Metagenomic Analysis during Co-Digestion Buffalo Sludge and Tomato Pomace Post Thermal Stress: A Case Study

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Abstract: The tomato industry and buffalo farming generate waste, including sludge (BS) and tomato pomace (TP), which can significantly impact their economic and environmental sustainability. The case study tracked changes in microflora composition after a thermal shock during anaerobic co-digestion. The inoculum-to-substrate ratio was 0.5 based on volatile solid content under mesophilic conditions. An Automatic Methane Potential Test System was used to monitor the process before and after thermal stress (50°C) occurred for three days. Next-generation sequencing analyzed the bacterial and archaeal communities. The pH decreased, and methane production plateaued due to the high volatile solid content (87 g/L). After thermal stress, the pH returned to neutral, and the batch resumed biogas production. The cumulative CH_4 production reached 3,115 Nml. The biogas had a maximum methane peak of 78.5% compared to 58.4% in BS. The taxonomic classification showed that Firmicutes (51.7%) and Bacteroidetes (29.9%) represented 81.6% of the total OTUs among the bacteria. Fonticella, the most abundant Clostridiaceae (average 4.3%), was absent in BS and increased (up to 17.1%) in TP during methane production. Methanocorpusculum was the most abundant in the archaeal community. However, Metanosarcina showed a stronger correlation with methane production. Brief thermal stress significantly altered bacterial and archaeal populations and allowed to resume biogas production.

Keywords: Biogas, agricultural by-products, anaerobic digestion, next generation sequencing.

1. INTRODUCTION

The accumulation of industrial process by-products and their disposal is becoming a problem of increasing economic and environmental burden [1,2]. Huge amounts of waste are generated yearly from the agri-food chain in all stages of production, either at the farm level or in food processing or distribution.

Nevertheless, food waste represents an inexpensive, renewable, and abundant feedstock for the sustainable production of a broad range of products (e.g., biochemistry, biomaterials, energy), which is playing an increasingly significant role.

Italy is the first producer of processed tomatoes in the Mediterranean Area and the third-largest producer worldwide [3]. According to data provided by FAO [4], in 2022, tomato production was 186.1 Mil T, with a cultivated area of 5.2 Mil ha, showing since 2011 a 15.5% increase in total production and 11.2% in cultivated area, respectively. Solid residues derive from both tomato production and process (7.0 - 7.5% of the total residue mass) [1,3], and tomato pomace constitutes the majority of it, around 2-5% of the total [4,5]. The management of tomato-processing residues (mainly peels) raises several concerns about the environmental impact and the economic cost. Accumulating high quantities may generate phenomena of uncontrolled anaerobic fermentation, while if not properly disposed of (i.e., left directly on the soil), it may cause liquid emissions and soil and odor problems. Moreover, landfilling represents a not negligible cost for the processing industry [5,6]. Bacenetti *et al*. [1] showed that the use of tomato by-products in anaerobic digestion gave a moderate, non-negligible reduction of the environmental load of tomato purée.

The wet pomace contains the following mass fractions: 33% seed, 27% peel, and 40% pulp. The fiber is the TP's major component on a dry matter basis (25.4 - 50.0%), followed by total protein (15.4% - 23.7%), total fat (5.4% - 20.5%, and mineral content (4.4% - 6.8%) [7,8]. TP has been proposed by many authors as a favorable substrate for AD due to its high content of fiber and volatile solids, up to 97% of total solids, and its slightly acidic pH (4.5). Within the range of fluctuations that slurry can withstand for its intrinsic

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alkalinity, the acidity enhances the early stages of hydrolysis and fiber degradation [5,9].

Non-woody biomass, such as animal manure, has a low lignin content compared to woody biomass sources and is classified as a waste material.

However, manure produced in intensive systems is highly concentrated in some regions and exceeds the needs of agricultural land. In this sense, excessive accumulation of organic waste, especially animal manure, causes land, water, and air pollution. Moreover, animal manure is a source of greenhouse gas emissions such as methane (CH_4) and nitrous oxide (N_2O).

One of the main issues of buffalo farms is manure management; an adult buffalo produces from 4 to 6 tons of wet manure per year, mainly used as fertilizer. The sustainability of this approach can be typically evaluated by referring to dairy cattle manure management. However, Faugno *et al*. [10] showed that the nutrient content of buffalo manure differs significantly from that of cattle manure concerning the amount of nitrogen, which makes buffalo manure less profitable as fertilizer, especially considering the European nitrate directive (Council Directive 91/676/EEC).

Anaerobic digestion for biogas production (methane) has been proven to be an efficient and green technology for disposing of sewage sludge, crop residues, food waste, and animal manure [11,12]. It has a whole range of benefits that include: the production of heat and electricity, high-quality fertilizer as digestate, and improvement of hygienic conditions through the reduction of pathogens [13].

Methane production from AD principally depends on pH, feedstock characteristics, and process temperature, which influence process stability.

There are limited papers on buffalo slurry in AD, which has by-products from the food industry that produce biogas. This paper reports a case study, part of a wider trial, on the co-digestion of buffalo sludge and tomato pomace for methane production [14,15]. During the experiments, after a temperature rise lasting for three days, it was observed that one of the batches resumed biogas production, reaching a cumulated high output.

The rationale of this study was to characterize the microbial population through next-generation sequencing techniques (NGS) before and after the thermal stress (50°C for three days).

2. MATERIAL AND METHODS

2.1. Collection of Substrates and Inoculum

A commercial variety of tomato (San Marzano cultivar type) was used. The fruits were produced by farms in the province of Latina that sell the product on local markets. One hundred fifty kilos of the product harvested at the beginning of August were washed and used to extract tomato pomace (TP). The TP was separated from the pulp by an electric tomato squeezer (Bialetti, Italy). The sauce was discarded, and the TP was stored at − 20°C before use [16]. A total of 6.8 kg of TP was obtained. For the methanation tests at 0.5 inoculum/substrate ratio, approximately 500 g of TP were taken from the frozen product and subdivided into three replications. Buffalo slurry (BS), a mixture of dung and urine from lactating buffalo cows (*Bubalus bubalis*), was collected at the CREA experimental farm in Monterotondo, Rome (Tor Mancina, 42° 05′ 26.0″ N12° 36′ 44.7″ E). Twenty-five liters of sludge samples were taken in July from the tank after a prolonged stirring (30 minutes). The BS was sieved through a 16 mm filter and stored at room temperature for 3 weeks prior to use.

Samples were collected at the beginning of the study and periodically during the trial for chemical analysis and microbial quantification by NGS.

2.2. Anaerobic Digestion Batch Tests

Automatic Methane Potential Test System (AMPTS) glass reactors (0.5 L) were fed with 0.4 L of TP and BS in an inoculum/substrate ratio of 0.5, based on volatile solid (VS), to a final content of 87 g SV/L. BS alone was used as control. The batches were flushed with dinitrogen gas (N_2) for 30'' to obtain anaerobiosis. The experiment was carried out at mesophilic conditions (39°C). The treatments were in triplicate.

AMPTS measures cumulated biogas production in NL (normal liter, 0°C, 1 atm) CH_4 day^{-1}. The study ended after 50 days when the gas production in the reactors was less than 10 mL per day. For an accidental electric break after 17 days of fermentation, the temperature rose to 50°C for three days during a weekend. In one single reactor of TP, fermentation restarted after 25 days. The behavior of this reactor was analyzed in detail according to the determinations described below. The rationale of the present study is represented in Figure **1**.

