generated from the substrate, the amount of CH_4 acquired from experimental vials were subtracted by the values obtained from the blank trials. All experiments were conducted in triplicate, and the CH_4 production was expressed as the volume of dry methane gas under standard conditions (273.15 K and 101.33 kPa) per mass of VS input ($NL_{CH4}kg_{VS}^{-1}$). The protocols for batch experiment performed in this study were according to the standard BMP test established by Holliger et al. [31].

Upon completion of the batch digestion, the digestates were analyzed for VS degradation and pH value. The VS degradation was calculated by subtracting the VS content of the blank from VS of the experimental digesters and then dividing this difference by the VS input from the substrate. The resulted VS degradation was expressed as percentage. The VS measurement was carried out following APHA [42] guidelines. The pH was determined with similar method and equipment described 2.1.

2.2.2. Kinetic parameters of batch AD

In order to assess the AD kinetics of whey permeate in batch experiments, the modified Gompertz model was used as a deterministic function based on non-linear regression [43]. The mean values from experimental data of triplicate batch experiments were fitted to this model. Non-linear regression was carried out using Solver Tool in Microsoft Excel. This model fit allowed for the estimation of the lag-phase, maximum CH_4 production rate and maximum CH_4 production potential. The modified Gompertz model is presented in Eq (1):

$$Pt = Pmax.exp\left\{ -\exp\left[\frac{Rmax^{e}}{Pmax}(\lambda - t) + 1\right]\right\} \quad (1)$$

where Pt represents the specific CH_4 yield at the given time ($NL_{CH4}kg_{VS}^{-1}$), P_{max} is the maximum CH_4 yield potential ($NL_{CH4}kg_{VS}^{-1}$), R_{max} is the maximum CH_4 production rate ($NL_{CH4}kg_{VS}^{-1}d^{-1}$), λ is defined as the x-axis intercept of the tangent of the cumulative CH_4 yield or the lag phase (day), t is the time point of observation (day), and e is Euler's number. Correlation coefficient (R^2) was calculated to determine model fitness.

2.2.3. Statistical analysis

To assess the relationship among ISR, initial pH and temperature and their effect on the monitored parameters, a univariate ANOVA with statistical significance assigned to $p < 0.05$ was performed with the use of SPSS (version 27) software. Fisher's Least Significant Difference test was employed as post-hoc test to determine significant between means.

2.3. Semi-continuous stirred tank reactor (s-CSTR) system

2.3.1. Operational conditions for s-CSTR

Four 5 L polyvinyl chloride reactors equipped with a heating jacket and mechanical stirrer operating at 30 rpm was used in these experiments. A heating jacket was connected to a water bath to maintain the system at a mesophilic temperature of 37 ± 1 °C. The digestate pH was monitored daily (Mettler Toledo™ SevenEasy™ Plus FP20 pH/mV pH meter). The CH_4 production was measured by using a gas flow meter from the reactor gas outlet connected with the CO_2 scrubber (1 N NaOH). The operational set up of the continuous experiment is presented in Table 1. Initially, the reactor underwent through an acclimatization period, where inoculum at a concentration of 58.83 $g_{VS}L^{-1}$ was added to the reactors. Whey permeate was then added at OLR of 0.5 $g_{VS}L^{-1}d^{-1}$ for a week and gradually increased with a rate of 0.5 $g_{VS}L^{-1}week^{-1}$ until target OLRs (2.5 $g_{VS}L^{-1}d^{-1}$ and 4 $g_{VS}L^{-1}d^{-1}$) were achieved. Deionized water was also added along with the whey permeate to maintain the volume of digestate throughout the experiment. Each target OLR

Table 1
Operational set up of the whey permeate AD under s-CSTR experiment.

Reactors	OLR ($g_{VS}L^{-1}d^{-1}$)	HRT (days)	Stirring mode	Observation period (d)
1	2.5	30	30 rpm, continuous	150
2	2.5	30	30 rpm, continuous	150
3	4	30	30 rpm, continuous	150
4	4	30	30 rpm, continuous	150

was operated in duplicate reactors. In addition, $NaHCO_3$ was used at the final concentration of 5 g L^{-1} to adjust the digestate pH at 7 ± 0.5. During the acclimatization period, it was observed that feeding at OLR of more than 4 $g_{VS}L^{-1}d^{-1}$ led to rapid acidification within the digester and system failure. Consequently, the maximum OLR for this experiment was set at 4 $g_{VS}L^{-1}d^{-1}$. The HRT was set for 30 d for each OLR, with an acclimatization period of 30 d. After this period, average biogas yields were measured for 150 d or 5 HRTs.

2.3.2. Biogas production and digestate monitoring

Temperature, pH and VS of the digestate were monitored daily. The VS was measured following APHA [42]. The daily biogas production volume was monitored using gas flow meter, and this data was then used to calculate the volume of dry methane produced under standard conditions (273.15 K and 101.33 kPa) per mass of VS input ($NL_{CH4}kg_{VS}^{-1}$).

2.3.3. Total organic carbon, nitrogen and volatile fatty acid determination

Total organic carbon (TOC), nitrogen and volatile fatty acids were measured every 3 d. The TOC and nitrogen content of the digestate were measured every 3 d using a Leco CHN628 instrument (LECO corp., USA). The volatile fatty acid (VFA) composition was analyzed from the effluent using a gas chromatograph (GC; Agilent 7890B) equipped with HP-5MS capillary column (~30 m × 0.25 mm I.D × 0.25 μm film thickness) and flame ionization detector (FID) with helium as the carrier gas. The injector and detector temperatures were set at 275°C. Before injection into the GC, digestate samples were centrifuged at 11,500×*g* at 20 °C for 30 min, and the supernatant was derivatized following the method described by Richardson et al. [44]. The observed VFAs analyzed were $C_2 - C_7$ acids, i.e, acetic acid, propionic acid, iso-butyric and n-butyric acid, iso-valeric and valeric acid, iso-caproic and caproic acid, and enanthic acid.

2.3.4. Microbial population analysis

In order to examine the impact of different OLRs on microbial communities, DNA samples were isolated from digestates which represent each HRT (day 1, 50, 91, 108 and 150) at both observed OLRs in duplicate. Additionally, samples from day 20 were also selected for microbial analysis as CH_4 production rates began to decline with halted carbon degradation throughout the remaining HRTs from this time point. The DNA was extracted and purified using QIAamp PowerFecal Pro DNA kit (Qiagen, Germany) according to the manufacturer's protocol. The DNA concentration of the extracted samples was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, US). The extracted DNA had at least ≥20 μL volume and 10 ng/μL template DNA concentration with 1.8–2.0 $OD_{260/280}$ value to be sequenced. The quantified extracted DNA samples were and stored at −20 °C prior to be sent for amplicon-based metagenomics sequencing (Illumina NovaSeq600) by Novogene Ltd. (Cambridge, UK). The PCR amplification targeted the V3 – V4 region of the 16S rRNA gene using primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) for bacteria. For archaea, the PCR amplification targeted the V4 region of the 16S rRNA gene using the primers Arch519F (5′-CAGCCGCCGCGGTAA-3′) and 915R (5′-GTGCTCCCCCGCCAATTCCT-3′). Sequencing of the amplified region libraries was performed using MiSeq next-generation sequencer (Illumina) with 2 × 250 nt paired-end technology using the v2 Illumina kit. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. The obtained raw reads of the amplicon were merged to obtain Clean Tags. The chimeric sequences in Clean Tags were detected and removed to obtain the Effective Tags which could be used for subsequent analysis. In order to study the microbial community composition in each sample, Operational Taxonomic Units (OTUs) were then obtained by clustering with 97% identity on the Effective Tags of all samples, and then identified. Based on the OTUs clustering, taxonomic annotation was performed by using Silva 138.1 as the database to obtain the corresponding taxa information and taxa-based abundance distribution. At the same time, OTUs were analyzed for α-diversity analysis to determine the microbial community diversity within the sample. Finally, *t*-test was employed to evaluate the sample diversity. All these bioinformatic analyses and data interpretation were performed in Qiime2 platform. The raw 16S rRNA sequencing data have been deposited in the NCBI Sequence Read Archive under accession number PRJNA1192434.

