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Performance Assessment and Operational Evaluation of a Ternary Anaerobic Co-Digestion System

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Abstract

One of the most challenging yet promising technologies combining waste management with waste utilization for the production of a renewable fuel (biogas) is anaerobic co-digestion. This study investigated the anaerobic co-digestion of three organic waste streams: cheese whey wastewater (CWWW), pig slurry (PS), and food waste leachate (FWL). FWL and CWWW were combined to form a base mixture of constant composition (BM), which was subsequently blended with diluted PS at three BM/PS mass ratios: 25/75, 50/50, and 75/25 (% v/v). The mixtures were evaluated under mesophilic conditions through laboratory-scale batch experiments to assess the influence of substrate composition on biomethane production and organic matter removal. Physicochemical characterization of the individual substrates and mixtures included pH, volatile fatty acids, alkalinity, chemical oxygen demand (COD), and volatile solids (VS). Biochemical methane potential (BMP) assays were conducted, with daily and cumulative methane production systematically monitored throughout the experimental period. Methane production kinetics were evaluated by fitting the modified Gompertz model to the experimental data, enabling estimation of maximum methane production potential, maximum production rate, and lag-phase duration. Experimental results demonstrated that the 75/25 BM/PS mixture achieved the highest specific biomethane yield (525.9 CH_4 Nml/g VS_{added}) and maximum production rate (μ_m = 36.3 CH_4 Nml/g VS_{added}/d), alongside the highest removal efficiencies for COD (83.0%), TOC (87.9%), and VS (63.7%), compared with the 50/50 (309.9 CH_4 Nml/g VS_{added}) and 25/75 (156.5 CH_4 Nml/g VS_{added}) mixtures. The integrated assessment of methane yield, kinetic parameters, and organic matter removal enables comparative evaluation of the three mixtures and identifies the 75/25 BM/PS formulation as the most favorable mixing ratio for efficient co-digestion of these waste streams.

Keywords: anaerobic co-digestion; food waste leachate; cheese whey wastewater; pig slurry; biochemical methane potential; substrate mixing ratio; biodegradability; modified Gompertz model

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1. Introduction

The treatment of organic waste through anaerobic digestion can combine the production of renewable energy from biogas with the recovery of digested residue for nutrient recycling. Its contribution to the circular economy and climate-change mitigation depends

on the feedstock, biogas recovery and use, and digestate storage and application practices [1]. Anaerobic digestion of municipal organic waste can also contribute to the United Nations' Sustainable Development Goals (SDGs), such as affordable and clean energy (SDG 7), responsible consumption and production (SDG 12), and climate action (SDG 13). To achieve these benefits, the effective management of waste and its by-products is required [2].

Liquid waste from livestock farming, cheese production, and food distribution contain organic matter that can be recovered through anaerobic digestion. Their suitability for anaerobic digestion depends on their composition and biodegradability. There have been reports of substantial differences in biochemical methane potential (BMP) among substrates like pig manure, food waste, and cheese waste [3]. The composition and degradability of the substrate are, consequently, important criteria during the selection of feed mixtures and the interpretation of the differences in methane production [4].

Of these wastes, the leachate from fruit and vegetable waste (FWL) has been investigated for the production of hydrogen and methane [5], as has co-digestion with cheese whey waste (CWWW) for methane production [6]. FWL should be distinguished from whole food waste, and liquid dairy waste should be sorted based on the specific waste flow from which it is collected. These classifications are crucial, as the properties of the substrate and the operating conditions both affect digestion efficiency. In experiments conducted with cheese whey, the rapid formation of acids disrupted the balance between acidogenesis and methanogenesis, with temperature and organic load both affecting the formation of intermediate products and the risk of acidification [7].

Co-digestion with animal manure can provide nutrients and buffering capacity, reducing pH fluctuations during the decomposition of readily biodegradable waste. This has been successfully applied to cheese whey mixed with livestock manure slurry [8] or the liquid fractions of manure from dairy livestock [9], despite the fact that effective performance is affected by the mixture ratio and the organic load. In mixtures of pig manure and food waste, the mixing ratio affected methane production and the rate of production, with the initial volatile fatty acids (VFAs) promoting rapid methane production during the early stages of digestion [10]. An increase in the manure ratio may also increase the nitrogen content, which depends on the composition of the waste. This must be taken into account, as inhibition of methane production by ammonia has been observed during the digestion of pig manure [11].

Ternary anaerobic co-digestion has been studied for various waste mixtures, including cattle manure, food waste, and pig manure [12], as well as pig waste slurry, dairy whey, and the organic fraction of municipal solid waste [13]. Studies under mesophilic conditions have also examined combinations containing cheese whey. Hallaji et al. [14] reported that adding a premixed combination of fruit waste and cheese whey to waste-activated sludge increased methane yield by 31% relative to sludge digestion alone. Dareioti and Kornaros [15] investigated sorghum silage, whey, and liquid cow manure in a two-stage system at 37 °C and found that methane production was sensitive to hydraulic retention time. Jo et al. [16] investigated slaughterhouse waste, cattle manure, and pig manure and found that increasing the proportion of slaughterhouse waste improved biodegradability, increased the methane production rate, and shortened the lag phase. These findings indicate that the performance of ternary mixtures depends on both the substrate composition and the operating conditions. This observation is consistent with broader evidence showing that co-substrate selection, feedstock characteristics, and mixture composition are major determinants of food-waste anaerobic co-digestion performance [17]. The methane yield and kinetic behavior of an FWL/CWWW mixture combined with pig slurry therefore require evaluation under the specific conditions used in the present study.

Kontodimos et al. [6] reported a methane yield of 616.7 Nml CH_4/gVS_{added} for a mixture containing 25% FWL and 75% CWWW by volume. This composition was selected as the base mixture (BM) for evaluating the addition of diluted pig manure in the present study. However, while the binary 25/75 FWL/CWWW mixture (BM) exhibits high biodegradability, it is characterized by a strongly acidic pH (4.13), high soluble organic load (COD of 43.3 g/L), and low ammoniacal nitrogen content (0.15 g/L), which pose a substantial risk of rapid VFA accumulation and reactor souring if digested without external buffering [6,7]. Consequently, a critical scientific knowledge gap exists regarding how the incorporation of aged, diluted pig slurry (PS) into this acidic FWL/CWWW base mixture regulates the trade-off between bicarbonate/ammonia buffering stability and overall mixture biodegradability.

We hypothesized that blending the carbohydrate-rich, acidic BM with diluted PS in a ternary co-digestion scheme would supply sufficient endogenous alkalinity and ammoniacal nitrogen to neutralize early-stage acidogenesis and prevent persistent pH inhibition without chemical pH adjustment, while maintaining high specific methane recovery at elevated BM proportions. To test this hypothesis across a representative operational gradient [18], three discrete BM/PS volumetric mixing ratios, 25/75 (manure-dominated/high-buffer regime), 50/50 (balanced regime), and 75/25 (BM-dominated/high easily degradable load regime), were systematically evaluated.

