The Effects of Using Evogen Biogas Additive on the Microbiome and Performance of Full-Scale Biogas Plant

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Article

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Abstract: Biogas production from organic waste is a promising renewable energy source, but achieving optimal production and digester stability can be challenging. This study investigated the impact of the Evogen microbial additive on biogas production and digester quality through microbial abundance and physicochemical parameter analysis. Two biogas plants, BG01 and BG02, were examined using 16S rRNA profiling to assess microbial abundance. Simultaneously, physicochemical parameters, including FOS/TAC ratio, total solids, volatile solids, biogas production, and VFA profile, were measured to evaluate digester performance. Results revealed distinct microbial community shifts in Evogen-treated digesters. Increased abundance of methanogenic archaea and hydrolytic bacteria indicated improved anaerobic digestion. Evogen supplementation also positively affected digester performance, with higher FOS/TAC ratios indicating enhanced acidification and methanogenesis. Reductions in total solids and volatile solids demonstrated improved organic matter degradation. Significantly higher biogas production was observed in Evogen-treated digesters, highlighting its potential as a microbial additive. Furthermore, VFA profiling demonstrated improved process stability and reduced substrate inhibition in Evogen-treated digesters. In summary, Evogen microbial additive positively influenced microbial dynamics, improving biogas production and digester quality. These findings contribute to optimizing biogas production systems and understanding the complex microbial interactions within anaerobic digesters.

Keywords: anaerobic digestion; hydrolytic bacteria; biogas additives; evogen biogas additive; supplements

1. Introduction

Biogas, a renewable energy source (RES) that is produced through anaerobic digestion (AD) of organic matter in biogas plants is a mixture of methane and carbon dioxide [1]. These plants can utilize a wide range of feedstocks, including agricultural waste, food waste, and sewage sludge, making them a versatile and sustainable energy option [2]. However, biogas plants face several challenges that can hinder their efficiency and effectiveness. One major issue is the variation in feedstock quality and quantity, which can lead to fluctuations in biogas production [3]. Additionally, the presence of toxic compounds or the accumulation of other compounds can lead to operational problems and reduced output [4]. To ensure the optimal functioning of biogas plants, it is essential to address these challenges and develop strategies for improving their performance.

In addition to the challenges posed by varying feedstock quality and toxicity, biogas plants can also face a range of operational problems that can impact their efficiency and profitability. One common issue is the high solid content in the digester, which can reduce biogas production and damage equipment [5]. Inadequate mixing or agitation of the feedstock can also lead to uneven digestion and reduced gas output [6]. Another problem is the accumulation of hydrogen sulfide and other corrosive compounds in the biogas, which can damage pipelines and other components of the

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system [7] and reduce at the same time significant bacterial populations as it is toxic like gaseous ammonia. Additionally, biogas plants can experience issues related to odor control, as the breakdown of organic matter can release unpleasant odors that can be a nuisance for nearby communities [8]. Addressing these operational problems requires careful monitoring and maintenance of the system, as well as the implementation of effective control measures to prevent or mitigate issues as they arise [9].

The use of biogas additives has gained increasing attention as a means of improving the efficiency and effectiveness of full-scale biogas plants [10]. Biogas additives are substances that are added to the feedstock or the digester to enhance the performance of the system [11]. These additives can address various challenges associated with biogas plant management, including poor feed quality, recalcitrant biomass, and VFA accumulation. By optimizing the conditions within the digester, biogas additives can help to increase biogas production, reduce operational problems, and improve overall plant performance [12,13].

One of the primary challenges facing biogas plants is the variability in feedstock quality, which can impact the availability of micronutrients and other essential components for microbial digestion [10]. Biogas additives can address this problem by providing a source of these nutrients, which can help to maintain the stability of the microbial community and enhance biogas production [14]. Additionally, the use of additives such as enzymes and acids can improve the hydrolysis of recalcitrant biomass, such as silage and corn, which can be difficult to digest through microbial action alone [13].

Other operational problems that can be resolved through the use of biogas additives include VFA accumulation, acetate inhibition, H_2 partial pressure, H_2S accumulation, NH_3 accumulation, temperature, and heavy metal toxicity [10]. For example, the addition of acidic buffer solutions or alkaline agents can help to maintain the pH balance within the digester, reducing the accumulation of VFAs and preventing acetate inhibition [15,16]. Similarly, the use of bioaugmentation agents can help to optimize the microbial community, reducing the accumulation of H_2S and other problematic compounds [17]. By addressing these challenges, biogas additives can improve the stability and efficiency of biogas plants, making them a more reliable and sustainable source of renewable energy.

Biogas production is a complex process that involves the breakdown of organic matter by a diverse community of microorganisms. While many factors can impact the efficiency and effectiveness of biogas plants, one key challenge is the variability of feedstock quality and composition. This variability can lead to imbalances in the microbial community, resulting in reduced biogas production and operational problems [4]. To address these challenges, biogas plants can benefit from the use of microbial additives, which can optimize the microbial community and enhance biogas production [18]. These additives can be chemical or biological in nature and can improve the stability and efficiency of the biogas production process [11,14,15].

Bioaugmentation involves the addition of specific microorganisms to the digester to enhance the performance of the system [19,20] or to increase the population of a species or even a family. These microorganisms can be selected for their ability to degrade specific types of organic matter or to improve the overall efficiency of the microbial community. Bioaugmentation can be achieved by adding microbial cultures, microbial consortia, or microbial enzymes, or the selective/favored cultivation of dominant species [21,22]. By introducing these microorganisms into the digester, biogas plants can optimize the microbial community and improve biogas production.