Table 2
Physicochemical characteristics of biogas effluent and whey permeate.

Parameters	Biogas Effluent	Whey Permeate
Total solids (%)	8.31 ± 0.04	94.06 ± 0.19
Volatile solids (%)	6.21 ± 0.03	86.35 ± 4.08
Ash (%)	0.58 ± 0.11	5.31 ± 0.25
pH	8.53 ± 0.14	5.52 ± 0.03
[a]Total Organic Carbon (%)	41.63 ± 1.32	40.36 ± 0.09
[a]Nitrogen (%)	4.49 ± 0.01	0.47 ± 0.01
C/N ratio	9.26 ± 0.20	85.74 ± 0.79
Lactose (%)	n/d	83.80 ± 0.38
Protein (%)	n/d	2.13 ± 0.01
Fat (%)	n/d	0.12 ± 0.01

[a] In dry matter; n/d: not determined.

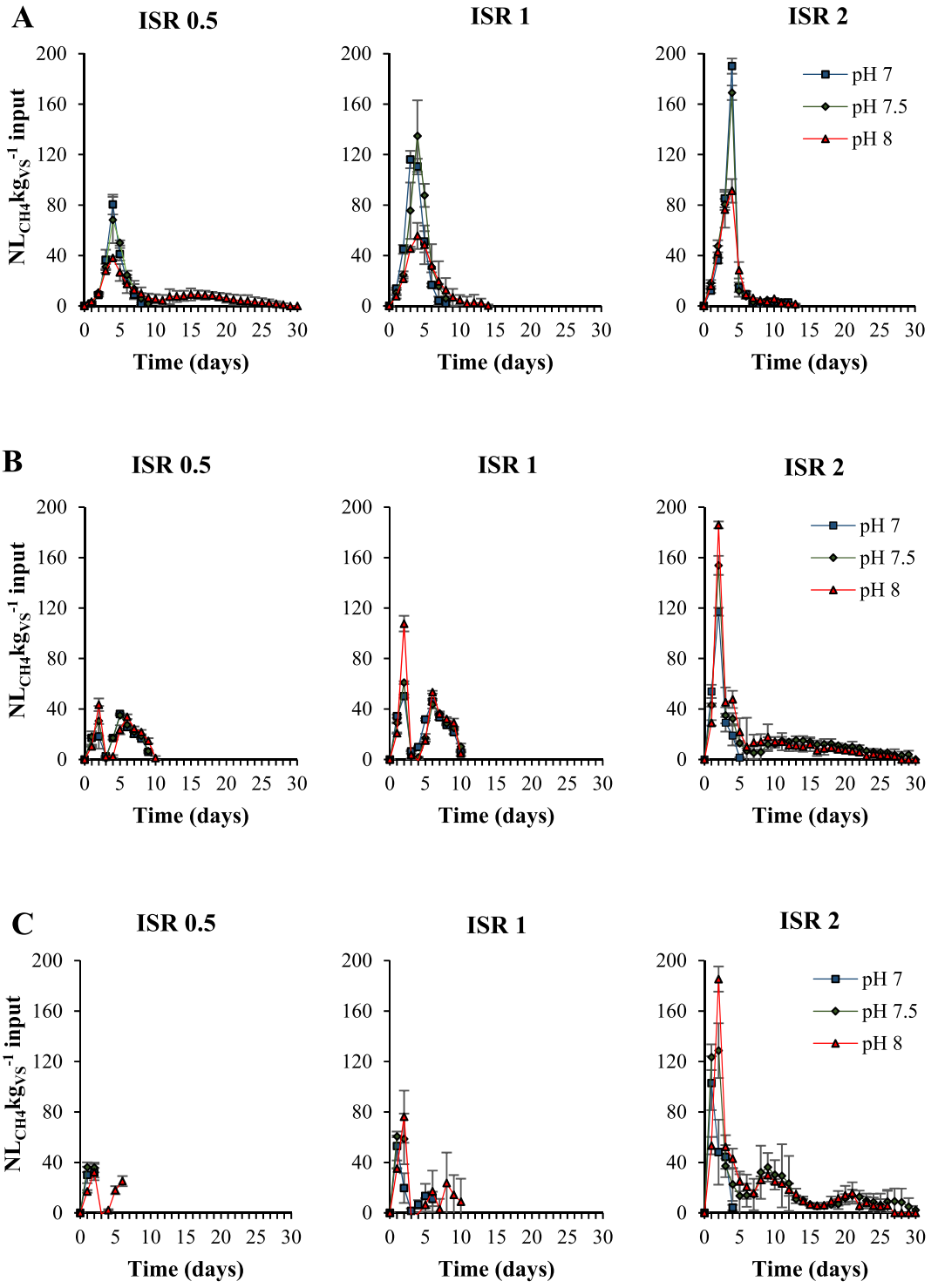

Fig. 1. Daily CH_4 production of whey permeate batch at various pH values and ISRs under (**A**) 20 °C; (**B**) 30 °C and (**C**) 37 °C. Error bars indicate standard deviation.

