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Optimizing Methane Production from Anaerobic Digestion of Dairy Cow Manure: The Potential Use of Carica (*Carica pubescens*) Seeds as a Co-Substrate

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ABSTRACT

A method to increase methane production in dairy cow manure (DCM) is to co-digest DCM with nutritious biomass. This study aimed to determine the methane yield during the anaerobic co-digestion of DCM and carica seeds meal (CSM). Four continuous stirred tank reactors were operated with treatments P0 (100% DCM), P1 (98% DCM and 2% CSM), P2 (96% DCM and 4% CSM), and P3 (94% DCM and 6% CSM). The results demonstrated that the presence of CSM as a co-substrate of DCM significantly increased ($p<0.05$) methane production. The average methane production resulting from P0, P1, P2, and P3 in units of mL/g substrate and mL/g volatile solid ($VS)_{added}$ were 10.05, 20.54, 32.26, and 19.29 mL/g substrate and 171.49, 278.96, 357.92 and 179.30 mL/g VS_{added}, respectively. Thus, the highest methane production was obtained at P2. Treatment P3 contained a substrate containing excessively high protein and organic content, negatively affecting anaerobic microorganisms' activity. The presence of CSM as a co-substrate enhanced methane production by 91.94%–221.06% compared with the control. The presence of CSM as a co-substrate significantly increased ($p<0.05$) volatile fatty acid and total ammonia nitrogen (TAN) concentrations and the pH of digested slurries but did not affect VS reduction. The co-digestion of DCM and CSM must consider the proportion of organic material in the mixed substrate. In this study, the mixed substrate with a VS proportion of 51.68% was the best-mixed substrate.

Keywords: biogas; carica seeds; co-digestion; dairy cow manure; methane

INTRODUCTION

Manure management is essential in mitigating greenhouse gas (GHG) emissions because manure contributes around 12.8% of GHG emissions from the livestock sector. In 2015, methane produced by livestock manure was 484 million tons, while CO_2 and N_2O were 309 million tons (Emmerling *et al.*, 2020). Even though the emissions of methane and N_2O were less than CO_2, these two gases are 21 and 310 times more powerful than CO_2 in terms of global warming potential, respectively (Thangarajan *et al.*, 2013). Manure management through anaerobic digestion (AD) can reduce CH_4 emissions by preventing unwanted fermentation during manure storage in the gutter and in the manure storage tank (Yan *et al.*, 2024). In addition, biogas production in the AD process can also reduce fossil fuel consumption, chemical fertilizers, and emissions from subsequent digestion storage (Kaparaju & Rintala, 2011).

Handling dairy cattle manure (DCM) in AD as a single substrate faces the main challenge of low biogas production per ton of biomass. Sutaryo *et al.* (2023) and Li *et al.* (2021) found that methane production per ton of DCM as a mono-substrate is low because DCM contains high water, ash, and crude fiber contents. Therefore, a substrate with high nutrition content and high digestibility is recommended for co-substrate with DCM in AD. Anaerobic co-digestion is an effort that can be made to increase biogas production by combining various types of waste-containing organic materials. Anaerobic co-digestion has several benefits, including: (i) dilution of toxic compounds in the substrate, (ii) synergistic effect on microbial growth, (iii) improving nutrient balance, (iv) increased organic loading rate, (v) increased methane yield, and (vi) modification of buffer capacity (Li *et al.*, 2024). The organic material contained in the co-substrate will increase nutrient concentration so that microorganisms can utilize it to produce higher biogas. On an industrial scale, the biogas produced by DCM as a single substrate is 10-20 m^3 CH_4/ton of processed waste, while if combined with other biomass, production can reach 30-500 m^3 CH_4/ton of processed waste, depending on the type of combined waste used (Angelidaki *et al.*, 2003).

