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Research Paper

Effect of feeding frequency on the anaerobic digestion of berry fruit waste

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ABSTRACT

On-site anaerobic digesters for small agricultural farms typically have feeding schedules that fluctuate according to farm operations. Shocks in feeding, particularly for putrescible waste can disrupt the stable operation of a digester. The effect of intermittent feeding on the anaerobic digestion of rejected raspberries was investigated in four 3L reactors operated in semicontinuous mode for 350 days at 38 °C with a hydraulic retention time of 25 days and an organic loading rate (OLR) of 1gVS/L/d. During the acclimatisation period (147 days) the organic loading was 5 feeds per week. The feeding regime of two reactors was then changed while maintaining the same OLR and HRT to one weekly feed event in one reactor and 3 equal feeds per week in another. The feeding regime did not significantly affect specific methane yield (369 ± 47 L/kgVS on average) despite very different weekly patterns in methane production. Volatile fatty acids (VFA) comprised >83 % of the organics in the effluent, while the rest included non-inhibitory concentrations of phenolic compounds (515–556 mg gallic acid/L). The microbial composition and relative abundance of predominant groups in all reactors were the archaeal genera *Methanobacterium* and *Methanolinea* and the bacterial phyla *Bacteridota* and *Firmicutes*. Increasing the OLR to 2gVS/L/d on day 238 resulted in failure of all reactors, attributed to the insufficient alkalinity to counterbalance the VFA produced, and the pH decrease below 6. Overall results suggests that optimal digestion of raspberry waste is maintained despite variations in feeding frequency, but acidification can occur with OLR changes.

1. Introduction

The global annual production of berry fruit is approximately 11 million tonnes (FAOSTAT, 2021), of which over 110,000 tonnes are produced in Australia (Horticulture Innovation Australia Limited, 2021). Because of stringent regulations and consumer standards, 20 to 40 % of this fruit is rejected prior to retail (Dee et al., 2018), resulting in a significant amount of waste requiring management or transport to landfill (Nichols, 2018).

A potential solution to the management of rejected berry fruit is on-site anaerobic digestion (AD) which can provide energy and fertilizer for the producer. This could reduce fossil fuel dependency and greenhouse gas emissions due to uncontrolled waste fermentation, provide local jobs, cleaner energy and more sustainable communities, in line with the United Nations Sustainable Development Goals 7, 8, 9, 12 and 13 (Nations, 2015).

AD technology is widely implemented in Australia for treating several high strength wastes from the agri-food industry (e.g. abattoirs, food processors, piggeries, dairies and poultry farms), there has been no uptake of this technology by the Australian fruit industry (Government, 2017). Challenges that limit the implementation of AD to treat fruit waste include the high risk of acidification, the unbalances in C/N ratio as well as the presence of potential inhibitors. The use of co-digestion, pre-treatment and/or multi-phase systems are the common strategies for overcoming these challenges (Table 1). However, on-site digestion systems need to be cost-effective, simple to operate and able to tolerate feeding that is seasonal and inevitably occasional and irregular within a season, the latter because of weather effects, harvesting schedules and unplanned disruptions. Therefore, understanding the effect of feeding frequency on digester performance is highly relevant in order to effectively operate small scale on-site digesters to avoid overloading and potential acidification (Li et al., 2011).

Several authors have studied the effect of feeding frequency on the extent of solubilisation of organic compounds and methane production

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Table 1
Described challenges and proposed overcoming strategies in the anaerobic digestion of fruits and vegetables.

Reference	Described challenges for AD of fruits and vegetables	Proposed solutions
Agrawal et al. (2023)	• Low pH of the substrates • Low nitrogen content in the substrates • Risk of acidification and VFA accumulation due to the quick hydrolysis of the substrates • Difficulty in operating at neutral pH values	• Co-digestion • Multi-phase systems • Pre-treatment of the substrate prior to AD
Assis and Gonçalves (2022)	• Accumulation of ammonia and VFAs	• Co-digestion • Multi-phase systems • Operation in wet/dry AD
Azevedo et al. (2023)	• Fast production and accumulation of VFA • High C/N ratio	• Co-digestion with sewage sludge
Fermoso et al. (2018)	• Presence of bioactive compounds with inhibitory potential • High risk of acidification due to the low pH of the substrates • A post-treatment required after AD for the total stabilization of the organic matter • Nonoptimal nutrient balance	• Pre-treatments for bioactive compounds removal • Composting for a final organic matter stabilization • Co-digestion
Ji et al., (2017a)	• Process acidification • Presence of pesticides • Adequate addition of microelements	• Co-digestion • Two-phase systems • Thermal pre-treatments
Komilis et al. (2017)	• Low methane yield and a high incidence of process instability due to the accumulation of volatile fatty acids	• Co-digestion • Pre-treatment of the substrate prior to AD
Zhu et al. (2023) Zia et al. (2022)	• Low pH of the substrates • Risk of AD acidification • Process acidification by overloading • VFA inhibition of methanogenic activity	• Co-digestion with some swine faeces and urine • Two-phase systems • Co-digestion with slaughterhouse waste • Pre-treatment can improve the AD process, but it should be able to justify the input cost with the increased biogas yield

VFA, volatile fatty acids; AD, anaerobic digestion.

of lignocellulosic substrates such as rice straw, manures, food waste, easily degradable substrates such as acetate and glucose, and at feeding frequencies ranging from multiple feeding events in a day (Conklin et al., 2006; Svensson et al., 2018) to feeding intervals of multiple days (Manser et al., 2015; Piao et al., 2018; Zealand et al., 2017). However, less is known about the effect of varying the feeding frequency on the long-term reactor performance when digesting substrates with high sugar content as in berry waste and on the structure of the reactor's microbial community. The latter is especially important as it determines the degradation pathways, which reflect the range of phenotypes present in the reactor, many of which are highly sensitive to environmental conditions. The absence of methanogens for example leads to the accumulation of volatile fatty acids (VFA) (Franke-Whittle et al., 2014; Hendriks and Zeeman, 2009) and other anaerobic inhibitors such as phenols (Poirier and Chapleur, 2018).

The study presented here evaluates the influence of feeding frequency on the performance of stirred anaerobic digesters fed with berry fruit, while maintaining the same average organic loading rate (OLR) and hydraulic retention time (HRT). The composition of the microbial community for each stabilised operating condition is also characterised to see at what taxonomic level changes might be induced by feeding frequency.