Another approach to biogas plant management is the use of multifunctional additives, such as a combination of mineral-based powder carrier and *Bacillus* microorganisms (Evogen Biogas Additive) [23]. These additives can help to enhance biogas production by improving the hydrolysis of complex organic compounds and optimizing the microbial community [11]. Zeolites are natural minerals that have a high surface area and can absorb and release water, making them effective carriers for microbial additives [24]. By binding microbial cultures to zeolite particles, Evogen Biogas Additive can improve the survival and performance of the microorganisms in the digester [23]. This approach can help to reduce the accumulation of problematic compounds, such as H_2S, and improve the overall efficiency of the biogas production process [24].

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This study investigated the yields of two full-scale biogas plants, the microbiome alternations, and the physicochemical characteristics' variations when using the multifunctional additive, zeolite-bound Bacilli.

2. Materials and Methods

2.1. Description of Evogen Biogas Additive

Evogen Biogas Additive (Genesis Biosciences, Ltd., Cardiff, UK) is a powder product that optimizes the anaerobic digestion process. It uses a mineral carrier to support methanogen colonization and improve electron transfer. Bacillus strains in the additive, increase hydrolysis and fermentation through the secretion of anaerobic enzymes. This combination enhances biogas production and reduces sludge volume. Evogen Biogas Additive is manufactured according to ISO 9001 standards, ensuring quality and stability. It provides both a physiochemical and biological response, strengthening microbial components and improving the degradation of complex compounds.

The product promotes methane production, system stability, and productivity. It operates effectively across different pH ranges and temperatures, making it suitable for various industries. The Bacillus strains in the additive also have plant growth-promoting properties. The innovative carrier absorbs compounds like ammonia and hydrogen sulfide, reducing their inhibitory effects. The Bacillus strains' enzymatic activity liquefies sludge, facilitating further degradation and methane generation.

2.2. Operation of the two full-scale biogas plants under investigation

The BG01 biogas plant has maintained a stable feedstock composition for a long time, giving it a stabilized microbial community and a higher probability of decomposing organic biomass into biogas. The primary digester of BG01 (D1) with an active volume of 4000 m^3 operates at 44°C with a pH of 7.9 from on-site reading. The FOS/TAC ratio ranges from 0.31 from the electrode readings (on-site) and 0.21 from the laboratory readings. The daily feed of D1 comprised 40,5 tn of silage, 5 tn of solid digestate (recirculation of digestate from storage tank back to D1), 15 tn of liquid digestate (recirculation of D2 digestate), and 5 kg/d of Evogen Biogas Additive (Table 1). The recommended additive dosage introduced daily, is 0.15% of the total solid content of the feedstock, much more than the dosage applied. The second digester of BG01 (D2) received material from D1 at the frequency of OLR, which is 1.9 tn per half an hour. It has a volume of 2700 m3 and operates at a mesophilic temperature. The pH from the on-site meter and laboratory measurements is in the range of 7.7. The FOS/TAC ratio is 0.46. The wet daily feed to D2 is 21.5 tn of whey, 3.5 tn of soapstock and 8 tn of glycerol. The stirring of the two digesters is continuous. As a result, the plant reaches its target of 2.1 MW with a 60% CH4 yield (24 hours x 920 to 1040 m3 biogas) (Table 1).

Table 1. Technical specifications and operational parameters amongst the three biogas plants (BG01, and BG02).

Biogas Plant	BG01	BG02
Electrical Power Capacity	2 MW	1 MW
Pre-tank	1 pre-tank	1 pre-tank
Daily Supply	45 tn/d: corn silage 8 tn/d: LD recirculation from storage tank 8 tn/d: glycerol 21 tn/d: waste residues 20 tn/d: pomace (olive, fruits)	8 tn/d: mix corn silage, potatoes, sunflower 7 tn/d: Rye silage 3tn/d: Beetroot 30 tn/day: chicken manure (solid) 2tn/day: liquid digested residue (after separator)

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		20tn/d: Organic waste (food waste, etc.) 30tn/d: Whey 60tn/d: Cattle manure (liquid)
Feeding Rate	2,1 tn/ 30min	160 tn/day
1st Digester (D1)	4000 m^3	4250 m^3
Temperature	44 °C	41 °C
HRT	50 days	40 days
Stirring	Constantly	Constantly
2nd Digester (D2)	2.800 m^3	-
Temperature	39,5 °C	-
HRT	20 days	-
Recirculation of Digested Residue	Yes	Yes

The BG02 biogas plant has an average yield of 354,000 m^3/month biogas retrieved from one digester of 4250 m3 volume operated at 41±1°C. The hydraulic retention time (HRT) of this reactor is 26.6 days with feeding rate 6.7 tn /hour while there is continuous stirring for 45 min per hour. The daily feed recipe is 62 tn manure and recirculation of liquid digestate (LD), 18 tn silage, 30 tn chicken manure, 20 tn organic waste and 30 tn whey. The additive is introduced in a daily amount of 0.15% of the digester's daily feedstock supply in dry matter basis.

2.3. Determination of Total Solids

The method for TS determination was based on the Total Solids Dried at 103–105°C methodology: APHA 2540-B [37]. A quantity of sample was placed in a dried and pre-weighed dish, and the weight of the sample was recorded. The dish containing the sample was placed in the drying oven at 105°C, overnight. Afterward, the dish was cooled in a desiccator to ambient temperature and weighed. The percentage of total solids content was calculated as follows:

$$Total\ Solids\ \%\ (TS\%) = 100 \cdot \frac{m_2 - m_1}{m_3} \quad (1)$$

Where:
TS: Total Solids (also known as Dry Matter) (%)
m_1: weight of the dried empty capsule (g)
m_2: weight of the sample added within the empty capsule (g)
m_3: weight of the sample (g)

2.4. Determination of Volatile Solids

The method was based on Fixed and Volatile Solids Ignited at 550°C: APHA 2540-E [37].The sample was dried before being placed in the muffle furnace. The dish was weighed with the sample within, was ignited for 4 hours at 550 °C, was cooled in a desiccator and the weight was recorded. The percentage of volatile solids in the sample was calculated as follows:

$$Volatile\ Solids\ (\%), (VS\%) = \left[\frac{(W_T - W'_T)}{W_S} \times 100\right]$$