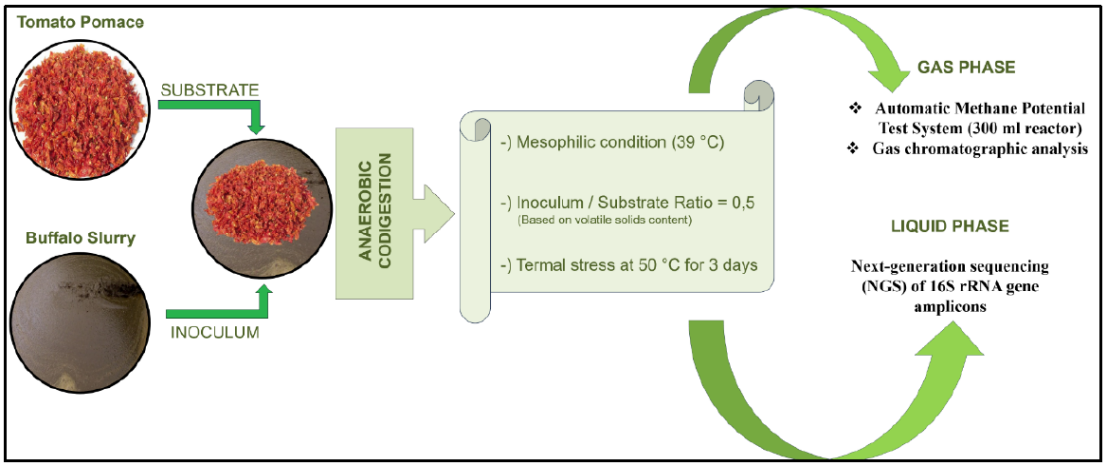

Figure 1: Graphical abstract.

2.3. Chemical Characterization of Feedstock

Inoculum and substrate were analyzed for total (TS) and VS at the start and end of the experimental period, according to the method 2090 APAT standard [17].

The fiber components (NDF and ADF, cellulose, hemicellulose, and lignin) were analyzed according to Van Soest *et al*. [18] modified according to Martillotti *et al*. [19]. The pH was recorded at the beginning of the experiment, 2 times a week, and at the end with a pH meter (GLP 21, Crison, Spain).

One ml of substrate samples was added with 50% of 0.1 N sulphuric acid, centrifuged at 14.000 rpm, 4°C per 10 minutes, 3 times, and was used to determine both the ammonia nitrogen (N-NH_3) and total nitrogen. In the ammonia nitrogen measurement, the supernatant was diluted with demineralized water to a final volume of 40 mL and then quantified using method 4030 [17]. Total nitrogen was determined using method 4060 on the APAT standard [17].

The Kjeldahl method was used to determine total nitrogen on TP since quantification is performed on solids (% DM).

The phosphorus as an orthophosphate (PO_4^{3-}) was determined in buffalo slurry using method 4110, which is based on the APAT standard [18].

All the determinations were performed in triplicate.

2.4. Biogas and Volatile Fatty Acids (VFAs) Measurements

The composition of biogas was analyzed using gas chromatography (MicroGC, GCX MPX, Pollution, Italy) with two channels. The first channel used argon as carrier gas and a Molsieve column (L 3 m; i.d. 320 µm; film 30 µm) to detect H_2, O_2, N_2, and CH_4. The second channel used helium as carrier gas and a PLOTQ column (L 8 m; i.d. 320 µm; film 10 µm) to detect CO_2 and H_2O. The analysis was conducted at isothermal conditions of 47°C and 50°C for the first and second channels, respectively. A thermal conductivity detector was used.

The HPLC analysis of VFAs was conducted using an Aminex 85 HPX-87H column on isotherm conditions at 40°C with a flow rate of 0.6 mL/min and isocratic 0.008 N H_2SO_4. The Shimadzu system (Japan) was used for the analysis, and a UV detector at 220 nm was employed.

2.5. Microbial Analysis

Effuent's samples were collected when biogas production was detected by AMPTS, immediately frozen and stored at -20°C prior to DNA extraction. Frozen samples were thawed and dispersed, and cells were concentrated by centrifugation at 14,000 rpm for 5 minutes and resuspended in 400 µl PBS (Sigma, USA). DNA was extracted using the Tissue DNA Purification Kit (Promega, USA) on a Maxwell16® instrument (Promega, USA) according to the manufacturer's instructions. DNA quality and quantity were determined using Nano Photometer TM Pearl (Implen GmbH, Germany) and Quantity-one fluorimeter (Promega, USA). The 16S rRNA gene amplicons were generated with the U341F (CCTAYGGGRBGCASCAG) and U806R (GGACTACNNGGGTATCTAAT) primers targeting the hypervariable V3–V4 regions for both bacteria and archaea. Amplicons from individual samples were pooled at equal molar ratios and purified.

About 100 ng of each pool was used to construct sequencing libraries using the FastQC v0.11.9 (Simon Andrews. FastQC A Quality Control tool for High Throughput Sequence Data. URL: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)

The sequencing libraries were pooled, and size selected by preparative gel electrophoresis and directly sequenced at 300 bp paired-end reads by using the Illumina MiSeq V3 sequencer (Illumina, USA) for 5 million read pairs.

2.6. Metagenomic Data Analysis

Samples were processed and analyzed with the following procedure: pre-processing and operational taxonomic units (OUT) picking from amplicons with Mothur 1.35.1, alignment against the 16S Mothur-Silva SEED r119 reference alignment, filtering of short alignments; sequencing error reduction by pre-clustering. Chimeras' sequences were removed using the Uchime algorithm, and taxonomical classification was performed against the Silva reference classification. OTUs were picked at a 97% identity level using the average neighbour method. OTU diversity was conducted using the Quantitative Insights Into Microbial Ecology (QIIME v1.9.0) open-source pipeline [20].

2.7. Statistical Analysis

As stated in the Introduction and described in Material and Methods, this case study has been focused on one single reactor TP and three reactors BS. Thus, considering the peculiarity of the investigation, a statistical analysis was not possible.

3. RESULTS AND DISCUSSION

3.1. Chemical Analysis of Feedstock

Feedstock chemical analysis is reported in Table **1**. The composition varied in terms of solids content and fibre composition. As reported by different authors, fibre is the main component of tomato by-products up to 50% DM or more [6,21]. ADL was higher in BS, but NDF, ADF and especially cellulose were higher in TP.

The AD process is influenced by the raw material's cellular components. This is because the cellular structure includes the outer cell wall, which contains molecules such as lignin and cellulose. Triolo *et al*. [22] observed a negative correlation between lignin content and Biochemical Methane Potential (BMP) when comparing different organic substrates. The correlation value was higher for animal manure than energy crop residues because plant cell walls partially break during animal digestion, facilitating the action of bacterial populations. Also, in this case, the different compositions of substrates (TP and BS) affected the anaerobic process.