The following study examines the anaerobic co-digestion of CWWW, PS, and FWL, initially focusing on the physicochemical characterization of the feedstock mixtures and the changes observed following the anaerobic digestion process. The performance of the co-digestion process is subsequently evaluated in terms of organic matter removal and biomethane production, together with the determination of key kinetic parameters. Particular emphasis is placed on the analysis and interpretation of organic matter degradation, cumulative biomethane production, biomethane production rates, and the kinetic behavior of the process. Finally, the experimental setup, substrate preparation, analytical procedures, and methodological framework employed to conduct the experimental campaign and evaluate the obtained results are described in detail.

The novelty of this study extends beyond the standard empirical evaluation of co-digestion performance, focusing specifically on the mechanistic interactions within a ternary system that combines a rapidly biodegradable, highly acidic base mixture (FWL/CWWW) with a buffered, nitrogen-rich co-substrate (PS). While our previous work established the biomethane potential of the binary FWL/CWWW mixture [6], the present study addresses a clear knowledge gap by quantitatively assessing how the incorporation of aged pig slurry regulates the VFA-to-alkalinity balance, mitigates early-stage acidification, and governs the transition from monophasic to multiphasic methane production kinetics. Rather than solely reporting biomethane yields and standard organic matter removal, this work provides new mechanistic insights into the endogenous buffering requirements and free ammonia nitrogen (FAN) dynamics necessary to prevent reactor souring when valorizing acidic agro-industrial waste streams. Furthermore, the simultaneous valorization of these complementary waste streams supports the development of integrated, robust waste-management strategies within the framework of the circular bioeconomy.

2. Results

The chemical characteristics of the three BM/PS assays differed at the beginning of the trials, particularly to pH, VFAs, the organic loading, and the buffering capacity. The initial pH was 7.93, 7.26, and 7.26 for the 25/75, 50/50, and 75/25 ratio, respectively. The alkalinity content was 12.5, 12.5, and 10.0 g/L $CaCO_3$, whereas the VFA concentrations followed an increasing pattern among the three ratios, ranging from 3.75 to 5.48 g/L HAc_{eq}. Similarly,

the initial VFAs/Alk ratios were 0.30, 0.35, and 0.55 for the three BMP tests. The starting values of the bench scale tests are presented on Table 1.

Table 1. Starting values of physicochemical and organic load parameters of bench scale tests.

Parameter (Unit)	25/75	50/50	75/25
pH (-)	7.93	7.26	7.26
Alkalinity (g $CaCO_3$/L)	12.5	12.5	10.0
VFAs (g HAc_{eq}/L)	3.74	4.34	5.48
NH_4^+ (g/L)	1.92	1.98	2.23
FAN (g N/L)	0.145	0.034	0.018
TS (g/L)	42.3	35.6	39.2
VS (g/L)	31.5	25.1	28.4
COD (g/L)	12.3	21.8	32.4
TOC (g/L)	3.2	9.2	13.2
VFAs/Alk	0.30	0.35	0.55

On the other side, at the end of the BMP trials, the pH values increased to 8.07, 7.97, and 8.02 for the 25/75, 50/50, and 75/25 ratio, respectively. The total alkalinity decreased to 4.6, 4.1, and 4.2 g/L $CaCO_3$, while VFAs declined to 0.6, 0.38, and 0.58 g/L HAc_{eq}. The corresponding final VFAs/Alk ratios exhibited 0.13, 0.09, and 0.14, indicating a "healthy reactor" [18]. In Table 2, the final values of the characteristics of the BMP tests are illustrated.

Table 2. Final values of physicochemical and organic load parameters of bench scale test.

Parameter (Unit)	25/75	50/50	75/25
pH (-)	8.07	7.97	8.02
Alkalinity (g $CaCO_3$/L)	4.6	4.1	4.2
VFAs (g HAc_{eq}/L)	0.6	0.38	0.58
NH_4^+ (g/L)	2.30	2.32	2.45
FAN (g N/L)	0.212	0.174	0.204
TS (g/L)	27.5	25.8	22.6
VS (g/L)	15.2	12.7	10.3
COD (g/L)	5.9	6.1	5.5
TOC (g/L)	1.2	1.5	1.6
VFAs/Alk	0.13	0.09	0.14

2.1. Organic Load Removal

The reduction in organic loading indicators was most pronounced as the quantity of the BM rose. COD biodegradability increased from 52.0% to 83.0%, while TOC reduction increased from 62.5% to 87.9%. VS biodegradability exhibited 51.7%, 49.4%, and 63.7% for the three mixing ratios of BM/PS, whilst VFAs biodegradability remained high in all anaerobic assays, reaching values 84.0%, 91.2%, and 89.4% for the 25/75, 50/50, and 75/25 trials, respectively. On the contrary, NH_4^+ content increased during the AD process on the three assays. The increase was 0.38 and 0.34 g/L for the 25/75 and 50/50 BM/PS ratios, respectively. The 75/25 ratio had a smaller increase on the NH_4^+ concentration of 0.22g/L. In Table 3, the variations in the parameters after the AD procedure are presented.

Table 3. Variations in physicochemical parameter values after anaerobic digestion.

Parameter (Unit)	25/75	50/50	75/25
pH_{raise} (-)	0.14	0.71	0.76
$VFAs_{biodegradability}$ (%)	84.0	91.2	89.4
$NH_4{}^+{}_{raise}$ (g/L)	0.38	0.34	0.22
$TS_{biodegradability}$ (%)	35.0	27.5	42.3
$VS_{biodegradability}$ (%)	51.7	49.4	63.7
$COD_{biodegradability}$ (%)	52.0	72.0	83.0
$TOC_{reduction}$ (%)	62.5	83.7	87.9

2.2. Biomethane Generation and Kinetic Analysis

Biomethane generation was affected by the BM/PS ratio. The cumulative gas production was 695.0 ± 12.0, 1218.0 ± 38.5, and 1799.0 ± 22.6 Nml for the 25/75, 50/50, and 75/25 ratios, respectively. The corresponding yields were 156.5 ± 2.7, 309.9 ± 9.8, and 525.9 ± 9.0 Nml/g VS_{added}. These values compare favorably with the literature on swine manure and food waste co-digestion: the yield of the 50/50 mixture (309.9 ± 9.8 CH_4 Nml/g VS_{added} aligns closely with the 323 CH_4 Nml/g VS_{added} reported by Han et al. [19] for FWL and piggery wastewater, whereas the 75/25 mixture (525.9 ± 9.0 CH_4 Nml/g VS_{added}) exceeded the 409.5 CH_4 Nml/g VS_{added} reported by Tian et al. [20] for kitchen waste and pig manure and matched the highest yield (521 ± 29 CH_4 Nml/g VS_{added}) reported by Dennehy et al. [10] at a 1:4 pig manure-to-food waste ratio. The 25/75 assay reached the endpoint after 15 days, while the other two investigated ratios reached the endpoint after 29 days. Table 4 demonstrates the net biomethane production and the obtained yield of the BMP assays. Figures 1 and 2 illustrate the net biomethane production and the obtained yield of the three investigated ratios.

