Article

Population Dynamics of Methanogenic Archea in Co-Digestion Systems Operating Different Industrial Residues for Biogas Production

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Abstract: This study aimed to analyze the population dynamics of methanogenic archaea in co-digestion systems operated under different concentrations of industrial waste such as ricotta whey and brewery waste sludge in association with bovine manure. It was believed that the association of these residues from the food industry combined with bovine manure can contribute to improve the production of biogas. To identify the archaea, DNA extractions and the sequencing of the 16s rRNA gene were performed from 38 samples of influents and effluents. The results indicated that *Methanosaeta* and *Methanosarcina* were predominant in the co-digestion of ricotta cheese whey and that *Methanosaeta*, *Methanocorpusculum*, and *Methanobrevibacter* prevailed in the co-digestion of residual brewery sludge. The three ricotta cheese whey biodigesters demonstrated efficiency in methane production; in contrast, residual sludge of brewery biodigesters only showed efficiency in the system operated with 20% co-substrate.

Keywords: biogas; methane; microbial ecology; ricotta cheese whey; residual brewery sludge brewery

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1. Introduction

Anaerobic biodigestion (AB) is an important tool for the treatment of organic waste used to generate byproducts (biogas and biofertilizer) from substrate stabilization, contributing to the production of renewable energy [1–4]. AB is a metabolic process with multiple syntrophic relationships that occur between microorganisms in the steps of hydrolysis, acidogenesis, acetogenesis, and methanogenesis under specific environmental conditions [5,6].

Methanogenesis is a crucial step to methane (CH_4) production [7], and methanogenic archaea are the key microorganisms of this step. These species are divided into three groups, according to the substrate used for their growth. Hydrogenotrophic methanogens use hydrogen (H_2) and carbon dioxide (CO_2) as substrates; acetoclastic methanogens use acetate structures; and the methylotrophic methanogens use methyl groups [8].

The identification of these microorganisms in samples of biodigesters is possible through metagenomics analysis [9]. As a technique that is independent of cultivation, metagenomics allows the identification of the microbiological diversity from a complex environmental sample [10,11]. This is possible by the investigation of the hypervariable regions (V1 to V9) of the 16s rRNA of prokaryotes.

An increase in the number of studies involving methods for improving the AB was seen in recent years [12], with emphasis on anaerobic co-digestion (AcoD) [13]. AcoD is a

simultaneous and homogeneous biodigestion of two or more complementary substrates to produce biogas rich in methane. This practice is recommended especially for recalcitrant substrates [14] as well as industrial waste.

The food industry, for example, is an expanding sector that generates pollutant byproducts that need treatment before disposal. Ricotta cheese whey (RCW) and residual brewery sludge (RSB) are examples of byproducts with high polluting potential [15].

RCW is a byproduct of the ricotta cheese manufacturing process, considered a highly polluting waste due to a large amount of lactose present in its composition [15]. This byproduct has a low nutritional value, making it difficult to reuse for food purposes [16]. Furthermore, the RCW has high values of BOD (50 g/L) and COD (80 g/L) requiring the environmentally correct measures before its disposal into the environment [15].

RSB is a byproduct generated in a brewery through wastewater treatment plants (WWTP) after the reuse of process leftovers such as spent grains, hops, and yeast. It is considered a semi-solid sludge, with a low benefit that must be disposed of in landfills [17], and its properties may vary according to the type of beer produced and the operations carried out at the WWTPs [18].

In general, the brewing industry wastes have a high organic load and, for this reason, are considered as potential polluters [19].

According to Xia, et al., 2012 [20], a specialized group of Archaeas produce methane. Methane is mainly produced from acetate by acetoclastic or from H_2/CO_2 by methanogenic hydrogenotrophic archaea. The coordinated and well-tuned interaction between microbial groups and their substrates in each of the biochemical processes is crucial for the efficiency of biogas production in anaerobic reactors [21]. The association of substrates during the AcoD promotes the system balance, increases the production of biomethane, and contributes to environmental preservation [4,22]. Therefore, both RCW and RSB are candidate substrates for AcoD.

The combination of these industrial wastes with bovine manure (BM) in AcoD may contribute to the production of biogas, awakening a new possibility for its disposal. Knowing the microorganisms present in AcoD systems, as well as their dynamics, it is important to model and design efficient and productive processes. When comparing the two co-substrates (RSB and RCW) it was possible to identify a population like the control. This population was considered a competent inoculum for methane production. Thus, the population dynamics found among archeas in co-digestion with ricotta cheese whey indicates that its presence is essential for the proper functioning of the anaerobic digestion process. Therefore, this work aimed to identify the population dynamics of methanogenic archaea during the co-digestion of industrial waste under different concentrations.

2. Materials and Methods

2.1. Pilot-Scale Biodigester

Seven biodigesters with a continuous and manual supply were used, with a total capacity of 60 L each. These biodigesters are constructed with PVC tubes and do not have an agitation system such as that described by Resende, et al., 2016 [23]. At the bottom of each biodigester, three sampling valves were installed. Each biodigester is coupled to a gasometer made of PVC tubes where the biogas produced during biodigestion is stored.