2. Materials and methods

2.1. Substrate and inoculum

The substrate used in this study was rejected raspberry fruit considered substandard for the market. The fruit was collected from Sunny Ridge® and Pinata® farms located in the Caboolture region, Queensland, Australia. To avoid fermentation prior to characterisation and usage, the fruit waste was blended and stored in a freezer at −15 °C. Two batches of fruit waste were collected from the farms. The first batch was used during the first 213 days of reactor operation, while the second batch was used for the rest of the study.

The microbial inoculum used in the reactors was methanogenic granular sludge collected from an UASB industrial wastewater treatment plant of the XXXX Brewery Company (https://www.xxxx.com.au/), located in Brisbane (Queensland, Australia). The characteristics of each batch of fruit waste and the inoculum are shown in the Supplementary Material (Table S1).

2.2. AD reactor conditions and operation

Four 4 L stirred tank reactors were operated in parallel at a 3 L working volume and a mesophilic temperature of 38.0 ± 0.5 °C. The reactors were inoculated with 10 g VS/L (VS, volatile solids) of methanogenic granular sludge and then operated at a fixed HRT of 25 days, an OLR of 1 g VS/L/d, and a feeding frequency of five times per week (5/7d) during a 147-day acclimatisation phase (Phase 0). The influent media used was distilled deionised water with 7.36 g $NaHCO_3$/L added with the feed that allowed to maintain the total alkalinity above 4380 mg $CaCO_3$/L in the reactors as recommended by other authors using a fruit waste with similar characteristics (Siles et al., 2013). Trace elements were added to the influent media from day 64 onwards at concentrations prescribed by Zhang et al. (2012) and macronutrients were added to the influent media on day 72 at varying concentrations, and from day 93 onwards, at the levels recommended by Angelidaki et al. (2009).

After the acclimatisation period (day 147 to 350), each reactor was operated at a different feeding frequency while maintaining the same OLR and HRT in all reactors. Two reactors were operated at a feeding frequency of 5 times per week (5/7d) to assess the reproducibility of the results, one reactor was fed three times per week (3/7d) and another one once per week (1/7d).

The reactors were mixed intermittently at a stirrer speed of 60 rpm for 10 min every 4 h. Digester feeding and discharge occurred according to the feeding frequency and followed a draw-fill mode, where digester effluent was removed prior to feeding. This was done during reactor stirring to ensure proper mixing. Feeding was administered with a 50 mL plunger via a valve located on top of the reactor. Effluent samples were collected for chemical analyses daily in the acclimatation phase and twice per week afterwards, that is pH, total solids (TS), VS, alkalinity, soluble chemical oxygen demand (sCOD). VFA analyses were performed once per week on samples collected after the weekend period during which the reactors were not fed. Biogas production and biogas composition was measured each day, Monday to Friday.

2.3. Analytical techniques

TS, VS, sCOD, alkalinity and pH of the effluent samples were measured in accordance with Standard Methods (APHA, 2005), while soluble phenols were determined through the Folin-Ciocalteau method (Lamuela-Raventós, 2017). Prior to sCOD, VFA and soluble phenol analysis, effluent samples were centrifuged at 4500 rpm for 20 min. The supernatant was filtered (0.22 μm) for soluble determinations. sCOD was measured using a Merck COD Spectroquant® test kit (range

25–1500 mg/L and 500 to 10,000 mg/L) and a (Spectroquant®Prove300) spectrophotometer at 620 nm. The VFA profile (C2–C6) was determined using gas chromatography (Agilent Technologies 7890A GC System, CA, USA). pH was measured using a SPER scientific pH probe. Total phenol was measured by colorimetric adsorption at a wavelength of 655 nm after the addition of the Folin-Ciocalteau reagent.

Biogas samples were collected with a 50 mL SGE Luer lock glass syringe for gas composition analysis. CH_4, CO_2, N_2, and O_2 concentrations were measured using gas chromatography (PerkinElmer® Clarus 590 GC) equipped with a packed-column injector, a HSN6-60/80 Sulfinert packed column (7′ x 1/8″ OD), a MS13X4-09SF2 40/60 packed column (9′ x 1/8″ OD) and thermal conductivity and flame ionization detectors (with 10^7 linear dynamic range), Argon was used as the carrier gas.

2.4. Microbial analysis

Samples for phylogenetic analysis were collected from each reactor once stable and repeatable performance was achieved. Prior to analysis, samples were stored at −20 °C. Genomic DNA was extracted using the FastDNATM SPIN kit for soil (MP Biomedicals, USA). Extractions were conducted according to the manufacturer's protocol. The V6 to V8 region of the 16S rRNA gene was amplified using the universal primers 926F (5′- AAACTYAAAKGAATTGACGG-3′) and 1392R (5′-ACGGGCGGTGWGTRC-3′) (Engelbrektson et al., 2010). Genomic DNA was submitted to the Australian Centre for Ecogenomics for 16S rRNA amplicon sequencing (https://ecogenomic.org/). Sequencing was performed on the DNA using the Illumina MiSeq platform (Illumina, USA) (Hölzer and Marz, 2019). 16S rRNA amplicon sequences were processed with the DADA2 (v.1.10.0) pipeline (Callahan et al., 2016) implemented in RStudio (v.3.5.1). Forward reads were trimmed at 250 bp to retain sequences with a quality score >30. Reads with >2 expected errors were discarded. Dereplication, amplicon sequence variants (ASV) inference and chimera removal were run with DADA2 default parameters. Taxonomic assignment of the ASVs was performed using assigned taxonomy function in DADA2 with a minimum bootstrap confidence of 50 % using SILVA v.138.2 database (Quast et al., 2012).

2.5. Data analysis

The reactor results data was analysed statistically using SigmaPlot™ version14.0, Systat Software and Microsoft Excel 2010 Software. The t-student and ANOVA tests (5 % significance threshold) were used to identify significant effects in considered performance variables between the reactors. Microbial communities were explored using vegan (v.2.5.3) and phyloseq (v.1.26.0) packages (McMurdie and Holmes, 2013). Relative abundance of archaeal and bacterial types was expressed as fractions of those respective domains. Differences in community composition and dynamics were investigated by principal component analysis (PCA). The influence of FF on microbial communities was evaluated with PERMANOVA using Bray-Curtis dissimilarity.