Carica (mountain papaya) has the scientific name *Carica pubescens* (A. DC.) Solms-Laub., *Carica candamarcensis* Hook.f., *Carica cestriflora* Solms or *Carica cundinamarcensis* Linden and grows at an altitude of

1.400–2.400 m above sea level with low temperatures and high rainfall. One of the carica-producing areas in Indonesia is the Dieng Plateau in Central Java. Carica fruit production in Dieng in 2019 reached 60.993 tons. Wonosobo subdistrict (Indonesia) is the area where the majority of carica fruits are processed. There are 54 carica processing business units spread across the subdistrict (BPS Wonosobo Regency, 2021). Carica fruit has been developed into an important commodity with considerable economic value, one of which is the carica-candied product in syrup (Idayanti *et al.*, 2024). In making candied carica in syrup, the industry uses fruit flesh only; therefore, carica seeds (CS) are waste. CS meal (CSM) has high nutritional content, i.e., 31.84% crude protein, 24.41% crude fiber, and 30.22% carbs/briones (Briones-Labarca *et al.*, 2015). However, up to now, CS has only been thrown away, so it poses a risk of polluting the environment. CS accounts for 25.59% of the total fruit weight; thus, the CS amount in Dieng in 2019 reached 15.608 tons. As part of the handling and utilization efforts, CS can be used as a co-substrate DCM and methane production via anaerobic digestion (AD).

Anaerobic co-digestion of CS and DCM can process two wastes simultaneously. The high nutritional content, availability of waste, and the lack of utilization of CS have led to its high potential to be used as a co-substrate with DCM to produce renewable energy through AD. Therefore, utilizing CS as a co-substrate can overcome the problem of low methane production from the digestion of DCM and increase farmers' interest in processing DCM anaerobically for biogas production. However, considering the high crude protein content in CS, the proportion of CS in the final substrate must be considered. One of the results of protein decomposition is ammonia, which in high concentrations, is toxic to microorganisms (Sutaryo *et al.*, 2014). Ammonia inhibition is related to the concentration of free ammonia (FA). FA has generally been shown to be the main cause of ammonia inhibition due to its permeability to microbial cell membranes, especially methanogens. Methanogens, especially acetoclastic methanogens, are more susceptible to ammonia inhibition (Siles *et al.*, 2010). Whittmann *et al*. (1995) stated that there are some ammonia inhibition mechanisms, including changes in the intracellular pH, increased maintenance energy requirements, and inhibition of certain enzyme reactions. Methanogen inhibition can cause process instability due to VFA accumulation and decreased pH, ultimately affecting the decreased biogas production or even failure of the AD process (Yellezuome *et al.*, 2022).

To the best of our knowledge, there has been a lack of information on using CS as a co-substrate in the AD of DCM to increase methane production. The use of CS as a co-substrate with DCM in AD can be a model for the utilization of other fruit seeds that are waste and have not been utilized yet. Hence, this research aimed to provide scientific information regarding using CS as a co-substrate in the AD of DCM for methane production. A much higher nutrient concentration of CS compared to that in the DCM, therefore utilization of CS as a co-substrate of DCM is expected to improve the nutrient concentration of the mixed substrate so that this synergistic effects of CS as a co-substrate of DCM allegedly can enhance the activity of critical enzymes and microorganisms, promoting the digestion of organic waste and methane generation.

MATERIALS AND METHODS

Experimental Setup

This research was experimentally conducted using four continuous stirred tank reactors (CSTRs). Each CSTR with a total volume of 7 L and an active volume of 75% (or 5.250 L) was operated at an incubator temperature of 37 **°C** (Figure 1). The research began by filling the empty CSTR with a starter until 75% of the total volume was filled. Then, during the adaptation stage, the slurry was removed, and the DCM substrate was entered into the CSTR at the same volume at 238.6 g/day. The adaptation stage was conducted for 22 days or 1 hydraulic retention time (HRT), after which it was continued with the data collection stage for 3 HRT. The following treatments were applied: P0 (100% DCM), P1 (98% DCM and 2% CSM), P2 (96% DCM and 4% CSM), and P3 (94% DCM and 6% CSM) (Table 2). The total solid (TS) content of the substrate in treatment P0 (control) was 6.77%. This value was agreed with Song *et al.* (2023), who reported that DCM contains 5%-12% TS.