Where:
W_T: final weight of the dish with the sample, in grams
W_S: weight of the sample, in grams
W'_T: final weight of the dish with the ignited sample, in grams

2.6. Determination of FOS/TAC Ratio

The FOS/TAC ratio serves as a measure to evaluate fermentation processes. TAC represents the estimated total inorganic carbon in the sample, while the ratio reflects the alkalinity buffer capacity, and the FOS value corresponds to the content of volatile fatty acids. The calculation of this ratio

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follows the Nordmann method, which involves titrating a 5 mL sample of fermentation substrate with 0.1 N sulfuric acid solution (H_2SO_4) until pH 5.0 to determine the TAC value, expressed in mg/L of calcium carbonate ($CaCO_3$). Subsequently, a second titration is performed between pH 5.0 and pH 4.4 to obtain the FOS value, expressed in mg/L of acetic acid (CH_3COOH) [13].

2.7. Determination of Volatile Fatty Acids Profile (VFAs)

The centrifugation process in the Eppendorf minispin table centrifuge involves spinning a 1.5 ml sample in a 2 ml Eppendorf tube at 12,000 rpm for 10 minutes. To ensure that the VFAs (acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate) are in their acidic form and to saturate the basic sites on the analytical column, the sample is acidified with 100 µL of ortho-phosphoric acid to reach a pH of 2 before centrifugation. For the gas chromatography analysis, 100 µL of the injection standard and 1 mL of the sample are added to the GC vial. A Shimadzu GC - 2010 Plus High-End gas chromatography system equipped with a flame-ionization detector (FID) is used to inject the liquid phase. The column used is an Altmann Anaytik AS-FFAP EXT, with dimensions of 30m × 0.25mm x 0.25µm. Helium of grade 99.999% is used as the carrier gas at a flow rate of 1.9 mL/min. The injection volume is 1 µL with a split ratio of 1:10, and the injector temperature is set at 250 °C. The detector temperature is also maintained at 250 °C. The temperature program consists of an initial oven temperature of 100 °C, held for 2 minutes. It then increases at a rate of 10 °C/min to 220 °C, without holding time. In the final step, the temperature is raised at a rate of 30 °C/min to 240 °C, with a hold time of 12 minutes. The total run time for the analysis is 27 minutes. The concentration of VFAs is determined using a linear calibration curve obtained from calibration standards and adjusted with the injection internal standard.

2.8. Biochemical Methane Potential (BMP) assay

A BMP test was carried out according to Bioprocess Control's protocols [Bioprocess Control Sweden AB, Scheelevägen 22, SE-223 63 Lund, Sweden]. The Bioprocess Gas Endeavour AMPTS® III (S/N: 1100-2100-5100-1235) set-up held 15 identical 500 ml Duran Schott bottles, tightening Teflon caps, and a gas outlet connected to a measuring cylinder submerged in water and a thermostatic water bath. Each of the bottles was filled with the sample and inoculum, with a working capacity of 400 ml and a headspace of 100 ml. The bioreactors were filled and then purged with nitrogen gas for 2 min to establish anaerobic conditions. After that, the bioreactors were submerged in a thermostatic water bath that was set to a mesophilic temperature of 40 °C and operated at a hydraulic retention time (HRT) of 30 days. Daily, the volume of biogas yield was monitored.

2.4. DNA extraction and 16S rRNA gene amplicon sequencing

Genomic DNA was extracted from the biofilm suspensions with the DNeasy PowerSoil Pro Kit (QIAGEN, Hilde, Germany) according to the manufacturer's instructions. The quantity and quality of the extracted DNA were then estimated using a V-630 Spectrophotometer (JASCO, Inc, Japan). Library preparation was performed following the standard guidelines of the 16S Metagenomic Sequencing Library Preparation protocol (Illumina™, Inc., San Diego, CA, United States). In brief, DNA was amplified using the HotStarTaq® Master Mix Kit (QIAGEN, Hilde, Germany) with the addition of the 341f/805r primer pair, which targets the bacterial and archaeal V3–V4 hypervariable regions of the 16S rRNA gene (341f 5′-CCTACGGGNGGCWGCAG-3′, 805r 5′-GACTACHVGGTATCTAATCC-3′). The PCR mixture (25 µL) contained 12.5 µL of HotStarTaq Master Mix, 5 µL of each primer, and 2.5 µL of DNA (5 ng/µL). Thermal cycling conditions included an initial 3-minute step at 95°C, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 30 sec and a final extension step at 72°C for 5 min. PCR amplicons were cleaned up by AMPure XP beads (Beckman Coulter, CA, USA) to remove unbound primers and primer dimers. Next, dual indices and Illumina sequencing adaptors were attached with an index PCR using the Nextera XT Index Kit (Illumina Inc., San Diego, CA, USA). The PCR reaction mixture (50 µL) comprised 25 µL of HotStarTaq Master Mix, 5 µL of each index, 10 µL of PCR Grade

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Water and 5 μL of the previous PCR product, and the cycling conditions remained the same as that of the first PCR reaction except that the number of iterative cycles was reduced to 8. Afterwards, Indexed PCR amplicons were cleaned up using the AMPure XP beads (Beckman Coulter, CA, USA). The produced DNA libraries were quantified with the Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and their size was verified via a 1.5 % agarose gel electrophoresis. Equimolar concentrations of the libraries were then pooled together, and a quantitative PCR was performed using the QIAseq Library Quant Assay Kit (QIAGEN, Germany) for library concentration evaluation. The pooled library was subsequently spiked with 25% phiX control library (Illumina Inc., San Diego, CA, USA), denatured and diluted to a final concentration of 6 pM. Sequencing was performed on an Illumina MiSeq™ platform with the MiSeq Reagent Nano Kit version 2 (500-Cycle)/ MiSeq Reagent Kit version 3 (600-Cycle) chemistry for a paired-end, 2D250-bp/2x300 cycle run.