Table 4. Biomethane production.

Parameter (Unit)	25/75	50/50	75/25
CH_4 (Nml)	695.0 ± 12.0	1218.0 ± 38.5	1799.0 ± 22.6
CH_4 (Nml/g VS_{added})	156.5 ± 2.7	309.9 ± 9.8	525.9 ± 9.0
Test days (days)	15	29	29

The daily cumulative methane generation profiles differed markedly among the three BM/PS mixtures (Figure 3). The 25/75 assay exhibited a relatively compact production profile, with the methane production increasing during the first seven days, reaching a maximum of 76 Nml/d on day 7. Thereafter, the production rate progressively decreased until no biomethane production was observed (day 15).

The 50/50 trial showed a higher maximum daily production rate of 130 Nml/d, similarly recorded on day 7. Following the main production peak, methane generation gradually declined, although a second increase was observed on day 17. After day 17, the daily production rate approached lower values.

The 75/25 test exhibited the highest and the most extended methane production profile. Daily methane production increased rapidly after day 4, reaching a maximum of 195 Nml/d on day 9. The rate subsequently decreased until day 17. On day 17, a second production phase developed until day 21. After day 21, the production declined progressively towards the end of the assay. Figure 3 illustrates the daily methane production of the BMP trials.

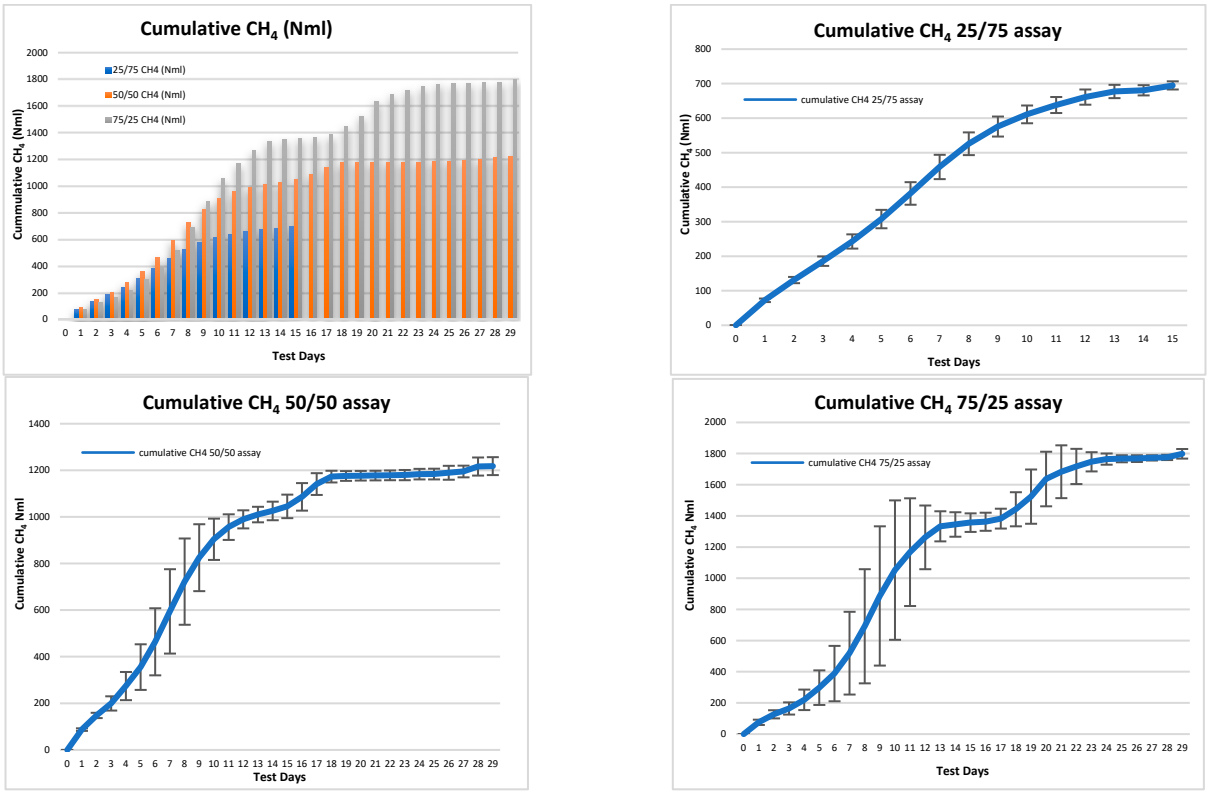


Figure 1. Cumulative CH_4 production for the three assays.