Table 1: Chemical, Physical Composition of Feedstocks

	BS	TP
TS	37.6 g L^{-1}	103.5 g Kg^{-1}
VS	25.54 g L^{-1}	97.81 g Kg^{-1}
pH	8.15	4.42
$N\text{-}NH_3$ (mg/L)	976.6 ± 0.01	3.1± 0.14
PO_4 (mg/L)	104.38 ±5.1	ND
Fibre content (% DM):		
NDF with ashes	40.92 ± 0.6	46.32 ± 0.11
NDF pure	35.67 ± 0.5	44.96 ± 0.4
ADF	29.67 ± 0.2	36.99 ± 0.4
Hemicellulose	5.94 ± 0.2	7.78 ± 0.4
ADL	13.95 ± 0.4	11.50 ± 0.3
Cellulose	8.72 ± 0.5	25.26 ± 0.5

BS: buffalo sludge; TP: tomato pomace; TS:Total solid; VS:Volatile Solid; DM:Dry Matter; NDF: Neutral Detergent Fiber; ADF:Acid Detergent Fiber; ADL: Acid Detergent Lignin.

3.2. Effect of Temperature

Methanogens, the group of microorganisms responsible for methane production, are thought to be sensitive to temperature change, and it has already been proven that the communities performing methanogenesis under mesophilic and thermophilic conditions are different [23].

The thermophilic condition, even for a short period, allowed the resumption of biogas production when a high concentration of VS was fermented (60% of the initial concentration). This result is consistent with the literature. Kabaivanova *et al*. [24] showed that the process at 55 °C tolerated a higher substrate load, up to 45 g/L, almost half of the TP in our study.

The increase in temperature proved beneficial to ferment high organic loadings, potentially allowing the recycling of more tomato waste with practical and environmental benefits. However, the correct balance between high TP recovery and increased energy costs of the process should also be taken into consideration.

Various authors report that at mesophilic conditions, the AD of lignocellulosic wastes occurs at low rates and

Table 2: pH, CH_4 % in Biogas (Methane Peak), TOT VFA, N-NH_3 Determination in Tomato Pomace and Buffalo Sludge at the Start and the End of the Trial and Prior and after Thermal Stress

	TP				BS			
	start	**Prior thermal stress**	**after thermal stress**	**end**	**start**	**Prior thermal stress**	**after thermal stress**	**end**
pH	7.38	5.50	6.50	7.47	8.15	8	8.5	7.65
CH_4 (%) ($CH_{4\ peak}$)	ND	53.31	ND	73.52 (78.54)	ND	23,81	58.44 (58.44)	ND
TOT VFA (mM)	86.04	186.61	172.96	14.6	5.34	2.72	ND	3.66
N-NH_3 g/L	0.82	1.6	1.14	1.24	1.12	1.26	1.27	1.31

TP: tomato pomace; BS: buffalo sludge; VFA: Volatile Fatty acid; CH_4: methane; ND: not determined.

is highly helped by thermal, chemical or biological pretreatments [25, 26], while at elevated temperatures, these processes take place faster and with higher biodegradation [14].

This could be due to the enhanced substrate swelling of the lignocellulosic material, decreasing the integrity and increasing the accessibility, and consequently, the greater availability of the substrates for the microbial cellulase enzyme systems' biodegradability, which is favored by higher temperature during the fermentation [24, 27, 28].

3.3. pH and Biogas Production

Table **2** reports the data about pH, biogas production, and VFAs.

Carotenuto *et al*. [29] studied the effect of temperature and pH on biomethane production in the anaerobic digestion of water buffalo manure, observing that the optimum pH was between 7 and 7.5. Since the pH was not optimal in our trial but was always above 8, this could be the reason why the BS production was low (Table **2**). The authors suggested that the digestion process of the naturally basic substrate can begin without any manipulation of the substrate pH. Water buffalo dung can help to maintain a neutral pH during digestion, even when starting at a pH of 5.0 or 6.0. This property is particularly useful in co-digestion processes with acidic waste, like TP, because it eliminates the need for additional buffering or pH control measures [5,9].

These results are consistent with our research. The high VS content of the TP acidified the reaction medium, with a pH shifting from 5.5 before the temperature rise to 6.2 after the thermal stress and showed a stable value of 7.5 at the end.

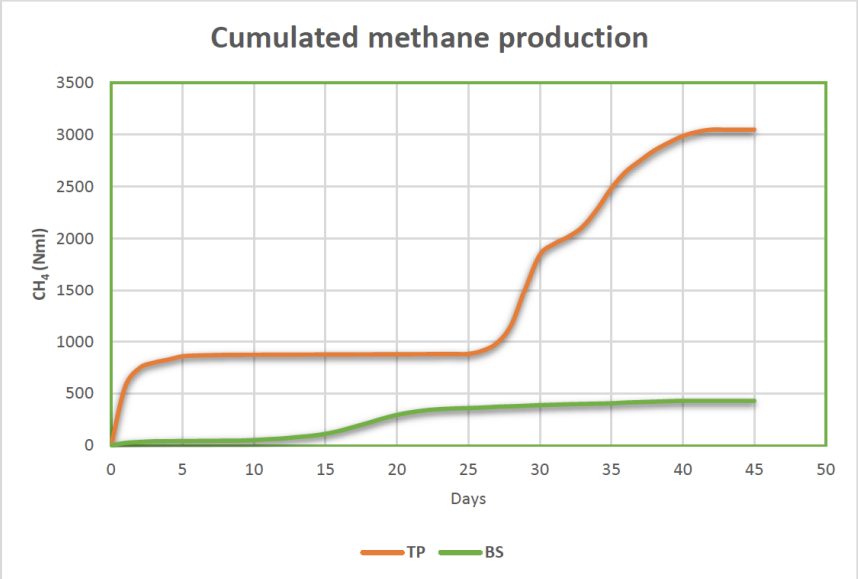

Figure 2: Cumulated methane production curves for Tomato pomace (TP) and Buffalo sludge (BS).

The cumulated production curves of TP and BS are reported in Figure **2**.

The TP value of methane reached a plateau on the 7th day (0.875—normal liter, dry gas, 0°C, 1 atm—NL CH_4) before the temperature rose. It resumed on the 25th day and ended at 3,115 NL CH_4. The small quantity of methane produced in TP under mesophilic conditions was due to the acidification of the media, which creates unfavorable conditions for the methanogen population to thrive [30,31].

In contrast, BS had a scarce cumulated production (0.112 NL CH_4) before the temperature rise, which increased to 0.495 NL CH_4 afterward. These results are consistent with those reported by other authors [32,33]. The methane production curves of the BS batches (Figure **2**) did not seem to be affected by the temperature increase. As observed by Saghouri *et al*. [33] in the anaerobic digestion of tomato processing waste, a pH drop in the first stage is expected due to the production of VFAs. In the second stage, when methanogenic bacteria predominate, the VFAs are converted to CO_2 and CH_4, and the pH returns to higher values, around 7-8.