Starter and Substrates

The starter was obtained from a biogas reactor at the Friesian Holstein (FH) Cowshed, Faculty of Animal and Agricultural Sciences, Diponegoro University, Indonesia. The starter contained TS 3.99%, volatile solids (VS) 3.42%, and pH 7.43. In this study, digested slurry from an active biogas digester was used as a starter because it contains active anaerobe microorganisms; hence, it can accelerate the adaptation period during the experiment. DCM was prepared by dissolving dairy cow feces in water at a ratio of 1:1.6 (w/w). The dairy cow feces were obtained from dairy cows in the lactation period, which were fed forage and concentrated in the FH Cowshed, Faculty of Animal and

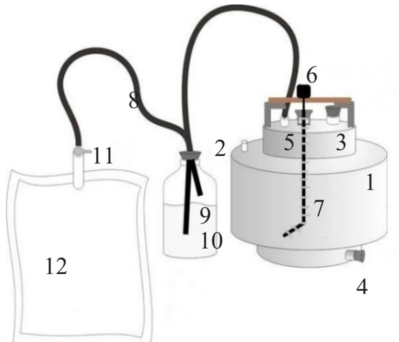

Figure 1. Continuous stirred tank reactor configuration (1. Reactor, 2. Rubber stopper, 3. Substrate inlet, 4. Digested slurry outlet, 5. Biogas outlet, 6. Motor, 7. Stirrer, 8. Teflon tube, 9. Infusion bottle, 10. NaOH solution, 11. Valve, and 12. Tedlar gas bag) (Saputra *et al.*, 2018).

Agricultural Sciences, Diponegoro University. CS was obtained from the Carica Gemilang Factory, which is located in Bojasari Village, Kertek District, Wonosobo Regency, Central Java, Indonesia (Figure 2).

First, the CS was cleaned with running water to separate the seeds from the sarcotesta layer. In the sorting process, the clean seeds were soaked in water to remove the floating seeds. The seeds were then drained and dried in the sun. Subsequently, they were blended and stored as stock. The CS flouring process made it easier to use, considering the reactor was laboratory-scale. CS was then analyzed for their nutrient contents, which are presented in Table 1.

Analytical Methods

Methane production data were collected every day at 10:00 AM local time for 3 HRT. Methane production was measured by passing biogas from the reactor with a 5-mL-diameter Teflon pipe into a 500 mL glass bottle filled with 4% NaOH solution (Merck®, Cat No. 1064981000). Furthermore, the methane gas was stored in a 10-L Tedlar gas bag (Hedetech-Dupont, China). Methane gas volume was measured using the liquid displacement method reported by Sutaryo *et al*. (2020).

Table 1. Characteristics of carica (*Carica pubescens*) seed meal

Variables (DM)	Values
Water content (%)	7.68
Dry matter (%)	93.72
Crude fiber (%)	16.05
Total solid (TS) (%)	92.32
Volatile solid (VS) (%)	87.76
Ash content (%)	4.57
Crude protein (%)	29.83
Crude fat (%)	37.48
Neutral detergent fiber (NDF) (%)	47.18
Acid detergent fiber (ADF) (%)	39.80
Hemicellulose (%)	7.38
Lignin (%)	29.16
Cellulose (%)	10.53
C/N	10.39

A digital pH meter (Ohaus® ST300) was used to measure liquid pH. The TS concentration of the starter and substrates was determined using the thermogravimetric method using an oven for 7 h at 105 °C, followed by ashing at a temperature of 550 °C for 6 h. The difference between the TS and ash content was used to calculate the VS concentration (APHA, 2005). The total ammonia nitrogen (TAN) concentration was determined using a NOVA 60 A Spectroquant® with Spectroquant® ammonium test reagent (Cat. No. 1.00683.0001). The volatile fatty acid (VFA) concentration was analyzed using gas chromatography-mass spectrometry (Bruker SCION 436-GC).

The C/N ratio was computed by comparing total organic carbon and total nitrogen. Total organic carbon was analyzed using a method described by Syaichurrozi (2018). Crude fat was analyzed using Soxhlet extraction. Protein content was analyzed using the Kjeldahl method. The nitrogen (N) value was then calculated from the crude protein content divided by 6.25. Crude fiber analysis was performed using the gravimetric method. Carbohydrate analysis was conducted using the volumetric method. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) analysis were performed using the Van Soest *et al*. (1991) method. Hemicellulose content was determined by the difference between the NDF and ADF values. Cellulose and lignin analyses were conducted using the gravimetric method. Experimental data were analyzed using analysis of variance with a significance level of 5%. If the results were significantly different, Duncan's multiple range test was continued.

