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Carina Malinowsky, Matheus Cavali, Igor Marcon Belli, Paulo Belli Filho, Rémy Bayard, et al.. Evaluation of the co-anaerobic digestion of food and garden wastes in a two-stage pilot system. Waste and Biomass Valorization, 2024, 10.1007/s12649-024-02803-x . hal-04934861

HAL Id: hal-04934861

<https://hal.science/hal-04934861v1>

Submitted on 7 Feb 2025

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Evaluation of the co-anaerobic digestion of food and garden wastes in a two-stage pilot system

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Abstract:

Purpose: The treatment and final disposal of municipal solid waste (MSW) can be an issue from environmental and social perspectives. In the search for MSW treatment technologies, anaerobic digestion (AD) emerges as a strategic technology to treat and convert solid organic waste into energy (biogas/methane). Thus, several advances have been proposed to reduce complexity and increase AD feasibility, such as Co-AD and two-phase AD. Accordingly, this work aimed to examine the efficiency of biogas production from Co-AD of food waste (FW) and garden waste (GW) in a two-phase pilot system.

Methods: To test the two-phase Co-AD of FW and GW in a pilot system under mesophilic conditions at different hydraulic retention times (HRT: 2 and 3 days) and organic loading rates (OLR: 4, 6, 8, and 10 kg_{VS}/m³/d).

Results: The highest biogas and CH₄ production was achieved with an HRT and an OLR of 2 days and 6 kg_{VS}/m³/d, respectively. The removal of total solids, total organic carbon, total nitrogen, phosphorus, and chemical oxygen demand was 68%, 27%, 45%, 22%, and 69%, respectively.

Conclusion: Co-AD of FW and GW through biogas/methane production in two phases represents a promising approach for the valorization of these wastes.

Keywords: Two-phase anaerobic digestion. Volatile fatty acids. Methane. Biogas. Waste valorization. Biofuel

Abbreviations: AD – anaerobic digestion; GHG – greenhouse gas; Co-AD – Co-anaerobic digestion; FW – food waste; GW – garden waste; VFA – volatile fatty acids; OLR - organic loading rate; HRT – hydraulic retention time; TS – total solids; VS – volatile solids; total TKN – total Kjeldhal nitrogen; COD – chemical oxygen demand; TOC – total organic carbon; SBY – specific biogas yield; SMY – specific methane yield.

1. INTRODUCTION

Rising energy costs, stricter environmental legislation, and concerns about climate change have led the industrial and public sectors to seek sustainable means of energy production and to use waste to provide renewable fuels to replace fossil ones. From a circular economy perspective, anaerobic digestion (AD) is a suitable process for converting waste into energy (biogas). This approach can reduce the environmental problems (e.g., greenhouse gas (GHG) emissions) associated with the disposal of these materials in landfills [1]. In addition, AD produces a stabilized, nutrient-rich digestate that can be either used to enrich soils or as feedstock for another process, such as biochar production [2–4]. Therefore, several advances have been reported to reduce the complexity and increase AD processes' technical and economic feasibility. For instance, one can cite co-digestion and two-phase AD as alternatives to improve this process.

Co-anaerobic digestion (Co-AD) is a well-established technology for increasing biogas production [5]. By combining two or more substrates, this approach is useful in improving the availability and balance of macro and micro-nutrients, diluting inhibitory compounds, strengthening the buffering capacity of the system, broadening the microbial community during the digestion process, and adjusting the proportion between carbon and nitrogen. Such improvements can, therefore, lead to higher methane (CH₄) yields [6, 7]. For example, food waste (FW) can undergo AD with other wastes, such as sewage sludge [8], algae [9], manure [10], leachate [11], and lignocellulosic wastes [12].

Regarding two-phase AD, this approach, unlike the single-phase AD, involves two reactors. Hydrolysis and acidogenesis take place in the first reactor (phase I) to produce the acids that are conveyed to the second reactor (phase II) in which methanogenesis reactions occur [13]. This process increases stability by limiting the accumulation of volatile fatty acids (VFA) in the reactor. The first reactor's environmental conditions are controlled to promote the growth and proliferation of acid-forming bacteria. In contrast, the second reactor has its conditions adjusted to be optimal for methanogenic archaea. Thus, two-phase AD offers a better level of control, as each reactor can be optimized independently, resulting in better regulation of pH, higher methane yield, and fewer volatile solids (VS) [14]. Additionally, two-phase AD can work efficiently at comparatively high organic loading rates (OLR). Therefore, considering its advantages compared to the single-stage AD process, this approach has been used for the conversion of FW into CH₄ [15].

Although Co-AD of FW and garden waste (GW) has been widely researched [16–19], as mono-digestion can present several potential inhibitors to the process due to the relatively low carbon-to-nitrogen (C/N) ratio [18], the investigations about Co-AD of FW and GW in two stages and at a pilot scale are less common [20]. Therefore, against this background, this study aimed to examine the efficiency of biogas production from Co-AD of FW and GW in a two-phase AD pilot system under mesophilic conditions. Additionally, albeit great progress has been made in understanding the main parameters of the AD process of FW, there are still notable deficiencies in understanding the structure and function of microbial communities in AD processes [21]. Thus, this study also investigated the composition of the microbial communities in the two phases of the process. Accordingly, this work might contribute to the expansion of the two-stage Co-AD system by combining two wide-available substrates (FW and GW), in addition to being a step towards improving waste valorization techniques from a circular economy perspective.

2. MATERIALS AND METHODS

2.1. Inoculum and Substrate

The two-stage AD pilot system was designed to use an inoculum with a high concentration of microorganisms. Bovine manure has high-quality microorganisms, a neutral pH, and adequate humidity [22–28]. Fresh bovine manure was used as the inoculum, collected at the Agricultural Sciences Experimental Farm of the Federal University of Santa Catarina (UFSC), Brazil, and it was diluted with water in a 1:3 ratio. The moisture, pH, total solids (TS), and volatile solids (VS) of the inoculum were, respectively, 98.9%, 7.3, 1.19 g/L, and 0.8 g/L.