The high concentration of VS in the TP reactors hindered a smooth transition from the acidogenic to the methanogenic phase. Therefore, correct substrate

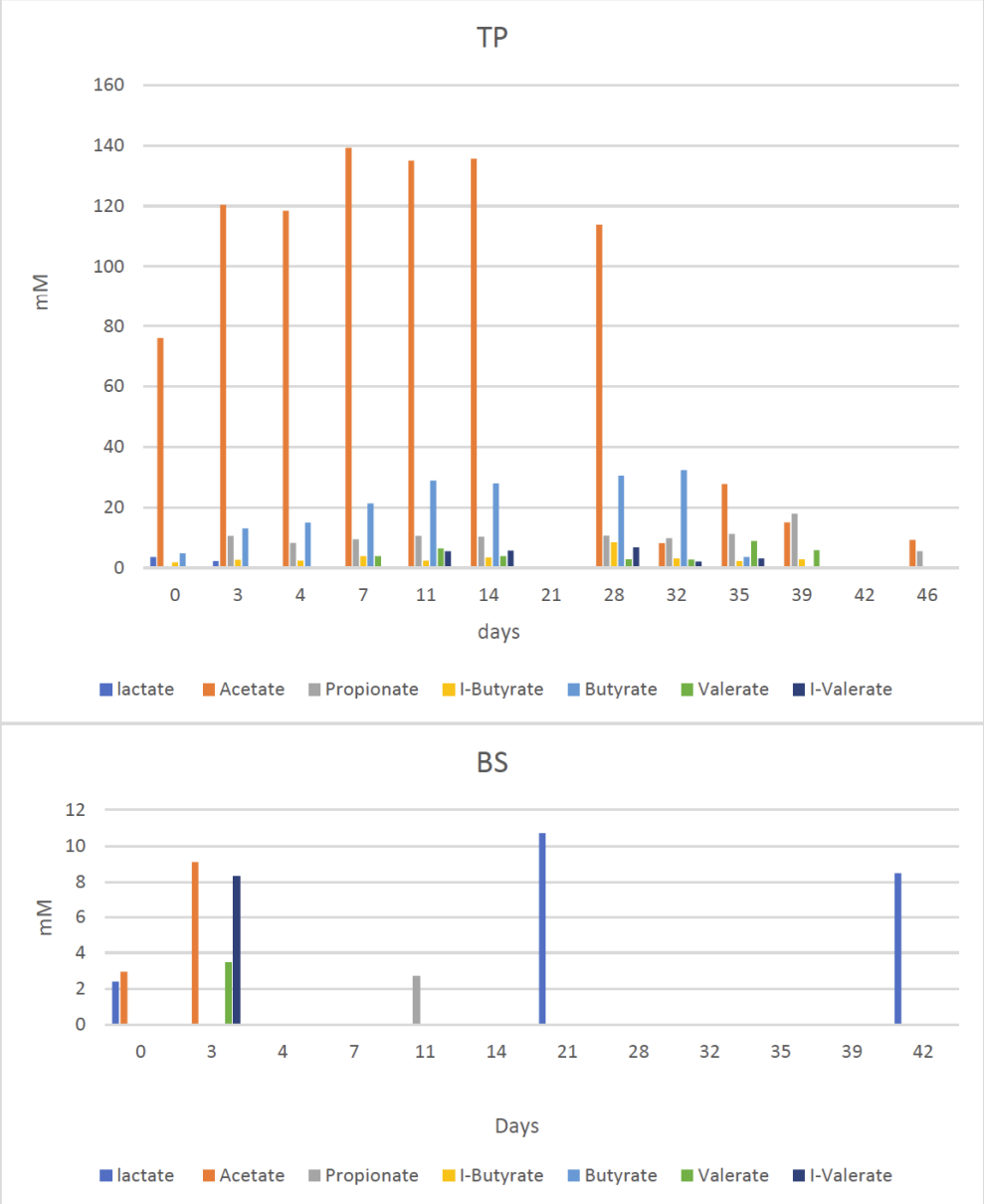

Figure 3: Volatile fatty acids (VFAs) measured during the trial.

dosing in co-digestion must be emphasized to ensure optimal conditions.

The biogas analyzed using gas chromatography showed consistent results with cumulative methane production. The TP had the highest value at 78.5%, while the maximum peak in BS was approximately 58.4% (Table **2**).

3.4. VFA and N-NH_3

Figure **3** reports the VFAs measured during the trial. The accumulation of VFAs in TP caused a significant reduction in pH, dropping to 5.5, as reported in Table **2**. However, the buffering capability of buffalo sludge as feedstock allowed for restoration of the pH level within the range for methanogens to produce methane throughout the fermentation process, as also reported by Weiland and Tenca *et al*. [34,35]. The total VFA value reached its peak (188.4 mM) in TP after 14 days due to the failure in biogas conversion. Acetate was the most abundant VFA, and its concentration was inversely correlated to methane production. It increased during fermentation when no methane was produced before decreasing towards the end when it was converted to biogas. The accumulation in BS reactors of Lactate, a significant intermediate in biogas production, could explain the low biogas production of BS not converted [36].

The N-NH_3 values in mesophilia increased from the beginning to the end of the BS batch trial but remained within the safe range reported in the literature [37]. The highest N-NH_3 value was recorded in TP (1.6 mg/L) before the temperature rise, but it decreased soon after. It does not appear to be correlated with TP content (see Table **2**).

The higher N-NH_3 values in TP compared to BS (Table **2**) confirmed what was observed in the literature [38].

3.5. Metagenomic Analysis

Following pre-processing, 1,666,429 combined reads were retained for subsequent analysis. The 16S rDNA amplicon sequencing depth of the 12 analyzed samples ranged from 38,210 to 151,373 paired reads. Singleton OTUs were removed, and the data from each sample were rarefied at 38,210 to ensure maximum sample retention. A total of 2,571 OTUs were obtained from all samples.

The Kruskal-Wallis test did not reveal any statistically significant differences in the abundance of OTUs among the various sample groups. Our initial analysis aimed to provide a comprehensive description of the microbial community composition present in all samples.

Most sequences belonged to Firmicutes (51.4% in TP and 26.0% in BS), of which Clostridia were 49.5% and 24.1% in TP and BS samples, respectively, and Bacteroidetes (28.2% in TP and 50.3% in BS), with a

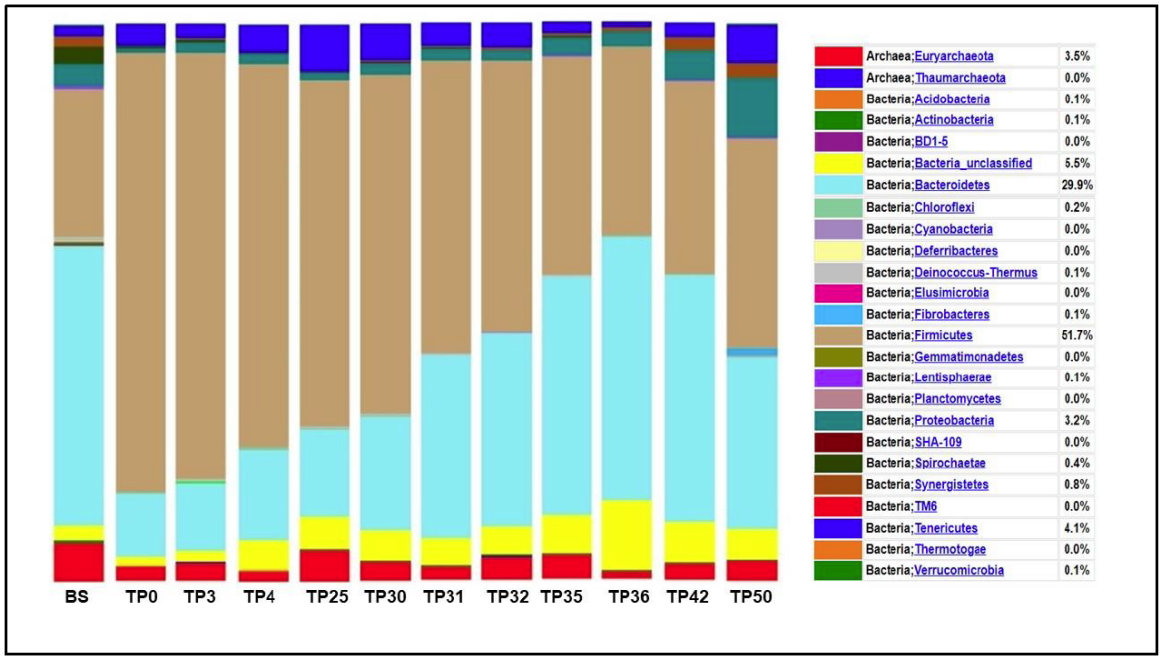

Figure 4: Taxonomic analysis at the phylum level (control Buffalo sludge BS – Tomato pomace-TP sampling days).