RESULTS

Methane Production

Methane production during the AD process is displayed in Figure 3 and Table 3. The average methane


(a) (b) (c)

Figure 2. The presentation of carica (*Carica pubescens*) fruit seed used as co-substrate with dairy cow manure in this recent study. a= Carica seeds fruit, b= Carica seeds before cleaning, and c= Carica seeds after cleaning.

Table 2. Properties of the mixed substrate, dairy cow manure, and carica (*Carica pubescens*) fruit seed in different levels combination used in this experiment

Treatments	TS (%)	VS (%)	Crude protein (%)	Proportion of VS in substrate (%)	pH	C/N ratio
P0	6.77±0.06	5.86±0.03	0.94	0.00	7.69	21.64
P1	8.35±0.16	7.36±0.07	1.38	34.38	7.57	18.52
P2	10.14±0.23	9.01±0.14	1.84	51.68	7.31	17.01
P3	11.96±0.08	10.76±0.10	2.35	62.10	7.18	15.89

Note: Data total solid (TS) and volatile solid (VS) are presented as mean ± standard deviation. P0= 100% dairy cow manure (DCM); P1= 98% DCM and 2% carica seeds meal (CSM); P2= 96% DCM and 4% CSM; and P3= 94% DCM and 6% CSM.

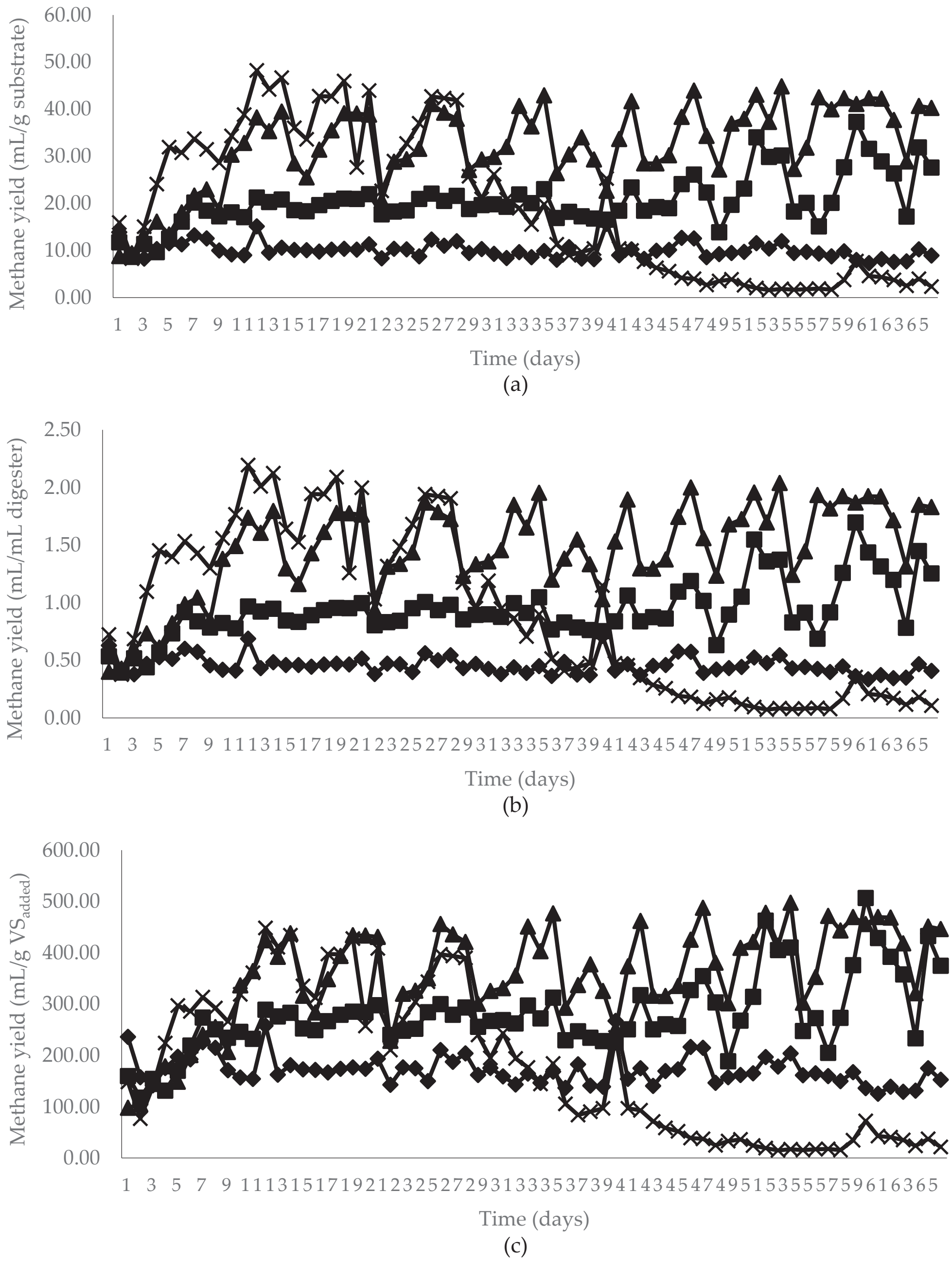

Figure 3. Methane production from digesters with mixed substrates of dairy cow manure and carica seeds (*Carica pubescens*) at different levels combinations and operating at 37 °C. (a) mL/g substrate, (b) mL/mL digester, and (c) mL/g VSadded. (-♦- = P0; -■- = P1; -▲- = P2; -ж- = P3). P0= 100% dairy cow manure (DCM); P1= 98% DCM and 2% carica seeds meal (CSM); P2= 96% DCM and 4% CSM; and P3= 94% DCM and 6% CSM.

production for treatments P0, P1, P2, and P3 in units of mL/g digester volume, mL/g substrate, and mL/g VS_{added} was 0.46, 0.93, 1.47, and 0.88 mL/g digester volume; 10.05, 20.54, 32.26, and 19.29 mL/g substrate; and 171.49, 278.96, 357.92, and 179.30 mL/g VS_{added}, respectively. The results showed that the utilization of CS as a co-substrate significantly affected ($p<0.05$) methane production.

Variables in Digested Slurry