The substrate used in the experiments came from the university restaurant of the UFSC, which was composed of FW. This waste was collected monthly and then pre-treated by diluting (1:1 with water) and grinding it twice in a TRAPP model TR200 organic waste grinder. This process aims to increase the specific surface area and facilitate microbial action. GW from the flowerbeds of the UFSC, collected during campus cleaning periods, was used as a co-substrate. After collection, they were shredded five times in a TRAPP organic waste grinder, stored in plastic bags, and frozen so that they could be mixed with the organic material. The collection of the substrate and co-substrate

did not follow a fixed schedule. Due to limited storage space, the substrate and co-substrate were collected according to feed demand, with intervals ranging from 15 to 90 days.

Immediately after collection, a sample of each waste was separated for analysis of pH, TS, and VS. After determining the VS of the substrate and co-substrate, both wastes were mixed and homogenized, following a 1:9 ratio between GW and FW. This ratio was established based on a previous study [29]. The resulting mixture was analyzed for total Kjeldhal nitrogen (TKN), chemical oxygen demand (COD), total organic carbon (TOC), and phosphorus. Finally, once the VS value of the mixture had been determined, individual portions were separated according to the OLR applied to the system, placed in plastic bags, and frozen. One portion was taken out of the freezer every day and kept under refrigeration for 24 hours in order to thaw before feeding the pilot reactor. Table 1 presents physical and chemical characterizations of the inflow waste.

Table 1. Physical and chemical characterization of the mixture (food waste and garden waste) used in the feeding system.

Parameter	Average	Median	Minimal	Maximum
pH	3.8	3.7	2.9	5.0
TS (g/kg)	254	228	184	398
VS (g/kg)	241	180	209	367
TS (%)	26	23	18	41
VS (%)	94	94	92	96
VS/TS (%)	94	94	91	96
COD (g/kg)	235	186	117	461
P (%)	0.13	0.06	0.01	0.46
TOC (%)	45	47	44	50
TKN (%)	11	11	4	16
C/N	8	10	3	12

2.2. Experimental Plan

The first stage of the system (acidogenic module) can be visualized in Figure 1. It was made of stainless steel and had a working volume of 38 L (total volume of 85 L); the heating system consisted of an external resistor located in the central part of the reactor, which operated at mesophilic temperatures (35 to 38 °C). In addition, it had a mechanical

homogenization system, consisting of three rotating blades and automatic activation at a frequency of 38 rpm, with agitation for 15 minutes per hour. The reactor had a sampler (diameter 150 mm) located at the bottom and three side samplers (diameter 75 mm) arranged at three heights to collect representative samples of the internal biomass.

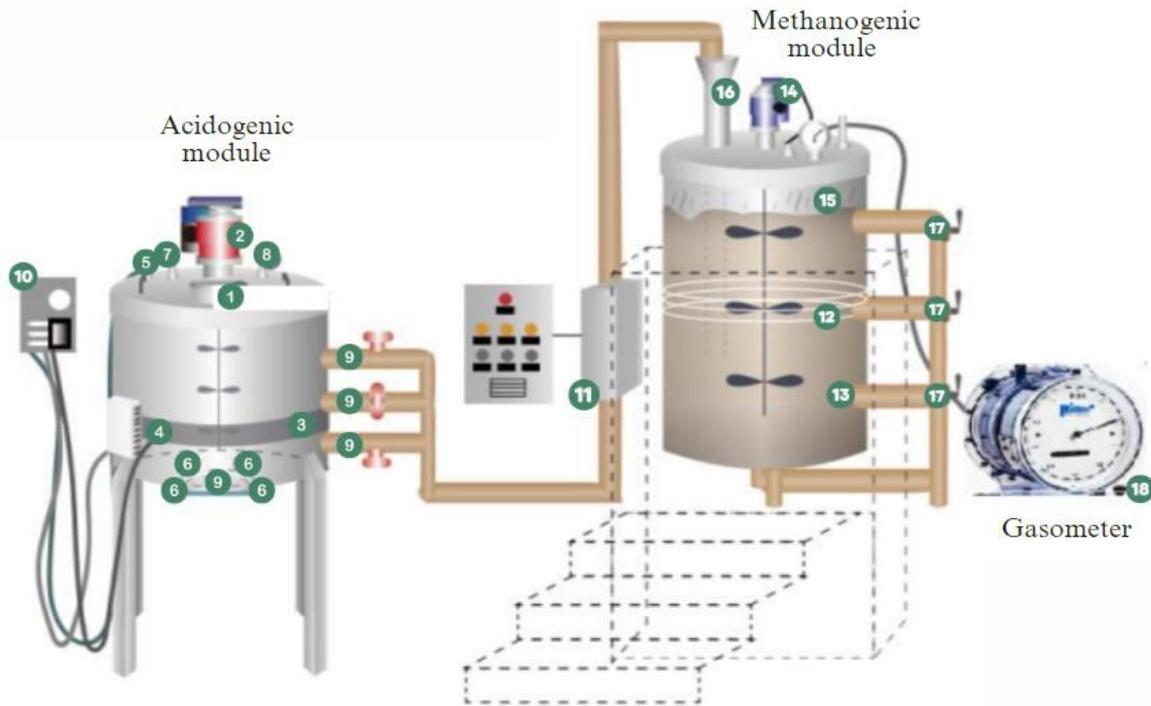


Figure 1. Pilot two-phase anaerobic digestion (AD) system. (1) Entrance; (2) Agitation System; (3) Heating air system; (4) Temperature sensor; (5) pH sensor; (6) Injection system; (7) Biogas measurer; (8) Pressure controller; (9) Samplers; (10) Control panel; (11) Control panel; (12) Heating system; (13) Digestate exit; (14) Shaking system; (15) Biogas reservoir; (16) Substrate entry; (17) Samplers; (18) Gasometer;