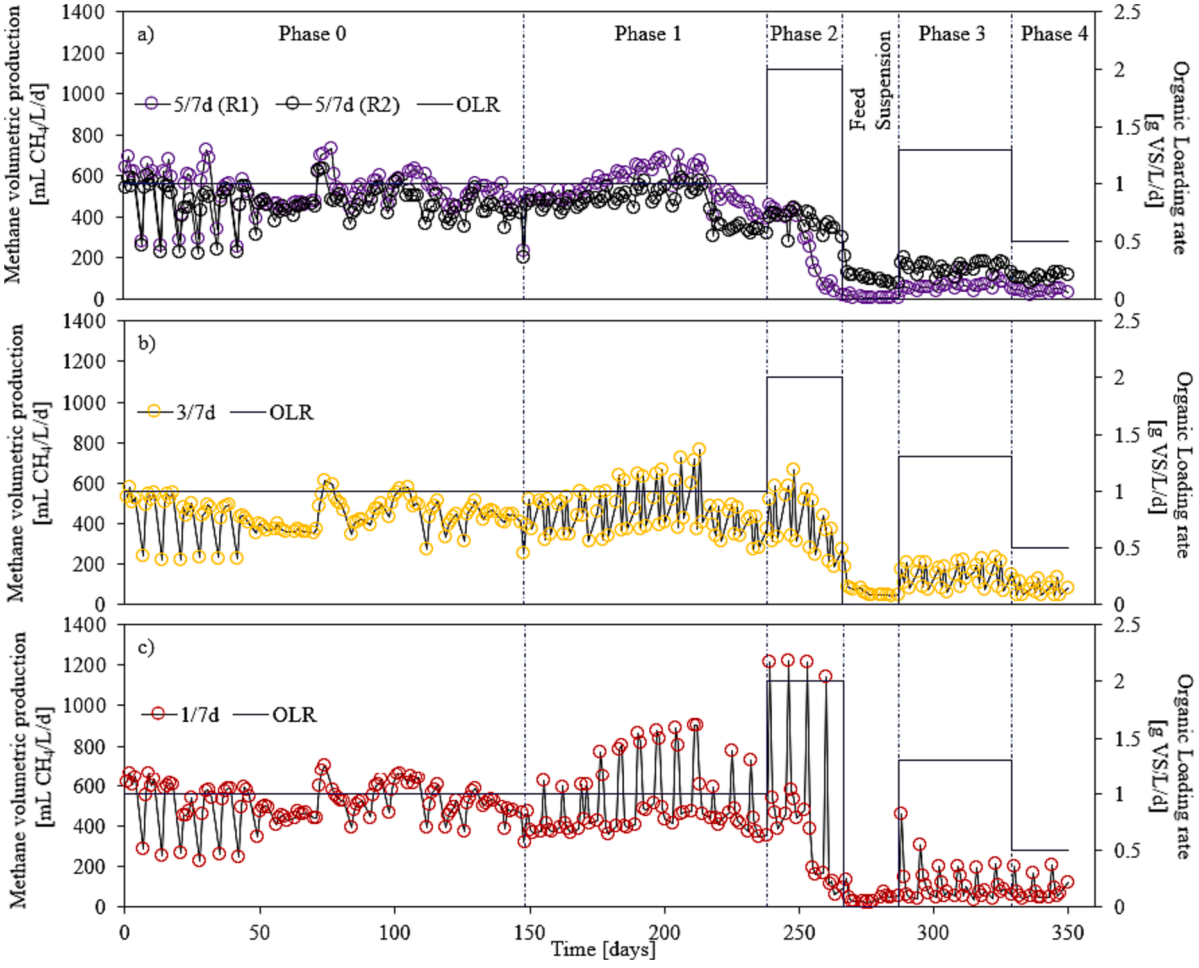

Fig. 1. Effect of feeding frequency of a) 5/7d; b) 3/7d and c) 1/7d on the daily volumetric methane production of anaerobic digestion of berry fruit waste at different organic loading rates of 1 g VS/L/d (Phase 1), 2 g VS/L/d (Phase 2), 1.3 g VS/L/d (Phase 3) and 0.5 g VS/L/d (Phase 4). R1, R2 = duplicate reactors feed at a frequency of 5/7d.

3. Results and discussion

3.1. Acclimatisation phase of the reactors

The objective of the acclimatisation period (Phase 0) was to provide replicate stable conditions in all reactors fed according to the 5/7d feeding frequency before changing the feeding frequency of two reactors to 3/7d and 1/7d. Methane production followed a repeatable weekly cycle, consisting of baseline daily methane production over the weekend when the reactors were not fed followed by a sharp increase in daily methane production over the 5 days that the reactors were fed (Fig. 1). This cyclic trend changed to steady methane production from day 43 where the fall in methane production over the weekend was no longer discernible and there was a slight decrease in average methane yields from 488 ± 133 mL CH_4/L/d, prior to day 43, to 446 ± 63 mL CH_4/L/d for the period days 43 to 63. This, along with the increase in VFA concentrations (Fig. 2) indicated a decrease in biodegradation and accumulation of a fraction of the fruit waste. In response, trace elements were added to the buffered media from day 64 and throughout all the reactor operation, and the concentrations of Ni (0.20 ± 0.07 mg/L), Co (0.47 ± 0.18 mg/L), Mo (0.04 ± 0.01 mg/L), Se (0.04 ± 0.03 mg/L), Zn (5.45 ± 0.78 mg/L) and Fe (21.46 ± 2.43 mg/L) were maintained within methanogenic stimulatory concentrations (Romero-Güiza et al., 2016).

The addition of trace elements had no effect on the daily methane production which remained steady over feeding and non-feeding days for 7 days. Macronutrients then were added on day 72, which immediately resulted in a spike in methane production as well as ammonia, at concentrations similar to the ones in the digester start-up (410 mg NH_3-N /L). Both values declined again without further macro-nutrient addition and therefore macro-nutrients were added to the feeding media from day 93 onwards which resulted in the re-establishment of the weekly cycle where daily methane production peaked on feeding days. The mean daily methane production from day 93 until the end of the establishment phase (Phase 0) for the four reactors was 543 ± 79 mL CH_4/L/d with no significant difference between the four reactors (Fig. 1). The effect of feeding frequency on methane production could then be explored.