The digested slurry of treatments P0, P1, P2, and P3 had average VFA concentrations of 8.14, 1.75, 1.11, and 40.58 mM; average TAN concentrations of 137.50, 316.00, 467.22, and 807.27 mg/L; VS reduction percentages of 31.42%, 33.42%, 35.93%, and 37.47%; and average pH values of 7.39, 7.51, 7.68, and 6.67. The utilization of CS as a co-substrate in the AD of DCM had a significant effect ($p<0.05$) on the VFA and TAN concentrations and liquid pH (Table 3) but had no significant effect ($p>0.05$) on VS reduction.

DISCUSSION

Carica Seeds Nutritional Content

CS has a high nutrient content, including protein. Based on the analysis results, the protein content in CS was 29.83% (Table 1). According to Hartadi *et al.* (1980), materials containing at least 20% protein, whether from animals or plants, are called protein sources. In AD, protein will be hydrolyzed by microorganisms into amino acids. However, substrates with high protein levels are not recommended for AD because they increase the risk of inhibiting methane production due to the accumulation of NH_3 (Kovács *et al.*, 2015).

An increase in protein content in the mixed substrate was directly proportional to an increase in the CS content in the mixed substrate. The higher the CS portion, the higher the protein content in the mixed substrate; therefore, the C/N ratio in the substrate tended to decrease (Table 2). Choi *et al.* (2020) reported that the C/N ratio must be ideal because it affects methane production. Methane production will be stable at a C/N ratio of 15–30:1 for mesophilic bacteria (Zhang *et al.*, 2014; Shrestha *et al.*, 2023). Substrates with a low C/N ratio (i.e., 15) cause an increase in the TAN concentration. The accumulation of ammonium ions (NH_4) in high concentrations can damage bacterial cells and negatively affect biogas production (Choi *et al.*, 2020; Shrestha *et al.*, 2023). This result agrees with the research results shown in Figure 3. Based on Figure 3, methane production from treatment P3 in HRT 1 remained high, whereas that in HRTs 2 and 3 decreased.