The design of the second stage of the system (methanogenic reactor), with a total capacity of 115 L and a useful capacity of 100 L, is made of stainless steel and has dimensions of 0.4 m in diameter by 1 m in height (Figure 1). This equipment includes a heating system with a bayonet-type electric heater positioned in the center of the digester. It also has a mechanical homogenization mechanism using a three-bladed agitator driven by a geared motor, which ensures adequate heating and efficient mixing of the substrate with the biomass. The reactor operates with mixing cycles of 30 min every three hours, at a speed of 30 rpm. The feed system for organic waste is mounted on the top of the reactor cover and plunges 0.3 m into the digester, ensuring a submerged intake of material

and preventing oxygenation. The effluent discharge process is facilitated by three side samplers and a bottom outlet with a nominal diameter of 100 mm.

A Ritter® model TG05 gasometer was used to measure the biogas, and the system was fed semi-continuously (once a day). The useful volume of the reactors was filled with inoculum and feeding and analysis began once the inoculum's biogas production had stabilized. The feeding process was determined by the organic loading rates (OLR) and the defined flow rate according to Table 2. The definition of the hydraulic retention time (HRT) and OLR were based on previous research [30, 31]. The tests were named based on the HRT and OLR of the acidogenic reactor. For example, the system whose acidogenic reactor had an HRT of 2 days and an OLR of 4 kgvs/m³/d was named S2D4KG. Once the amount of substrate to be added to the reactor had been defined, a complementary volume of water was added to this substrate to complete the flow volume. Table 2 indicates the different values applied to the system. An HRT was applied to the methanogenic reactor; the acidogenic reactor completed several cycles within the time of the two-phase system. The system was started with a fixed HRT and a gradual increase in OLRs. Upon reaching the maximum OLR supported by the system, the HRT of both reactors varied. The interactions between the parameters were evaluated throughout the process, but the main objective was to obtain the highest conversion of organic material into CH₄.

Table 2. Summary of the volumetric organic loading rates (OLRs) and hydraulic retention time (HRT) applied in each system.

Test	Acidogenic			Methanogenic		
	HRT (days)	OLR (Kgvs/m ³ /d)	Application (L)	HRT (days)	OLR (Kgvs/m ³ /d)	Application (L)
S2D4KG	2	4	20	8	1.8	12
S2D6KG	2	6	20	20	3.5	5
S2D10KG	2	10	20	100	1.9	1
S3D4KG	3	4	13.3	10	4.4	10
S3D6KG	3	6	13.3	20	4.3	5
S3D8KG	3	8	13.3	3.6	2.7	3

The system was fed in the morning and at times when the reactors were stirring. Finally, the output flow removed from the acidogenic reactor was added to the methanogenic reactor. The substrate was added to the acidogenic reactor according to the

corresponding volume of each OLR. The excess volume was applied for analysis. CH₄ and CO₂ concentrations were quantified using a GEM5000 portable gas analyzer (LANDTEC) and evaluated in the qualitative analysis.

2.3. Analytical Methods

The analyses of pH, TS, VS, TKN, NH₃, phosphorus, and COD of the substrate, co-substrate, and outflow were performed according to the Standard Methods for The Examination of Water and Wastewater [32]. Two tests determined the specific CH₄ yield (SMY) and the specific biogas yield (SBY). Test 1 (SMY1, SBY1) was expressed as the CH₄ and biogas produced per unit mass of the initial VS added to the methanogenic reactor. Test 2 (SMY2) was calculated per unit mass of the initial VS added to the first reactor (acidogenic) to evaluate the performance of the entire system [5]

The determination of VFA was conducted by gas chromatography using HP 5890 Series II equipment equipped with Flame Ionization detection and DB-WAX capillary column (polar). The samples were extracted by adding 1g of sodium chloride, 200 μ L of sulfuric acid, 200 μ L of crotonic acid (internal standard), and 600 μ L of diethyl ether (solvent). To identify the microorganisms at the beginning and end of the application of OLR in the reactor, approximately 15 mL of the outflow was collected from each reactor and centrifuged at 3,000 rpm for 5 minutes. The supernatant was discarded, and the pellet was stored at around 3 ± 1 °C until the DNA analysis, which was quickly performed. For the DNA sequencing, the samples were sent to the company Neoprosecta Microbiome Technologies. The methodology applied in brief was a highly sensitive optimized screening of the 16S rRNA gene V3-V4 hypervariable region, amplified with standard hot-start DNA polymerase, according to the methodology reported by Christoff et al. [33].

3. RESULTS AND DISCUSSION

3.1. Volatile Fatty Acids (VFA) Production

The concentration of VFA in the acidogenic reactor is presented in Table 3. The highest VFA concentrations were observed at organic loads of 6 and 10 kgvs/m³/d. For the system operated at 2 days with OLR of 2, 4, and 10 kg the total VFA concentrations were 12957.2, 50581.8, and 24606.0 mg/L, respectively. Acetic acid had the highest

proportion followed by butyric in the S2D4KG and S2D6KG systems and by propionic acid in the S2D10KG system. In the acidogenic reactor with 3-day HRT, a predominance of valeric acid over other VFA was observed at all OLR. Batstone et al. [34] reported that valeric acid is mainly associated with protein fermentation and as HRT increases, the concentration of long-chain VFA (such as valeric acid) may also increase, as shown by Malinowsky et al. [35]. In addition, Elbeshbishy and Nakhla [36] carried out a study on the production of VFA from a mixture of bovine serum albumin and starch, concluding that the degradation of most of the proteins occurred within three days. Measuring the concentration of the products resulting from acid fermentation is fundamental to assessing whether the hydrolytic-acidogenic effluent is suitable for the methanogenic phase [37]. This analysis is essential to ensure that the fermentation process is occurring effectively for the effluent to be effectively converted into methane in the subsequent stage.