3.2. Effect of feeding frequency on the reactor's performance at OLR of 1 g VS/L/d

The feeding frequency in two of the four reactors was changed to 1/7d and 3/7d on day 143 to start Phase 1 of the experiment (Fig. 1). The methane yield in all reactors increased steadily in the period day 143 to day 214 in all four reactors. Variation in the daily methane production increased as the frequency of feeding reduced. That is, the most frequent feeding of 5/7d had the least variation in methane production followed by 3/7d, while the infrequent feeding of 1/7d had the largest weekly oscillations (Fig. 1). Methane production was reduced to a baseline level in the 1/7d reactor before the same pulse of fruit waste one week later. The high response in methane production to the more concentrated pulse of substrate in the 1/7d reactor indicates that biodegradation activity in the reactors was substrate limited. Despite the difference in the size of the oscillations between reactors in daily methane production, the weekly methane yield was not significantly affected by changes in the feeding frequency, that is, it ranged from 343 ± 32 for the 3/7d reactor to 383 ± 60 mL CH_4/gVS for the 1/7d reactor, with the yield for the 5/7d reactors lying between those values. Zealand et al. (2017) applied the same feeding regimes of 1/7d, 3/7d and 5/7d in semi-continuous digestion experiments on rice straw at an OLR of 1 g VS/L/d also observed that methane yield was unaffected by the feeding regime. Their methane yield ranged from 112 to146 mL/gVS/d) which was lower than the yields in this study (Table 2) because of the

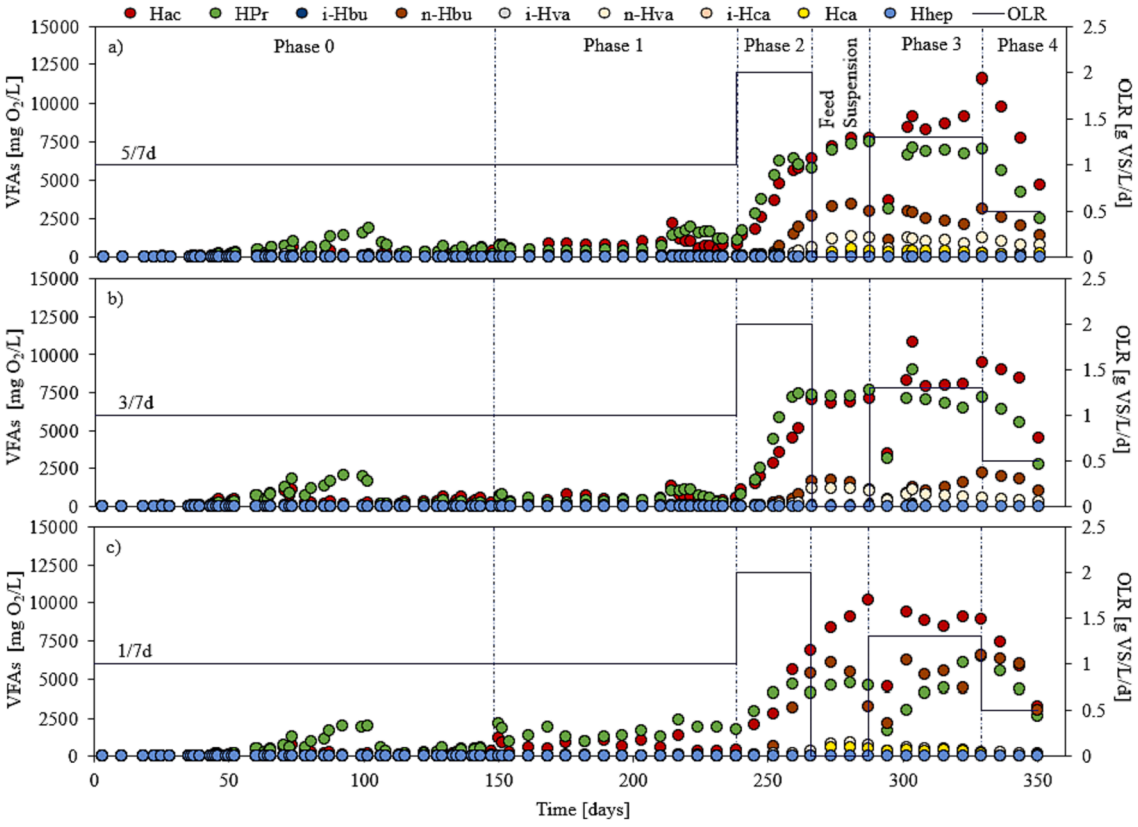

Fig. 2. Effect of feeding frequency of a) 5/7d; b) 3/7d and c) 1/7d on the VFA production in the anaerobic digestion of berry fruit waste at different organic loading rates (OLR) of 1 g VS/L/d (Phase 1), 2 g VS/L/d (Phase 2), 1.3 g VS/L/d (Phase 3) and 0.5 g VS/L/d (Phase 4). Hac (Acetic acid), HPr (Propionic acid), i-Hbu (Isobutyric acid), n-Hbu (Butyric acid), i-Hva (Isovaleric acid), n-Hva (Valeric acid), i-Hca (Isocaproic acid), Hca (Caproic acid), Hhep (Heptanoic acid).

Table 2
Mean effluent characteristics and reactors performance data for the AD of berry fruit waste at different FF and OLR of 1 (Phase 1) and 2 (Phase 2).

Feeding Mode [feed/week] [a]	5/7		3/7		1/7	
Organic Loading Rate (g VS/L/d)	1.0	2.0	1.0	2.0	1.0	2.0
Biogas Production [mL/L/d]	1425 ± 647	1471 ± 519	1323 ± 737	1685 ± 995	1521 ± 645	2001 ± 1549
CH_4 content [%]	57.2 ± 5.8	36.0 ± 9.7	58.6 ± 5.3	43.6 ± 7.5	55.9 ± 11.7	44.5 ± 14.4
CH_4 Production [mL CH_4/L/d]	538 ± 69	363 ± 98	491 ± 122	451 ± 134	546 ± 184	562 ± 364
CH_4 Yield [mL CH_4/g VS]	381 ± 49	138 ± 37	343 ± 32	168 ± 19	383 ± 60	213 ± 62
pH [-]	7.3 ± 0.1	6.8 ± 0.5	7.3 ± 0.1	7.0 ± 0.3	7.1 ± 0.2	6.7 ± 0.5
sCOD [mg O_2/L]	1868 ± 802	9658 ± 5778	1228 ± 514	7630 ± 5035	2545 ± 661	10616 ± 5870
VFA Total [mg O_2/L]	1568 ± 1089	8499 ± 4065	1075 ± 545	6766 ± 4012	2471 ± 736	9257 ± 4371
Acetic [mg O_2/L]	889 ± 666	3310 ± 1734	558 ± 304	2572 ± 1278	778 ± 330	3485 ± 1885
Propionic [mg O_2/L]	567 ± 376	4395 ± 1867	454 ± 250	4102 ± 2658	1451 ± 434	3932 ± 894
Alkalinity Total [mg $CaCO_3$/L]	5817 ± 381	6680 ± 680	6043 ± 417	6744 ± 758	5784 ± 402	6556 ± 932
VFA/Alkalinity [b]	0.27 ± 0.19	1.37 ± 0.78	0.18 ± 0.09	1.06 ± 0.75	0.41 ± 0.11	1.50 ± 0.94
VFA% in sCOD [%]	83 ± 19	94 ± 14	89 ± 29	92 ± 9	97 ± 11	90 ± 10
Phenol Soluble [mg Gallic acid/L]	515 ± 169	452 ± 69	513 ± 203	428 ± 82	556 ± 203	513 ± 120