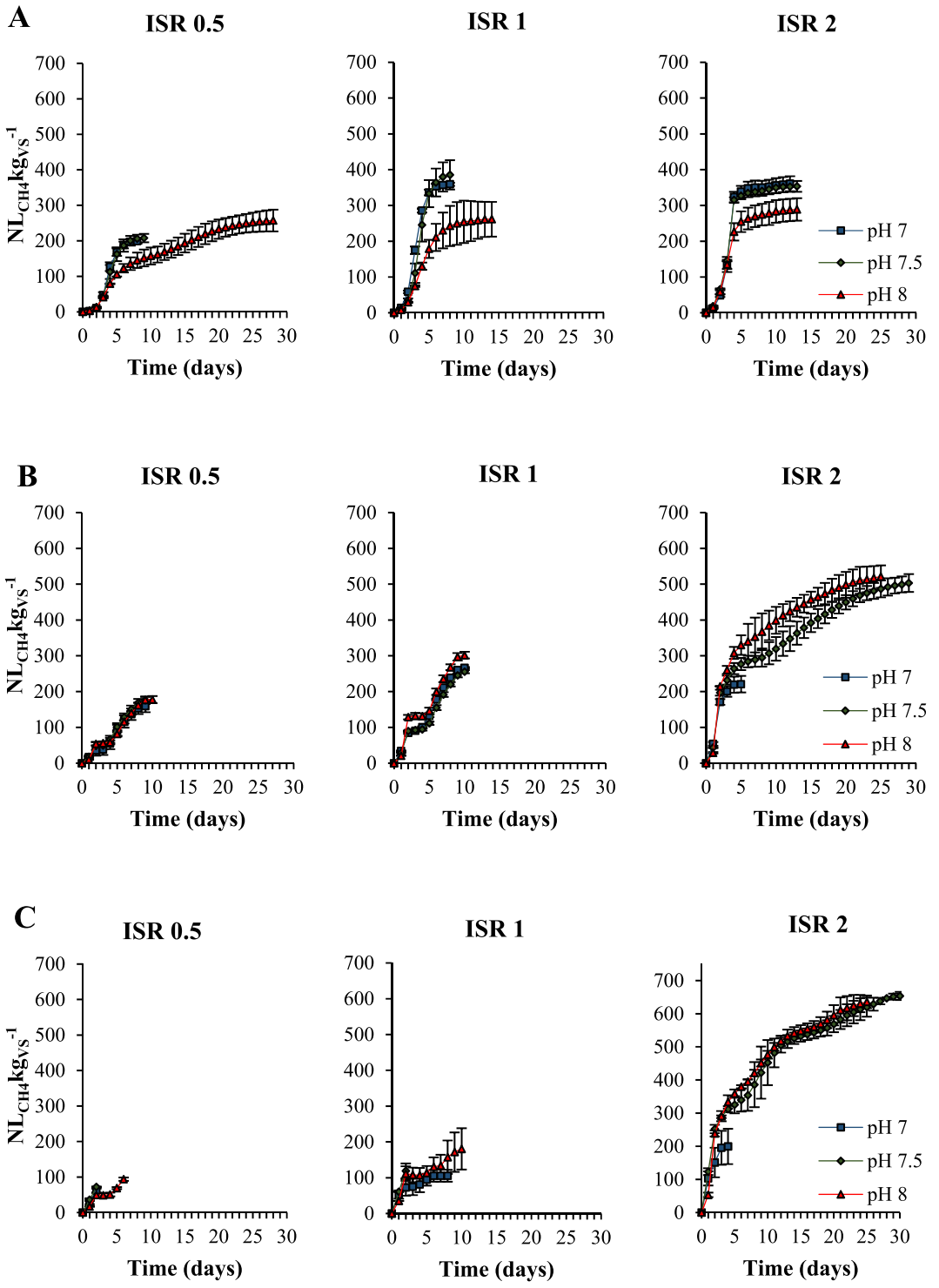

Fig. 2. Cumulative CH_4 production of whey permeate batch AD at various pH values and ISRs under (**A**) 20 °C; (**B**) 30 °C and (**C**) 37 °C. Error bars indicate standard deviation.

3. Results and discussion

3.1. Whey permeate and biogas effluent characterization

The characterization of whey permeate showed high TS, VS and carbon to nitrogen (C/N) ratio and low pH (Table 2). TS and VS values of the whey permeate were comparable to those typically found in energy crops [45–47], indicating its suitability as substrate or feedstock for AD. Typically, TS and VS values for dairy waste are low, as previously indicated by Demirel et al. [48], Pilarska et al. [49] and Traversi et al. [50]. The higher TS and VS of whey permeate in our study as compared to other type of dairy wastewaters were due to water removal prior to being used as AD feedstock, which enabled higher organic matter concentration per volume of whey permeate.

Whey permeate is a dairy waste generated from a series of fermentation and filtration steps that remove proteins and other solids. As a result, whey permeate has high C/N ratio. The ideal C/N ratio for bacterial growth in AD systems has been reported to be between 20 and 30, although this optimal range can vary depending on the type of feedstock being digested [51]. The C/N ratio of whey permeate in our study had a C/N ratio significantly higher than the optimal for AD feedstock. Feedstocks with higher than optimal C/N ratio such as switchgrass [46], corn straw [47], or rice straw [45] are commonly fed in AD systems. However, it is important to consider the physicochemical properties of the feedstock, as C/N ratio alone does not directly indicate its bioavailability in the digester. For instance, feedstock with high lignocellulosic composition has a particularly rigid structure, making its organic matter more difficult for anaerobic microorganism to degrade [52]. In the case of whey permeate, such organic matter degradation inhibitor is not present. Combined with its low pH, rapid acidogenesis of whey permeate during AD is anticipated. Therefore, specific environmental conditions need to be evaluated for the AD of whey permeate, as VFAs accumulation and acidic conditions could inhibit methanogenic microorganisms [53,54].

3.2. Batch anaerobic digestion and kinetics

In our study, significant variations in daily CH_4 production rate (Fig. 1), cumulative CH_4 production (Fig. 2) and CH_4 production kinetics (Table 3) were observed. The variations indicate that ISR, initial pH and temperature have shown to affect the AD performance of whey permeate. CH_4 production started immediately from day 1 in all vials, with the highest daily CH_4 production rate of 190.16 ± 6.05 $NL_{CH4}kg_{VS}^{-1}$ achieved at day 4 of digestion at ISR 2, initial pH 7 and 20 °C. Furthermore, it was also shown that the duration to reach the <BMP1% varied from 2 to 29 d. The optimum temperature for hydrolysis process of cellulosic substrate AD is ranged between 30 and 50 °C [55], while energy gain from syntrophic VFAs degradation declines at < 30 °C [56]. Similarly, our study showed that the hydrolysis and acidification processes were hindered at 20 °C, as indicated by the longer lag period (Fig. 1). The reduced

Table 3
Kinetic analysis performance of the whey permeate under batch reactors.

Digesters			*Pmax*	*Rmax*	λ	R^2	$T_{80\text{-}90}$
ISR	Temp	pH					
0.5	20	7	177.61	69.48	3.32	1	5
		7.5	196.35	61.70	3.46	1	6
		8	150.47	9.70	5.52	0.97	17
	30	7	146.87	16.14	4.56	0.99	7
		7.5	238.15	24.55	4.43	0.99	7
		8	215.30	19.40	4.93	0.97	8
	37	7	64.93	50.40	0.90	1	2
		7.5	113.06	91.33	0.88	1	2
		8	149.80	17.49	2.93	0.90	6
1	20	7	188.78	68.99	2.63	1	5
		7.5	288.20	96.36	3.17	1	5
		8	184.62	39.64	3.35	1	6
	30	7	183.24	15.87	4.59	0.98	8
		7.5	311.50	21.34	5.70	0.96	8
		8	367.03	31.43	4.15	0.94	8
	37	7	64.95	30.42	0.71	0.93	5
		7.5	157.29	129.80	0.87	1	2
		8	128.21	25.07	1.36	0.88	8
2	20	7	154.06	132.71	2.99	0.99	4
		7.5	220.56	89.92	2.63	0.99	4
		8	308.42	97.99	2.53	1	5
	30	7	114.33	70.11	1.20	1	3
		7.5	273.42	13.40	3.37	0.93	16
		8	291.69	28.38	2.44	0.93	12
	37	7	113.26	56.83	0.80	0.99	3
		7.5	272.86	15.71	3.11	0.96	14
		8	272.86	22.49	2.85	0.95	12

Temp = °C; *Pmax* = $NL_{CH4}kg_{VS}^{-1}$; *Rmax* = $NL_{CH4}kg_{VS}^{-1}.d^{-1}$; λ and $T_{80\text{-}90}$ = Technical digestion time, day(s).

hydrolysis rate allowed batch reactors with low concentration of inoculum to convert the acidified organic matter into CH_4 while avoiding VFAs accumulation. This was evident as ISR 0.5 and 1 exhibited longer CH_4 production period at 20 °C compared to 30 and 37 °C. The final pH of the digestate (Fig. 3) and VS degradation (Fig. 4) post-AD data also confirmed this finding, as lower pH and VS degradation were noted at 30 and 37 °C with ISR 0.5 and 1. This indicates that reducing temperature could serve as an alternative strategy to prevent AD system failure due to rapid acidification in easily degradable substrates with low concentrations of inocula.

However, hindering the acidification process was unnecessary when higher ISR was applied. As can be seen from Figs. 1 and 2, ISR 2 resulted in higher CH_4 levels compared to lower ISRs, regardless of the incubation temperature. The highest CH_4 production of whey permeate in our study has been shown to be 63.25% higher than waste milk AD [57] or 11% higher than cheese whey AD [58], where both referred studies utilized co-digestion strategy at different ISRs. Furthermore, our finding also showed higher cumulative CH_4 production and VS degradation when compared to that of AD of several energy crops, such as corn stover [59] and wheat straw [60]. The highest cumulative CH_4 production of 653.64 ± 12.16 $NL_{CH4}kg_{VS}^{-1}$ ($p < 0.05$) was achieved at 37 °C, where ISR 2 at initial pH of 7.5 was applied. This was expected as higher ISR allows for higher microbial abundance to degrade the organic matter of whey permeate and subsequently produce CH_4. The results of our study further confirm the finding of Koch et al. [61] who showed that ISR could affect the efficiency of the AD process through under- or overloading of the system. Consistent with the cumulative CH_4 production data, the highest VS degradation ($p < 0.05$) was also achieved at an ISR of 2, with an initial pH of 7.5 at 37 °C, where 85.85 ± 2.46% of VS was degraded via the AD process. This suggests that organic matter in whey permeate was converted to CH_4 more efficiently under these conditions, compared to other scenarios investigated in our study. The substantial organic load in dairy wastewaters represents a significant environmental pollutant when improperly disposed [21], while eliminating this organic load from dairy wastewaters incurs additional costs for the industry [62]. The results of our study thus suggest that AD is an effective and cheap approach for dairy wastewater treatment, where significant amount organic load could be reduced and converted into renewable energy.