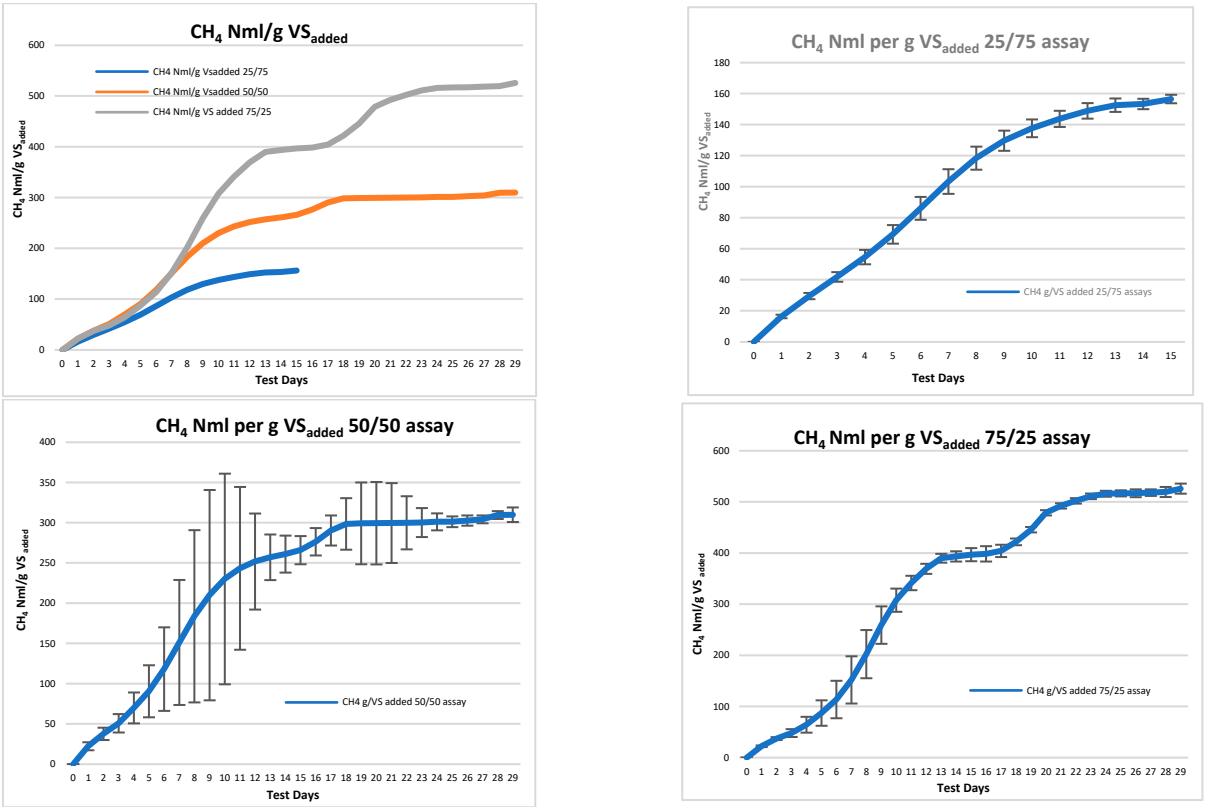


Figure 2. CH_4 production per g VS_{added} for the three assays.

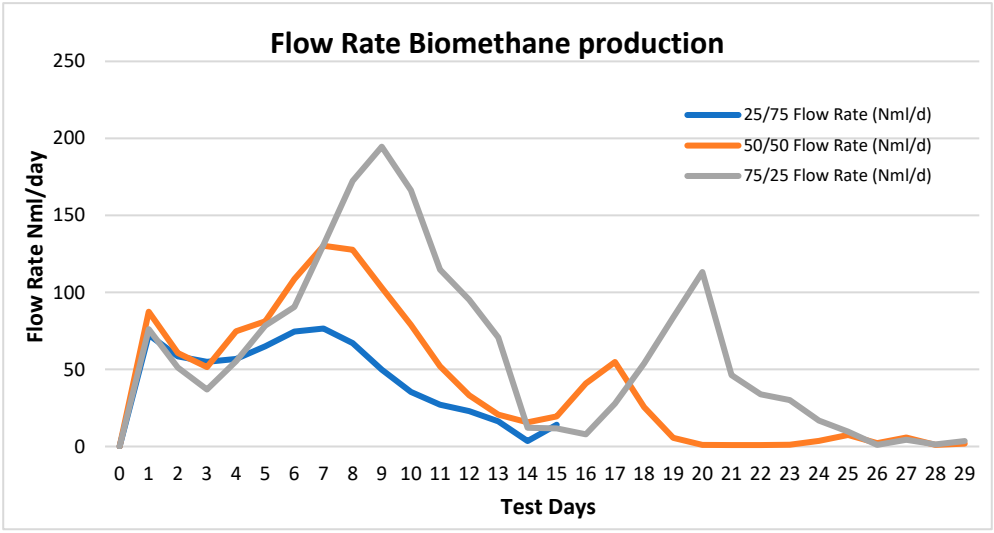


Figure 3. Flow Rate of biomethane production.

The cumulative biomethane production curves were fitted using the modified Gompertz model and the derived kinetic parameters are presented in Table 5. The model provided an excellent fit for all BM/PS ratios, R^2 of 0.997, 0.996, and 0.989 for the 25/75, 50/50, and 75/25 ratios, respectively.

Table 5. Kinetic parameters of methane production determined by the modified Gompertz model for the three BM/PS co-digestion ratios.

Substrate Ratio (BM/PS)	BMPexp (Nml/g VS_{added})	A (Nml/g VS_{added})	μ_m (Nml/g VS_{added} per Day)	λ (Days)	R^2	RMSE
25/75	156.5	165.8	17.1	0.71	0.997	2.77
50/50	309.9	304.5	27.9	1.44	0.996	5.91
75/25	525.9	524.6	36.3	2.32	0.989	18.40

The predicted maximum methane production potential (A), increased markedly as the BM contribution increased, from 165.8 for 25/75, 304.5 for 50/50, and 524.6 for 75/25. A similar trend was observed for the maximum methane production (μ_m), which increased from 17.1 to 27.9 and 36.3 Nml/g VS_{added} per day, respectively. The estimated lag phase (λ), also increased as the BM content increased, from 0.71d, 1.44d, and 2.32d for the 25/75, 50/50 and 75/25 mixture, respectively.

The experimentally determined biomethane yield was 156.5, 309.9, and 525.9 Nml CH_4/g VS_{added} for the three investigated mixing ratios.

The 75/25 formulation had both the highest predicted methane potential and the highest maximum methane production rate, whereas the 25/75 trial exhibited the shortest estimation lag phase.

3. Discussion

3.1. Process Stability and VFAs-to-Buffer Balance

The initial acid–base conditions of the ternary mixtures were established naturally upon mixing the substrates with the highly buffered inoculum, without any external pH adjustment. As detailed in the analytical framework, these initial baseline parameters were determined from representative composite samples. The initial pH values ranged favorably between 7.26 and 7.93, falling well within the optimal methanogenic window (6.8–8.0). Notably, the 75/25 mixture exhibited the highest initial VFA/alkalinity ratio of

0.55. While VFA/alkalinity ratios exceeding 0.4 are conventionally considered indicators of potential instability or souring in continuous-flow anaerobic digestion [21,22], batch co-digestion assays relying on robust, well-acclimatized inoculum can sustain significantly higher initial organic acid loadings. A healthy inoculum sourced from a well-functioning digester, combined with nitrogen-rich co-substrates, provides substantial alkalinity and buffering capacity [23,24], which ensures that a neutral pH is maintained and VFA inhibition is prevented [21]. As previous batch studies have demonstrated, VFA concentrations at these levels do not directly inhibit methanogenesis when adequate buffering is present [22]. In our system, the inherent buffering capacity was entirely sufficient to neutralize the acidic input from the base mix. Consequently, rather than causing methanogenic inhibition, this elevated initial VFA availability was rapidly converted to methane during the first days of digestion [10], serving as an immediately accessible and highly biodegradable substrate for methanogens, ultimately culminating in the highest observed specific biomethane yield.

Total alkalinity (TA) represents the acid-neutralizing capacity of the liquid phase [25]. While governed mainly by the carbonate/bicarbonate system, TA quantification by standard titration to pH 4.3 inherently encompasses the contribution of volatile fatty acids, commonly referred to as VFA alkalinity, alongside ammonia species [23,26]. The substantial initial TA (10.0–12.5 g/L $CaCO_3$) observed in the assays was partially inflated by the extremely high initial VFA concentrations (up to 5.48 g HAceq/L), whose conjugate bases buffer effectively in this titration range. As methanogenesis actively consumed these organic acids, this VFA-derived alkalinity was analytically removed.