CS also had a high crude fat content of 37.48% (Table 1). It is suspected that the high crude fat content will inhibit methane production. Shrestha *et al.* (2023) and Elsamadony *et al.* (2021) stated that fat will be hydrolyzed into long-chain fatty acids (LCFAs). Accumulation of LCFAs in high concentration will inhibit the AD process. LCFAs will form a layer that can block bacterial cell walls, thereby inhibiting or reducing the entry of nutritional intake and reducing the ability to balance the intracellular pH of bacteria.

Methane Production

Based on the research results (Table 3), the utilization of CS as a co-substrate in the AD of DCM significantly enhanced ($p<0.05$) methane production in units of mL/g digester volume, mL/g substrate, and L/g VS_{added} compared with the control (without CS addition). Thus, the presence of CS as a co-substrate with different percentages can enhance methane production. This finding follows that of previous research by Mustikasari *et al.* (2023), who reported that the presence of a co-substrate of *Imperata cylindrica* in AD increased the quality of organic materials, thereby increasing methane production by 35.52%–45.44% in a unit of mL/g substrate.

In a unit of mL/g VS_{added}, the utilization of CS in treatments P1 and P2 significantly enhanced ($p<0.05$) methane production compared with treatments P0 (control) and P3. However, methane production at treatment P3 was not significantly different from that at the control. This may be due to the limited ability of methanogenic microorganisms to convert VFAs (especially acetate) into biogas (Table 4). This was confirmed by the highest concentrations of acetate and VFAs found in the digested slurry of treatment P3. High VFA concentrations also affected the low pH at treatment P3 (Tables 3 and 4). Mao *et al.* (2015) reported that the VFA concentration is correlated with the pH. When the pH is 6.0, hydrolytic enzyme activity increases, leading to the production of high VFAs. High VFA content will have a negative effect on methane production. In AD, acidogenic and methanogenic microbes have different optimal pH conditions. Methanogenesis is efficient at pH 6.5–8.2, with an optimum pH of 7.0. The optimum pH for acidogenesis is 5.5–6.5. The digested treatment P3 slurry had the lowest pH value. Low pH can prevent methanogens from converting acetate into biogas.

Table 3. Methane yield, TAN concentration, VFA concentration, VS reduction, and pH value of digested slurry of each digester treating different level combinations of dairy cow manure and carica (Carica pubescens) fruit seed

Treatments	Methane production			VFAs (mM)	TAN (mg/L)	VS reduction (%)	pH
	mL/mL digester volume	mL/g substrate	mL/g VS_{added}				
P0	0.46 ± 0.78^a	10.05 ± 1.71^a	171.49 ± 29.11^a	8.14 ± 13.51^a	137.50 ± 23.36^a	31.42 ± 4.35	7.39 ± 0.19^b
P1	0.93 ± 0.25^b	20.54 ± 5.42^b	278.96 ± 73.60^b	1.75 ± 1.23^a	316.00 ± 77.67^b	33.42 ± 3.22	7.51 ± 0.15^b
P2	1.47 ± 0.40^c	32.26 ± 8.84^c	357.92 ± 98.12^c	1.11 ± 0.32^a	467.22 ± 151.87^b	35.93 ± 5.78	7.68 ± 0.15^b
P3	0.88 ± 0.70^b	19.29 ± 15.46^b	179.30 ± 143.71^a	40.58 ± 37.90^b	807.27 ± 318.81^c	37.47 ± 8.85	6.67 ± 0.91^a

Note: ^{abcd} means in the same column with different superscripts differ significantly ($p<0.05$). Data are presented as mean ± standard deviation. TAN= total ammonia nitrogen; VFA= volatile fatty acid; VS= volatile solid. P0= 100% dairy cow manure (DCM); P1= 98% DCM and 2% carica seeds meal (CSM); P2= 96% DCM and 4% CSM; and P3= 94% DCM and 6% CSM.