predominance of Bacteroidia (29.0%). The two phyla accounted for 81.6% of the total OTUs. Bacteroidetes and Firmicutes microorganisms are widely distributed in a variety of anaerobic habitats, both natural and artificial. They can degrade a wide range of complex organic macromolecules, including both proteins and carbohydrates [39-41].

The abundance of minor bacterial phyla, such as Actinobacteria and Spirochaetae, was below 3% (Figure **4**).

Several authors report the first two main phyla together with Tenericutes as part of a core group [42-45] in AD of cattle manure and sludge. As part of the core group, some authors [46-48] also reported Cloroflexi. However, we found it to be present at only 0.03%.

Maus *et al*. [49] reported that Firmicutes and Bacteroidetes were the main bacterial phyla in mesophilic conditions of biogas plants with cow manure as inoculum. Firmicutes are important because they are involved in several metabolic processes, including the degradation of carbohydrates and the utilization of fatty acids. Campanaro *et al*. [48] have reported either the use of the Ljungdahl pathway (WLP) for homoacetogenesis or the use of syntrophic acetate oxidation (SAO). Bacteroidetes are mainly related to the degradation of proteins, fats, and polysaccharides [50-52].

Concerning Clostridiaceae, *Fonticella* was the most represented bacteria (4.3% on average). It was absent in the BS sample and increased (up to 17.1%) in the TP sample when methane was resumed, as reported by Shiratori *et al*. [53] (Figure **5**). The second most represented *Incertae Sedis* belongs to the Ruminoccoccaceae, with 3.8% and 2.6% in TP and BS samples, respectively. It was previously identified as a functional bacterium associated with cellulose and hemicellulose degradation [54]. Therefore, the microbial community containing these bacteria enabled the enhancement of methane production [55].

Among the Clostridiales, we detected *Defluviitalea,* which has been reported to be abundant and metabolically active in reactors operating at highly

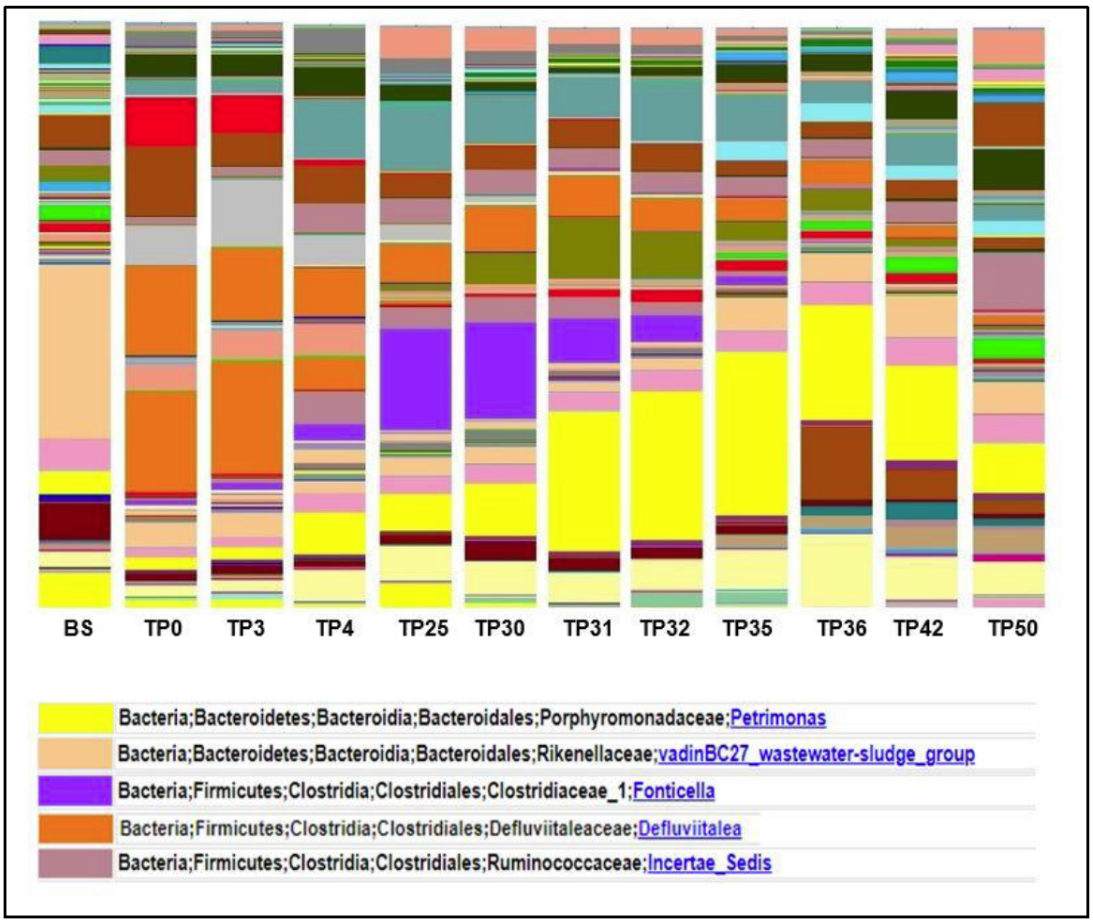

Figure 5: Taxonomic analysis at the genus level (control Buffalo sludge BS – Tomato pomace TP sampling days).

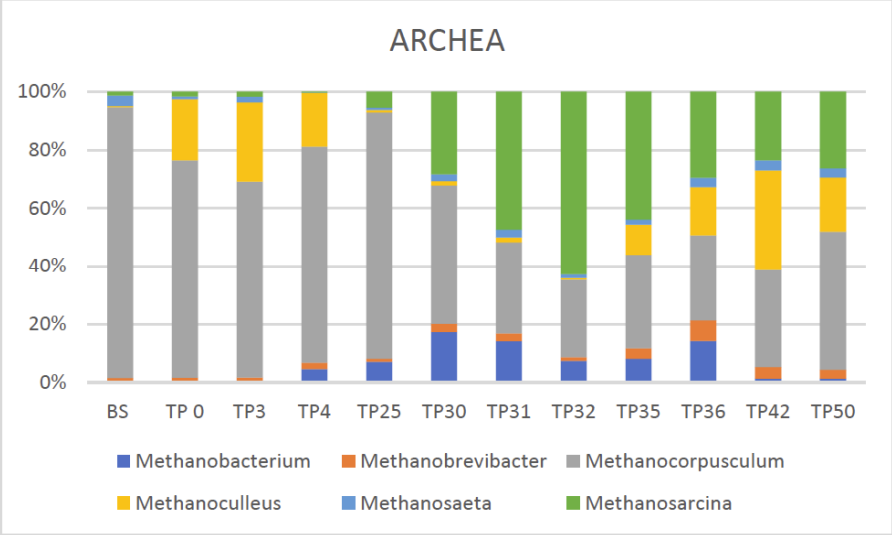

Figure 6: Taxonomic analysis at the genus level of the most representative Archea (control Buffalo sludge BS – Tomato pomace TP sampling days).

efficient process conditions, supporting its importance for the hydrolysis of the raw substrate [56]. When substrates were hydrolyzed and acetate was produced, it was absent in BS and present at 17.2% and 19.1% in TP2 and TP3, respectively. Afterward, the *Defluviitalea* disappeared.