2.5. Bioinformatics

The primer sequences were removed and reads with a low-quality score (average score, <20) were filtered out using the FASTQ toolkit within BaseSpace version 2.2.0 (Illumina). The 16S Metagenomics application (version 1.0.1) within BaseSpace was used to perform a taxonomic classification, which uses an Illumina-curated version of the GreenGenes taxonomic database and the RDP naive Bayes taxonomic classification algorithm with an accuracy of >98.2% at the species level [25].

3. Results and Discussion

3.1. Physicochemical results during additive administration

Biogas plants are vital for sustainable energy production as they convert organic waste into biogas through anaerobic digestion. The performance and efficiency of biogas plants can be influenced by various parameters and the addition of specific additives. In this study, we analyzed several parameters, including pH, FOS or Volatile Fatty Acids (VFA), TAC or Total Inorganic Carbon, FOS/TAC ratio (alkalinity buffer capacity), Total Solids (TS), Volatile Solids (VS), NFE-Nitrogen Free Extracts (proximate analysis), Crude Ash, Crude Fat, Crude Protein, Crude Fibers, Theoretical gas yield estimation, Methane, Moisture, and Ammonium Nitrogen ($N\text{-}NH_4^+$), for two different biogas plants (BG01 and BG02) and their respective additives (Table 2).

Before supplement addition, the BG02 biogas plant had a slightly alkaline environment (pH 7.9) with FOS concentration of 4451 mg (CH_3COOH)/L and TAC concentration of 14377 mg($CaCO_3$)/L. The FOS/TAC ratio was 0.31, indicating stability (Table 2). TS content was 9.44%, VS content was 7.01%, and various components were analyzed (Figure 1B). The theoretical gas yield of LD was 35.5 L (Biogas)/kg with 62.5% methane showing that recirculation of LD could be applied since there was excess of VFAs that could produce more energy.

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Table 2. Physicochemical parameters measured in two Biogas stations (BG01 and BG02) during the Evogen Biogas additive administration.

Biogas Plant	Sampling Day		pH (μoph)	FOS (mgc/l)	TAC ($mgCaCO_3$)	FOS/TAC ratio	TS (%)	VS (%)	NFE (%DM)	Fats (%)	Proteins (%)	Fibers (%OM)	Theoritical gas yield (L/kg)	Methane (%)	N-NH4 (mgN/L)
BG01 (D1)	Day 0		8.10	2321	10592	0.22	9.36	7.37	-	-	-	-	-	-	2608
	Day 15		7.70	2562	10595	0.24	8.91	7.10	-	-	-	-	-	-	1547
	Day 40		7.40	2498	10408	0.24	8.66	6.93	-	-	-	-	-	-	3156
BG01 (D2)	Day 0		7.70	4432	9533	0.47	7.60	5.49	-	-	-	-	-	-	944
	Day 15		7.70	4342	6348	0.68	7.50	5.31	-	-	-	-	-	-	1079
	Day 40		8.50	3724	11748	0.32	7.47	5.09	-	-	-	-	-	-	2045
BG02 (D1)	Day 0	Before	7.90	4451	14377	0.31	9.44	7.01	14.80	0.56	3.89	1.16	37.00	62.30	2609
	Day 15	Evogen	8.10	2368	12203	0.19	8.56	6.10	4.95	0.51	3.75	1.41	30.20	63.90	1559
	Day 30	During	8.10	2689	14344	0.19	8.57	6.09	-	-	-	-	-	-	3044
	Day 40	Evogen	8.20	2985	14270	0.21	8.58	6.24	8.10	0.32	3.62	1.61	30.70	62.50	2861

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Figure 1. Total Solids, Volatile Solids and Total Biogas in the two biogas plants during the Evogen Biogas Additive administration. The total biogas is been demonstrated as a grey line with a secondary versital axis.

During additive application, pH increased slightly (8.2) and FOS concentration decreased to 2689 mg/L, while TAC remained at 14344 mg/L. The FOS/TAC ratio slightly decreased to 0.19. TS content decreased to 8.57% and VS content to 6.09 %, while the total biogas was increased after the additive administration (Figure 1B).

The BG01 (D1) biogas plant had also a slightly alkaline environment (pH 7.9) with FOS concentration of 2321 mg/L and TAC of 10592 mg/L (Table 2). TS and VS slightly decreased from 9.36 to 8.66 % in contrast with an increase at the production of biogas (Figure 1A) due to the higher hydrolysis of biomass and further production of biogas.

The analysis of biogas plant parameters and additives provided valuable insights into their performance and efficiency. The BG02 biogas plant exhibited changes in pH, FOS, TAC, and other parameters after the introduction of the additive with an increased biogas production with less total solids. BG01 (D1) biogas plant showed variations in the measured parameters during the continuous administration of Evogen. These findings highlight the potential effects of additives on biogas production and composition, emphasizing the importance of careful selection and optimization [26].

Additionally, the analysis of organic acid concentrations revealed significant variations under different conditions. Acetic acid and its equivalents were analyzed using GC-FID. The concentrations of acetic acid and its equivalent were expressed in parts per million (ppm). In the “BG02” condition, the concentration acetic acid equivalent slightly decreased during the additive application period (Figure 2B).