The results of kinetic analyses demonstrated high correlation coefficients ($R^2 = 0.9$–1; Table 3) of modified Gompertz model towards the cumulative CH_4 production data. This indicates that the modified Gompertz model effectively represented the kinetics of AD in our study. In theory, ISR should not influence the cumulative CH_4 yield and only impact the kinetics of CH_4 production. However, our experimental data demonstrated that ISR can impact both factors. Study by Owamah et al. [63] found that ISR negatively impacted both λ and Rmax, with higher ISR resulting in increased λ and decreased Rmax in the AD of food waste and maize husk. The highest Rmax in our study was found at ISR 2, initial pH 7 under 20 °C incubation. However, while this condition had the highest Rmax, it was not followed by its Pmax value. This shows that while the organic matter of whey permeate can be quickly converted into CH_4 in low pH and temperature, a lot of the organic matter might remain undegraded. In addition, our study revealed that ISR alone could not explain the variations in λ and Rmax, as temperature and initial pH levels played a significant role in the AD processes. The pH level of the digestate is shown to influence AD kinetics. It is reported that microbial enzymatic reactions are affected by the pH of the digester, where lower or higher pH than the optimal, can both negatively affect methanogenesis [22,64]. Other studies have demonstrated that factors such as inoculum source and pre-treatments [65], as well as substrate type [66] can also influence kinetic parameters in AD. However, the impact of initial pH levels on AD kinetics is less explored. Our findings indicate that initial pH levels should also be considered as a factor in determining the kinetics of AD in batch systems. In field applications, this highlights the importance of co-digestion strategies with careful attention to initial pH levels, as co-digested substrate might affect the pH of the AD system.

3.3. Semi-continuous stirred tank reactor (s-CSTR) anaerobic digestion

Theoretically, parameters observed in batch experiments can be translated into continuous systems, such as using ISR to determine OLR and technical digestion time ($T_{80\text{-}90}$) to determine HRT. Batch experiments showed that the most optimal CH_4 production

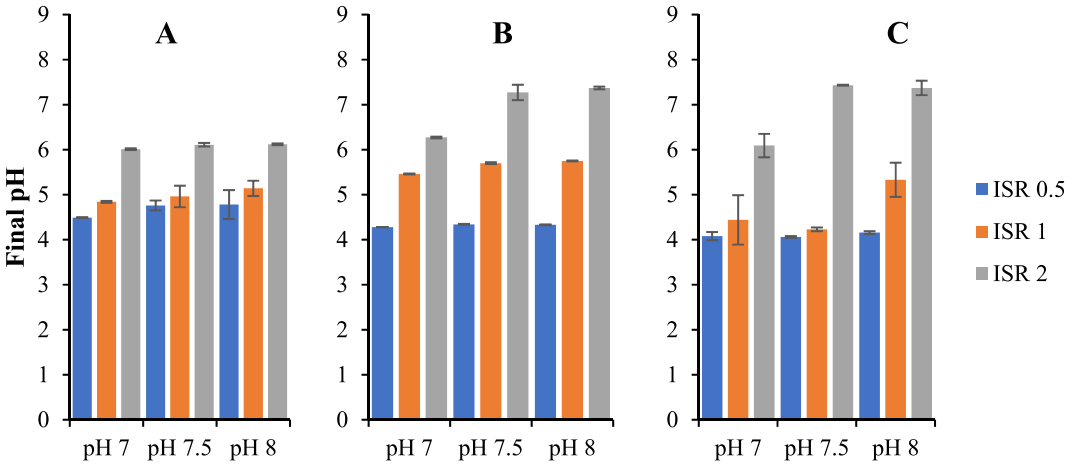

Fig. 3. Final pH of the digestate post-batch AD under various pH values and ISRs at (**A**) 20 °C; (**B**) 30 °C and (**C**) 37 °C. Error bars indicate standard deviation from triplicates.

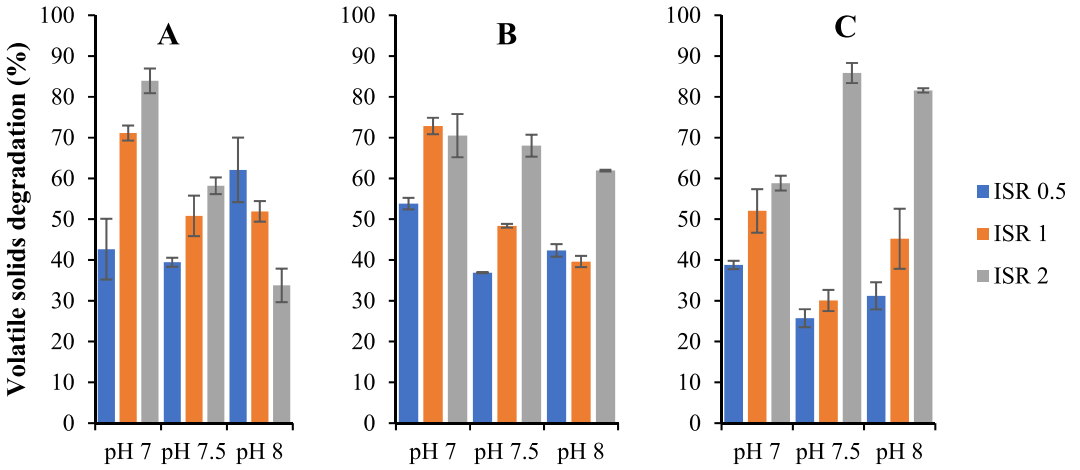

Fig. 4. Volatile solids (VS) degradation of whey permeate post-batch AD under various pH values and ISRs at (**A**) 20 °C; (**B**) 30 °C and (**C**) 37 °C. Error bars indicate standard deviation from triplicates.

occurred at an ISR of 2 at 37 °C. In our s-CSTR set up, these conditions correspond to an OLR of approximately 2.5 $g_{VS}L^{-1}d^{-1}$ with an HRT of 14 d. Additionally, the batch analysis indicates that AD could still be performed at an ISR as low as 0.5, or equivalent to an OLR of 23.5 $g_{VS}L^{-1}d^{-1}$ with an HRT of 5 d. However, rapid acidification of whey permeate AD in the s-CSTR system occurred when the OLR was increased to more than 4 $g_{VS}L^{-1}d^{-1}$ during the acclimatization period of our study. Additionally, reducing the HRT to match the $T_{80\text{-}90}$ value from our batch experiments led to significant reduction in CH_4 production. In both cases, the system ultimately failed before 1 HRT was reached. This highlighted a limitation of biochemical methane potential (BMP) tests, as they could not account for long-term effects of substrate in AD, as noted by Koch et al. [67]. Consequently, it was decided that the s-CSTR experiment would proceed with an OLR of 2.5 and 4 $g_{VS}L^{-1}d^{-1}$ with HRT of 30 d.