Concurrently, the rise in pH (from initial 7.26–7.93 to final 7.97–8.07) and the continuous stripping of CO_2 into the biogas phase shifted the inorganic carbon equilibrium. At pH levels approaching 8.0, the precipitation of calcium and magnesium carbonates—minerals typically abundant in pig slurry—is thermodynamically favored, physically removing bicarbonate alkalinity from the soluble phase [25]. Furthermore, while ammonification increased the total ammoniacal nitrogen, at a final pH of 8.0, the predominant species is the ammonium ion (NH_4^+, pKa = 9.25). This ion does not consume acid during titration to pH 4.3 and thus does not contribute to the measured TA [21,26].

Consequently, the observed TA reduction to 4.1–4.6 g/L $CaCO_3$ does not indicate a deterioration of process stability but rather reflects the analytical removal of VFA alkalinity and the transition to a stable, bicarbonate-regulated system [27]. When this occurs simultaneously with VFA reduction, recovery of pH, and a decline in the VFA/Alkalinity ratio, it confirms successful process stabilization [28,29]. PS-based systems are particularly relevant in this respect because manure contributes endogenous bicarbonate buffering and mineral nutrients that can partly offset the acidification tendency of food-derived co-substrates [10,30].

3.2. Organic Matter Conversion

The effect of substrate composition of BMP trials was also evident in the extend of organic matter conversion. Increasing the BM concentration was associated with greater COD and TOC removal, indicating more extensive conversion of the organic carbon introduced with the food-derived fraction. This behavior is consistent with the high biodegradability of FWL and CWWW, which contain substantial amounts of readily biodegradable organic matter [7,23,31].

The different removal patterns observed for COD, TOC, and VS should not be regarded as contradictory. In the 25/75, 50/50, and 75/25 BM/PS trials, COD removal increased from 52.0% to 72.0% and 83.0%, respectively, while TOC reduction increased from 62.5% to 83.7 and 87.9%. In contrast, VS biodegradability followed a less uniform trend, reaching 51.7%, 49.4%, and 63.7%, respectively. These differences are expected because COD, TOC,

and VS quantify different characteristics of the organic matrix and therefore do not necessarily exhibit proportional reduction during anaerobic degradation [32]. A comparable divergence between COD and VS removal has also been reported in anaerobic co-digestion procedure [33], observing COD reduction of approximately 67–68% compared with only 19–37% VS removal.

The storage conditions of the PS may also have affected its biodegradability process. The PS used in the present study was obtained from an outdoor semi-liquid manure stockpile. A previous study [34] has shown that prolonged slurry storage promotes degradation and loss of the more readily biodegradable organic fraction, thereby altering the residual substrate composition and its subsequent biodegradability. This may partly explain the lower conversion observed when PS represented the largest fraction (25/75 BM/PS) of the ternary mixture.

3.3. Ammonium Evolution During Anaerobic Procedure

The increase in NH_4^+ content during the digestion process is consistent with net ammonification of organically bound nitrogen. The degradation of proteins, amino-acids, and other nitrogen-containing organic compounds release ammoniacal nitrogen during anaerobic procedure [35,36]. In our study, NH_4^+ concentration increased from 1.92 to 2.30 g/L in the 25/75 BM/PS assay, from 1.98 to 2.32 g/L in the 50/50 trial, and from 2.23 to 2.45 g/L in the 75/25 test. Thus, the absolute NH_4^+ increase was progressively lower as the PS contribution decreased (Table 3). This trend is compatible with a greater content of manure at higher PS proportions. However, the measured NH_4^+ concentration reflects the net balance between ammonium formation, microbial uptake, and NH_4^+/NH_3 equilibrium and therefore cannot be used as a direct quantitative measure of gross ammonification [35,36].

Despite the increase in NH_4^+ content, no clear evidence of severe ammonia-related inhibition was observed under the tested conditions. VFAs decreased substantially from 3.74 to 0.60, 4.34 to 0.38, and 5.48 to 0.58 g/L HAc_{eq} in the 25/75, 50/50, and 75/25 assays, respectively. The concurrent VFA consumption and biomethane generation are consistent with the sustained methanogenic activity despite the increase in NH_4^+. This is particularly evident for the 75/25 trial, which reached the highest final NH_4^+ concentration (2.45 g/L) while also exhibiting the highest biomethane yield (525.9 CH_4 Nml/g VS_{added}) and substantial VFA removal. To quantitatively evaluate potential ammonia inhibition, free ammonia nitrogen (FAN), the membrane-permeable species primarily responsible for methanogenic toxicity, was calculated using the aforementioned equilibrium Equations (3) and (4). At day 0, owing to lower initial pH values (6.93–7.93), FAN remained low (0.018–0.145 g N/L). By the end of the assays, the rise in pH to 7.97–8.07 increased the calculated FAN concentrations to 0.212 g N/L (25/75), 0.174 g N/L (50/50), and 0.204 g N/L (75/25). According to the literature, initial ammonia inhibition thresholds typically lie around 0.15–0.22 g FAN/L for unacclimated inoculum [20] whereas acclimated manure-digesting consortia tolerate 0.45–1.10 g FAN/L without severe process failure [11,30]. Therefore, while the final FAN levels approached the lower threshold where mild kinetic stress may occur, they remained well below acute inhibitory levels, explaining the absence of VFA accumulation at the end of the digestion. The final NH_4^+ concentrations of all BMP assays range in the same order as the ammonia–nitrogen range reported by Tian et al. [20] for kitchen-waste/pig-manure co-digestion.

3.4. Biomethane Generation and Production Rate

Biomethane recovery increased markedly with increasing the BM contribution. The specific methane yields were 156.5, 309.9, and 525.9 Nml CH_4/g VS_{added}, for the 25/75, 50/50, and 75/25 BM/PS trials, respectively. This pattern is consistent with the progressively greater

COD and TOC removal. The higher BM concentration indicates that substrate composition strongly influenced the fraction of organic matter ultimately converted to biomethane. The obtained methane performance of the 75/25 assay (525.9 CH_4 Nml/g VS_{added}) is comparable with the upper range (521 CH_4 Nml/g VS_{added}) reported for co-digestion of food waste and pig manure systems [10]. However, another study [19] reported approximately 323 CH_4 Nml/g VS_{added} for co-digestion of FWL and piggery wastewater. Interestingly, the methane yield of the present 50/50 test (309.9 CH_4 Nml/g VS_{added}) was close to that reported by Han et al. [19], whereas the 75/25 trail reached a substantially higher value. Tian et al. [20] reported a lower maximum of 409.5 CH_4 Nml/g VS_{added} for kitchen waste/pig manure, illustrating how strongly the biomethane potential depends on the biochemical character of the food-derived fraction and the manure age.