2.2. Substrates

BM and wastewater were weekly collected in the production system of the José Henrique Bruschi experimental field of Embrapa Dairy Cattle, in Coronel Pacheco, Minas Gerais, Brazil. To obtain the liquid fraction of the BM, it was diluted with wastewater and sieved manually to achieve a concentration close to 6% of total solids [23]. This material was stored at 4 °C for up to one week. Before use, each portion was removed from the refrigerator to reach room temperature (≈20 °C).

A dairy company in the city of Juiz de Fora supplied the RCW. RCW samples were collected weekly, transported to the rumen microbiology laboratory, homogenized, bottled,

and frozen at −20 °C. Before use, each sample was defrosted for 24 h in a refrigerator, and then kept at room temperature. The pH value was corrected with the addition of 59 mL of 4.2% limewater ($Ca(OH)_2$) for each liter of RCW to obtain a pH close to neutrality [24].

An industrial brewery in Juiz de Fora supplied the RSB. After receiving the RSB, it was sieved and stored in a refrigerator at 4 °C. Before use, each sample was removed from the refrigerator to reach room temperature.

2.3. Experimental Design for the Analysis of Archaea Dynamics

The experiment lasted 165 days and was divided into three phases called competent inoculum (phase 1), acclimatization (phase 2), and anaerobic co-digestion (phase 3). The graphic scheme of the sample preparation is presented in Figure S1.

During the competent inoculum phase, seven biodigesters were completely supplied with diluted BM, reaching their full capacity (60 L). Flame tests were carried out daily after the start of biogas production. The collection of biogas for chromatographic analysis was performed after positive flame tests for biogas production. The substrate remained in the biodigester until the concentration of biogas in the system reached at least 60% CH_4 [25].

Subsequently, the acclimatization phase started. In this phase, one biodigester was selected as control and continued the supply exclusively with BM. Three biodigesters started the supply with the mixtures of RCW + BM and other three with mixtures of RSB + BM (Table 1). Biodigesters were identified according to the name and concentration of the co-substrate used in AcoD. The daily supply (2 L/day) was performed according to the values of the mixtures indicated in Table S1 with the supply, the same volume of effluent (2 L) was removed, keeping the system operating at its maximum capacity.

Table 1. Characterization of supply mixtures and physicochemical characteristics.

Biodigester	Substrate Characterization	Physicochemical Characteristics	
		pH	Ammoniacal Nitrogen (mg/L NH_3-N)
BM	100% BM (Control)	6.20	134.67
RSB20	20% RSB + 80% BM	5.98	81.22
RSB40	40% RSB + 60% BM	5.89	145.04
RSB80	80% RSB + 20% BM	5.48	121.83
RCW20	20% RCW + 80% BM	6.09	133.44
RCW40	40% RCW + 60% BM	6.08	98.63
RCW80	80% RCW + 20% BM	6.01	98.63

The anaerobic co-digestion phase lasted 120 days. Daily supply continued according to the concentrations indicated in Table 1. The control biodigester worked only with BM, so the term co-digestion does not apply to it.

2.4. Analytical Techniques

Ammoniacal nitrogen (NH_3-N) and pH analysis of effluents were performed every 2 weeks. The pH meter Tec-3MP (Tecnal, Piracicaba, Brazil) was used to measure the pH values of effluents, and NH_3-N was determined according to the standard methods and methodologies [26].

The analysis of the biogas concentration was performed weekly to measure the CH_4 concentrations in biogas samples. These procedures were conducted by the Chromatography Laboratory at Embrapa using the Agilent 7820 A Chromatograph System and the EzChrom Elite interface software [27]. As indicated by Collins, et al., 1997 [27], a split-splinter type 50:1 injector used in 120 °C with a Separation System with two columns: (i) an HP-Plot/Q 30 m × 0.530 mm × 40.0 mm (ii) and an HP-Moleisieve 30 m × 0.530 mm × 25.0 mm. H_2 was carrier gas, with flow of 8.3 mL/min. The equipment used a TCD Detector under the following operating conditions: 250 °C heating; 25 mL/min flow/reference; 0.5 mL/min flow (H_2) complementary; 8.8 mL/min column

ϸ complementary constant. FID Detector with standardized conditions were as follows: 270 °C heat; 15 mL/min H2 flow; 350 mL/min air flow (synthetic); 20 mL/min complementary flow. Metanador at 375 °C heating was used. Oven temperature was regulated for 55 °C/45 min to completion. The chromatograph calibration occurs with standard gas, certified Linde™, with methane (CH_4) concentrations of 5.05; 10.2; 14.7; and 20.1. Furthermore, carbon dioxide values were (CO_2) 20.2; 39.7; 58.3l and 79.9. The methodology used for the chromatographic analysis was based on da Silva, et al., [24], with adaptations.

2.5. Collection and Conservation of Samples

During the acclimatization phase, one influent sample was collected and after the CH_4 production (15 days), the effluent samples from the BM biodigester were collected. All effluent samples were collected on sampling valves. Before the samples were collected, the sampling valves were open for some minutes to flush out the valves. Thereafter, the effluent samples for microbial analysis were collected in sterile glass bottles and immediately sealed to maintain the anaerobic condition.

During the anaerobic co-digestion phase, influent samples were collected only for the biodigesters that operated in the co-digestion system (RSB20, RSB40, RSB80, RCW20, RCW40, and RCW80). In this phase, the collection of effluent samples occurred weekly until the end of the experiment.