Over the course of 150 d of observation (5 HRT), the average CH_4 production rate of both OLR 2.5 and 4 $g_{VS}L^{-1}d^{-1}$ declined over

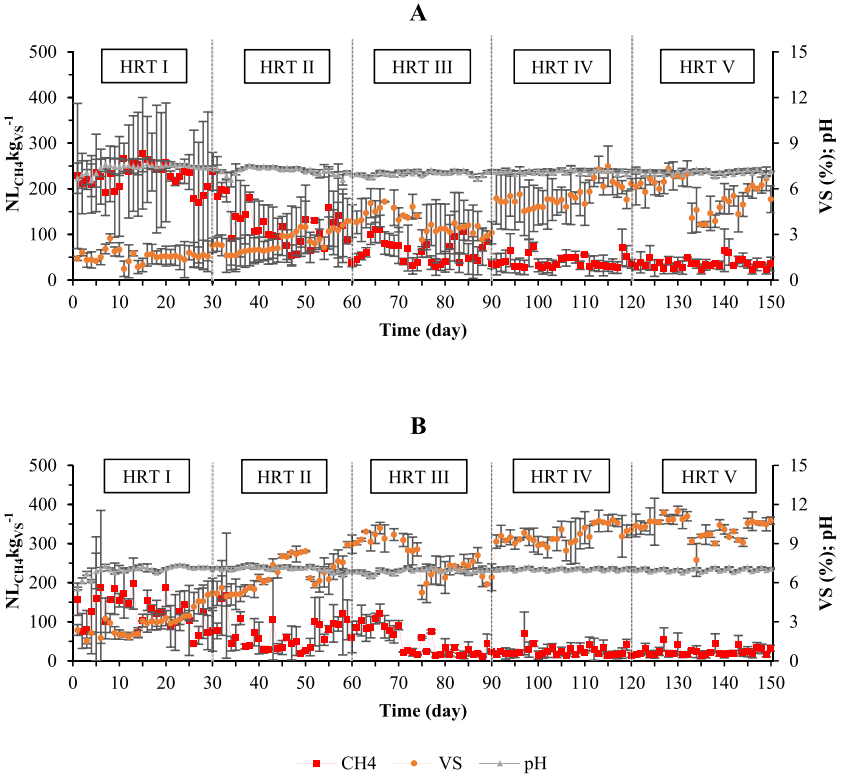

Fig. 5. Methane production, VS and pH of whey permeate AD under s-CSTR. (**A**) OLR 2.5 $g_{VS}L^{-1}d^{-1}$; and (**B**) OLR 4 $g_{VS}L^{-1}d^{-1}$. Error bars indicate standard deviation.

time and finally stabilized during the 4th and 5th HRT (Fig. 5). The average CH_4 production rate at OLR 2.5 $g_{VS}L^{-1}d^{-1}$ was 227.84 ± 83.49 $NL_{CH4}kg_{VS}^{-1}d^{-1}$ during the 1st HRT, followed by declined CH_4 production throughout the 2nd and 3rd HRT, and then stabilized at 40.37 ± 6.28 and 36.03 ± 3.80 $NL_{CH4}kg_{VS}^{-1}d^{-1}$ during the 4th and 5th HRT, respectively. Similarly, declining CH_4 production rate over time was also observed at OLR 4 $g_{VS}L^{-1}d^{-1}$, where the 1st HRT showed the CH_4 production rate of 125.04 ± 22.10 $NL_{CH4}kg_{VS}^{-1}d^{-1}$, then halved during the 2nd and 3rd HRT. The CH_4 production rate at OLR of 4 $g_{VS}L^{-1}d^{-1}$ was then stabilized at 25.35 ± 3.70 and 24.30 ± 2.33 $NL_{CH4}kg_{VS}^{-1}d^{-1}$ during the last two HRTs of the study. On the contrary, the VS content of the digestate was increased over time both for OLR of 2.5 and 4 $g_{VS}L^{-1}d^{-1}$ (Fig. 5).

Furthermore, VFAs analysis showed that volatile acids accumulation did not occur. As seen in Fig. 6, total VFA was relatively stable at OLR 2.5 $g_{VS}L^{-1}d^{-1}$ and even lower at OLR 4 $g_{VS}L^{-1}d^{-1}$. This further confirmed that hydrolysis and acidification of whey permeate was inhibited in the s-CSTR system. This finding is rather unexpected, given that whey permeate is typically considered an easily degradable substrate as demonstrated in the VS degradation under batch system (Fig. 4). The VFA composition is important as it offers useful insights with regards to the degree of hydrolysis and acidification. At both OLRs, acetic, propionic and butyric acid were the three main VFAs produced in the digestate. Furthermore, butyric acid production declined and ceased after three HRTs, while propionic acid production increased and became the predominant VFA during the 4th and 5th HRTs.

Different VFAs may be produced during the AD process, with each having unique individual or combined effect on the process. As a result, there are differing views on which specific VFA acts as the key indicator of reactor acidification, impending process failure. However, it is often reported that propionic acid has a more significant inhibitory effect on biogas production compared to other VFAs, due to the low acetogenic rate of the volatile acid [68,69]. Our study supports this finding, as declining CH_4 production rate was observed along with increased propionic acid concentration in the digestate (Figs. 5 and 6).

A study on the acidification of dairy wastewater has shown that propionic acid production is favoured at pH 4.0–4.5, whereas acetic and butyric acids are favoured at pH 6.0–6.5 [70]. However, high proportion of propionic acid was observed despite maintaining the digester at pH 7. This contradictory outcome indicates that digestate pH is not the definitive factor in determining VFAs composition. Acetic, propionic and butyric acids are directly produced from the acidification of soluble proteins, carbohydrates, and lipids [71], whereas iso/n-valeric acid is primarily produced from protein degradation. In our study, the production of iso/n-valeric acid was observed to be very low, and even ceased entirely at OLR 4 $g_{VS}L^{-1}d^{-1}$ after 3 HRT. This could be attributed to the low nitrogen and high C/N ratio of whey permeate, which supplied insufficient nitrogen to produce iso/n-valeric acid. During the 150-day observation

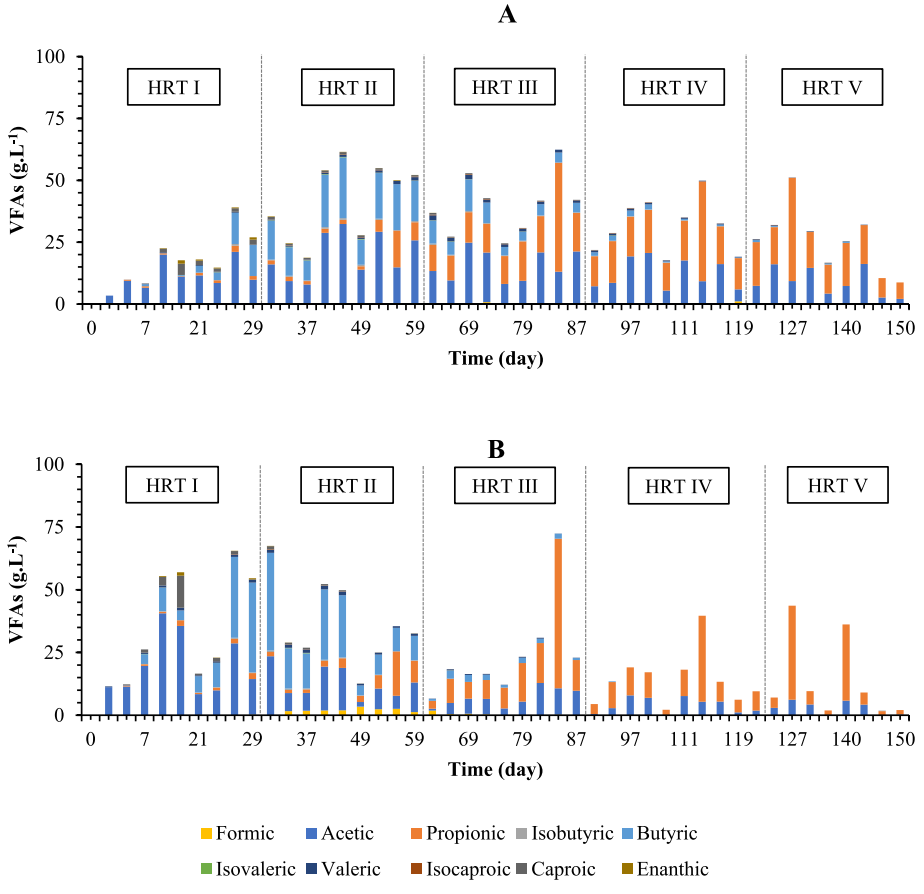

Fig. 6. Volatile fatty acids (VFAs) production of whey permeate AD under s-CSTR. (**A**) OLR 2.5 $g_{VS}L^{-1}d^{-1}$; and (**B**) OLR 4 $g_{VS}L^{-1}d^{-1}$.

period, the nitrogen content in the digestate decreased by more than 90% (Fig. 7).