The daily methane production profiles add an important kinetic distinction. Whereas the 25/75 mixture displayed a single production peak on day 7, the secondary production phase observed in the 50/50 trial and, more clearly, in the 75/25 treatment (days 17–21) indicates a multiphasic conversion behavior. While intermediate metabolites were not kinetically tracked over time to definitively prove the underlying mechanism, this behavior is hypothesized to result from a combination of substrate heterogeneity, hydrolysis limitations, and dynamic microbial activity.

First, inherent substrate heterogeneity across the ternary mixture can lead to the sequential conversion of substrate pools with different biodegradation rates. Dissolved carbohydrates in CWWW/FWL and pre-existing acetate are presumably methanized rapidly during the first week, while more complex proteins, lipids, and particulate organics undergo slower enzymatic hydrolysis, which progressively becomes the rate-limiting step [6,10]. Second, changes in microbial activity and metabolic pathways may occur. As demonstrated by Tian et al. [20] and Dennehy et al. [10], high proportions of food waste (>50%) can promote the early accumulation of propionic and butyric acids. Because the syntrophic β-oxidation of these heavier acids is thermodynamically restricted until hydrogen partial pressure and acetate concentrations decline, their delayed conversion—as the microbial consortium acclimates—typically generates a secondary methane peak during the third week. Conversely, in the 25/75 assay, the predominance of aged PS (75%)—whose labile organic fraction had already been depleted during outdoor storage [32]—provided a smaller pool of readily degradable organics that was exhausted within a single phase, inherently preventing initial VFA overload and subsequent multiphasic behavior.

3.5. Kinetic Model, Modified Gompertz

The modified Gompertz model provided a satisfactory description of cumulative methane production for all three BM/PS formulations, with R^2 values 0.997, 0.996, and 0.989 for the 25/75, 50/50, and 75/25 assays, respectively. The fitted asymptotic methane potential (A) increased as the BM contribution increased, from 165.8 to 304.5 and 524.6 Nml CH_4/g VS_{added}, respectively. A similar trend was observed for the apparent maximum methane production rate (μ_m) which increased from 17.1 to 27.9 and 36.3 Nml CH_4/g VS_{added} per day. These kinetic trends are consistent with the higher experimental methane recovery and the greater extent of organic matter conversion observed as the BM contribution increased.

The lag parameter (λ) also increased progressively, from 0.71 d for 25/75 trial to 1.44 d for 50/50 and 2.32 d for 75/25. Mechanistically, this progressive elongation of λ is directly explained by the higher initial VFA concentration (rising from 3.74 to 5.48 g HAc_{eq}/L) and higher initial VFA/alkalinity ratio (rising from 0.30 to 0.55) introduced by increasing proportions of the acidic BM (Table 1). As shown by Tian et al. [20], elevated initial VFA loads impose transient acidogenic stress on methanogenic archaea, requiring an extended metabolic acclimation period before exponential methanogenesis can proceed. However,

the longer apparent lag at higher BM contributions should not be interpreted as direct evidence of process inhibition. In fact, the 75/25 assay exhibited the longest fitted lag while subsequently reaching both the highest μ_m and the highest biomethane yield. For the 25/75 test, the Gompertz asymptote was only about 5.9% higher (Table 5) than the experimental values, suggesting that some additional methane generation could have occurred beyond the experimental endpoint. The 25/75 assay requires a specific qualification because net biomethane production practically ceased by day 15. As the readily biodegradable fraction of this heavily manure-dominated mixture (75% aged pig slurry) was, rapidly exhausted, gross biogas generation halted. Consequently, the standard volumetric subtraction of the continuously degassing blank inoculum resulted in artificial negative net daily methane values beyond this point. To prevent mathematical deflation of the cumulative specific yield due to this background endogenous interference, the assay was practically terminated. As a result, the strict mathematical validation of the <1% threshold over three consecutive additional days was not formally recorded [21,37,38]. Therefore, while it accurately reflects the depletion of accessible substrate, its measured value is classified here as a day-15 natural endpoint rather than an unequivocally validated ultimate BMP. However, this limitation is in accordance with the observations by Dennehy et al. [10], who reported two distinct methane generation phases in a food waste/pig manure co-digestion system, with rapid VFA-associated methane production during the first days followed by slower degradation of the remaining organic matter.

While the goodness-of-fit metrics (high R^2 and low RMSE) and the random distribution of residuals confirm that the modified Gompertz model adequately captures the individual methanogenic profiles, the comparative interpretation of the kinetic parameters must acknowledge certain mathematical limitations. Specifically, the unequal test durations—15 days for the 25/75 assay versus 29 days for the 50/50 and 75/25 assays—limit the direct, side-by-side comparability of the fitted parameters. The natural cessation and subsequent truncation of the 25/75 assay restrict the model's formulation of an extended asymptotic phase. This potentially introduces a degree of uncertainty in the absolute comparative evaluation of the maximum production rate (μ_m) and lag phase (λ) against the assays that were monitored over the full 29-day baseline.

4. Materials and Methods

4.1. Materials

4.1.1. Anaerobic Sludge

Anaerobic sludge (AS) was used as the microbial inoculum for all BMP assays (Figure 4). The sludge was obtained from a full-scale mesophilic anaerobic digestion facility, located in Ptolemaida area, Western Macedonia, Greece. The AS was stored at 4 °C for 10 days [39] to preserve the activity of the microbial consortium [37]. The physicochemical characteristics of the inoculum are illustrated on Table 6. Prior to the initiation of the BMP tests, the AS was pre-incubated for 5 days at 35 °C to degas and reduce the impact of the methane production [21].

4.1.2. Food Waste Leachates

Food waste leachates (FWL) were obtained from biodegradable fruit and vegetable residues collected from the catering facilities of CERTH (Figure 5). Prior to leachate (Figure 6) generation, the collected residues were manually sorted to remove nonbiodegradable materials and were reduced in size. The prepared food waste consisted of a 1/1 (w/w) mixture and was transferred into closed containers maintained at 4 °C until a sufficient liquid fraction had accumulated. The main characteristics of the FWL are shown in Table 6. FWL constituted 25% of the BaseMix (BM).

Table 6. Physicochemical characteristics of the inoculum and substrates.

Parameter (Unit)	AS	FWL	CWWW	BM	PS
pH (-)	7.82	3.25	3.34	4.13	6.37
TS (g/L)	45.0	27.5	22.5	22.3	81.6
VS (g/L)	30.0	22.5	17.5	19.0	66.0
Alkalinity (g $CaCO_3$/L)	12.6	7.5	5.0	8.7	10.4
VFAs (g HAc_{eq}/L)	2.31	- [1]	- [1]	- [1]	0.3
COD (g/L)	10.1	57.5	38.9	43.3	9.8
TOC (g/L)	2.8	20.7	14.9	17.3	3.2
NH_4^+ (g/L)	2.6	0.53	0.32	0.15	1.7

[1] The VFAs content could not be determined by means of potentiometric titration due to low pH values of the FWL, CWWW, and BM.