After each collection, the samples were frozen at −80 °C. The samples were submitted to a freeze-drying process, which was conducted by the Chromatography Laboratory using the Liotop model L120 freeze dryer (Liobras, São Carlos, Brazil). After freeze-drying, all samples were stored in Falcon 50 mL conical tubes.

2.6. Sample Selection for Molecular Microbiology Analysis

To characterize the acclimatization phase, samples of the influent and effluent from the control biodigester were selected. From the material collected in the sampling valves, sample pooling was made to obtain greater representativeness of the content of the biodigesters. Therefore, each effluent collection had a single pooled sample representative of the biodigester instead of three samples. The preparation of sample pooling of effluent samples occurs from the lyophilized material.

Seventy mg of lyophilized effluent was weighed corresponding to the material collected from the V1 sampling valve and the same volume for the effluent of V2 and V3 valves. Then this material was homogenized and transferred to a microtube, representing a pooled sample with 210 mg of volume. The graphic scheme of the sample preparation is presented as a Figure S2.

For a better representation of this phase, the selection of at least one collection/month for each biodigester was prioritized. The effluent samples corresponding to the collections of days 1, 36, 78, 99, and 120 of the anaerobic co-digestion phase were chosen as representatives for molecular analysis. Sampling pooling of the selected effluent samples was also performed.

The characterization of the anaerobic co-digestion influents was also made through a lyophilized sample pooling. To characterize the RCW influents, were weighed 70 mg of the 20% RCW sample, 70 mg of the 40% RCW sample, and the same volume for the sample with 80% of RCW. Then this material was homogenized and transferred to a microtube, representing a pooled sample with 210 mg volume. The same procedure was carried out for RSB influents.

2.7. DNA Extraction

A pre-preparation of samples was made using enzyme lysis buffer (500 mM NaCl; 50 mM Tris-HCl, pH 8; 50 mM EDTA; 4% SDS) in association with mechanical lysis using the "Mini-Bead Beater 16" (BioSpec Products®, Bartlesville, OK, USA) with zirconia beads (0.1 mm and 0.5 mm) [28,29]. Subsequently, the samples were submitted to the QIAamp DNA stool mini kit protocol (QIAGEN, Heidelberg, Germany), following the manufacturer's

recommendations. The determination of concentration and purity of DNA was performed in NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Metagenomic Analysis—Sequencing and Bioinformatics

The extracted DNA was sent to a service provider company responsible for executing the sequencing and bioinformatics steps. For amplification of the polymorphic region (V4) of the 16S rRNA gene, PCR was conducted in triplicate using the following oligonucleotides: 515 F—'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA' and 806 R—'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT' [30,31]. The bioinformatics analysis was performed on the QIIME2 platform, version 2019.7 [32]. The sequences were filtered by quality and grouped into Operational Taxonomic Units (OTUs) using 97% of the identity between them. The sequences were also compared with the Silva 132 database [33] for taxonomic analysis. An OTU table selected by genus is presented as a Table S1.

2.9. Statistical Analysis

To investigate an association between the performance of the biodigesters and the composition of the archaeal microbiome, a principal component analysis (PCA) was performed. The PCA tried to identify if the selected physicochemical variables (concentration of CH_4 and NH_3-N, and pH value) could justify the variation in the occurrence of OTUs in the biodigesters. This analysis was conducted on the statistical software JMP 14.0 (SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1. Archaea Acclimatization

To analyze the structure of the archaeal community, the taxonomic classification of sequences was performed to the genus level. In the influent samples, the most abundant genus was *Methanobrevibacter* (Figure 1), showing a high relative abundance for the three samples. The relative frequency of *Methanobrevibacter* in the influent samples of BM (Figure 1A), RCW (Figure 1C), and RSB (Figure 1D) were 94%, 95%, and 92%, respectively.

The abundance of *Methanobrevibacter* in the BM supply samples is in accordance with previous studies that mention it as the most profuse hydrogenotrophic methanogen in the bovine ruminal system [34–36]. Therefore, the high frequency of *Methanobrevibacter* in influent samples of RSB and RCW may be justified due to it being prepared with mixtures containing high amounts of bovine manure.

Senés-Guerrero, et al., 2019 [37], compared the abundance of this genus in rumen and biodigester samples, highlighting the taxon predominance in the rumen sample and its subsequent disappearance in the biodigester. Similarly, after 15 days of HRT from the acclimatization phase, the analysis of the BM effluent (Figure 1B) had a reduction in the *Methanobrevibacter* frequency, from 94% in the initial sample to 3% in the digestate.

The reduction of this genus was followed by an increase in *Methanosarcina*, *Methanocorpusculum*, and *Candidatus Methanoregula* abundance. In influent samples, the genera *Methanosarcina* and *Methanocorpusculum* showed a relative abundance of 1% and 0.5%, respectively, while the genus *Candidatus Methanoregula* was not identified. After 15 days of HRT, the relative abundance of the genera *Methanosarcina*, *Methanocorpusculum*, and *Candidatus Methanoregula* increased to 64%, 25%, and 5.5%, respectively.