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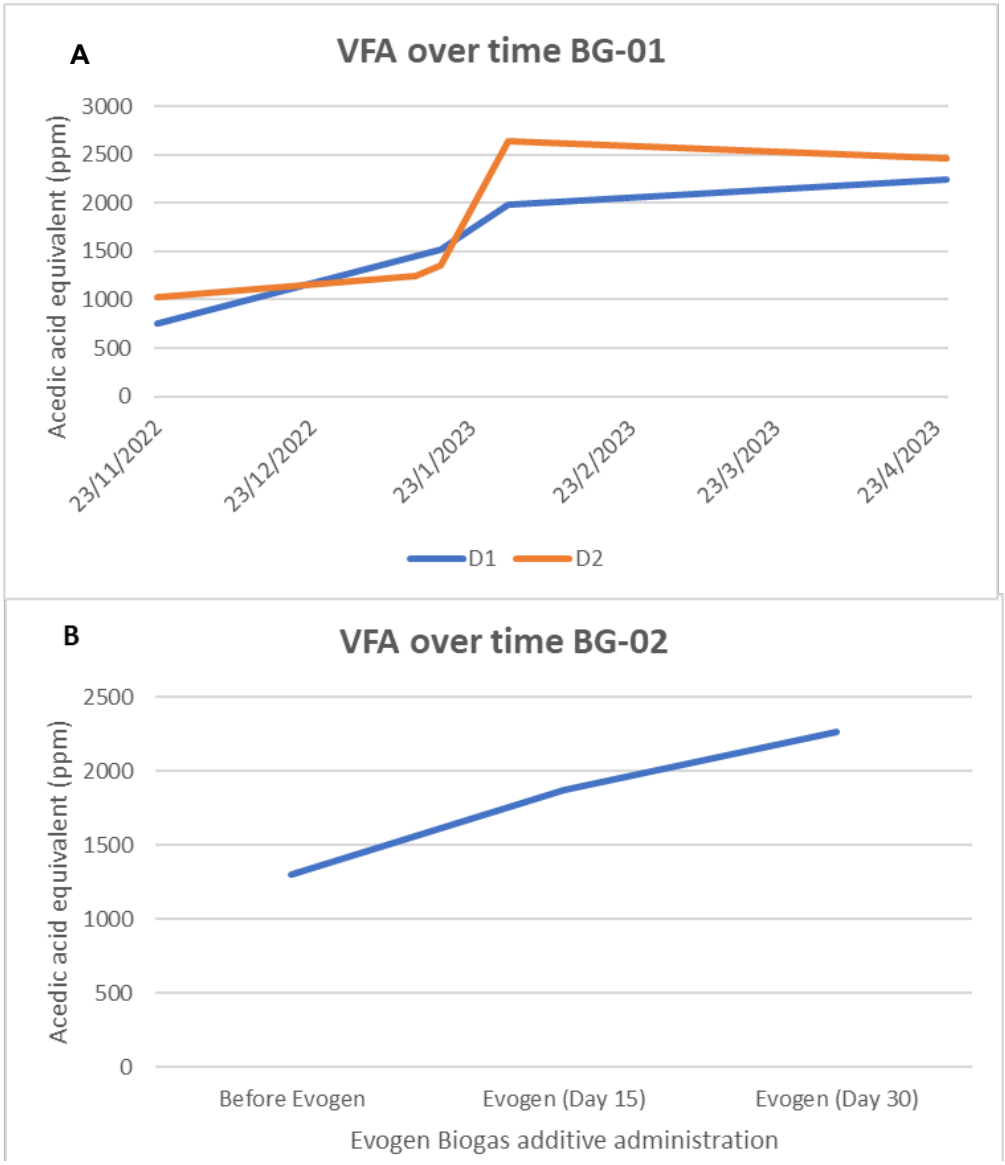

Figure 2. Acedic acid equivalent concentrations in the two biogas stations, A) the two digesters of BG01 are been demonstrated; with orange the D1 and with blue line the D2 (secondary vertical axis), B) the acedic acid equivalent concentration in BG02.

Comparing the "BG02" results with the "BG01 (D1)" conditions, it was observed that the concentrations of acetic acid and its equivalents were significantly lower in the "BG01 (D1)" samples (Figure 2A). The acetic acid concentration in "BG01 (D1)" was 583 ppm, while the acetic acid equivalent concentration remained the same at 583 ppm, indicating a decrease compared to the initial "BG02" condition. The latter was an indication that the biomass within the D1 could be decomposed further in VFAs showing that there was more biogas potential than it was produced.

In contrast, the "BG01 (D2)" samples exhibited much higher concentrations of acetic acid and its equivalents. The acetic acid equivalent concentration in "BG01 (D2)" was 1054 ppm, and that was due to the easily degradable feedstock fed to D2 which requires less HRT in order to be decomposed (Figure 2A).

Overall, this analysis provides insights into the concentrations of acetic acid and its equivalents, as well as other organic acids, under different conditions. The significant variations observed between the "BG02" and "BG01 (D2)" samples highlight the impact of additives on the concentrations of these organic acids, which can have implications for various applications in industries such as food, fermentation, and biochemistry [26].

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In conclusion, there was an improvement in the total biogas production for both biogas plants (BG01 and BG02). BG01 proved to have a big improvement in D1 where the feedstock needed more HRT in order to be decomposed by hydrolytic bacteria speeding up the rest metabolic pathways for biogas production. This occurred in the D2 too but since the rate of decomposition was already high, the difference before and after the application of the supplement by means of biogas production was not so significant. D2 in in this plant was already operating in its maximum potential and in a critical FOS/TAC ratio. Though, a small increase in biogas production was recorded followed by a small decrease in daily feedstock, as it is depicted in the TS and VS contents too. On the other hand, for BG02 a slight improvement was noticed in total biogas production but still it was not so significant since the plant was in a "recovery mode" from a previous inhibition incident. After rough estimations we calculated an increase of +9 % for BG02 and +16% for BG01 based on reduction in average daily feedstock intake.

In order to get more reliable results, this study will be continued, and the period of Evogen Biogas Additive effect will be increased for 6 to 12 months (monitoring period) while the steady state of 2 months of the biogas plants will be a pre-requisite for the supplement introduction. Feedstock variations should be avoided so that the daily intake reduction could be recorded in a reliable way.