Among the members of the phylum Bacteroidetes, order Bacteroidales, particularly Dysgonomonadaceae, the genus *Petrimonas*, was present on an average percentage of 3.8% and 13.4% in BS and TP, respectively. Moreover, TP reached a concentration of 27.8% when methane production was resumed on day 36. The Dysgonomonadaceae (formerly Porphyromonadaceae) families are often abundant in mesophilic biogas reactor systems in connection with the formation of VFA [57] and increased ammonium concentrations [58-60]. Biogas plants are frequently fed with food waste or protein-rich and poorly digestible substrates, such as straw. This can result in unstable processes, with variations in biogas/methane yields and elevated nitrogen/ammonia levels [61,45].

The vadinBC27_wastewater-sludge_group, belonging to the Bacteroidales, is a typical hydrolytic bacterium that produces CO_2 and NH^{4+} as terminal metabolites [62]. It was present in BS samples (29.4%) rather than in TP (3.8% on average). Nevertheless, it increased up to 5.6% when methane was produced. Li *et al*. [62] suggested that vadinBC27_wastewater-sludge_group, with its electrochemical activity, might participate in diet with Methanosarcina, which can accept electrons from the carbon-based mediator.

All the Archea species identified in this study were attributed predominantly to phylum Euryarcheota (99% of Archea; 3.5% on average on total sequences). Considering the group's analysis, Euryarcheota included 4 orders: Methanobacteriales, Methanomicrobiales, Methanosarcinales, and Thermoplasmatales, as found in previous studies [47,59]. The most abundant among Archea were Metanomicrobiales (65%), particularly Methanocorpusculaceae (46.4%, 1.9% on average on total sequences), followed by Methanosarcinales (24.5%) with three genera Methasarcina (22.8%), Methanosaeta (1.65%), and Methanomicrococcus (0.02%).

Methanocorpusculum was the most prevalent microorganism among the Metanomicrobiales, accounting for 6.4% in control (BS) and 1.5% in TP on average. This confirms the findings of Pan *et al*. [63], who reported that H_2/CO_2 methanation is the fastest methanogenic step in digested manure and sewage sludge systems, with Methanomicrobiales as the dominant methanogens.

However, Metanosarcinales, and in particular Metanosarcina, showed the strongest correlation with methane production (see Figure **6**). In the BS sample, it was present at 0.08%, compared to the TP sample, which had an average percentage of 2.7%. This increased to 8.8% on the 35th day of fermentation. In systems with high acetate concentrations, such as our reactor (see Figure **3**), the dominance of Metanosarcina spp. is promoted [63]. Conversely, Methanosaeta spp. thrives in low acetate concentrations, as reported by Karakashev *et al*. [64].

4. CONCLUSION

The case study presented in this paper demonstrates that even short exposure to thermophilic conditions can significantly increase biogas yield, thereby improving the co-digestion of BS and TPs. The temperature change activated a distinct microbial environment, leading to a significant shift in the bacterial and archaeal populations. *Fonticella* genus, among bacteria, and *Metanosarcina* genus, among archaea, were found to closely follow the methane production curve.

The new milieu proved to be capable of fermenting high organic loads, which may allow the recycling of a greater quantity of tomato waste, with practical and environmental advantages. However, the correct trade-off between high TP recovery and increased energy cost of the process should also be considered.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The data that supports this study may be shared with the corresponding author upon reasonable request.

REFERENCES

[1] Bacenetti J, Duca D, Negri M, Fusi A, Fiala M. Mitigation strategies in the agro-food sector: The anaerobic digestion of tomato purée by-products. An Italian case study. Sci Total Environ 2015; 526: 88-97. https://doi.org/10.1016/j.scitotenv.2015.04.069

[2] Paritosh K, Kushwaha SK, Yadav M, Pareek N, Chawade A, Vivekanand V. Food Waste to Energy: An Overview of Sustainable Approaches for Food Waste Management and Nutrient Recycling. Biomed Res Int 2017; 1-19. https://doi.org/10.1155/2017/2370927

[3] The 2023 Processed Tomato Yearbook. Special issue of www.tomato.news.com_Edited by Tomato News sas 2024; ISSN 1145-96.

[4] FAOSTAT, 2024. https://www.fao.org/faostat/en/#data (accessed February 2024)

[5] Boccia F, Di Donato P, Covino D, Poli A. Food waste and bio-economy: A scenario for the Italian tomato market. J Clean Prod 2019; 227: 424-433. https://doi.org/10.1016/j.jclepro.2019.04.180

[6] Calabrò PS, Greco R, Evangelou A, Komilis D. Anaerobic digestion of tomato processing waste: Effect of alkaline pretreatment. J Environ Manage 2015; 163: 49-52. https://doi.org/10.1016/j.jenvman.2015.07.061

[7] Del Valle M, Cámara M, Torija ME. Chemical characterization of tomato pomace. J Sci Food Agric 2006; 86: 1232-1236. FAOSTAT, 2019. http://www.fao.org/faostat/en/#data/QA (accessed January 10, 2021). https://doi.org/10.1002/jsfa.2474

[8] Lu Z, Wang J, Gao R, Ye F, Zhao G. Sustainable valorisation of tomato pomace: A comprehensive review. Trends Food Sci Technol 2019; 86: 172-187. https://doi.org/10.1016/j.tifs.2019.02.020

[9] Li Y, Xu F, Li Y, Lu J, Li S, Shah A, Zhang X, Zhang H, Gong X, Li G. Reactor performance and energy analysis of solid state anaerobic co-digestion of dairy manure with corn stover and tomato residues. Waste Manag 2018; 73: 130-139. https://doi.org/10.1016/j.wasman.2017.11.041

[10] Faugno S, Pindozzi S, Infascelli R, Okello C, Ripa MN, Boccia L. Assessment of nitrogen content in buffalo manure and land application costs. Journal of Agricultural Engineering 2012; 43(2): e13. https://doi.org/10.4081/jae.2012.18

[11] Li Y, Chen Y, Wu J. Enhancement of methane production in anaerobic digestion process: A review. Appl Energy 2019; 240: 120-137. https://doi.org/10.1016/j.apenergy.2019.01.243

[12] Hoyos-Sebá JJ, Arias NP, Salcedo-Mendoza J, Aristizábal-Marulanda V. Animal manure in the context of renewable energy and value-added products: A review. Chemical Engineering and Processing-Process Intensification 2024; 196: 109660. https://doi.org/10.1016/j.cep.2023.109660

[13] Kougias PG, Angelidaki I. Biogas and its opportunities—A review. Front Environ Sci Eng 2018; 12-14. https://doi.org/10.1007/s11783-018-1037-8

[14] Santangelo E, Calì M, Rossi E, Scalella R, La Mantia MC, Chiariotti A. Anaerobic co-digestion of tomato pomace and buffalo slurry. Procedia Environmental Science, Engineering and Management 2022; 9(4): 979-985.