As illustrated in Fig. 7, both carbon and nitrogen levels of the digestate were reduced during AD of whey permeate which reflected the organic matter degradation through the AD process. Furthermore, the degradation rates of both compounds were found not to be linear. The carbon level decreased during the 1st HRT and then remained constant for the subsequent HRTs at both OLRs of 2 and 4 $g_{VS}L^{-1}d^{-1}$, resulting in a degradation of 34 and 35% of the carbon content, respectively. On the other hand, the nitrogen level continuously decreased until the 3rd HRT, leaving approximately 10% and 7% of the initial nitrogen level for OLRs of 2 and 4 $g_{VS}L^{-1}d^{-1}$, respectively. This resulted in increased levels of C/N ratio for the digestate of both OLRs. There are conflicting findings on whether C/N ratio impacts propionic acid production, with some studies suggesting an effect [72–74] while another indicates no influence [75]. The VFAs composition and C/N ratio data from our study suggest a positive correlation between C/N ratio and propionic acid production by methanogenesis inhibition as reflected by declining CH_4 production.

The reduction in CH_4 production due to an increase in OLR beyond a certain threshold has also been found in other studies [28,46]. In our study, we found that OLR 2.5 $g_{VS}L^{-1}d^{-1}$ would be preferable, as the generated cumulative CH_4 production over the course of 150 d was 65% higher than that at OLR 4 $g_{VS}L^{-1}d^{-1}$. This result aligned with the batch experiment, where a higher ISR, or equivalent with lower OLR, yields higher CH_4. Additionally, the CH_4 production rates declined for both observed OLRs until eventually stabilized on the 4th and 5th HRT. During this period, there were increases in VS content, C/N ratio and propionic acid concentration of the digestate. This suggests the lack of nitrogen to support the hydrolysis and acidogenesis process in the s-CSTR experiment, leading to a reduced VS degradation and shift of the microbial population towards propionic acid-type fermentative pathway.

3.4. Microbial communities

The observed number of OTUs (operational taxonomy units, indicating the total number of microorganisms in the sample) and Shannon index (indicating the microbial diversity in the sample) were used to assess the α-diversity of the AD system of our study (Fig. 8 A). Consistent with the CH_4 production rate data, a decline in OTUs and Shannon index values was also observed starting from day 20 in s-CSTR AD. Previous research has indicated that a decrease in α-diversity is associated with instability of the AD system [76]. The reduction in α-diversity was accompanied by a decrease in AD performance, highlighting the limited resilience and redundancy of microbial stability [77]; this was also confirmed by our study, since a positive correlation between AD performance and α-diversity was

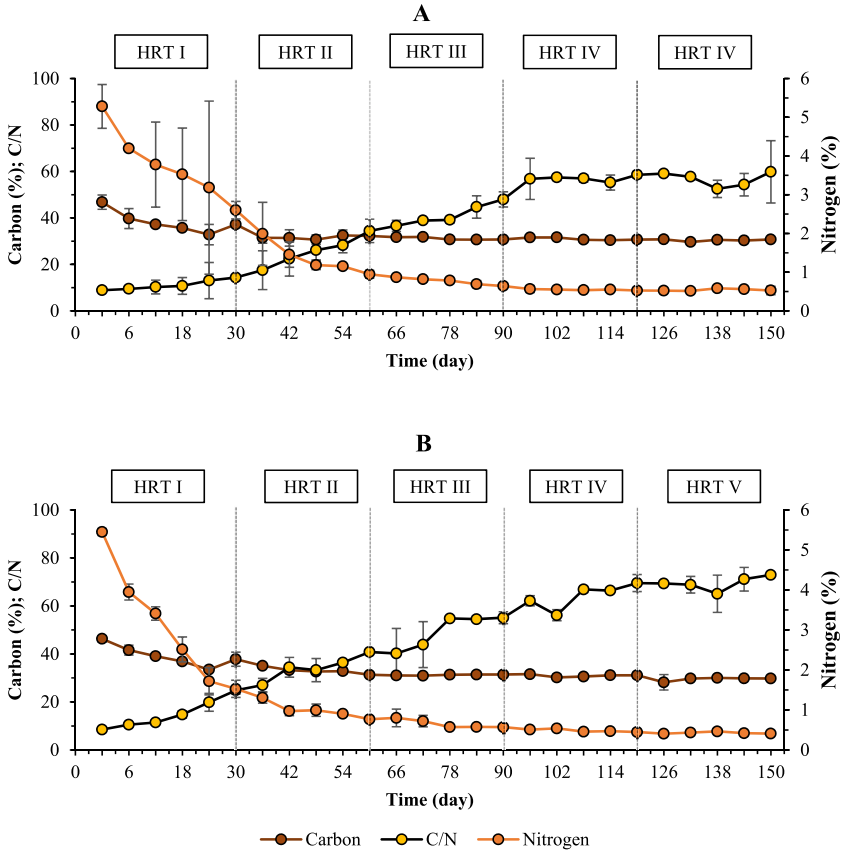

Fig. 7. Carbon, nitrogen and C/N ratio of whey permeate AD at s-CSTR with (**A**) OLR 2.5 $g_{VS}L^{-1}d^{-1}$; and (**B**) OLR 4 $g_{VS}L^{-1}d^{-1}$. Error bars indicate standard deviation.

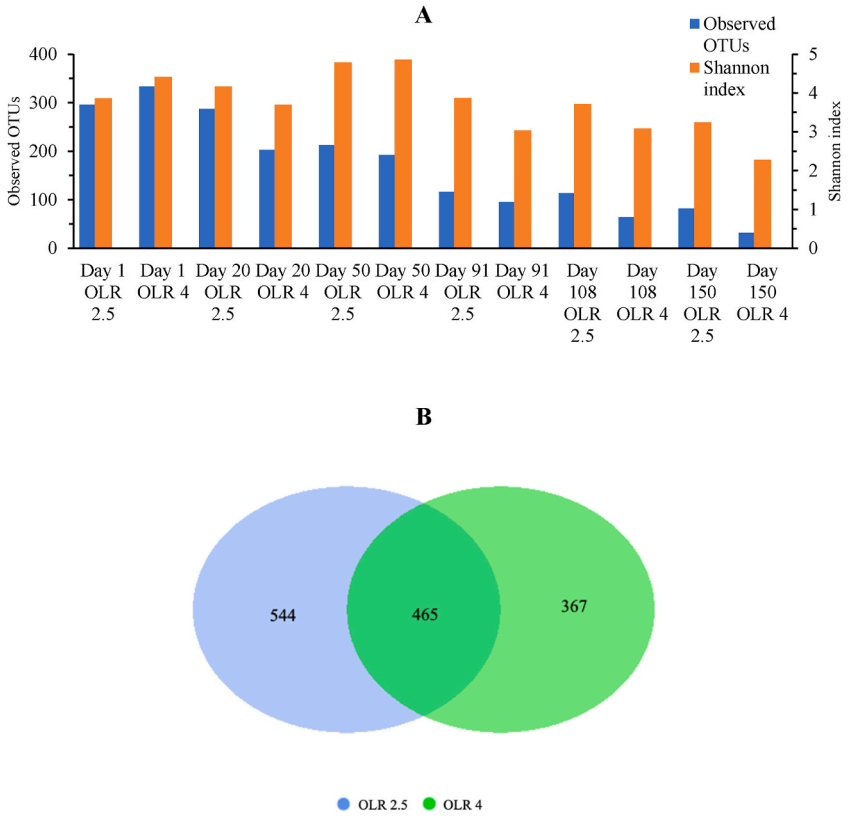

Fig. 8. (**A**) The bacterial α-diversity across different OLRs, as analyzed by OTUs and Shannon index. (**B**) Venn diagrams reflect the number of species (OTUs) shared and unique in at different OLRs observed in the study.

observed. In addition, day 150 had the lowest α-diversity, with 82 observed OTUs and Shannon Index of 3.25 for OLR 2.5 $g_{VS}L^{-1}d^{-1}$, while 32 OTUs and Shannon Index of 2.28 were observed at OLR of 4 $g_{VS}L^{-1}d^{-1}$. The final OTUs were 72.29% and 90.42% lower compared to their respective initial OTUs for OLR 2.5 and 4 $g_{VS}L^{-1}d^{-1}$. This suggests that mono digestion of whey permeate at the observed OLRs and HRT in our study leads to declined AD performance in the continuous system.