It was considered that the hydrogenotrophic pathway was predominant during the acclimatization phase. This is because the increase in the abundance of the facultative acetoclastic methanogen, *Methanosarcina* [38,39], occurred concomitantly with the increase of two exclusively hydrogenotrophic genera, *Methanocorpusculum*, and *Candidatus Methanoregula* [40,41]. According to Venkiteshwaran, et al., 2017 [39], the *Methanosarcina* genus has less affinity for acetate, reinforcing the idea that the hydrogenotrophic pathway was the propellant of methanogens during the acclimatization phase. Furthermore, previous stud-

ies suggest that the hydrogenotrophic pathway is the main route for methane production from a biomass with bovine manure [42].

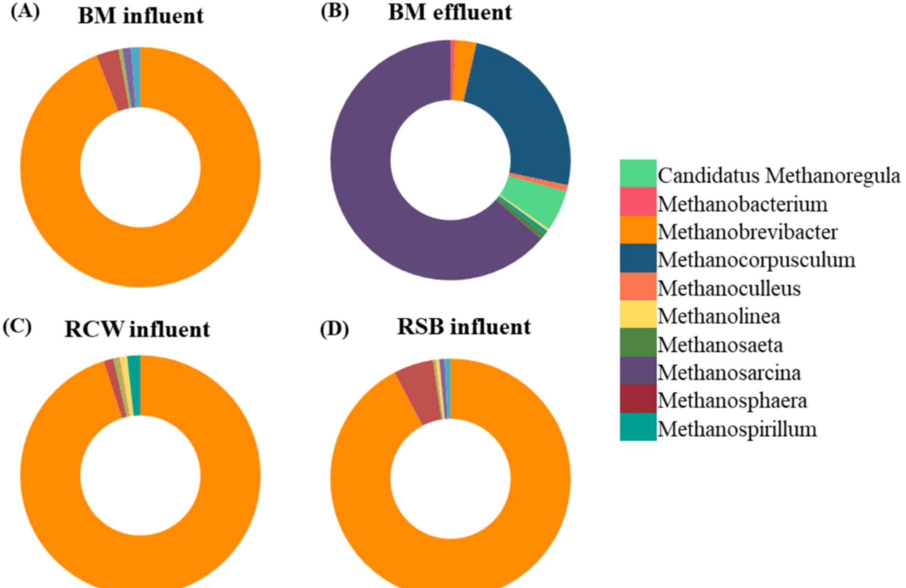

Figure 1. Phylogenetic distribution of genera presents in the competent inoculum and acclimatization phases. (**A**) Influent of the control biodigester. (**B**) Effluent from control biodigester after 15 days of retention. (**C**) Pool of influent samples of RCW biodigesters. (**D**) Pool of influent samples of RSB biodigesters.

3.2. Physicochemical Analysis of Effluents from the Anaerobic Co-Digestion Phase

The methane concentration (% v/v CH_4) in biogas samples remained above 54% during the experiment, except for the RSB40 biodigester that declined the methane concentration from the 36th day of anaerobic co-digestion, reaching a concentration of 40% methane. The RSB80 biodigester declined from the start process of anaerobic co-digestion and stopped biogas production on the 99th day (Figure 2A).

The CH_4 average concentration in BM biodigester was 58% (±1.66). These results are in accordance with authors who point out a variation between 55% and 70% in the CH_4 concentration for biogas samples generated from the AB of bovine manure [23,25,43]. According to Enitan, et al., 2014 [44], and Chen, et al., 2016 [45], the methane concentration in biogas samples of the brewery residues of biodigestion can vary from 59% to 69% CH_4. The RSB20 biodigester was the only one that showed results within this threshold with a CH_4 average production of 59% (±2.46). The RSB40 and RSB80 biodigesters showed an average concentration of 54% (±11.08) and 30% (±20.21), respectively. In biogas produced by biodigesters co-digested with RCW, this average was 58% (±2.96) for RCW20, 57% (±2.65) for RCW40 and 57% (±3.99) for RCW80. These results are as expected once the average value can vary between 50% and 65% of CH_4 in the cheese whey biodigestion [46–48].

The pH did not show high variations and remained close to neutrality in all biodigesters, presenting an overall average of 7.1 (±0.24) (Figure 2B). According to Lemmer et al., 2017 [49], it is possible to achieve the optimal methane yield keeping the pH value in the system between 6.8 and 7.2. From this result, it is possible to infer that the biodigesters showed favorable conditions for the survival of methanogenic archaea [50].

It is possible to observe in Figure 2C a gradual increase in the NH_3-N concentration during the biodigestion of BM. The BM biodigester showed a minimum value of 128 and a maximum of 736 mg/L of NH_3-N, with an average of 493 mg/L of NH_3-N (±226). De Mendonça, et al., 2017 [25], also reported this progressive increase in the concentration of NH_3-N during the bovine manure biodigestion in a full-scale biodigester. The RCW biodigesters showed close values for the ammoniacal nitrogen concentration of the digested.