Table 4. Partial volatile fatty acid concentrations of digested slurry from digesters treating mixed substrate of dairy cow manure and carica (*Carica pubescens*) fruit seed in different level combinations

Treatments	VFA concentrations (mM)					
	Acetic acid	Propionic acid	Iso-butyric acid	N-butyric acid	Isovaleric acid	N-valeric acid
P0	7.49 ± 12.92	0.47 ± 0.62	0.06 ± 0.05	0.06 ± 0.05	0.01 ± 0.01	0.04 ± 0.05
P1	1.32 ± 0.88	0.26 ± 0.30	0.07 ± 0.08	0.05 ± 0.05	0.01 ± 0.02	0.04 ± 0.05
P2	1.07 ± 0.69	0.13 ± 0.07	0.05 ± 0.06	0.03 ± 0.04	0.01 ± 0.01	0.04 ± 0.05
P3	22.86 ± 17.65	13.34 ± 12.27	1.50 ± 1.26	3.20 ± 4.14	2.12 ± 1.82	1.28 ± 1.77
Total	32.74 ± 8.04	14.20 ± 3.31	1.69 ±0.36	3.35 ± 1.07	2.16 ± 0.46	1.41 ± 0.48

Note: Data are presented as mean ± standard deviation. TAN= total ammonia nitrogen; VFA= volatile fatty acid; VS= volatile solid. P0= 100% dairy cow manure (DCM); P1= 98% DCM and 2% carica seeds meal (CSM); P2= 96% DCM and 4% CSM; and P3= 94% DCM and 6% CSM.

In all unit measurements, methane production in P1 and P2 was significantly higher ($p<0.05$) than in P0. This fact can be that applying CS as a co-substrate with DCM in the AD process provides a better balance of substrate nutrients than a single digestion of DCM since CS has a better nutrition content (Table 2). During the AD process, several various operational variables can affect the microorganism activity, such as ambient temperature, pH, organic loading rate, HRT, nutrients, alkalinity, ammonia toxicity, carbon to nitrogen ratio (C/N ratio), etc. (Nayeri *et al.,* 2023). No positive effect of the utilization of CS as co-substrate with DCM in P3 was due to the high nitrogen content in the co-substrate, so the C/N ratio with carbon needs to be balanced. The high nitrogen content causes the C/N of the co-substrate to be low, confirmed by the C/N ratio in the co-substrate (Table 2) and the high TAN concentration (Table 3) in P3. Therefore, the substrate combination process must consider the C/N ratio because biogas production will increase when there is a positive interaction between the co-substrate and the microbial population, otherwise, methane production will decrease (Ayaji-Banji & Rahman, 2022). Although the use of co-substrate in AD has many advantages, this process can also have an inhibiting effect due to the improper substrate mixing ratio, which causes acidification, excess organic matter, and process failure (Pramanik, 2022; Siddique & Wahid, 2018). This result showed that the TAN concentration in P1 and P2 has not yet had a negative impact on methane production. On the other hand, the TAN concentration at P3 began to harm methane production. Jiang *et al.* (2019) reported that the inhibitory threshold for TAN concentration is 1.500–7.000 mg/L. TAN concentrations in P1, P2, and P3 were below the ammonia inhibition threshold; however, it seems that the higher TAN concentration, along with the low pH value in P3 (Tabel 3), disrupted the growth of anaerobic microorganisms and caused suboptimal methane production in P3.

Variables in the Digested Slurry

The presence of CS as a co-substrate in treatment P3 significantly ($p<0.05$) increased the VFA concentration compared with the control and other treatments. The high VFA content concentration in the digested slurry was related to the high organic matter and nutrient content in the substrate of P3. This finding agrees with that of Tampio *et al.* (2019), who reported that the characteristics of substrates can influence the concentration of VFAs in digested slurries. VFAs are intermediate compounds resulting from the degradation of proteins, carbohydrates, and fats. According to Nwokolo *et al.* (2020), there are four phases in the biological decomposition of substrates during AD. The first phase is hydrolysis, in which complex biopolymer compounds such as carbohydrates, proteins, and lipids are converted into simpler compounds. The second phase is acidogenesis, in which acidogenic bacteria consume glucose, amino acids, and lipids to produce VFAs, CO_2, and H_2. The most significant VFA resulting from this phase is acetate (CH_3COOH), which functions as a substrate for methanogenic microorganisms. The third phase is acetogenesis, in which VFAs with more than two carbon atoms are metabolized to produce acetate, CO_2, and H_2. Furthermore, in the final phase (methanogenesis), the acetate (CH_3COOH) is converted into CH_4 and CO_2. The CO_2 will react with H_2 to produce CH_4, whereas CH_3CH_2OH undergoes decarboxylation to produce CH_4. Agnihotri *et al.* (2022) reported that VFA concentration is related to ammonia concentration. An increase in ammonia concentration can be balanced by an increase in VFA concentration. However, the accumulation of high VFA concentrations inhibits methane production because the liquid pH decreases to low. According to Shrestha *et al.* (2023), methanogenic bacteria produce methane by consuming VFAs, especially acetate.