[a] Data is the mean of analysed samples during the reactor operation, except VFA/Alkalinity [-] and VFA% in sCOD [%], which considered data from days on which both parameters were analysed. [b]mg O_2/L/ mg $CaCO_3$/L.

recalcitrant nature of the rice straw where digestion is limited by hydrolysis (Ji et al., 2017b; Shrestha et al., 2017). Comparing the methane volumetric production, other authors have found a significantly higher methane production (6300 ± 600 mL/L/d and 2700 ± 300 mL/L/d) with frequent feeding of 10 times per day (Svensson et al., 2018), and infrequent feeding of 4 times per day (Vital-Jacome and Buitrón, 2021), while digesting steam exploded food waste and winery waste, respectively, which may be due to both, the difference in substrate characteristics and a higher OLR applied in those studies (10.7 and 2.7 g VS/L/d).

The methane production decreased when a new source of raspberry waste (Fig. 1) was introduced on Day 214. The introduction of the new substrate resulted in an increase in VFA concentrations in all reactors which then decreased within 30 days to the stabilised levels observed prior to the substrate change. This indicates that the decreased methane yield was due to the biodegradability of the substrate rather than the stability of the reactors.

The feeding frequency of 1/7d also resulted in the highest weekly oscillations in pH (Fig. 3). The same weekly oscillations in VFA concentrations are not evident in Fig. 2 because VFA was analysed from the reactor once per week. VFA concentrations were higher in 1/7d reactor compared to the other reactors, even though the reactor was not fed for 7 days prior to collecting a sample for analysis. VFA comprised 97 % of the sCOD in the effluent from the 1/7d reactor compared to 83 % and 89 % for the 3/7d and 5/7d reactors (Table 2). The average total VFA concentration in the effluent from 1/7d reactor over Phase 1 was 2471 ± 736 mg O_2/L, compared to 1568 ± 1089 mg O_2/L for the 5/7d reactor and 1075 ± 545 mg/L for the 3/7d reactor (Table 2). The COD of the VFA in the effluent from the 1/7d reactor was 6 % of the total COD leaving the reactor as either methane or sCOD. Despite having the highest VFA concentrations, the methane yield from the 1/7d reactor was marginally higher than that from the 3/7d and 5/7d reactors, which indicates that VFA did not inhibit the methanogenic activity (Li et al., 2011).

The concentration of the individual VFA compounds maintained a stable profile except for a slight fluctuation observed on day 220 (Fig. 2) in response to the switching of the source of raspberry waste on day 214. The highest VFA concentrations were again in the 1/7d reactor, where propionic acid was the predominant acid (59.57 % of the VFA COD, Figure S1) at a concentration of 1451 ± 434 mg O_2/L, while the predominant VFA in the 5/7d and 3/7d reactors was acetic acid (Table 2). Although propionic acid accumulation is indicative of unbalanced digestion conditions, the methane yield from the 1/7d reactor was not less than the 5/7d and 3/7d fed reactors. This may be because the propionic acid concentration in the 1/7d reactor was still less than what is proposed to be an inhibitory level of 3000 mg/L (Ji et al., 2017b).

Phenolic compounds were identified in all the reactors (Table 2, Figure S1). Interestingly, the concentration of these compounds was similar in all reactors and independent of the feeding frequency (515 ± 169 mg/L (5/7d), 513 ± 203 mg/L (3/7d), and 556 ± 203 mg/L (1/7d) even with the change in OLR on the following phases of the reactor's operation. This indicates that the accumulation of these compounds was dependent only of the OLR. The concentration throughout this study were below the inhibitory limit of 1000 mg/L (Fedorak and Hrudey, 1984; Siles et al., 2013) and therefore it was unlikely that process inhibition occurred due to the release of these compounds.

As expected, the total alkalinity was the lowest in the 1/7 d reactor (5784 ± 402 mg $CaCO_3$/L, Table 2) as this parameter decreases with VFA accumulation (Drosg, 2013). Nevertheless, the VFA/Alkalinity ratio was below the instability threshold of 0.3–0.4 (Chen et al., 2008).

3.3. Effect of increasing the OLR on the reactor's performance

Increasing the OLR to 2 gVS/L/d while maintaining the same HRT and feeding frequencies resulted in declining methane production in all reactors, with a commensurate fall in pH and an accumulation of VFA (Figs. 1-3). The 1/7d reactor was more resilient to the higher organic loading, but methane production continued to trend downwards while an OLR of 2 gVS/L/d was maintained. This may be attributed to the spikes and accumulation of VFA in the reactors (Zealand et al., 2017). Others have reported OLR limits below 2 gVS/L/d for various fruit and vegetable waste digested in single stage continuous digesters. (Cubero-Cardoso et al., 2023; Trujillo-Reyes et al., 2023; Zhang et al., 2022). Cubero-Cardoso et al. (2023) described a drop in methane yield during the anaerobic digestion of strawberry extrudate waste when the OLR increased from 1.0 to 1.5 g VS/L/d due to the acidification of the reactors. Trujillo-Reyes et al. (2023) found the CSTR digestion of a blend of mixed fruit and vegetables could be maintained at an OLR of 1gVS/L/d, but failed with a sharp drop in pH and methane production when the OLR was increased to 3 gVS/L/d. A more moderate increase in OLR to 1.5 gVS/L/d after re-inoculation also induced instability. Trujillo-Reyes et al. (2023) attributed this failure to the inhibitory effect of monoterpenes, particularly limonene which is found in the peel of citrus fruit. Of relevance to the current work is that α-pinene, also a monoterpene, which is an important flavour component in raspberries (Trujillo-Reyes et al., 2019). The digestion of banana peel failed when the OLR was increased from a stable operation at 0.41 gVS/L/d to 0.83gVS/L/d (Odedina et al., 2017). Similarly, the stable digestion of whole bananas could not be maintained in a fed-batch digester when the OLR was increased from 0.6 kgVS/L/d to 1.6 kgVS/L/d (Clarke et al., 2008). Zhang et al. (2022) found the operation of CSTR fed with apple pomace