The averages of the ammoniacal nitrogen concentrations were 460 mg/L NH_3-N (±78) in RCW20, 394 mg/L NH_3-N (±58) in RCW40, and 315 mg/L NH_3-N (±85) in RCW80. The results differ from those reported by Comino, et al., 2012 [46], during the co-digestion of cattle slurry and cheese whey, in which values over 800 mg/L of NH_3-N were found. This may have a relation to the type of cheese whey used and to the addition of limewater to stabilize the RCW pH [24]. The slight increase of NH_3-N in BM and RCW biodigesters did not cause toxicity to the system since the increase of this compound did not affect methane production. The RSB biodigesters showed a high concentration of ammoniacal nitrogen (>700 mg/L NH_3-N) from the first digested collection. The average concentration of RSB20 was 949 mg/L NH_3-N (±116).

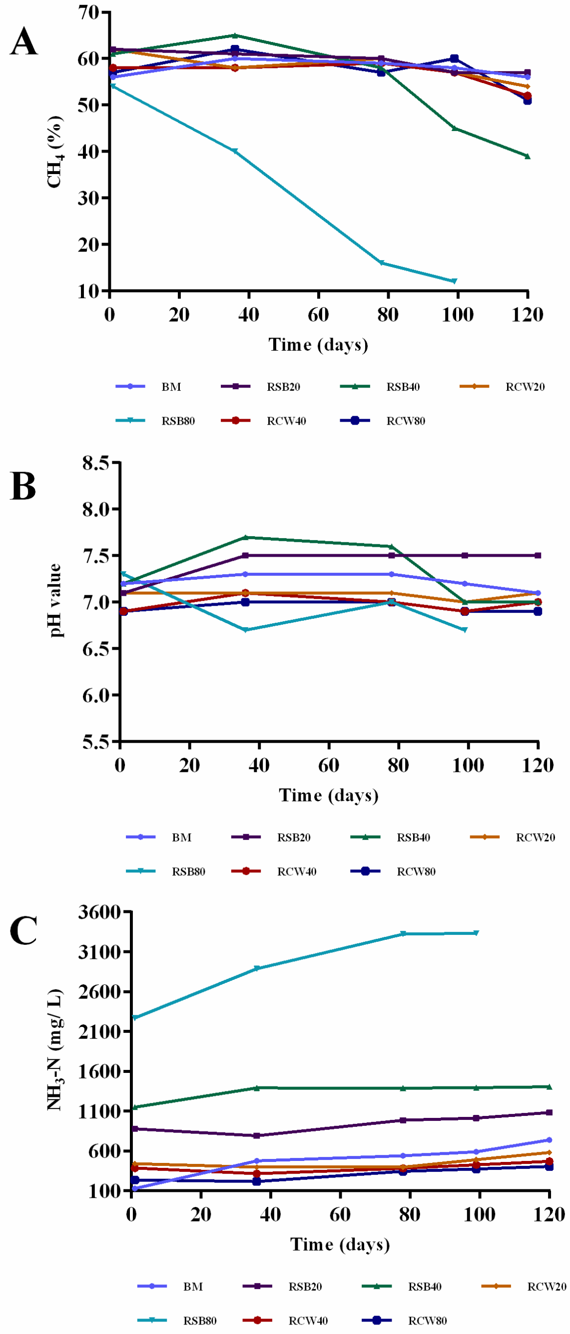

Figure 2. Results of physicochemical analyzes in AcoD phase. (**A**) Methane concentration (% v/v CH_4) in biogas. (**B**) pH values of effluents. (**C**) Ammoniacal nitrogen concentration (mg/L NH_3-N) in the effluents.

The RSB40 and RSB80 biodigesters showed the highest concentrations of ammonia nitrogen, with average values of 1331 mg/L NH_3-N (±121) and 2952 mg/L NH_3-N (±501), respectively. It is possible that this considerable increase in the ammoniacal nitrogen concentration is related to the decline in the methane concentration of these biodigesters.

3.3. Archaea Identification during Anaerobic Co-Digestion

The improvement in the generation of biogas rich in methane is the central point of AcoD processes, in which an appropriate selection of co-substrate and mixing ratio is necessary [51]. Inappropriate choices can lead to an imbalance in the system, limiting the methane generation. The results showed in Figure 3 reveal the dynamics of methanogenic microbiota in different biodigesters during the anaerobic co-digestion phase. A high abundance of *Methanosarcina* genus is also observed in the BM samples during the initial anaerobic co-digestion phase with a relative abundance of 76% on the first day (Figure 3A). However, there is a notable reduction in the frequency of this genus during biodigestion, from 62% on the 36th day to 14% on the 120th day. Simultaneously, there is a considerable increase of *Methanosaeta* from 6% on the 36th day to 42% on the 99th day, which registered its highest frequency.