Figure 4. Anaerobic sludge (AS).

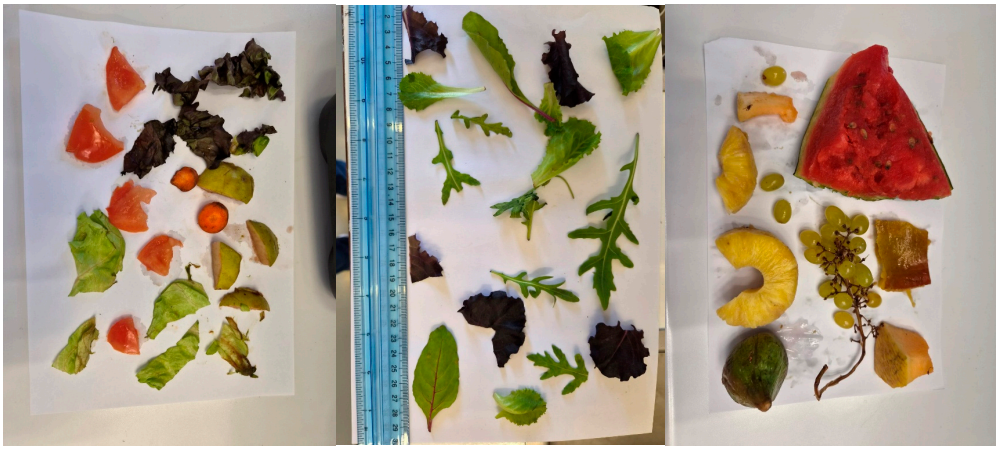


Figure 5. FW surpluses and leftovers used for the tests.



Figure 6. Food waste leachates (FWL).

4.1.3. Cheese Whey Wastewater

Cheese whey wastewater (CWWW) was sourced from a small- to medium-sized cheese manufacturing facility, established in the Kozani area, Western Macedonia, Greece (Figure 7). Following collection, the CWWW delivered to the lab and stored at 4 °C until use. The chemical characteristics of the CWWW are demonstrated on Table 6. CWWW accounted for 75% of the BM in this study.



Figure 7. Cheese whey wastewater (CWWW).

4.1.4. Pig Slurry

Pig slurry (PS) was collected from a semi-liquid manure stockpile, located in an outdoor storage area of a pig farm unit, established in the Kozani region, Western Macedonia, Greece (Figure 8). Due to the outdoor storage, the material was considered aged rather than freshly excreted slurry. This distinction is relevant because the chemical composition of the PS may vary considerably as a function of seasonal conditions and on-farm management

practices, as reported by Rodríguez-Abalde et al. [30]. Furthermore, outdoor storage may include substantial biological transformation of the organic matter and consequently affect its biodegradability [34]. Following collection, the PS was stored at 4 °C in the lab until its use. Prior to the BMP assays, the PS was subjected to a two-fold dilution with tap water, reducing its VS content and facilitating the establishment of a SIR 0.5. The main characteristics of PS are presented in Table 6.



Figure 8. Pig slurry (PS).

4.1.5. Experimental Procedure

The biochemical methane potential (BMP) assay design was based on the technical framework of VDI 4630 and established international recommendations for BMP standardization and validation [26,37,38,40]. The substrate-to-inoculum ratio (SIR) was expressed on a volatile solids (VS) basis as g $VS_{substrate}$/g $VS_{inoculum}$. In a previous study [6], the co-digestion and synergy index of FWL and CWWW were investigated. Among the co-digestion test, the 25/75 (FWL/CWWW) ratio exhibited the most favorable performance and was therefore selected as the reference mixture for the present study. This binary mixture is hereafter referred to as "BaseMix (BM)".

To study the effect of the incorporation of a livestock-derived co-substrate, the BM was subsequently combined with pig slurry (PS), resulting in a ternary substrate system consisting of FWL, CWWW, and PS. Prior to its use, the PS was diluted in a two-fold dilution (1/2) in order to reduce its VS content and facilitate the implementation of a SIR near to 0.5. To ensure comparability and maintain identical hydrodynamic conditions across all assays, a fixed working volume of 400 mL was utilized, comprising 250 mL of inoculum and 150 mL of combined substrates. The ternary mixtures were formulated on a volumetric basis (% v/v) by proportionally dividing this 150 mL substrate volume to achieve the three discrete BM/diluted-PS ratios: 25/75, 50/50, and 75/25 (% v/v). Because of the inherent minor differences in the initial VS concentrations of the BM and the diluted PS, this fixed volumetric blending resulted in actual SIR values spanning a tight operational window (0.46 to 0.59), successfully centering around the target nominal SIR of 0.5. The exact added volumes and corresponding organic loads are detailed in Table 7.

Table 7. Experimental protocol and operational parameters of the BMP assays.

BMP	25/75	50/50	75/25
Target SIR (g VS/g VS)	0.5	0.5	0.5
Actual SIR (g VS/g VS)	0.59	0.52	0.46
g VS Substrate	4.44	3.93	3.42
g VS Inoculum		7.5	
Total Volume (mL)	500	500	500
Working Volume (mL)	400	400	400
Inoculum added (mL)	250	250	250
BM added (mL)	37.5	75.0	112.5
Diluted PS added (mL)	112.5	75.0	37.5
Headspace	Flushed with N_2		
Mixing Regime	Automated intermittent mechanical stirring (10 min ON/5 min OFF cycle, at 40% speed)		
Temperature (°C)	35	35	35
Test Days	15	29	29

4.1.6. Biomethane Potential Test

Bench-scale biomethane potential (BMP) tests were performed in triplicate employing AMPTS II system. Each glass reactor of the AMPTS II system had a total volume of 0.5 L, comprising a working volume of 0.4 L and a headspace of 0.1 L. No pH adjustment was applied during the BMP assays. Prior to the incubation, the glass bottles were flushed with N_2 to achieve anaerobic conditions. The reactors were placed in a water bath operating in mesophilic conditions 35 ± 2 °C.

The generated biogas of each reactor passed through an individual absorption unit containing 3M NaOH-thymolpthalein solution to capture CO_2 and other impurities. Following the biogas upgrade, the purified stream was transferred to the corresponding flow cells, where biomethane production was continuously quantified by water displacement.

The complete experimental protocol and operational parameters of the BMP assays are detailed in Table 7.

4.1.7. Kinetic Analysis

A kinetic study of the three experimental conditions was conducted to provide a kinetic interpretation of the BMP tests, applying the modified Gompertz model as described by Pasciucco et al. [41] and Nielfa et al. [42].

$$BMP(t) = A \times exp\left\{-exp\left[\frac{\mu_m \times e}{A}(\lambda - t) + 1\right]\right\} \quad (1)$$

where:

$BMP(t)$ = cumulative biomethane production at time t;
A = maximum biomethane potential (Nml/g VS_{added});
μ^m = maximum biomethane production rate (Nml/g VS_{added} per day);
λ = lag phase duration (days);
t = time (days);
e = exp (1).