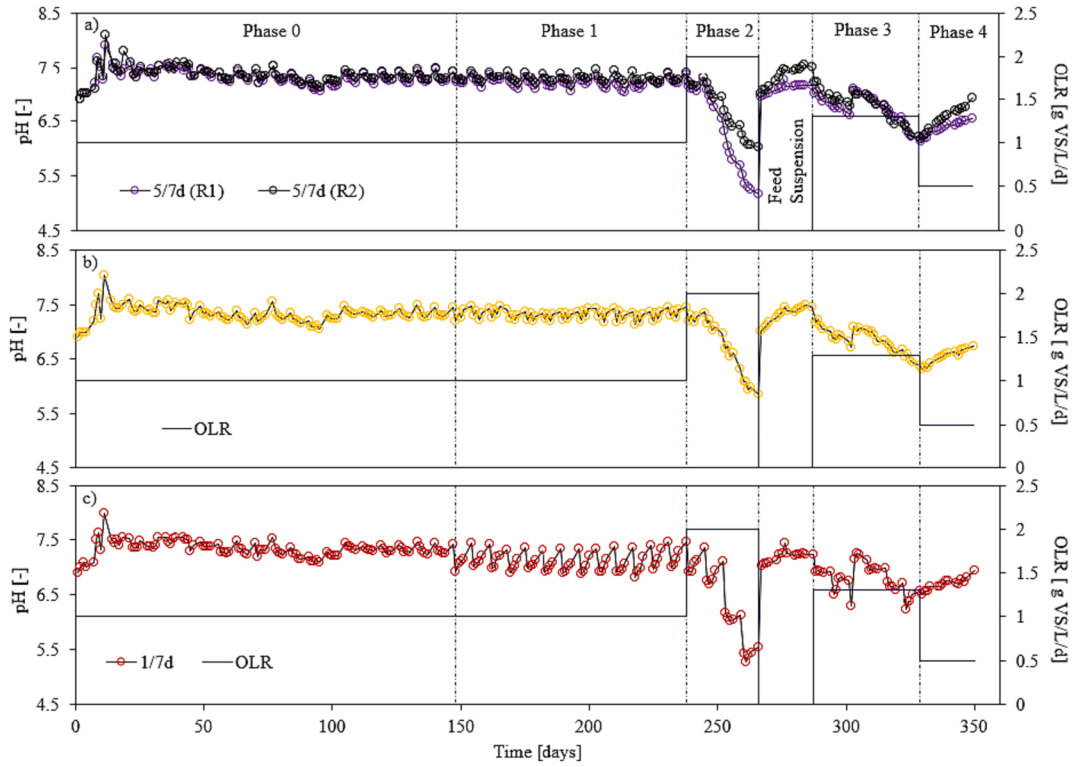

Fig. 3. Effect of feeding frequency of a) 5/7d; b) 3/7d and c) 1/7d on pH in the anaerobic digestion of berry fruit waste at different organic loading rates of 1 g VS/L/d (Phase 1), 2 g VS/L/d (Phase 2), 1.3 g VS/L/d (Phase 3) and 0.5 g VS/L/d (Phase 4). R1, R2 = duplicate reactors feed at a frequency of 5/7d.

at an OLR of 1.44 kgVS/L/d and varying amounts of waste activated sludge (WAS) soured if the OLR of WAS was less than 0.48 kgVS/L/d. A similar OLR limitation has even been observed for rice straw where methane yields could not be maintained at an OLR of 2 gVS/L/d if a single stage reactor was fed at a frequency less than once per week.

The high concentration of VFA in all the reactors suggested an organic overload and imbalance in the anaerobic methanogenic pathways. More than 90 % of the soluble organic matter, measured as sCOD was metabolised into VFA compounds in all reactors (Table 2), reflecting uninhibited activity by acidogenic bacteria (Serrano et al., 2020). The highest accumulation of propionic and butyric acid occurred in the 1/7d reactor (Fig. 2, Figure S2).

The rapid acidification that occurred in response to feeds is also evident in the pH trend for the 1/7d reactor during Phase 2, where this parameter fell sharply and irrecoverably each time the reactor was fed (Fig. 2). The pH fell in reactors to well below the optimal pH range of 7.3–7.8 for methanogenic activity (Fannin, 1987; Serrano et al., 2014; Wheatley, 1990). This might be remedied by supplementing the raspberry waste feed with a higher concentration of $NaHCO_3$ which was set at 7.36 g/L throughout the operation of all reactors.

The total VFA concentrations accumulated to levels between 6500 and 10,000 mg/L and was mainly composed of acetic and propionic acid in all reactors by the end of Phase 2 (Fig. 2). Such levels should not be inhibitory in well buffered conditions (Ji et al., 2017b). The average total alkalinity in the reactors (Table 2) was always well above 5000 mg $CaCO_3$/L, a buffering level that maintained neutral pH conditions for the digestion of pre-treated and raw strawberry waste (Siles et al., 2013), although the digestion and therefore the OLR of raw strawberries was inhibited by the presence of achenes, the ligneous seeds and associated phenolic compounds on the skin of the strawberries. A large proportion of the total alkalinity in the reactors was partial alkalinity from the buffering effect of VFAs at pH levels well below that suitable for sustainable methanogenic activity. This was confirmed by the intermediate/partial alkalinity (IA/PA) ratio, which was between 1.40 ± 1.68 and 2.99 ± 3.7 when the OLR in this study was 2 gVS/L/d, far higher than the limit of 0.8 for stable digester operation (Drosg, 2013).

Volatile fatty acids are present in undissociated form when the reactors pH range between 5 and 6. The undissociated form of VFAs can permeate bacterial cells and adversely affect microorganisms (Agrawal et al., 2023). Consequently, the lack of response of the digesters to the $NaHCO_3$ and low OLR interventions (day 266-Fig. 2) may have been because of the earlier attrition of critical functional groups as the result of high VFA levels in low pH conditions. Therefore, contrary to other authors (Kim and Lee, 2015), the application of the aforementioned strategies, followed by a new re-adjustment of the pH (day 302), and a slight decrease in the OLR to 1.3 g VS/L/d (Phase 3) were insufficient to recover the methanogenic activity. It was only after a further decrease in OLR to 0.5 g VS/L/d (Phase 4) that the reactors began to recover, evident by an increase in the pH and a decline in VFA concentrations (Figs. 2 and 3).