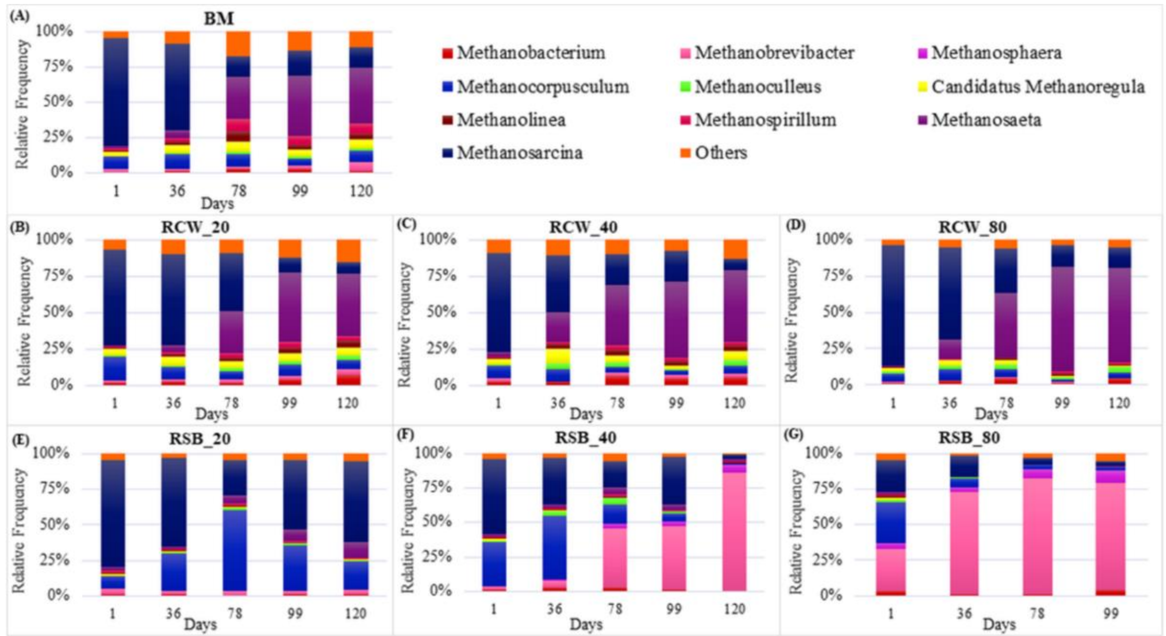

Figure 3. Distribution and relative abundance of OTUs in effluent samples of the AcoD phase. (**A**) Biodigester control supplied with bovine manure. (**B**) Biodigester supplied with 20% ricotta cheese whey. (**C**) Biodigester supplied with 40% ricotta cheese whey. (**D**) Biodigester supplied with 80% ricotta cheese whey. (**E**) Biodigester supplied with 20% brewery sludge. (**F**) Biodigester supplied with 40% brewery sludge. (**G**) Biodigester supplied with 80% brewery sludge.

Methanosarcina and *Methanosaeta* are responsible for producing a large part of the CH_4 generated during anaerobic digestion [8]. Individuals of the *Methanosaeta* genus are mandatory acetoclastic methanogens, able to contribute to more than 60% of CH_4 generated through the oxidation of acetic acid [52–54]. This variation in the prevalence of *Methanosarcina* is consistent with the results of Song, et al., 2015 [55], observing the co-digestion of a pretreated wheat straw with cattle manure, which shows a higher frequency of this group in the initial period of biodigestion and its subsequent decline. The expressive increase in the relative abundance of *Methanosaeta* may be indicative of its affinity for acetate, suggesting the acetoclastic pathway as the propulsive pathway of BM methanogenesis. However, with the increase of *Methanosaeta*, there is a slow increase of some hydrogenotrophic genera (*Candidatus Methanoregula*, *Methanocorpusculum*, and *Methanospir-*

illum). The increase of these genera, concomitant with the increase of *Methanosaeta*, may be evidence of the coexistence of hydrogenotrophic and acetoclastic pathways [54]. During the analysis of wetland samples, Zhang, et al., 2019 [54], found evidence that these two methanogenic pathways can coexist although one pathway may be more significant for methane production than the other one.

In this case, it was not possible to stipulate what was the exact propulsive pathway for methanogenesis from the 78th day on the BM biodigester, although it is believed that acetogenesis may have prevailed, based on the high abundance of the *Methanosaeta* genus. It was also noted that the RCW archaeal microbiota was quite similar to that found in the BM biodigester (Figure 3A–C). The relative abundance of *Methanosarcina* on the first day of anaerobic co-digestion phase was 66% for RCW20, 66% for RCW40, and 83% for RCW80.

As in the BM biodigester, the RCW20 and RCW40 biodigesters presented an increase in the relative abundance of *Methanosaeta* along with a slight increase of hydrogenotrophic methanogens during co-digestion. This coexistence between acetoclastic and hydrogenotrophic methanogens again raises the question about which pathway promotes the methanogenesis in RCW20 (Figure 3B) and RCW40 (Figure 3C) co-digestion. Differently, the RCW80 (Figure 3D) biodigester did not show a considerable increase in hydrogenotrophic methanogens. However, the genus *Methanosaeta* was dominant during anaerobic co-digestion, reaching 66% relative abundance.

Chen, et al., 2015 [56], demonstrated the prevalence of *Methanosaeta* over *Methanosarcina* in mesophilic biodigesters supplied with dairy residues under high organic loading rates. Saha, et al., 2019 [57], described similar results during the digestion of sludge obtained in a WWTP and augmented with a mixed waste of fruit, where the prevalence of *Methanosaeta* over *Methanosarcina* was observed. However, Chen, et al., 2017 [58], demonstrated that the prevalence of *Methanosaeta* can occur due to the competitiveness of this genus under high acetate concentrations.