Table 3. Concentration of acids in the acidogenic reactor.

Test	Acetic (mg/L)	Propionic (mg/L)	Isobutyric (mg/L)	Butyric (mg/L)	Isovaleric (mg/L)	Valeric (mg/L)	Total (mg/L)
S2D4KG	5234.7	1772.2	207.1	3187.0	238.0	2318.2	12957.2
S2D6KG	27839.2	3990.31	3212.5	10017.8	159.2	5362.8	50581.8
S2D10KG	8752.1	6496.1	3513.6	3014.8	867.6	1961.8	24606.0
S3D4KG	57.3	450.5	50.3	1166.3	75.3	2731.1	4530.8
S3D6KG	1320.9	332.5	1083.7	636.2	407.8	3328.9	7110.0
S3D8KG	2143.1	906.8	611.4	2351.9	543.2	2223.7	8780.1

In the acidogenic fermentation process, pH is a crucial element in the hydrolysis and acidogenesis stages [38]. Baldi et al. [39] observed that VFA production was more pronounced at the beginning of the experiment, with butyric acid predominating, followed by acetic acid. During the fermentation phase, the average concentration of VFA was around 8000 mg/L, similar production to this study. It was observed, in the acidogenic reactor in this study, that the pH measured in the two HRT was below the ideal range of 5 to 6 (the pH in the reactor showed results between 3 and 5) [40–42]. Comparing single-phase and two-phase systems for semi-continuous anaerobic digestion of food waste, Jo et al. [43] also obtained a pH value outside the ideal range (between 3.3 and 3.4), despite it being much more acidic than in the present study. In contrast, in the large-scale treatment of food waste, Li et al. [44] observed a pH in the acidogenic phase ranging

from 6.4 to 8.3, a considerably high value when compared to acidogenic reactors in other studies (between 3.6 and 5.52) [39, 45]. This difference was attributed to the recirculation of the effluent. As a result, the concentration of VFA was between 874.4 mg/L and 8722.2 mg/L, similar concentrations to the current study. During hydrolysis and acidogenesis stages, acetic acid is one of the most important VFAs, acting as a precursor to approximately two-thirds of methane production [46]. However, high concentrations of acetic acid can inhibit methanogenic activity. Thus, pH control is vital, as it directly influences VFA production.

For the methanogenic reactor, Table 4 presents the concentration of VFA. The highest concentration of acids was in the reactor S2D4KG (20847 mg/L), dominated by butyric and valeric acid. In S2D4KG, the most prevalent acids were butyric and valeric, revealing that there was greater consumption of acetic acid, which is the main and fastest CH₄ production pathway. In S2D6KG, the prevailing acids were propionic and isobutyric. The increase in OLR can result in the rapid accumulation of VFAs, especially propionic acid, leading to a decrease in biogas production [43]. In the 3-day HRT methanogenic reactors, there was consumption of total VFAs and changes in the concentration of specific acids, suggesting transformation into biogas. Isovaleric acid increased, while acetic and butyric acids decreased. Valeric and butyric acids were predominant, in line with the behavior of the acidogenic reactor. The drop in valeric acid points to its use in biogas generation.

Initial tests indicated that the methanogenic reactor was unable to maintain the ideal pH of 6,5 to 7,5 when processing the effluent from the acidogenic reactor [47]. Therefore, except in the case of S3D4KG, sodium bicarbonate (NaHCO₃) was added at a concentration of 1g/L to the acidogenic effluent before it entered the methanogenic reactor. This value was defined by titrating the S3D4KG methanogenic effluent until it achieved pH 7. Baldi et al. [39] also had to introduce a NaOH solution to prevent a drop in pH values, which could significantly suppress microbial activity.

Table 4. Concentration of acids in the methanogenic reactor.

Test	Acetic (mg/L)	Propionic (mg/L)	Isobutyric (mg/L)	Butyric (mg/L)	Isovaleric (mg/L)	Valeric (mg/L)	Total (mg/L)
S2D4KG	970.3	2630.6	1056.0	9012.5	1583.2	5594.8	20847.4
S2D6KG	565.0	24.4	23.5	125.5	634.5	20.4	1393.3
S2D10KG	147.9	97.0	922.2	473.7	774.6	503.9	2919.3

S3D4KG	1696.0	1342.3	454.5	1898.3	723.6	3644.9	9759.6
S3D6KG	263.1	998.7	790.6	962.2	2002.6	6602.6	11519.8
S3D8KG	1243.9	1855.1	631.4	3017.7	1223.8	3433.0	11404.9

VFAs, including acetic, propionic, and butyric acids, represent crucial intermediate compounds in the anaerobic fermentation process [48, 49]. Wu et al. [50] found that the maximum proportions of acetic and propionic acids under alkaline conditions were 49% and 34%, respectively. Under neutral conditions, the growth of methanogens accelerated the consumption of acetic and propionic acids [51]. The concentration of acid fermentation products provides crucial information for determining their suitability for the methanogenic stage [52]. Butyric, isobutyric, valeric, and isovaleric acids are not used directly by methanogenic microorganisms, making their preliminary conversion to acetic acid necessary. Extreme pH conditions decreased the abundance of the methanogens [53], as propionic acid contributes up to 35% of CH₄ production but can cause system failure in excess. Xiao et al. [54] verifying the complete degradation of acetic acid at concentrations up to 4.2 g/L in methanogens. However, when the load reached 7.4 g/L, there was a decrease in methanogenic activity. For the degradation of propionic acid without the inhibition of methanogens was verified at concentrations of up to 4.6 g/L in the pH range of 6.40 - 7.30 [55].