3.2. Microbiome Alternation during additive administration

The biotechnology behind this additive is the combination of a novel mineral carrier and selected *Bacillus* strains. The vector acts in a multifaceted manner ultimately enhancing methanogenesis. The pores within the surface of the carrier allow for deep colonization, providing an extra layer of protection to microbes. Thus, they are more tolerant to pH changes and exposure to inhibitory compounds, such as ammonia. The carrier surface acts as an ion exchanger by facilitating electron transfer and absorbs compounds, such as ammonia and hydrogen sulphide, reducing their inhibitory effect on the system. Bacilli have been selected because of their diverse metabolic capacity and their ability to operate over a range of pH and temperature values. The ability of Bacilli to secrete hydrolytic enzymes under anaerobic conditions enhances the degradation of feed polymeric compounds, such as proteins, polysaccharides and fats [27]. In this way, complex organic compounds are converted into simpler and bioavailable compounds for further degradation to final methane production. Finally, the ability to form resistant *Bacillus* spores ensures that they will only germinate when the right conditions allow them to do so, providing long-term stability and specificity [26].

The results obtained from the 16S rRNA microbiome analysis of the samples collected at three timepoints (on day 0, 15, and 30) during operation with Evogen administration revealed valuable insights into the microbial composition and dynamics in the biogas digester BG02. The dominance of the phylum Firmicutes (64.2% to 58.3%) throughout the experiment indicated its significant role in biogas production (Figures 3 and 4). This phylum comprises members known for their involvement in the degradation of various substrates, such as proteins and polysaccharides, leading to the generation of acetate and propionate [28]. Furthermore, Firmicutes bacteria have been found to establish syntrophic relationships with acetoclastic methanogens, facilitating the overall methanogenic process [29].

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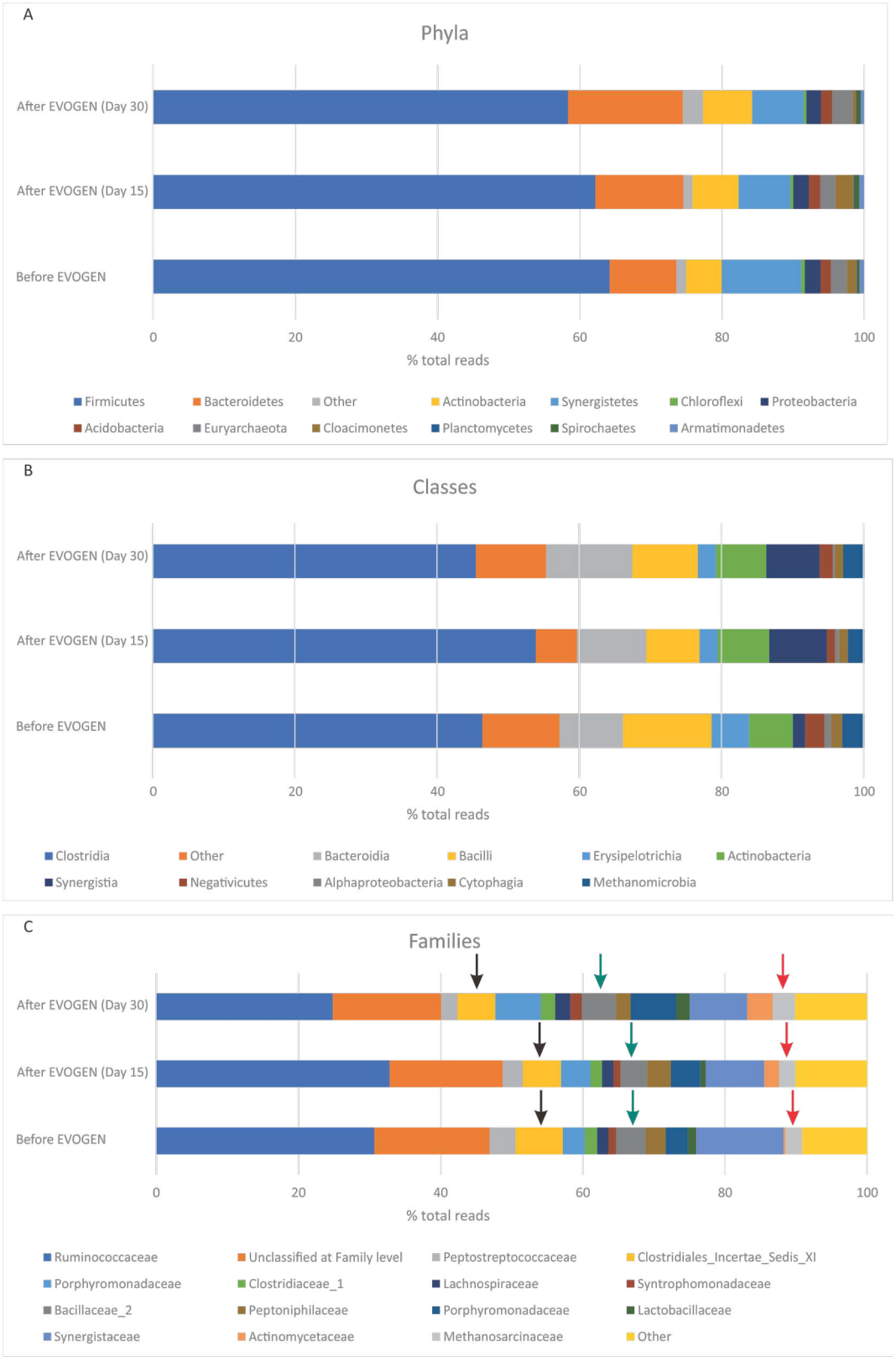

Figure 3. Abundance of the 16S rRNA operational taxonomic units in BG02 over Evogen Biogass additive administration classified in Phyla, Classes and Families ranks. Arrows are indicating hydrolising (black and green) and methanogen (red) bacteria families.