Based on the results, it is assumed that acetoclastic methanogenesis prevailed during the AcoD of ricotta cheese whey due to the abundance of acetoclastic over methanogenic genera. A deep study of the bacterial metagenome that was found in this study may provide us with a more effective answer on this issue. The biodigesters that used RSB as AcoD substrate differed from each other and the BM biodigester. *Methanosarcina* and *Methanocorpusculum* were the main representatives of the archaeal community in RSB20 (Figure 3E), indicating that these two genera led the methane production. The co-occurrence of these two genera suggests that the biodigester RSB20 followed the hydrogenotrophic pathway for methane production.

A noticeable change in the dynamics of archaeal microbiota was observed in the RSB40 biodigester (Figure 3F). The beginning of RSB40 AcoD is dominated by members of *Methanosarcina* and *Methanocorpusculum* genera with 55% and 32% of relative abundance respectively. Subsequently, there was a reduction in the abundance of these two genera and prevalence of *Methanobrevibacter* genus. *Methanobrevibacter* was also abundant in the RSB80 biodigester (Figure 3G). Associated with the increase of this taxon, it is also possible to observe a slight increase of *Methanosphaera* genus, a hydrogenotrophic methanogen equally abundant in the gastrointestinal tract of ruminants [35,59]. Principal component analysis (Figure 4) revealed that these two genera, *Methanobrevibacter* and *Methanosphaera*, are strongly related to the increase of the NH_3-N concentration.

Although ammoniacal nitrogen is crucial to microbial growth when in high concentration, it is considered an inhibitor of the system [60]. The NH_3-N increase is important evidence to justify the discrepant occurrence of *Methanobrevibacter* in RSB40 and RSB80 samples, followed by a reduction in the relative abundance of the other genera. High concentrations of this compound have been associated with *Methanobrevibacter* survival in previous studies [61,62].

Nevertheless, our results support those of Nguyen, et al., 2019 [8], who highlighted acetoclastic methanogens, the main responsible for methane production, as vulnerable to NH_3-N increase. The predominance of hydrogenotrophic methanogen under high

NH_3-N concentration may be considered as a response to the change from acetoclastic methanogenesis to hydrogenotrophic methanogenesis [63].

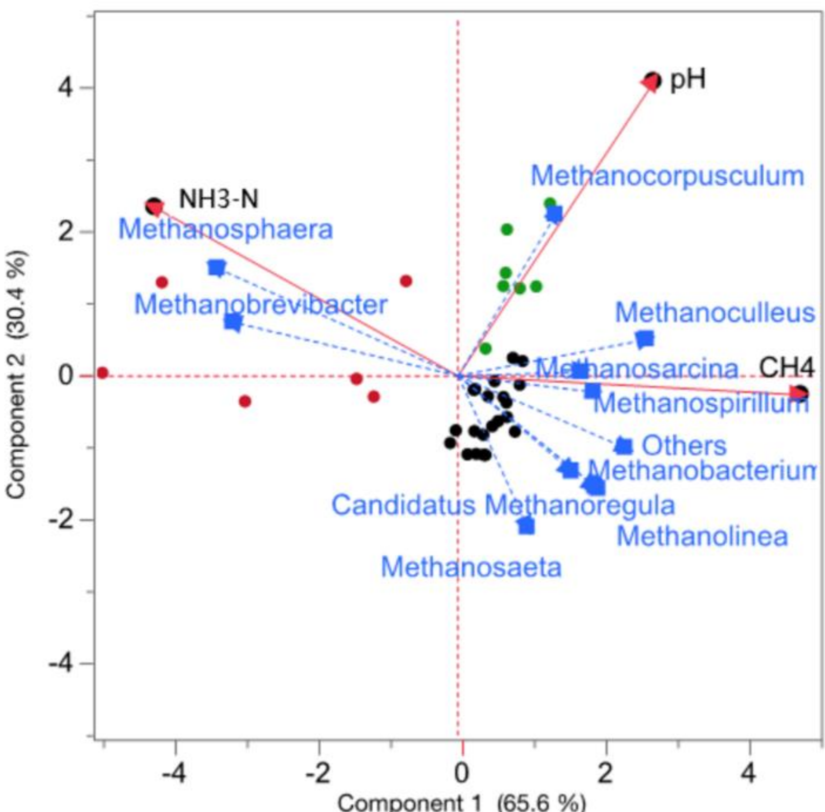

Figure 4. Association between the physicochemical variables and the relative abundance of OTUs, classified at the genus level. The physicochemical variables included in the PCA plot are the values of CH_4 (% *v*/*v*), NH_3-N concentration (mg/L), and pH.

Probably, the interruption in the production of biogas by the RSB80 biodigester before the end of the experiment is due to the increase of NH_3-N in the system, once high ammoniacal nitrogen values can also prevent the growth of microbiota, affecting the AcoD process [64,65]. The PCA (Figure 4) also revealed that the genus *Methanocorpusculum* has a connection with the pH value. Zhou, et al., 2016 [66], point *Methanocospusculum* as the main genus in laboratory-scale mesophilic reactors adjusted to pH 7. However, despite the PCA result, our study does not provide enough evidence to prove a relationship between the occurrence of *Methanocospusculum* and the pH variation. A heatmap (Figure 5) was created for a better visualization of the genera that dominated each biodigester. Therefore, it was observed that BM and RCW biodigestion is dominated by the genera *Methanosarcina* and *Methanosaeta*. RSB biodigestion, on the other hand, presents the genera *Methanosarcina*, *Methanocorpusculum*, and *Methanobrevibacter* as the propellants of methanogenesis. An OTU table selected by genus is presented in Table S1.