3.4. Microbial community richness and diversity analysis

Fig. 4 shows the alpha diversity plots of bacteria and archaea throughout the reactor's operation. The alpha diversity plots indicate that the number of bacterial amplicon sequencing variants (ASV) in the original inoculum was maintained in all reactors throughout the acclimatisation period (Phase 0). This diversity continued to be maintained in all reactors when two were switched to 3/7d and 1/7d feeding (Phase 1). The maintenance of the diversity was in line with the values of the Simpson Index which remained close to 1 indicating that a clear dominance of a bacterial group did not occur (Fig. 4). The number of archaeal ASV was also maintained throughout Phase 0, but then reduced in the 3/7d and 1/7d reactors during Phase 1 (Fig. 4). Most of the lost variants comprised a small percentage of the archaeal population, as shown by constant Simpson and Shannon Indices throughout Phase 0 and Phase 1. This slight decrease in the diversity would be a consequence of the adaptation of the inoculum to new operational conditions (Ferguson et al., 2016; Svensson et al., 2018).

The heat map of relative abundance indicates that among the

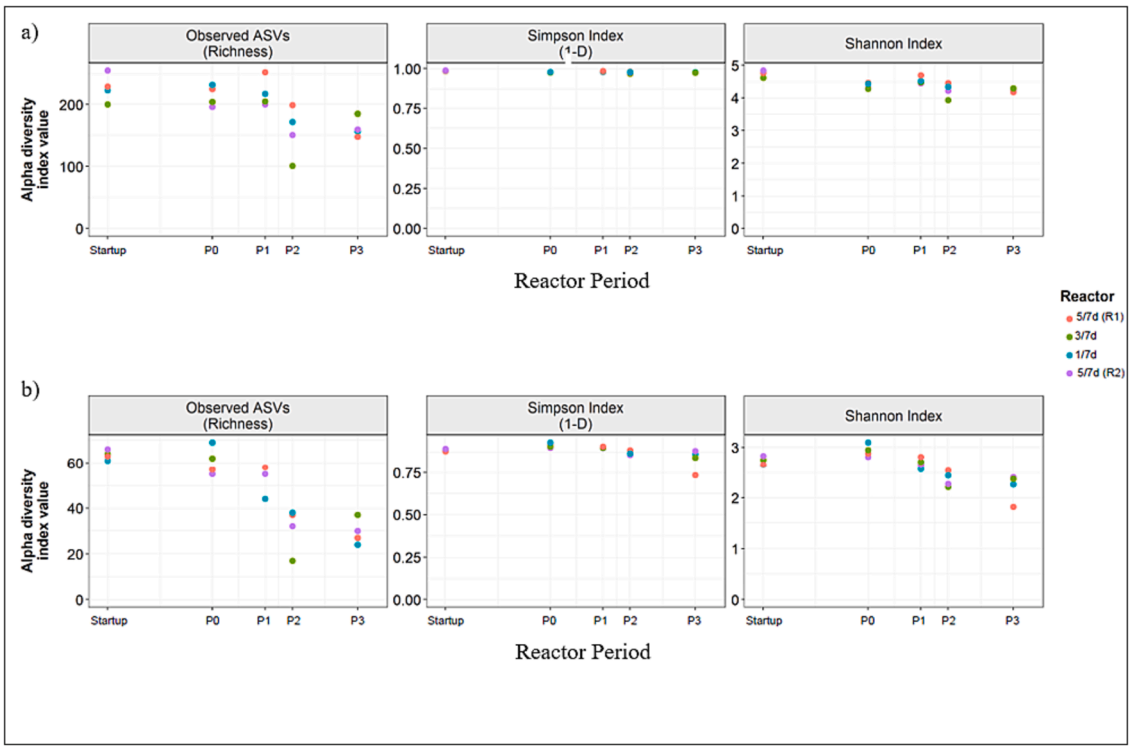

Fig. 4. Alpha diversity plots of a) Bacteria and b) Archaea from the anaerobic digestion of berry fruit waste at different feeding frequency and organic loading rates. P0 (end of Phase 0 - Acclimation), P1 (end of Phase 1 – 1 g VS/L/d), P2 (end of Phase 2 – 2 g VS/L/d), and P3 (end of Phase 3 – 1.3 g VS/L/d, were VS, volatile solids).

archaea population, the relative abundance of the identified hydrogenotrophic methanogens, i.e., Methanobacterium and to a lesser extent Methanolinea, were the most abundant methanogens in the digesters regardless of the feeding frequency (Fig. 5). In contrast, a higher decrease in the relative abundance was observed for *Methanosaeta*, the most abundant acetoclastic methanogen in the digester, which decreased from a relative abundance of 12 % to values below 4 % (Fig. 5). The higher sensitivity of the genus *Methanosaeta* to the concentration of acetic acid would explain the observed marked depletion (Conklin et al., 2006).

The overall decrease in the relative abundance of the methanogens was linked to an increase in the relative abundance of *Bacteridota* and *Firmicutes*, which represents more than 30 % of the total relative abundance of bacteria in the digesters, regardless of the applied feeding frequency (Fig. 5). Many species in phyla Firmicutes and Bacteroidetes are acidogenic bacteria (Eichorst et al., 2013). Moreover, Firmicutes has been reported to be involved in the hydrolysis of cellulose to sugars (Ni et al., 2022). The similar microbial population structure among the different feeding frequency modes (Fig. 5 and Figure S3) would explain the observed resilience in the 1/7d reactor, whose microbial consortia were able to recover and retain the system performance after each bulk feeding event (Fig. 1 – Phase 1). In agreement with this study, De Vrieze et al. (2013) obtained the same average methane production with similar bacteria richness from the AD of synthetic sewage sludge at different feeding frequencies (daily vs. every 2 day).

The relative abundance analysis showed an important increase in phylum Bacteroidetes, which reached relative abundances around 18 % at the end of Phase 2, regardless of the feeding frequency (Fig. 5). This, along with the results showing a higher accumulation of sCOD and its almost total conversion into VFA (Table 2) indicate that the acidogenic activity was not affected by the destabilisation of the reactors. Hydrolytic bacteria from phylum *Firmicutes* also showed an increase at the end of Phases 2 and 3 as compared to Phase 1 relative abundances (Fig. 5), in line with the observed high accumulation of sCOD (Table 2). The accumulation of VFA can also be related to a variation in the relative abundances of acetoclastic and hydrogenotrophic methanogens, such as *Methanobacterium*, increasing in abundance relative to the acetoclastic methanogens (Ni et al., 2022). As an example, acetoclastic methanogens *Methanosaeta* decreased from 1.6 % to 0.6 % from the end of Phase 1 to the end of Phase 3 for a feeding frequency of 5/7d (Fig. 5). The preponderance of the hydrogenotrophic methanogenic pathway would be explained by the higher tolerance of these microorganisms to acid pH conditions in comparison to acetoclastic methanogens (Wang et al., 2020). These results were in line with Trujillo-Reyes et al. (2023), which correlated the accumulation of VFA in an anaerobic reactor fed with wholesale market waste with a decline of acetoclastic methanogens and, at the same time, the promotion of a strict hydrogenotrophic methanogen classified as *Methanobrevibacter* genus. These authors also reported that the hydrogenotrophic methanogenic activity was not able to counterbalance the VFA accumulation in the digesters, like in the present research (Fig. 2). The low variations observed in the microbial communities among the reactors, along with the similarity in the performance of all reactors, indicates that feeding frequency did not strongly affect reactor behaviour, unlike OLR where a small increment of an additional 1gVS/L/d led to reactor failure in al reactors regardless of feeding frequency.