3.2. Biogas Production

Figure 2 illustrates the average daily values of biogas and CH₄ production, as well as the specific biogas (SBY) and CH₄ (SMY) yields, showing the actual efficiency of biogas and CH₄ production in the two-stage methanation system. The SBY1 and SMY1 consider only the grams of VS of the acidogenic outflow added in the methanogenic reactor. The SBY2 and SMY2 include the total amount of VS entering the system, i.e. the grams of VS introduced into the acidogenic reactor. Due to a technical problem with the gasometer used to measure the biogas, it was not possible to measure the biogas production of the S2D4KG reactor, therefore the results of this reactor are not shown in Figure 2.

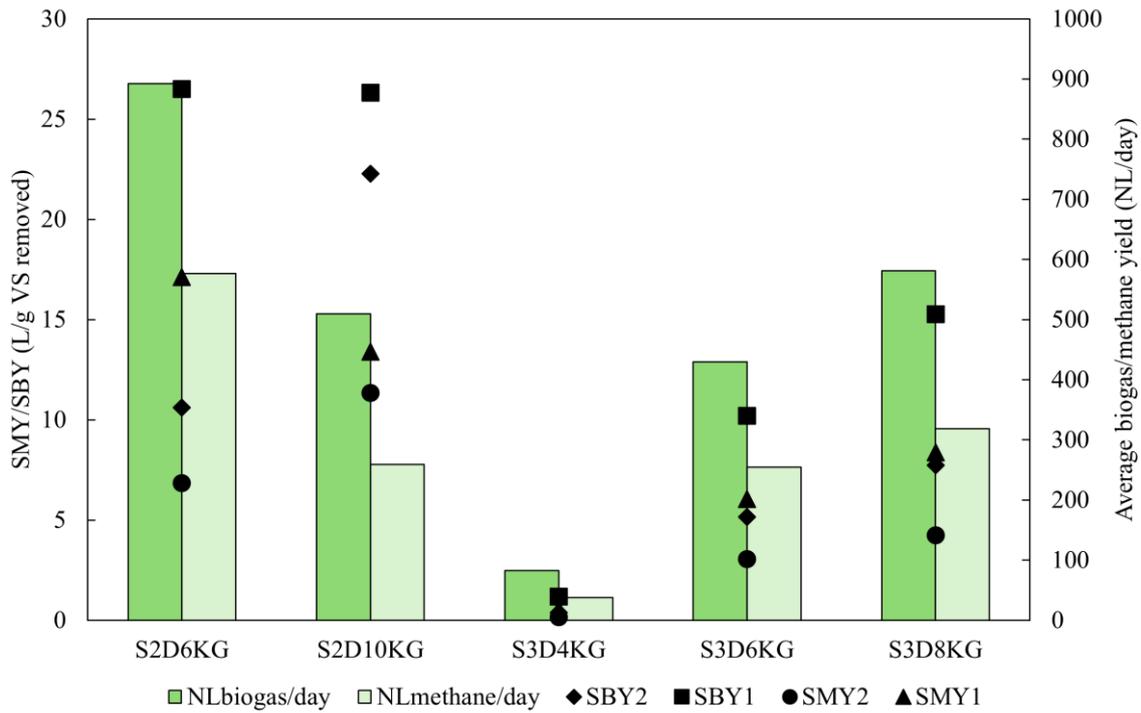


Figure 2. Average daily production of biogas and methane, and specific production of biogas and methane in the overall system and in the methanogenic reactor.

The best performances for SBY and SMY were observed in the 2-day HRT. S2D6KG had the highest SBY₁_{average} (0.88 L_{biogas}/g.VS_{added}) and the highest SMY₁_{average} (0.57 L_{methane}/g.VS_{added}). Thakur et al. [56] explored the Co-AD of FW and bio-flocculated sewage sludge (BFS) under mesophilic conditions in two reactors, namely, R1 (fed with 50% FW and 50% BFS; HRT of 4 days) and R2 (fed with 2% FW and 98% BFS; HRT of 35 days). The authors reported a maximum CH₄ yield of 142 and 225 mL_{methane}/g.VS_{added} in reactors R1 and R2, respectively. In contrast, Sosnowski et al. [57] evaluated the Co-AD of sewage sludge and organic fraction of municipal solid waste in a mixing ratio of 75:25 and reported 439 mL_{methane}/g.VS_{added}, which operated mesophilically.

Based on the parameters evaluated herein, CH₄ yield has a negative correlation with the concentration of valeric and isovaleric acids in the acidogenic reactor. The results for SBY₁ and SMY₁ demonstrated that the concentration of CH₄ in the biogas averaged > 60%, reaching a maximum value of 72%. The average biogas yield was 27.71 L/d. S2D10KG had the highest efficiency in converting VS to biogas, with SBY₁ of 0.74 L_{biogas}/g.VS_{added} and SMY₁ of 0.38 L_{methane}/g.VS_{added}. Average daily biogas production was 14.85 L/d and the concentration of CH₄ in the biogas was higher than that of CO₂. The reduction in SMY_{average} from 0.57 to 0.45 L_{methane}/g.VS_{added} with the increase

in OLR from 6 to 10 Kg.STV/m³.d can be explained by the imbalance in the methanogenic stage caused by the drop in pH at the higher load and the increase in the concentration of VFA in the reactor. Similarly to what was observed in this research, Leite et al. [58] and Flor [59] found an instability inside the reactor resulting in a reduction in SMY as the load increased.

In a 3-day HRT period, a reduction in biogas and CH₄ production was noted compared to the 2-day HRT. Instability was observed through fluctuations in pH and high levels of VFA, especially in the acidogenic reactor, which negatively affected the efficiency of VS conversion into biogas and CH₄. At certain times, the concentration of CO₂ exceeded that of CH₄ in the biogas. S3D4KG had the lowest performance in terms of biogas and CH₄ production. Changes in the waste fed to the reactor caused instability and a consequent drop or stagnation in biogas production, with a daily average of just 2.1 liters. In comparison, a longer HRT resulted in a decrease of around 50% in biogas and methane production for the same OLR of 6 kg_{VS}/m³/d.