Dhungana, et al., 2022 [67], studied the anaerobic co-digestion process, seeking to optimize to increase the recovery of resources from organic waste. The results indicate that the use of an optimized mixture of co-substrates together with the summer starter can remarkably improve energy generation; these results corroborate what we also have in our findings, where the co-substrate, in the case of RCW in the mixture was beneficial in the stability and productivity of the AcoD bioprocess.

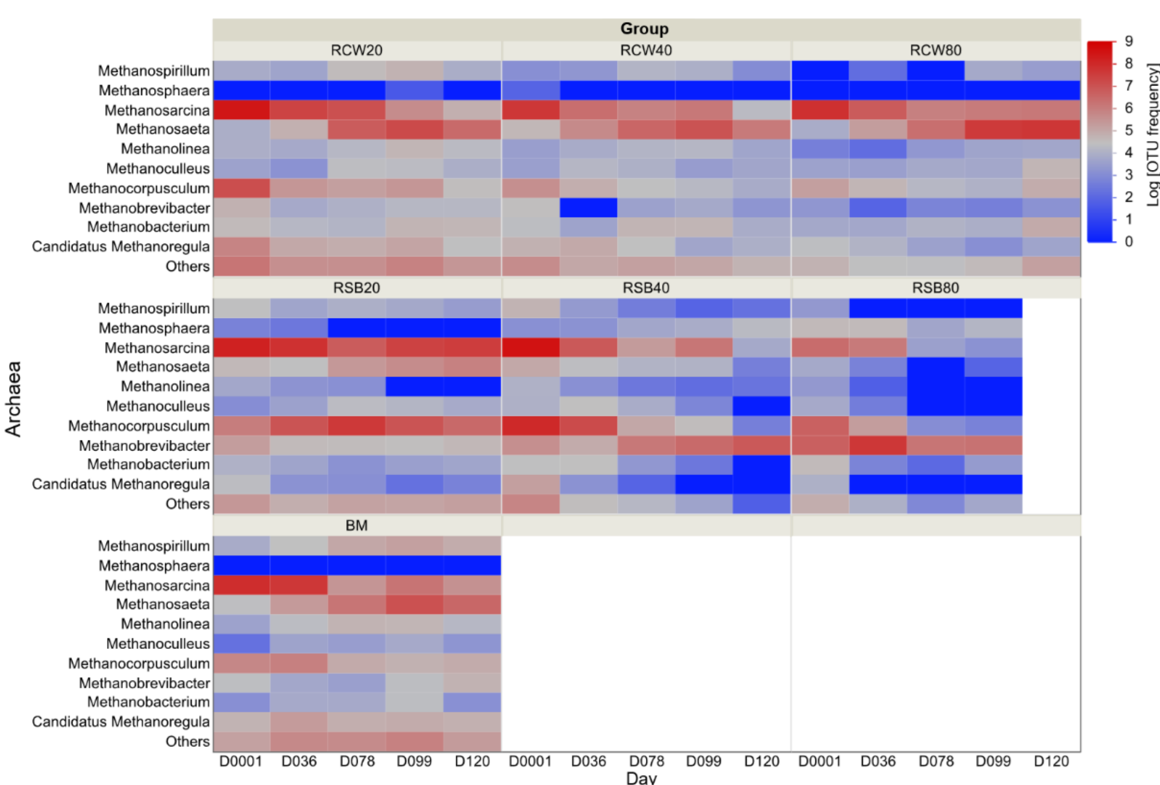

Figure 5. Heatmap of genera and relative abundance (log scale) of archaea in different treatments. Red colors indicate higher abundance and blue colors lower abundance.

4. Conclusions

It was observed that biodigestion of bovine manure may assume the acetoclastic and hydrogenotrophic pathway and sometimes this path can coexist. The three treatments containing RCW showed good results in the production of CH4, proving its efficiency in mixtures containing up to 80% of it. Methanosarcina and Methanosaeta genera are the most abundant in the RCW systems. The AcoD of the RSB is mostly conducted by hydrogenotrophic archaea, especially the Methanocorpusculum and Methanobrevibacter genera, and the facultative Methanosarcina, indicating that the hydrogenotrophic pathway is dominated the CH4 production. The RCW biodigesters have shown better results than the RSB biodigesters. The biodigester that worked with 20% RSB showed a satisfactory result, indicating a possibility in the use of small proportions of this residue in the AcoD process. E-supplementary data of this work can be found in the online version of the paper.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su141811536/s1, Figure S1: Experimental design of the project. Biodigesters named according to the co-substrate concentration used; Figure S2: Scheme of sample pooling preparation for DNA extraction. (A) Pooling of lyophilized effluents from 70 mg of the sample collected in each biodigester valve (V1, V2, and V3). (B) Pooling of lyophilized influents from 70 mg of each supply mixture sample (20%, 40%, and 80%). Table S1: OTU table classified by genus level presenting the most abundant genera in influent and effluent samples. The column classified as "Others" contains OTUs that were not possible to be classified by gender or those with small numbers of representatives.

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