[15] Calì M, Rossi E, Scalella R, La Mantia MC, Santangelo E, Chiariotti A. Thermophilic anaerobic codigestion of tomato pomace and buffalo slurry. European Biomass Conference and Exhibition Proceedings 2022; 581-584.

[16] Manfredini A, Chiariotti A, Santangelo E, Rossi E, Renzi G, Dell'Abat, MT. Assessing the Biological Value of Soluble Organic Fractions from Tomato Pomace Digestates. J Soil Sci Plant Nutr 2020; 21: 301-314. https://doi.org/10.1007/s42729-020-00361-4

[17] APAT IRSA-CNR, 2003. Analytical methods for waters. Manual and Guideline 2003; vol III.

[18] van Soest PJ, Robertson JB, Lewis BA. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. J Dairy Sci 1991; 74: 3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

[19] Martillotti F, Antongiovanni M, Rizzi L, Santi E, Bittante G. Analysis methods to evaluate animal feeds. CNR, IPRA, Rome, Italy 1987.

[20] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA. QIIME allows the analysis of high-throughput community sequencing data. Nature Methods 2010; 7(5): 335-6. https://doi.org/10.1038/nmeth.f.303

[21] Coelho MC, Rodrigues AS, Teixeira JA, Pintado ME. Integral valorization of tomato by-products towards bioactive compounds recovery: Human health benefits. Food Chemistry 2023; 410: 135319. https://doi.org/10.1016/j.foodchem.2022.135319

[22] Triolo JM, Sommer SG, Møller HB, Weisbjerg MR, Jiang XY. A new algorithm to characterize biodegradability of biomass

during anaerobic digestion: Influence of lignin concentration on methane production potential. Bioresource Technology 2011; 102(20): 9395-9402. https://doi.org/10.1016/j.biortech.2011.07.026

[23] Ziembińska-Buczyńska A, Banach A, Bacza T, Pieczykolan M. Diversity and variability of methanogens during the shift from mesophilic to thermophilic conditions while biogas production. World Journal of Microbiology and Biotechnology 2014; 30: 3047-3053. https://doi.org/10.1007/s11274-014-1731-z

[24] Kabaivanova L, Petrova P, Hubenov V, Simeonov I. Biogas production potential of thermophilic anaerobic biodegradation of organic waste by a microbial consortium identified with metagenomics. Life 2022; 12(5): 702. https://doi.org/10.3390/life12050702

[25] Ning X, Shixun L, Fengxue X, Jie Z, Honghua J, Jiming X, Min J, Weilian, D. Biomethane Production From Lignocellulose: Biomass Recalcitrance and Its Impacts on Anaerobic Digestion. Front Bioeng Biotechnol 2019; 7: 191. https://doi.org/10.3389/fbioe.2019.00191

[26] Zhu N, Gao J, Liang D, Zhu Y, Li B, Jin H. Thermal pretreatment enhances the degradation and humification of lignocellulose by stimulating thermophilic bacteria during dairy manure composting. Bioresource Technology 2021; 319: 124-149. https://doi.org/10.1016/j.biortech.2020.124149

[27] Tu M, Pa X, Saddler JN. Adsorption of cellulase on cellulolytic enzyme lignin from lodgepole pine. J Agric Food Chem 2009; 57: 7771-7778. https://doi.org/10.1021/jf901031m

[28] Cazaudehore G, Monlau F, Gassie C, Lallement A, Guyoneaud R. Methane production and active microbial communities during anaerobic digestion of three commercial biodegradable coffee capsules under mesophilic and thermophilic conditions. Science of The Total Environment 2021; 784: 146972. https://doi.org/10.1016/j.scitotenv.2021.146972

[29] Carotenuto C, Guarino G, Morrone B, Minale M. Temperature and pH effect on methane production from buffalo manure anaerobic Digestion. Int J Heat Technol 2016; 34: S425-S429. https://doi.org/10.18280/ijht.34S233

[30] Zhang C, Su H, Baeyens J, Tan T. Reviewing the anaerobic digestion of food waste for biogas production, Renew. Sustain. Energy Rev 2014; 38: 383-392. https://doi.org/10.1016/j.rser.2014.05.038

[31] Yang L, Huang Y, Zhao M, Huang Z, Miao H, Xu Z, Ruan W. Enhancing biogas generation performance from food wastes by high-solids thermophilic anaerobic digestion: Effect of pH adjustment. Int Biodeterior Biodegrad 2015; 105: 153-159. https://doi.org/10.1016/j.ibiod.2015.09.005

[32] Almeida PV, Rodrigues RP, Gaspar MC, Braga ME, Quina MJ. Integrated management of residues from tomato production: Recovery of value-added compounds and biogas production in the biorefinery context. Journal of Environmental Management 2021; 299: 113505. https://doi.org/10.1016/j.jenvman.2021.113505

[33] Saghouri M, Mansoori Y, Rohani A, Khodaparast MHH, Sheikhdavoodi MJ. Modelling and evaluation of anaerobic digestion process of tomato processing wastes for biogas generation. J Mater Cycles Waste Manag 2018; 20: 561-567. https://doi.org/10.1007/s10163-017-0622-4

[34] Weiland P. Biogas production: current state and perspectives. Applied Microbiology and Biotechnology 2010; 85: 849-860. https://doi.org/10.1007/s00253-009-2246-7

[35] Tenca A, Schievano A, Perazzolo F, Adani F, Oberti R. Biohydrogen from thermophilic co-fermentation of swine manure with fruit and vegetable waste: maximizing stable production without pH control. Bioresource Technology 2011; 102(18): 8582-8588. https://doi.org/10.1016/j.biortech.2011.03.102

[36] Satpathy P, Steinigeweg S, Siefert E, Cypionka H. Effect of lactate and starter inoculum on biogas production from fresh maize and maize silage. Advances in Microbiology 2017; 7(5): 358-76. https://doi.org/10.4236/aim.2017.75030

[37] Daeschel MA, Andersson RE, Fleming HP. Microbial ecology of fermenting plant materials. FEMS Microbiol Lett 1987; 46: 357-367. https://doi.org/10.1111/j.1574-6968.1987.tb02472.x

[38] Holliger C, Alves M, Andrade D, Angelidaki I, Astals S, Baier U, *et al*. Towards a standardization of biomethane potential tests. Water Sci Technol 2016; 74: 2515-2522. https://doi.org/10.2166/wst.2016.336

[39] Bernardet JF, Bowman JP. The genus flavobacterium. The prokaryotes 2006; 7: 481-531. https://doi.org/10.1007/0-387-30747-8_17

[40] Slepechy RA, Hemphill HE. The genes *Bacillus*- non medical. In Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K (ed.), The Procaryotes, Springer, New York, NY 1992; II: 1663-1896.