The anaerobic digestion process requires the collaboration of various microorganisms to accomplish the key stages of hydrolysis, acidogenesis, and methanogenesis. The microorganisms involved in anaerobic digestion are divided into two groups: bacteria and archaea. Bacteria break down complex organic matters into VFAs, CO_2, and H_2, while archaea are responsible for CH_4 production. These two groups differ significantly in their physiology, biokinetics, and growth environments. The OTUs distribution pattern revealed distinct microbiota characteristics in the s-CSTR AD system (Fig. 8 B). Venn analysis showed that OLR 2.5 $g_{VS}L^{-1}d^{-1}$ had a total of 544 unique OTUs, nearly 1.5 times more than OLR 4 $g_{VS}L^{-1}d^{-1}$.

The microbial 16S rRNA sequencing analysis showed a shift in microbiota composition over 5 HRTs of whey permeate AD in s-CSTR system (Figs. 9 and 10). During the 1st HRT, *Erysipelatoclostridium*, *Trichococcus*, and *Sedimentibacter*, were the dominant bacteria for both at OLR of 2.5 $g_{VS}L^{-1}d^{-1}$ and 4 $g_{VS}L^{-1}d^{-1}$. These bacteria are recognized as hydrolytic bacteria. *Erysipelatoclostridium* belongs to the family of *Erysipelatoclostridiaceae*, a group of Gram-positive anaerobic bacteria with phenotypic features similar to members of the *Firmicutes* phylum. This indicates that *Erysipelatoclostridium* is an anaerobic fermentative bacterium that can ferment multiple substrates into lactate and other short-chain carboxylates [78]. *Erysipelatoclostridium* has also been found in cheese whey AD, with positive correlation to propionic acid production [79]. *Trichococcus* has important roles in the acidification and methanization processes by converting glucose into lactic, formic and acetic acid [80], and has shown its connection to the microbial volatile organic compounds metabolism [81]. *Sedimentibacter* is halotolerant, endospore-forming, and anaerobic bacteria, which utilizes pyruvate and amino acids to provide H_2 and acetic acid [82,83]. On day 20, both *Trichococcus* and *Sedimentibacter* were depleted in both OLRs. This depletion was accompanied with halted carbon degradation of the digestate, suggesting that both genera play an important role in whey permeate AD through carbon/lactose degradation. Similarly, positive correlation of *Trichococcus* [80,81] and *Sedimentibacer* [84,85] abundance to methanogenesis process has also been previously reported.

In the last 3 HRTs (during which CH_4 production rate was low and steady), the bacterial composition in reactors were dominated by *Actinomyces* and *Sporolactobacillus*. Furthermore, there was notable abundance of *Raineyella* in OLR of 2.5 $g_{VS}L^{-1}d^{-1}$, and *Acidipropionibacterium* in OLR of 4 $g_{VS}L^{-1}d^{-1}$. *Actinomyces* is a genus within the order of *Actinomycetalles*, and phylum *Actinobacteria* [86]. The genera naturally produce organic acids from glucose fermentation [87]. *Actinomyces* has been positively correlated with propionic acid production [88]. *Sporolactobacillus* are facultative anaerobic bacteria that perform homolactic fermentation [89] and have been

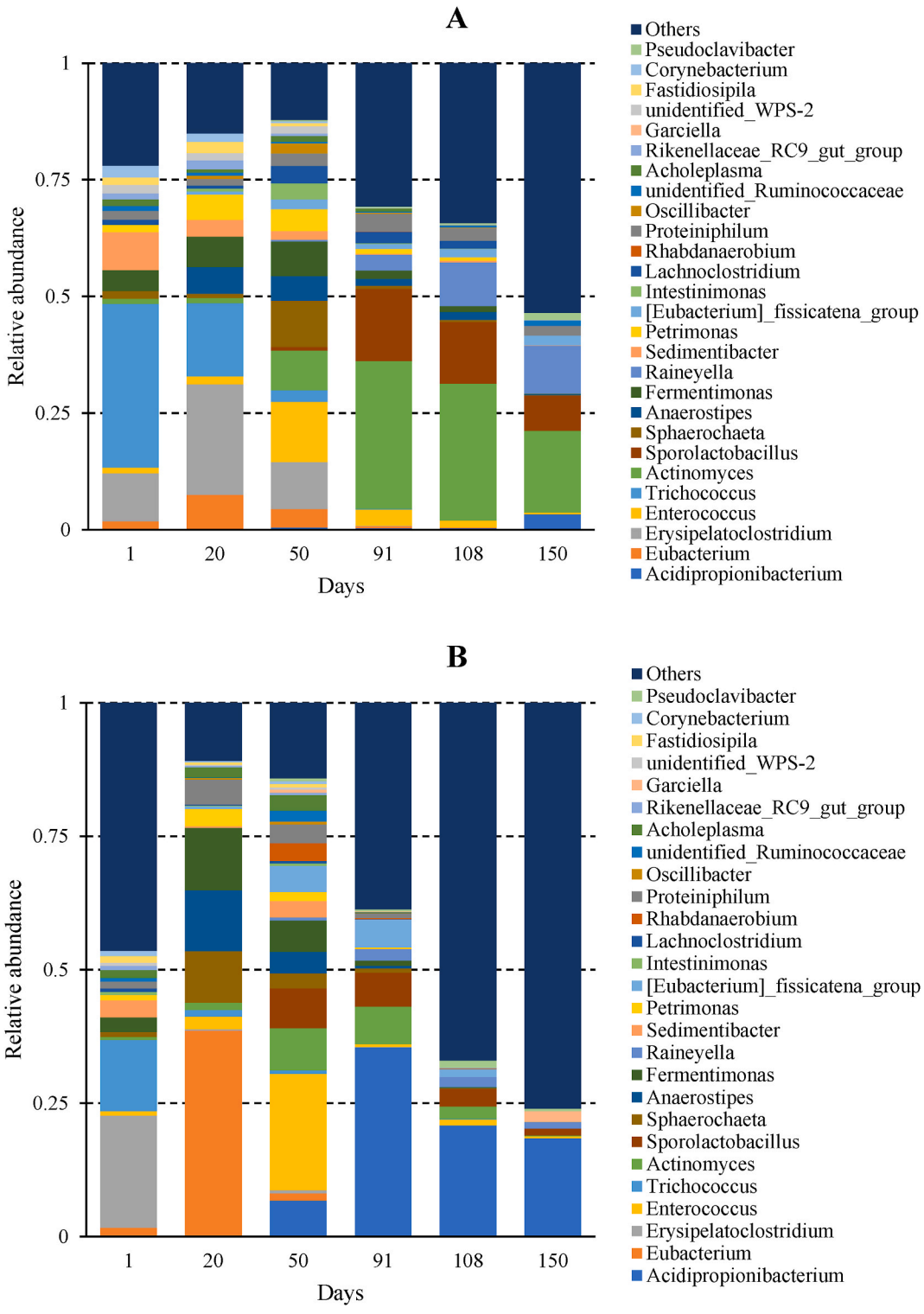

Fig. 9. Bacterial composition at Genus level in (**A**) OLR 2.5 $g_{VS}L^{-1}d^{-1}$; and (**B**) OLR 4 $g_{VS}L^{-1}d^{-1}$.