4. Practical implications

Considering the high putrescibility of berry fruit waste, the rejects need to be treated as they are produced with little scope for the farmer to store the waste. The findings from this study show that a reactor can be sized to accommodate the rate at that rejected berry fruit accumulates at a farm. This waste could be fed to the reactor continuously but more realistically semi-continuously, especially on small scale farms where the operation is likely to be less automated. Interestingly, despite the greater fluctuations in methane production in the digester operated at a feeding frequency of 1/7d, the mean methane yield from this reactor was higher, although not significantly more than the frequent feed reactors. This is promising because it indicates that a digester can absorb

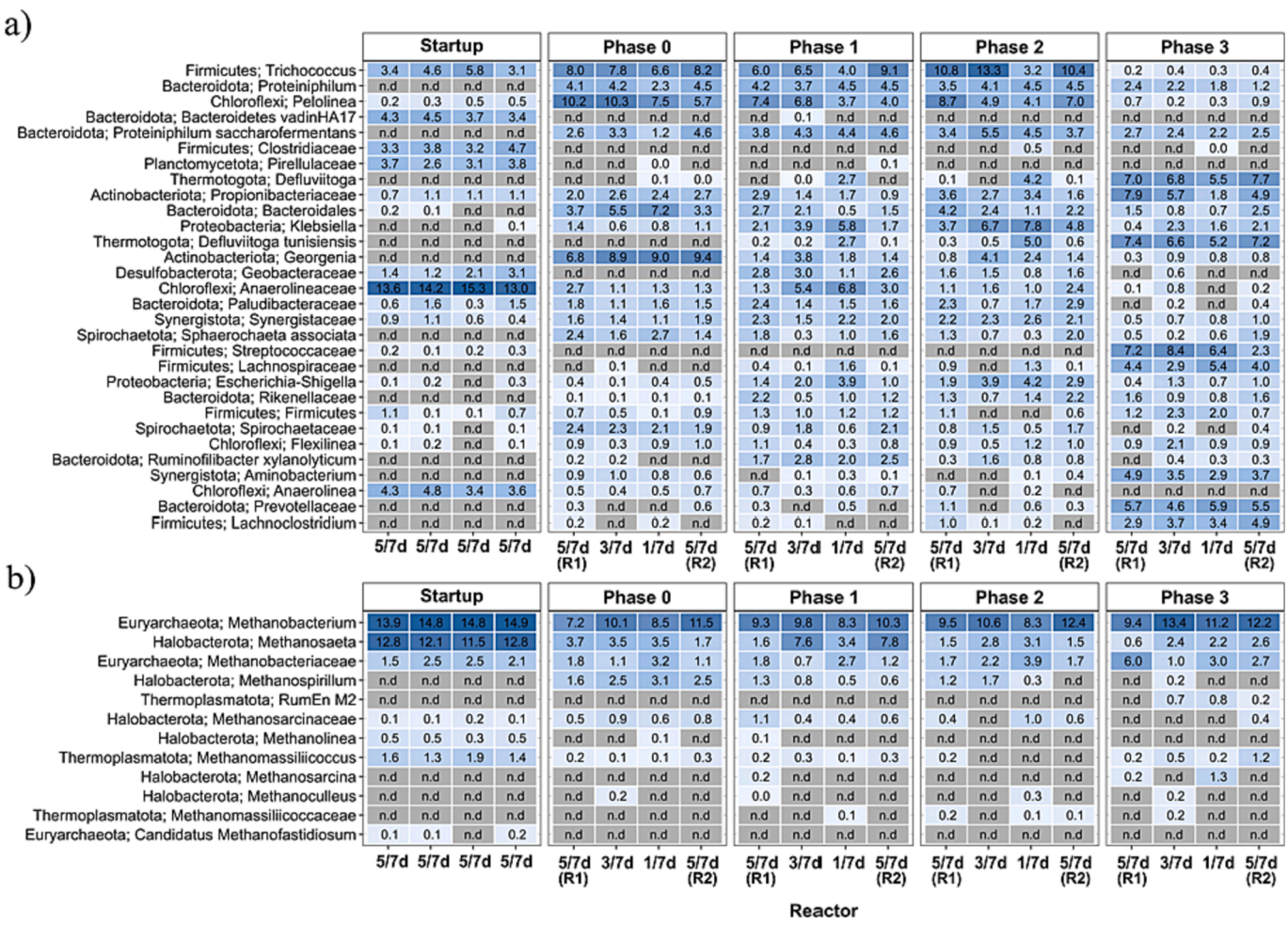

Fig. 5. Heat maps of relative abundance of taxa in each sample, a) bacteria and b) archaea.

intra-seasonal peaks of berry waste production from a farm without a significant impact on the stability of the reactor.

5. Conclusions

This study demonstrated that the digestion of raspberry waste can be sustained at different feeding intervals. The experiments demonstrated that stable and repeatable operation was achieved at feeding frequencies of 5/7d, 3/7d and 1/7d. Methane yield was maintained in the 1/7d reactor despite this reactor exhibiting the largest fluctuations in daily methane production in response to the same OLR applied in one concentrated weekly feeding pulse. The feeding frequency mode did not significantly impact the microbial composition and relative abundance. As opposed to feeding frequency, an increment of 1 gVS/L/d in the OLR lead to the failure of all reactors, regardless of feeding frequency, which may be caused by the insufficient buffer in the reactors to neutralise the increase in VFA production. Future studies should focus on finding other strategies that make on-site digesters for berry fruit waste suitable for coping with OLR higher than the optimal ones found in this study.

CRediT authorship contribution statement

Arinze Hycienth Ezieke: Data curation, Investigation, Methodology, Writing – original draft. **Antonio Serrano:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Miriam Peces:** Software, Methodology, Formal analysis, Data curation. **William Clarke:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Funding acquisition. **Denys Villa-Gomez:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2024.02.011.

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