[41] Wiegel J, Tanner RA, Rainey FA. An introduction to the family Clostridiaceae. Prokaryotes 2006; 4: 654-78. https://doi.org/10.1007/0-387-30744-3_20

[42] Abendroth C, Vilanova C, Günther T, Luschnig O, Porcar M. Eubacteria and archaea communities in seven mesophile anaerobic digester plants in Germany. Biotechnology Biofuels 2015; 8(87). https://doi.org/10.1186/s13068-015-0271-6

[43] Bassani I, Kougias PG, Treu L, Angelidaki I. Biogas upgrading via hydrogenotrophic methanogenesis in two-stage continuous stirred tank reactors at mesophilic and thermophilic conditions. Environmental Science & Technology 2015; 49(20): 12585-12593. https://doi.org/10.1021/acs.est.5b03451

[44] Duda R M, da Silva Vantini J Martins SL, de Mello Varani A, Lemons MVF, Ferro MIT, *et al*. A balanced microbiota efficiently produces methane in a novel high-rate horizontal anaerobic reactor for the treatment of swine wastewater. Biores Technol 2015; 197: 152-160. https://doi.org/10.1016/j.biortech.2015.08.004

[45] Goux X, Calusinska M, Lemaigre S, Marynowska M, Klocke M, Udelhoven T, Benizri E, Delfosse P. Microbial community dynamics in replicate anaerobic digesters exposed sequentially to increasing organic loading rate, acidosis, and process recovery. Biotechnology for Biofuels 2015; 8: 1-8. https://doi.org/10.1186/s13068-015-0309-9

[46] Riviere D, Desvignes V, Pelletier E, Chaussonnerie S, Guermazi S, Weissenbach J, Li T, Camacho P, Sghir A. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. The ISME Journal 2009; 3(6): 700-714. https://doi.org/10.1038/ismej.2009.2

[47] St-Pierre B, Wright AD. Comparative metagenomic analysis of bacterial populations in three full-scale mesophilic anaerobic manure digesters. Applied Microbiology and Biotechnology 2014; 98: 2709-2717. https://doi.org/10.1007/s00253-013-5220-3

[48] Campanaro S, Treu L, Kougias PG, De Francisci D, Valle G, Angelidaki I. Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnology for Biofuels 2016; 9: 1-7. https://doi.org/10.1186/s13068-016-0441-1

[49] Maus I, Tubbesing T, Wibberg D, Heyer R, Hassa J, Tomazetto G, Huang L, Bunk B, Sprӧer C, Benndorf D, *et al*. The Role of Petrimonas Mucosa ING2-E5AT in Mesophilic

Biogas Reactor Systems as Deduced from Multiomics Analyses. Microorganisms 2020; 8(12): 2024. https://doi.org/10.3390/microorganisms8122024

[50] Chen L, Xu D, Liang J, Zhang Y, Fang W, Zhang P, Zhang G. New insight into effects of waste scrap iron on sludge anaerobic digestion: Performances, microbial community, and potential metabolic functions. Journal of Water Process Engineering 2023; 55: 104230. https://doi.org/10.1016/j.jwpe.2023.104230

[51] Nabi M, Gao, Liang J, Cai Y, Zhang P. Combining high-pressure homogenization with free nitrous acid pretreatment to improve anaerobic digestion of sewage sludge. J Environ Manag 2022; 318: 115635. https://doi.org/10.1016/j.jenvman.2022.115635

[52] Wang J, Liu X, He J, Cheng G, Xu J, Lu M, Shangguan Y, Zhang A. Mechanism of dielectric barrier discharge plasma technology to improve the quantity of short-chain fatty acids in anaerobic fermentation of waste active sludge Front. Microbiol 2022; 13: Article 963260. https://doi.org/10.3389/fmicb.2022.963260

[53] Shiratori H, Ohiwa H, Ikeno H, *et al*. Lutispora thermophila gen. nov., sp. nov., a thermophilic, spore-forming bacterium isolated from a thermophilic methanogenic bioreactor digesting municipal solid wastes. Int J Syst Evol Microbiol 2008; 58: 964-969. https://doi.org/10.1099/ijs.0.65490-0

[54] Ecem Öner B, Akyol Ç, Bozan M, Ince O, Aydin S, Ince B. Bioaugmentation with *Clostridium thermocellum* to enhance the anaerobic biodegradation of lignocellulosic agricultural residues. Bioresour Technol 2018; 249: 620-625. https://doi.org/10.1016/j.biortech.2017.10.040

[55] Song C, Li W, Cai F, Liu G, Chen C. Anaerobic and microaerobic pretreatment for improving methane production from paper waste in anaerobic digestion. Frontiers in Microbiology 2021; 12: 688290. https://doi.org/10.3389/fmicb.2021.688290

[56] Rettenmaier R, Schneider M, Munk B, Lebuhn M, Jünemann S, Sczyrba A, Maus I, Zverlov V, Liebl W. Importance of Defluviitalea raffinosedens for hydrolytic biomass degradation in co-culture with Hungateiclostridium thermocellum. Microorganisms 2020; 8(6): 915. https://doi.org/10.3390/microorganisms8060915

[57] Zhang J, Loh KC, Lee J, Wang CH, Dai Y, Wah Tong Y. Three-stage anaerobic co-digestion of food waste and horse manure. Scientific Reports 2017; 7(1): 1269. https://doi.org/10.1038/s41598-017-01408-w

[58] Sun L, Pope PB, Eijsink VG, Schnürer A. Characterization of microbial community structure during continuous anaerobic digestion of straw and cow manure. Microbial Biotechnology 2015; 8(5): 815-27. https://doi.org/10.1111/1751-7915.12298

[59] Moset V, Poulsen M, Wahid R, Højberg O, Møller HB. Mesophilic versus thermophilic anaerobic digestion of cattle manure: methane productivity and microbial ecology. Microbial Biotechnology 2015; 8(5): 787-800. https://doi.org/10.1111/1751-7915.12271

[60] Maus I, Tubbesing T, Wibberg D, Heyer R, Hassa J, Tomazetto G, Huang L, Bunk B, Spröer C, Benndorf D, *et al*. The Role of Petrimonas Mucosa ING2-E5AT in Mesophilic Biogas Reactor Systems as Deduced from Multiomics Analyses. Microorganisms 2020; 8(12): 2024. https://doi.org/10.3390/microorganisms8122024

[61] Müller B, Sun L, Westerholm M, Schnürer A. Bacterial Community Composition and Fhs Profiles of Low- and High-Ammonia Biogas Digesters Reveal Novel Syntrophic Acetate-Oxidising Bacteria. Biotechnol Biofuels 2016; 9: 48. https://doi.org/10.1186/s13068-016-0454-9

[62] Li X, Li Q, He J, Zhang YF, Zhu NM. Application of activated carbon to enhance biogas production rate of Flammulina velutipes residues with composting pretreatment. Waste and Biomass Valorization 2021; 12: 823-831. https://doi.org/10.1007/s12649-020-01039-9

[63] Pan X, Angelidaki I, Alvarado-Morales M, Liu H, Liu Y, Huang X, Zhu G. Methane production from a formate, acetate, and H2/CO2; focusing on kinetics and microbial characterization. Bioresource Technology 2016; 218: 796-806. https://doi.org/10.1016/j.biortech.2016.07.032

[64] Karakashev D, Batstone DJ, Angelidaki I. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Applied and Environmental Microbiology 2005; 71(1): 331-338. https://doi.org/10.1128/AEM.71.1.331-338.2005

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