3.3. Removal efficiency of total and volatile solids

AD of food waste is a complex process in which anaerobic bacteria transform VS into biogas, biomass, and stable solids. Table 5 presents the average removal of TS and VS in the acidogenic and methanogenic reactors. In the S2D4KG operation, the initial concentrations of TS and VS were 8.47 and 8.0 g.VS/L, respectively; the concentration of VS in the outflow was 2.06 g.VS/L. Meanwhile, in S2D6KG, the values were higher, with 13.02 and 12.0 g.VS/L at the inlet and 2.89 g/L of VS in the effluent. For S2D10KG, there was an increase in TS and VS in the effluent compared to the inflow, indicating an accumulation of sludge. However, the average VS in the acidogenic reactor effluent was 4.07 g/L during monitoring, lower than the input average of 20 g/L.

Similarly to the samples with 2-day HRT, the 3-day monitoring revealed an increase in solids in the reactor collectors, resulting in increases in the levels of TS and VS in certain samples. In the S3D4KG, S3D6KG, and S3D8KG tests, the affluent TS levels were 13.06, 19.05, and 25.45 g.TS/L, respectively, while the affluent VS levels were 12.00, 18.00, and 24.00 g.VS/L, respectively. The effluent VS values at the end of the process were 3.07, 4.03 and 8.09 g.VS/L, respectively. During AD, partial mineralization of organic matter is expected, which leads to a reduction in TS and VS levels and an increase in fixed solids (FS), according to Metcalf and Eddy [60]. The

efficiency of the system in removing nutrients was measured by the percentage reduction of TS and VS in the acidogenic and methanogenic reactors, as well as the total removal of the system is detailed in Table 5.

Table 5. Average removal of total solids (TS) and volatile solids (VS) in the acidogenic and methanogenic reactors.

Test	TS removal (%)			VS removal (%)		
	Acidogenic reactor	Methanogenic reactor	System	Acidogenic reactor	Methanogenic reactor	System
S2D4KG	29.5	5.5	48	42	16	54
S2D6KG	58	27	68	59.5	9	76
S2D10KG	24	47	60	20	76	70
S3D4KG	53	25	63	49	36	69.5
S3D6KG	59	27	71	40.5	44.5	76.5
S3D8KG	48	17	52	48.5	10	66

The tests revealed that the highest VS removal rate was 76% for the S2D6KG and S3D6KG loads, while the lowest was 54% for S2D4KG. These results are the same line of approach as a study conducted on two-stage thermophilic anaerobic digestion, which found effective results although it had a sludge retention time (SRT) of 30 days. The VS reduction rate was 82% during the stable phase, and TS removal reached 77% [61]. Other studies, such as Wu et al. [62], have achieved VS removal yields of 75%, with HRT of 4.25 days and OLR of 4 kg_{VS}/m³/d.

With 2-day HRT, it was noted that the lowest load (S2D4KG) resulted in a less effective removal efficiency, with 48% for TS and 54% for VS. The removal efficiency increased with the OLR, reaching 68% for TS and 76% for VS in the S2D6KG test. However, an increase in OLR from 6 to 10 kg_{VS}/m³/day was harmful, decreasing the overall efficiency of the system. For a 3-day HRT, the average removal rates were 63%, 71%, and 52% for TS and 69%, 76%, and 66% for VS in the S3D4KG, S3D6KG and S3D8KG, respectively, with the 6 kg OLR (S3D6KG) presenting the best efficiency. The increase in OLR from 6 to 8 kg_{VS}/m³/d caused a reduction in nutrient removal efficiency in both reactors. In contrast to studies conducted in a two-stage system that worked efficiently at OLR 10-15 kg_{VS}/m³/d in the methanogenic reactor [63]. Parra-Orobio et al. [64] observed a drop in the removal of VS when the OLR went from 4.5 (average over

85%) to 9.0 kgvs/m³/d (under 75%) in an acidogenic reactor, and from 2.0 to 4.0 kgvs/m³/d in the methanogenic reactor.

3.4. Removal efficiency of total organic carbon, nitrogen, phosphorus, and chemical oxygen demand

The average concentration of TOC, TKN, phosphorus, and COD in acidogenic and methanogenic reactors, as well as system removal percentage, are presented in Table 6, Table 7, Table 8, and Table 9, respectively. Carbon, nitrogen, and phosphorus are fundamental nutrients for AD by being involved in cell synthesis, microorganism growth, and energy supply [65].

Regarding TOC (Table 6), in the 2-day HRT tests, the conversion value in the acidogenic reactor was below 10%, while in the methanogenic reactor, the conversion was close to 20%. When analyzing the system removal, S2D6KG and S2D10KG presented a TOC removal of 27% and 22%, respectively. The results of the 3-day HRT tests were comparable to those of the 2-day HRT. In all tests conducted in the acidogenic reactor, conversion did not exceed 8%. The overall system conversion was highest in S3D4KG, followed by S3D6KG, and the lowest conversion was observed in S3D8KG. It was observed that the TOC conversion did not follow the increase in OLR. For both HRT (2 and 3 days), the highest OLR showed the lowest results, indicating a possible accumulation of organic material in the system.

Table 6. Average concentration of total organic carbon (TOC) in relation to total solids (TS) evaluated in the acidogenic and methanogenic reactors.

Test	TOC (%)			System removal (%)
	Affluent	Acidogenic reactor	Methanogenic reactor	
S2D4KG	45	41	33.57	25
S2D6KG	45	43	33	27
S2D10KG	45.5	43	35.5	22
S3D4KG	4	42	30	34
S3D6KG	45.5	45	33	27
S3D8KG	45	42	37	18

Concerning nitrogen (Table 7), the percentage removal of TKN in the system was directly proportional to the increase in OLR in the 2-day HRT tests. The best efficiency was obtained with the reactor with the highest OLR (S2D10KG). However, in the 3-day

HRT tests, the percentage removal of TKN from the system was in the following order: S3D4KG > S3D6KG > S3D8KG. Sánchez et al. [66] reported that the increase in OLR does not considerably affect the concentrations of TKN in the effluent, as it was noted in the 3-day HRT tests. It is also important to highlight that when comparing the same charge volume under varying hydraulic retention times, the effectiveness in eliminating TKN is slightly reduced when the HRT is shorter. Arvin et al. [67], finding a maximum TKN removal performance of 92.4% in leachates, also observed a decrease in the efficacy of TKN elimination when the HRT was decreased, from 48 hours to 24 hours, and from 24 hours to 12 hours.