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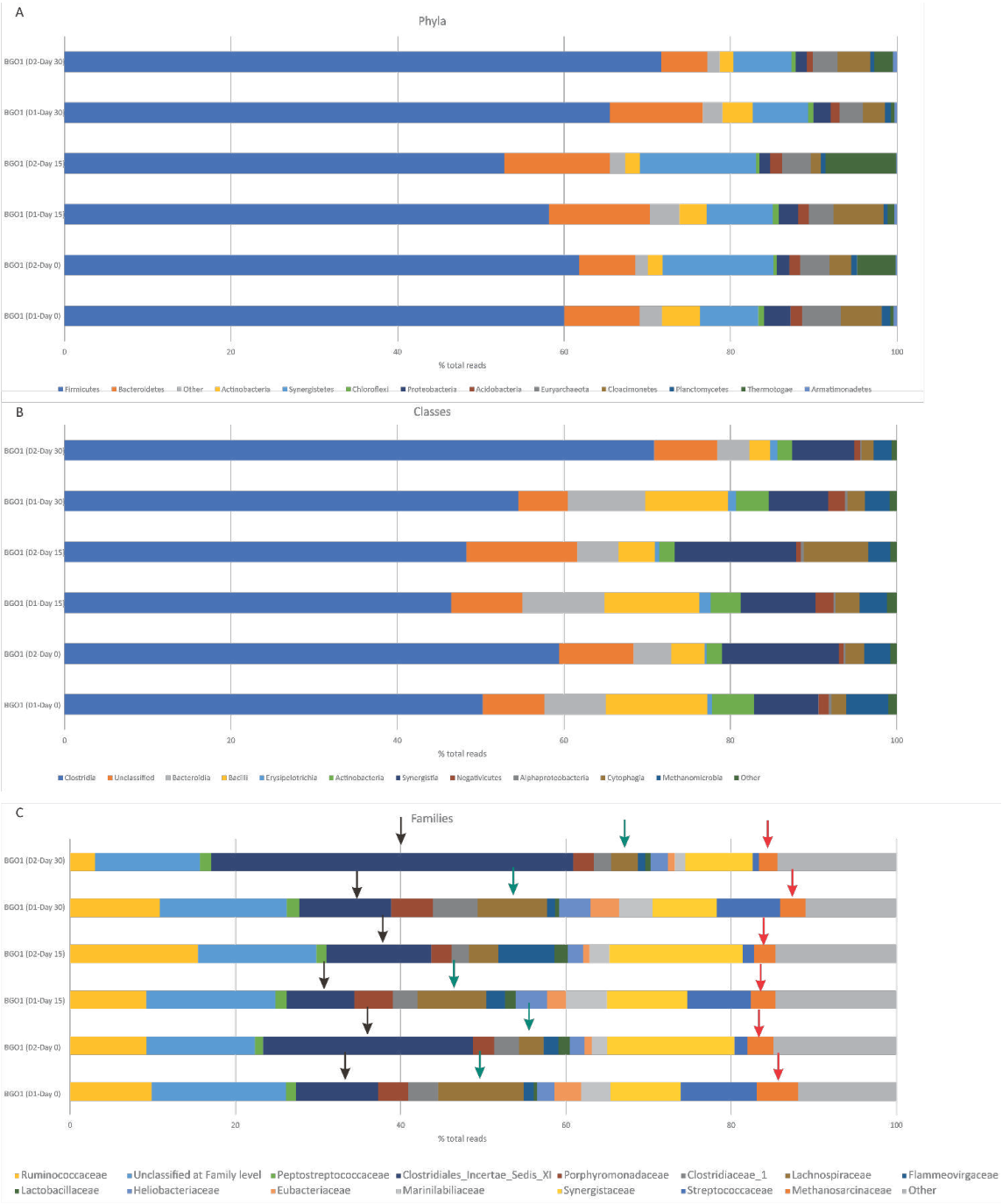

Figure 4. Abundance of the 16S rRNA operational taxonomic units in BG01 (D1 and D2) over Evogen Biogass additive administration classified in Phyla, Classes and Families ranks. Arrows are indicating hydrolising (black and green) and methanogen (red) bacteria families.

Another abundant taxon identified in the digester were Synergistetes (11.2% to 7.3%), which exhibited a high abundance at the beginning of the experiment but decreased on day 15, remaining relatively stable thereafter. Synergistetes are known for their ability to ferment long-chain and monocarboxylic fatty acids, producing acetate, H_2, and CO_2 [30] (Figure 3A). This metabolic activity contributes to the pool of substrates available for methanogenesis in the digester.

Proteolytic Bacteroidetes displayed an increasing trend (9.4% to 16.1%) throughout the experiment, indicating their involvement in protein degradation and subsequent biogas production (Figure 3A). These bacteria possess the capability to break down proteins into amino acids, which can be further metabolized into volatile fatty acids (VFA) and subsequently utilized by methanogens [30].

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Actinobacteria (5% to 6.9%) also exhibited an initial increase in abundance, followed by a relatively stable presence. Actinobacteria primarily function as acidogenic microorganisms, contributing to the accumulation of volatile fatty acids (VFA) in the digester (Figure 3A). Additionally, they have the ability to inhibit the growth of methanogenic bacteria, potentially affecting the overall biogas production [30].

At the family level, Ruminococcaceae from the class Clostridia was the most enriched family in the digester but showed a decrease from 30.6% to 24.8% after the extensive use of Evogen (Figure 3B). Ruminococcaceae bacteria are known for their hydrolytic and acidogenic functions, facilitating the breakdown of complex substrates (2). The decrease in their abundance may indicate a shift in the metabolic dynamics of the digester following the introduction of Evogen.