The kinetic parameters were estimated by non-linear least-squares regression analysis. These parameters correspond to the lag, exponential, and stationary phases of the anaerobic digestion process, reflecting the typical sigmoidal shape of the growth curve [43]. Parameter estimation was performed using non-linear least-squares regression via the generalized reduced gradient (GRG) optimization algorithm. To ensure biological and physical validity, non-negativity boundary constraints were applied to all parameters ($A > 0$, $\mu_m > 0$, $\lambda > 0$).

Because the coefficient of determination (R^2) can be artificially high in monotonically increasing cumulative curves, the model's adequacy was additionally evaluated using the root mean square error (RMSE) as an absolute goodness-of-fit metric. Furthermore, basic residual analysis (calculated as the difference between experimental and predicted values over time) was conducted to verify the random distribution of errors and the absence of systematic modeling bias across the distinct phases of the digestion.

4.2. Methods

The determinations of TS, VS, COD, TOC, NH_4^+, and alkalinity were carried out according to APHA Standard Methods [26]. The pH was determined using a digital pH-meter (Hanna, HI2260, Woonsocket, RI, USA). The VFAs content was calculated as described by Mota et al. [44] and expressed as acetic acid equivalents (HAc_{eq}). COD content and NH_4^+ concentration were quantified using a HACH DR2800 spectrometer (Hach Lange, Loveland, CO, USA). The quantification of TOC content of the materials was employed by a TOC analyzer (TOC-Shimadzu, Kyoto, Japan).

The organic matter removal efficiencies (for COD, TOC, TS, VS, and VFAs) were evaluated on a concentration basis using the following equation:

$$Removal\ (\%) = \frac{(Cinitial - Cfinal)}{Cinitial} \times 100\% \quad (2)$$

representing the parameter concentrations at day 0 (Table 1) and at the endpoint of the assay (Table 2), respectively. Given the nature of the closed-batch AMPTS II system operating at a fixed 400 mL working volume, water vapor loss and evaporative volume changes are practically negligible throughout the digestion period. Consequently, the concentration-based removal efficiencies accurately reflect the absolute mass-based biodegradability of the organic components.

The biomethane potential yield expressed as mL CH_4/g VS_{added}, was continuously quantified using the flow meter unit of the AMPTS II system. The instrument's software automatically normalized the recorded gas volumes to standard temperature and pressure on a dry basis. To rigorously account for the endogenous methane production of the inoculum, blank assays containing solely inoculum and deionized water were conducted in parallel. The cumulative methane volume generated by these blanks was proportionally subtracted from the total methane volume of each experimental assay prior to calculating the final net specific yield by dividing by the exact mass of substrate volatile solids (VS) added to the reactor. The calculation and reporting of BMP values followed established standardization principles, including correction for inoculum-derived methane and expression of dry methane volume under standard conditions per unit mass of VS added [37,38].

To evaluate potential ammonia toxicity, the concentration of free ammonia nitrogen (FAN, g N/L) was calculated from the measured NH_4^+ concentration (converted to total ammonia nitrogen), pH, and fermentation temperature, according to the chemical equilibrium equation reported by Hansen et al. [11], Rodríguez-Abalde et al. [30], and Tian et al. [20].

$$TAN = \left[NH_4^+\right] \times \frac{14.007}{18.038} \quad (3)$$

$$FAN = \frac{TAN \times 10^{pH}}{e^{\frac{6344}{273.15+T}} + 10^{pH}} \quad (4)$$

4.3. Data Presentation and Statistical Approach

To ensure experimental reproducibility and the reliability of the biomethane generation data, all biochemical methane potential assays were executed in independent triplicates.

The continuously monitored variables, specifically the cumulative biomethane production and the specific biomethane yield, are reported as the arithmetic mean accompanied by the corresponding standard deviation. The experimental variance across the triplicates is graphically depicted using error bars in the experimental temporal production profiles. Owing to the volumetric constraints of the bench-scale reactors and the requirement for substantial sample aliquots to conduct robust multiparametric physicochemical analyses, including chemical oxygen demand, volatile solids, volatile fatty acids, and alkalinity, both initial and post-digestion characterizations were performed on representative composite samples. These composites were meticulously formulated by proportionally pooling the digestate or substrate from the corresponding replicate reactors. This procedure represents a standardized methodological approach to preserve analytical accuracy in volume-limited batch assays. Consequently, the physicochemical state variables and their associated removal efficiencies are reported as singular, representative composite values.

5. Conclusions

This study investigated an alternative approach to the treatment and valorization of wastewater streams through ternary anaerobic co-digestion, extending beyond conventional co-digestion schemes based on two substrates. Three waste streams originating from different sectors and exhibiting distinct physicochemical characteristics were simultaneously treated to assess their compatibility and evaluate the overall performance of the anaerobic digestion process in terms of organic matter removal and biomethane production.

The evaluation of different substrate mixing ratios demonstrated that feedstock composition had a considerable influence on process performance. In particular, increasing the proportion of PS in the mixture resulted in reduced organic matter removal efficiency and lower biomethane production, highlighting the importance of substrate composition and mixing ratio in determining overall digestion performance. Nevertheless, stable anaerobic digestion was achieved under all the experimental conditions investigated, with no evidence of process failure across the different mixing ratios. The observed differences were therefore primarily associated with process efficiency rather than operational stability.

The experimental findings demonstrate the technical feasibility of the simultaneous anaerobic digestion of three compositionally distinct waste streams at laboratory scale, providing a basis for the further development of multi-substrate anaerobic digestion strategies. Such an approach could facilitate the integrated management and valorization of heterogeneous wastewater streams while simultaneously enabling renewable biomethane production. This concept may be particularly relevant to facilities where multiple waste streams are generated or available within a limited geographical area, such as industrial parks, agro-industrial facilities, and integrated waste-management systems. In such applications, centralized co-treatment could potentially reduce the need for separate waste-treatment infrastructure while promoting resource recovery and circularity.

Further research should focus on identifying compatible substrate combinations and determining absolute optimal mixing ratios through continuous reactor testing. As highlighted by Koch et al. [24], batch BMP tests are inherently designed to determine the maximum biochemical methane potential under ideal conditions; therefore, definitive conclusions regarding long-term process stability, chronic toxicity, or absolute operational optima of a continuous system cannot be firmly established solely from batch results. Consequently, while the 75/25 volumetric ratio is identified here as the most favorable formulation for ultimate yield among the discrete setpoints tested, continuous gradient testing is required to establish a true operational optimum. Future investigations must additionally incorporate continuous-flow validation and comprehensive techno-economic assessments to definitively determine the feasibility of this ternary co-digestion approach for industrial scale-up.

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