The presence of NH₃ was observed throughout the monitoring period. In the acidogenic reactor, the maximum average value observed was 63.5 mg/L in S2D10KG, while in the other tests, the concentration was less than 30 mg/L. In the methanogenic reactor, the highest average value was obtained in the S3D8KG test. The NH₃ concentration in both reactors increased directly to the OLR. This increase can be attributed to the organic material available for assimilation by the bacteria, since the ammonification of the medium occurs when bacteria convert proteins to ammonia, thereby increasing the concentration of this substance as the load increases [68]. Ammoniacal nitrogen in high concentrations can act as an inhibitor in anaerobic processes, causing instability in the process and leading to a decrease in biogas and methane yields; this can eventually fail the methanogenic reactor [69]. According to Prochazka and co-workers, the optimal ammoniacal nitrogen concentrations for CH₄ production lie between 2,100 mg/L and 3,100 mg/L. Ammoniacal nitrogen concentrations above 4,000 mg/L were found to inhibit methane production, whereas low concentrations (500 mg/L) were observed to cause a decrease in CH₄ production [70].

Table 7. Average concentration of total Kjeldahl nitrogen (TKN) and ammonia (NH₃) evaluated in the acidogenic and methanogenic reactors.

Test	TKN (mg/L)			NH ₃ (mg/L)		System removal of TKN (%)
	Affluent	Acetogenic reactor	Methanogenic reactor	Acetogenic reactor	Methanogenic reactor	
S2D4KG	332.72	289.8 ± 59	263.74 ± 82	7.68 ± 7	52.95 ± 22	21
S2D6KG	526.5	383.6 ± 187	290.71 ± 87	26.21 ± 32	217.82 ± 74	45
S2D10KG	840.9	598.2 ± 112	360.10 ± 35	63.58 ± 37	293.04 ± 27	57
S3D4KG	576.53	387.7 ± 163	303.32 ± 91	20.36 ± 6	202.14 ± 65	47
S3D6KG	665.25	429.1 ± 131	406.48 ± 127	26.61 ± 16	206.65 ± 70	40
S3D8KG	1030.24	831.95 ± 78	798.41 ± 598	33.19 ± 21	379.16 ± 150	23

Table 8 presents the average concentration of phosphorus evaluated in the acidogenic and methanogenic reactors. During the experiment, there was little variation in the concentration of phosphorus, which was already expected and justified by the low assimilation of this nutrient by the bacteria. The biological utilization of phosphorus occurs through the selection of bacteria capable of storing polyphosphate. However, in anoxic environments, this reduction tends to be very low [71]. The biological utilization of phosphorus occurs through selecting bacteria capable of storing polyphosphate. However, polyphosphate-accumulating organisms are typically cultured under alternating anaerobic and aerobic conditions [72]. Overall, AD has no significant effect on phosphorus removal [73, 74], although it can achieve a phosphorus reduction in the range of 10 to 30% [75]. In this study, the S2D4KG reactor had the best efficiency (42%), in accordance with the efficiency found by Costa et al. [76]. Furthermore, operating an anaerobic-aerobic reactor to treat the effluent from an anaerobic digester fed with organic food waste, Melo et al. [77] achieved a phosphorus removal efficiency of 23%, with a hydraulic retention time of 24 h and an organic load rate of 1 kg_{COD}/m³/d applied.

Table 8. Average concentration of phosphorus evaluated in the acidogenic and methanogenic reactors.

Test	Phosphorus (mg/L)			System removal (%)
	Affluent	Acetogenic reactor	Methanogenic reactor	
S2D4KG	11.39	7.68 ± 70	6.56 ± 21	42
S2D6KG	28.25	26.21 ± 32	22.11 ± 15	22
S2D10KG	72.49	63.58 ± 38	52.12 ± 3.5	28
S3D4KG	21.75	20.36 ± 60	18.37 ± 55	16
S3D6KG	35.16	26.6 ± 15.6	26.1 ± 22	26
S3D8KG	68.83	33.2 ± 20	65.8 ± 31	4

The results of COD removal efficiency are reported in Table 9. It was observed that, for 2-day HRT, the COD removal percentages were 51%, 69%, and 57% for the S2D4KG, S2D6KG and S2D10KG tests, respectively. The lowest efficiency was recorded for the lowest organic loading rate. As the OLR increased, there was an increase in COD removal, reaching a peak of 6 Kg.VS/m³.d. However, when the OLR was increased to 10 Kg.VS/m³.d, there was a drop in the system's COD removal efficiency. For 3-day HRT, the COD removal percentages found were 23%, 49%, and 35%, respectively, for the S2D4KG, S2D4KG and S2D4KG tests, with OLR of 4, 6, and

8 Kg.VS/m³.d. The maximum COD removal was obtained with an OLR of 6 kgvs/m³/d (69% for a 2-day HRT).

Table 9. Mean percentage removal of chemical oxygen demand (COD) evaluated in the acidogenic and methanogenic reactor.