The TAN concentrations of the digested slurry in all treatments are presented in Table 3. The utilization of CS as a co-substrate significantly ($p<0.05$) increased the TAN concentration compared with the control. Ammonia is a product of the decomposition of crude protein. The TAN concentrations in digested slurries of P1, P2, and P3 were higher than those of P0 (control). The increase in the TAN concentration in treatments P1, P2, and P3 agreed with the increase in the proportion of CS in the mixed substrates (Table 2). Proteins in the substrate are degraded by microorganisms to produce ammonia. Ammonia is a nitrogen source that can be consumed by microorganisms. The TAN concentration in all treatments was below the inhibition threshold. Treatment P3 had the highest TAN concentration and produced the lowest methane concentration among all treatments (Table 3). Jiang *et al.* (2019) reported that the inhibitory threshold for TAN concentration is 1.500–7.000 mg/L. TAN concentrations exceeding this threshold strongly inhibit methane production.

The utilization of CS as a co-substrate in the AD of DCM had no significant effect ($p>0.05$) on VS reduction. However, treatment P3 resulted in the highest VS reduction of all treatments (Table 2). The VS reduction value in this study was higher compared with that in previous studies by Sutaryo *et al.* (2014^a) and Sutaryo *et al.* (2022), who reported that the VS reduction in the AD of DCM using CSTR is 27%–33% and 35%–37%, respectively. According to Sutaryo *et al.* (2023), increasing the amount of nutrients in the substrate can increase the bacterial activity required for digesting organic material, thereby enhancing methane production.

The pH of treatment P3 was significantly ($p<0.05$) lower than that of treatments P0, P1, and P2. The pH values of P0, P1, and P2 were still within the recommended pH values for the AD process. However, the pH of P3 was not within the recommended pH range for methane production. According to Syaichurrozi *et al.* (2020), the recommended pH value for AD is 6.5–8.5, with an optimal pH of 6.8–7.4. During the AD process, the pH should be maintained because it is an important parameter in methane production. The other parameters that can influence pH values are VFA and ammonia concentrations. It was explained by Gerardi (2003), in which a rapid increase in the TAN concentration in the digester causes an increase in VFA, thereby causing a loss of alkalinity and a decrease in pH so that the pH value decreases even though the TAN concentration increases.

In this recent study, four combination treatments using continuous feeding digesters needed a lot of resources. Concerning obtaining an optimal comparison of organic matter CS and DCM in AD, further optimization studies using many more treatment combinations that can be carried out using a batch digester are needed to obtain the optimal point combination of the two substrates. Therefore, it is possible to use the high nutrient concentrations of CS for other purposes, including for animal feed (Idayanti *et al.,* 2024) and fat extraction for consumable oil production (Salsabila & Broto, 2023). Pretreatment in the form of germination can be applied to increase the nutrient concentration and the digestibility of CS before using it as a co-substrate with DCM in AD (Purwasih *et al.,* 2024; Abel *et al.,* 2024).

CONCLUSION

Anaerobic co-digestion of CS and DCM with the VS proportion of CS in the final substrate (51.68%) enhanced methane production by 91.94%–221.06% compared with the control (without CS addition). All digesters ran smoothly with stable methane production, TAN concentration, and pH in the normal range. However, when the VS proportion of CS in the final substrate was higher than that, this treatment caused inhibition, as indicated by a lower methane yield and pH value than in the control. The P2 treatment gave the best result, with methane production at 357.92 mL/g VS added, which corresponds to 108% higher (in terms of mL/g VS) than that in the control,

CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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