previously found in cheese whey wastewater AD [90]. *Raineyella* belongs to *Actinomycetota* which includes both mesophilic and neutrophilic microorganisms, able to utilize glucose to produce acetate, lactate and CO_2 [91]. A study by Su et al. [92] showed a significant positive correlation between *Raineyella* and elevated OLR, which contradicts our findings whereby *Raineyella* abundance was lower at OLR of 4 $g_{VS}L^{-1}d^{-1}$. However, it should also be noted that different substrate was used between the studies, which

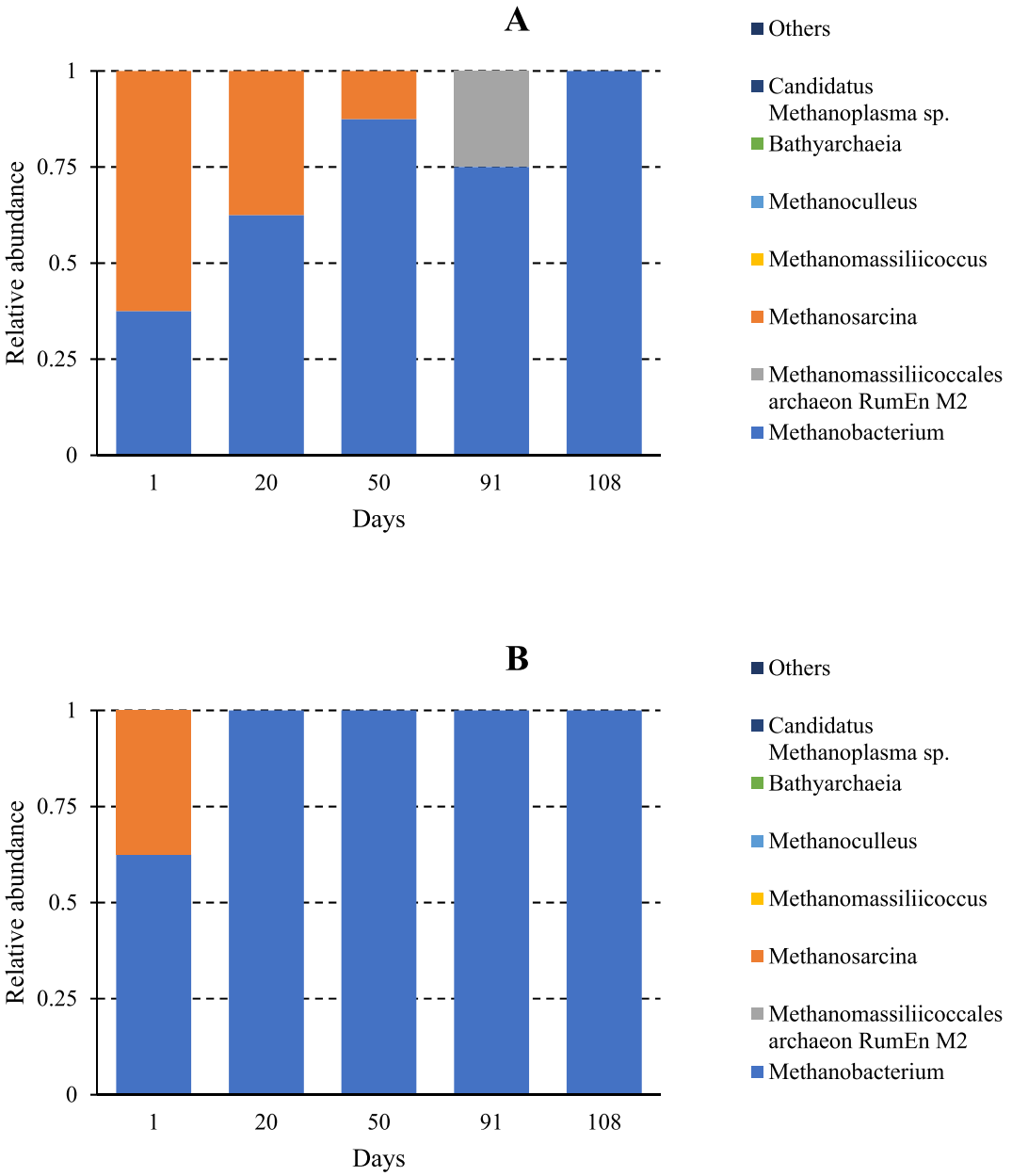

Fig. 10. Archaeal composition at Genus level. (**A**) OLR 2.5 $g_{VS}L^{-1}d^{-1}$; and (**B**) OLR 4 $g_{VS}L^{-1}d^{-1}$.

impacts on the growing environment of the genera. *Acidipropionibacterium* is a rod-shaped, Gram-negative, non-spore-forming, non-motile, and facultative anaerobic bacteria. This bacteria has been shown to be able to utilize a wide range of carbon sources [93] with the potential for propionic acid production via anaerobic fermentation [94]. Our study observed a shift in VFAs composition from being dominated by acetic acid in the earlier HRTs to propionic acid in the later HRTs. Correspondingly, there was an increasing abundance of propionic acid-producing bacteria, such as *Actinomyces* and *Acidipropionibacterium*, which explain the shift in VFAs composition towards higher concentrations of propionic acid.

The 16S rRNA sequencing for archaea in our study revealed that archaeal abundance significantly declined to zero at the final day of observation (day 150). Therefore, this time point was excluded from the presented archaeal relative abundance data (Fig. 10). At the genus level, *Methanobacterium* dominated all reactors throughout all HRTs. Initially, *Methanosarcina* was present during the 1st HRT in all reactors but decreased by the 2nd HRT for OLR 2.5 $g_{VS}L^{-1}d^{-1}$. For OLR 4 $g_{VS}L^{-1}d^{-1}$, *Methanosarcina* was depleted earlier (by day 20) and *Methanobacterium* became dominant. Wang et al. [83] demonstrated that the syntrophic relationship between *Methanosarcina* and *Sedimentibacter* was crucial in enhancing CH_4 production. In our study, the highest CH_4 production rate was observed during high abundance of *Sedimentibacter* and *Methanosarcina*. Both *Methanosarcina* and *Methanobacterium* are commonly detected in AD [95].

Methanosarcina are both acetoclastic and hydrogenotrophic methanogen [96], which have more extensive pathway that produced CH_4 from a wide variety of feedstocks, such as acetic acid, hydrogen and methylated compound [97]. On the other hand, *Methanobacterium* is a strictly hydrogenotrophic methanogen that can only synthesize CH_4 from H_2 and CO_2 [86]. The decreased abundance of *Methanosarcina* in our study then suggested a shift in methanogenesis pathway from acetoclastic to hydrogenotrophic. Such change in the archaea composition could be affected by the decreased availability of acetic acid and increased concentration of propionic acid for methanogenesis. It should be noted that hydrogenotrophic methanogenesis gives out negative free enthalpy ($\Delta G^\circ < 0$), while acetogenesis of propionic acid into acetic acid is an endergonic reaction, thus resulting in hydrogenotrophic methanogenesis as a more favourable pathway. The effect of OLR towards microbial diversity has been previously reported [76,98]. In our study, a change on both bacteria and archaea composition was observed which can subsequently affect the AD performance of whey permeate in a s-CSTR system.

4. Conclusions

Batch system with low amount of inocula can be benefited at 20 °C due to hindered acidification. However, increasing ISR was shown to improve AD performance regardless of incubation temperatures. It was found that directly applying kinetic parameters from batch to s-CSTR led to system failure. In s-CSTR system, CH_4 production rate declined until stabilized after 3 HRTs. Correspondingly, shift in microbial composition was observed, suggesting changes in methanogenesis pathway from acetoclastic to hydrogenotrophic pathway. To our knowledge, our study is the first to observe the correlation between the shift in microbial composition to the AD performance of whey permeate.

CRediT authorship contribution statement

Aldyon Restu Azkarahman: Conceptualization, Methodology, Investigation, Writing – original draft. **Denise Cysneiros:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing. **Afroditi Chatzifragkou:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing. **Kimon-Andreas G. Karatzas:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & edit.

Availability of data and materials

The data and materials are available from the corresponding author upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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