Test	Removal (%)		
	Acidogenic reactor	Methanogenic reactor	System
S2D4KG	40	19	51
S2D6KG	26	61.5	69
S2D10KG	20	53	57
S3D4KG	18.5	35	23
S3D6KG	27	24	49
S3D8KG	33	20.5	35

Leite et al. [58] treated thickened sludge from wastewater treatment plants (WWTPs) in the same reactor used in this study and obtained 60% removal with an OLR of 2.5 Kg.VS/m³.d. Increasing the OLR to 10 and 8 Kg.VS/m³.d, respectively, led to a drop in COD removal efficiency. Flor [59] also reported this phenomenon; when the maximum load applied was 17 Kg.VS/m³.d, there was a reduction in removal efficiency, which dropped from 60% to 48%. Xiao et al. [78], meanwhile, studied thermophilic AD with FW in a two-stage system and found that nutrient removal in the acidogenic reactor was < 4%; in addition, the removal values for COD were 77%.

Silva et al. [79] reported that, in the acidogenesis process, VS and COD removal efficiency did not exceed 10% and 20%, respectively. It is common to observe a modest reduction in COD during the acidogenic phase, as this is a period in decreasing organic compounds are predominantly transformed into soluble metabolites, such as volatile fatty acids (VFA) and alcohols. This partial removal of carbohydrates can be attributed to the complexity of the organic matter in the food waste, which includes a mixture of fruit, vegetables and meat [80]. During the methanogenic stage, a significant reduction in the concentration of organic matter was observed. COD removal ranged from 20% (S3D8KG) to 61% (S2D6KG). Around half of the organic content left over from acidogenesis was processed or stabilized in the methanogenic phase. The soluble organic matter remaining from the acidogenic stage was effectively used to produce CH₄.

3.5. Microbial Community Assessment

In the acidogenic reactor, the microbial community assessment showed that the *Firmicutes* phylum was the most prevalent for all the HRT and OLR. In S2D6KG and S2D10KG systems, the *Firmicutes* phylum had the highest relative abundance at the beginning and the end of the process. In general, the predominant phyla in the acidogenic stage in AD are the *Firmicutes* and *Bacteroidetes* [81], as reported in this study and by Duan et al. [82]. These microorganisms have the ability to produce lytic enzymes to decompose organic compounds, as well as being able to resist extreme environments and conditions [81]. For the S2D6KG system, the second-highest relative abundance was for *Actinobacteria* phylum at the beginning, while *Proteobacteria* was the second most prevalent at the end. Conversely, for the S2D10KG system, the *Proteobacteria* was replaced by the *Actinobacteria* with the second-highest relative abundance at the end of the process. Overall, the main genera found in the acidogenic reactors were *Bacteroides*, *Butyvirbio*, *Clostridium*, *Fusobacterium*, *Selenomonas*, *Streptococcus*, *Proteus*, *Peptococcus*, and *Bacillus*.

Previous studies have also observed organisms under different process conditions. The results showed that microbial communities are clearly influenced by process temperatures and ammonia concentrations [83]. In reactors operated under mesophilic conditions, *Levilinea*, *Syntrophomonas*, *Methanothrix*, and *Methanosphaerula* were dominant organisms detected during the process. In thermophilic reactors, *Levilinea*, *Ornatilinea*, *Methanosphaerula*, and *Methanomassiliicoccus* dominated [84]. Concerning archaea, *Methanosaeta*, *Methanobacterium*, and *Methanosarcinaceae* are commonly detected in high relative abundance in anaerobic digesters operated with different operating parameters [81].

This microbial diversity was also observed in the methanogenic reactor, the *Methanosarcina* and *Methanosaeta* prevailed equally in the S2D6KG initially, but the *Methanosaeta* genus predominated at the end. For S2D10KG, the *Methanosarcina* and *Methanosaeta* were the genus with the highest relative abundance at the beginning and the end, respectively. It also highlights that OLR and HRT are connected parameters and influence the ecological aspects of anaerobic digestion, which can alter the growth of microorganisms [85]. Regarding the 3-day HRT, the *Methanobacterium* genus

predominated at the beginning and the end of S3D6KG. For S3D6KG, *Methanosaeta* was the most prevalent genus at the beginning, changing to *Methanobacterium* at the end.

A positive correlation between anaerobic reactor performance and microbial diversity was reported by Liu et al. [86], with an observed increase in methane linked to five operational taxonomic units of *Firmicutes* and four of *Bacteroidetes* [82]. The chemistry of the substrate considerably influences the configuration of the microbial community in biogas reactors [81]. As for the microbiology involved in the production of VFAs in this study, *Clostridium*, *Staphylococcus*, *Acetivibrio*, and *Eubacterium* predominated in the hydrolysis of polysaccharides, while *Clostridium*, *Micrococcus*, and *Staphylococcus* predominated in the group involved in the breakdown of lipids. For protein degradation, the main genera found were *Bacteroides*, *Butyvirio*, *Clostridium*, *Fusobacterium*, *Selenomonas*, *Streptococcus*, *Peptococcus*, and *Bacillus*. These microorganisms are responsible for the production of acetic and propionic acids, which are necessary precursors for methane production, but also formic and butyric acids, as indicated by Harirchi et al. [87].

4. CONCLUSION

This study employed a two-phase evaluation of a pilot-scale Co-AD process, utilizing different HRT and OLR conditions. An elevated OLR proved advantageous for the formation of VFAs and the selection of the specific microbial community within the acidogenic reactor. In methanogenic reactors, a reduction in the concentration of acetic, butyric, and propionic acids in the effluent was observed to be associated with an increase in methane production. This indicates that these acids are more effectively consumed and converted into biogas. The most significant finding concerning biogas/methane production was observed at an HRT of 2 days and an OLR of 6 kg_{VS}/m³/d. In this system, the removal of total solids, total organic carbon, total nitrogen, phosphorus, and chemical oxygen demand was 68%, 27%, 45%, 22%, and 69%, respectively. Therefore, the valorization of FW and GW through biogas/methane production by Co-AD in two phases represents a promising approach for the valorization of these wastes.

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Statements & Declarations

Competing Interests: The authors declare no conflict of interest.

Data availability statements

Data will be made available on reasonable request.