Similarly, the family Synergistaceae (Synergistia) exhibited a decreasing trend, from 12.3% to 8.1%, suggesting a potential impact of Evogen on their population dynamics (Figure 3B). Synergistaceae bacteria have been associated with various mechanisms that can influence different phases of the production process [31].

On the other hand, Porphyromonadaceae (Bacteroidia) and Actinomycetaceae (Actinobacteria) showed an increase in abundance from 3.1% to 6.4% and from 0.2% to 3.6%, respectively (Figure 3B). Porphyromonadaceae bacteria are known as important fiber-digesting microorganisms, capable of enhancing the anaerobic digestion of lignocellulosic biomass. The observed increase in their abundance may be associated with the presence of lignocellulosic matter in blackwater-fed reactors [32]. Actinomycetaceae, on the other hand, may contribute to acidogenesis in the digester, aiding in VFA production [28].

At the genus level, *Oscillibacter* and *Clostridium_IV* were the dominant genera throughout the entire experimental period, although their abundances decreased from 13.3% to 11.9% and from 12.2% to 5.6%, respectively. *Oscillibacter* has been widely identified in cow manures and has been linked to the enhancement of the hydrogen reduction CO_2 pathway [33,34]. The positive correlation between the abundance of *Oscillibacter* and the H_2 flux suggests its potential contribution to the CO_2 reduction by hydrogen, ultimately leading to methane production.

Another notable genus, *Proteiniphilum*, displayed an upward trend from 2.9% to 5.1% throughout the experiment. The final production of CH_4 flux was significantly correlated with the abundance of *Proteiniphilum*. Conversely, the H_2 flux showed a negative correlation with the abundance of *Proteiniphilum* but a positive correlation with the abundance of *Oscillibacter*. *Proteiniphilum* has been found to produce acetate from proteins, and their interaction with acetate methanogens has been shown to promote methane recovery in digesters [34].

The methanogenic community in the digester was primarily composed of the genus *Methanosarcina*, belonging to the class Methanomicrobia. *Methanosarcina* species are known for their versatility in utilizing various substrates, including acetate, methanol, and methylamines, to produce methane [35] (Figure 3C). The presence of *Methanosarcina* in the digester indicates their essential role in the final step of biogas production, converting the accumulated substrates into methane gas [36].

In summary, the analysis of the microbial community dynamics in the biogas digester BG02 before and after the introduction of Evogen revealed several significant findings. The dominance of *Firmicutes*, along with the presence of Synergistetes, Proteolytic Bacteroidetes, and Actinobacteria, highlighted their roles in substrate degradation and VFA production. The changes observed in the abundance of specific families and genera, such as Ruminococcaceae, Synergistaceae, Porphyromonadaceae, and Actinomycetaceae, suggest potential impacts of Evogen on the microbial community composition. Moreover, the correlations observed between the abundance of *Oscillibacter* and *Proteiniphilum*, and the flux of methane and hydrogen provide insights into the complex interactions occurring within the microbial consortium during biogas production. Further studies are warranted to elucidate the specific mechanisms underlying these interactions and the effects of Evogen on the microbial dynamics in biogas digesters.

Additionally, the investigation focused on the microbial community dynamics in biogas production during the continuous addition of the additive Evogen in two biogas plants BG01 (D1 and D2) and BG02. By introducing this additive, due to higher hydrolysis rate which takes place

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mainly in the primary digester of a biogas plant, the required HRT for biomass decomposition becomes lesser, providing VFAs and subsequently acetic acid for methane production.

The results from the microbial analysis revealed interesting dynamics within the microbial community throughout the experiment. Firmicutes, specifically the dominant phylum, consistently accounted for a substantial portion of the bacterial community, ranging from 60% to 71%. Firmicutes are known for their ability to degrade various substrates, generating important intermediates for methanogenesis (Figure 4A). This finding aligns with previous studies highlighting the abundance of Firmicutes in anaerobic digestion processes. The persistence of Firmicutes throughout the experiment suggests their significant role in biogas production.

Bacteroidetes, another prominent phylum associated with anaerobic digestion, exhibited varying abundances ranging from 4.8% to 12.1%. These fiber-digesting bacteria contribute to the breakdown of lignocellulosic biomass (Figure 4A). The fluctuations observed in the abundance of Bacteroidetes may indicate changes in substrate availability or microbial interactions influenced by the addition of Evogen. At lower abundances, Actinobacteria, Synergistetes, and Proteobacteria were also detected in the microbial community. Actinobacteria are primarily involved in acidogenesis and can produce volatile fatty acids (VFA), while Synergistetes are known for their fermentative abilities. Proteobacteria represent a diverse group with various metabolic capabilities. Their presence suggests their involvement in the overall microbial community dynamics, but further investigations are needed to elucidate their specific roles in biogas production processes.

The analysis at the class, family, and genus levels further supported the findings. Clostridia, a dominant class within the phylum Firmicutes, consistently dominated the bacterial community throughout the experimental period (Figure 4B). This class is renowned for its ability to degrade complex organic matter and produce volatile fatty acids (VFAs) during anaerobic digestion. Other classes, families, and genera detected in the microbial community displayed varying abundances and metabolic functions, contributing to the complexity of the biogas production process.

Overall, the results demonstrate that the addition of Evogen influences the microbial community composition and dynamics in biogas production. The observed changes in the abundances of different microbial taxa indicate potential alterations in substrate utilization, metabolic interactions, and overall microbial community structure. The dominance of Firmicutes and Bacteroidetes highlights their importance in substrate degradation and biogas production. However, further studies are required to fully understand the specific mechanisms by which Evogen affects the microbial community dynamics and subsequent biogas production.

In conclusion, the investigation provides valuable insights into the microbial community dynamics during biogas production with the continuous addition of Evogen. The findings contribute to our understanding of the roles played by different microbial taxa at various taxonomic levels, shedding light on the complex interactions and processes involved in biogas production.

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