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Isolation and Identification of Some Bacteria Associated with Biogas Production from Food Waste and Rumen Content

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ABSTRACT

The goal of this study was to investigate the bacterial community associated with biogas production from food waste and rumen content. Anaerobic mono-digestion and co-digestion were performed using mixture design within Design Expert, resulting in 100 experimental runs. Parameters such as food waste and rumen content, water content, temperature, pH, number of digester agitation per day and retention time were varied during the anaerobic digestion processes. Classical microbiological techniques were used to isolate and identify strict anaerobic and facultative anaerobic bacteria from the food waste and rumen content before and after anaerobic digestion. Sixteen bacterial species belonging to 12 different generae were isolated and identified from the food waste, rumen content and composite digestates obtained from the 100 bio-digesters. These generae included *Acetobacterium, Bacteroides, Clostridium, Enterobacter, Escherichia, Lactobacillus, Pseudomonas, Ruminococcus, Staphylococcus, Streptococcus, Syntrophomonas* and *Syntrophobacter*. With the exception of *Escherichia coli* and *Pseudomonas* sp., all other bacterial species identified in the substrates were also found in samples of the composite digestates, suggesting that they may have played important roles in the anaerobic digestion process inside the 100 bio-digesters. Overall, this study provides valuable insights into the microbial community structure and function during biogas production from food waste and rumen content. The results could contribute to the development of more efficient and sustainable biogas production processes.

INTRODUCTION

Biogas production from food waste and rumen content is a promising renewable energy source that reduces environmental pollution and provides sustainable energy. However, the process is dependent on microbial activity and therefore, the isolation and identification of bacteria associated with biogas production is essential. Several studies have isolated and identified various types of bacteria species associated with biogas production organic substrates including food waste and rumen content. For example, a recent study by Li et al. (2021) found that *Lactobacillus* sp. played an important role in acidogenesis and acetogenesis during biogas production from food waste. Another study by Zhang et al. (2020) found that the *Clostridium* sp. was responsible for the degradation of cellulose and hemicellulose during biogas production from rumen content of a cow. Ma et al. (2020) found that *Methanobrevibacter* sp. was responsible for the production of methane gas during biogas production from food waste. Yuan et al. (2021) showed that *Acetobacterium* sp. was responsible for the production of acetate during

biogas production from food waste. A study by Chen et al. (2021) found that *Lactobacillus* sp., *Clostridium* sp. and *Methanobacterium* sp. were the dominant bacterial species associated with biogas production from food waste that was co-digested with chicken manure. Furthermore, Cui et al. (2020) showed that *Clostridium* sp. was the dominant bacterial species, while *Lactobacillus* sp., *Methanobacterium* sp. and *Methanosarcina* sp. were also present.

Han et al. (2021) investigated the microbial community structure during anaerobic digestion of corn straw for biogas production and found that *Lactobacillus* sp., *Clostridium* sp., and *Methanobacterium* sp. were the dominant bacterial species, while *Methanoculleus* sp. and *Methanosarcina* sp. were the dominant methanogenic species. Additionally, recent advances in metagenomic and transcriptomic sequencing technologies have facilitated the identification of previously unknown and uncultivable bacterial species associated with biogas production. For example, a study by Ju et al. (2021) used metagenomic sequencing to identify a novel bacterium, *Candidatus Cloacamonas acidaminovorans*, which plays an important role in protein degradation and amino acid metabolism during biogas production from food waste. Moreover, the study of the microbial community structure and function during biogas production from food waste and rumen content has implications for the optimization of the biogas production process. For example, a study by Wu et al. (2020) found that the microbial community structure and function in the anaerobic digestion of food waste varied with different operating conditions, such as temperature, pH, and substrate concentration. Therefore, understanding the microbial community dynamics and their responses to different conditions is important for the development of strategies to optimize biogas production.

Furthermore, understanding the interactions between different bacterial species in the microbial community during biogas production is essential for improving the efficiency and stability of the process. For example, a study by Wu et al. (2022) found that the co-cultivation of acetogens and methanogens increased the production of methane and improved the stability of the biogas production process. Moreover, the use of microbial additives, such as bacteria or enzymes, to enhance the biogas production process has gained attention in recent years. A study by Ma et al. (2021) found that the addition of *Bacillus* sp. *B4* increased the production of biogas and improved the degradation of organic matter in food waste. In addition, the use of microbial electrochemical technologies (METs) for biogas production has also been explored. A study by Zhu et al. (2021) found that microbial electrochemical system increased the production of methane by promoting the growth of methanogenic bacteria and improving the electron transfer efficiency in the microbial community.

Overall, further research is needed to fully understand the microbial ecology of biogas production and to develop technologies for improving the efficiency and stability of the biogas production process. This study aimed to investigate the bacterial community associated with biogas production from food waste and rumen content.

METHODS

Collection of the Substrates

Cattle rumen content (RC), also used as the main source of microbial inoculum for the anaerobic digestion process, was collected from the Dutse Central Abattoir in Dutse, Jigawa State of Nigeria. After collection, the rumen content was immediately transported to the experimental site in an air-tight, non-transparent 60 L-capacity plastic container. Food waste, which included cooked rice, cooked beans, cooked rice powder meal (Tuwo-Shinkafa), cooked corn powder meal (Tuwo-Masara), boiled yam, waste bread, wasted bean cake (Akara), and wasted rice and corn cakes (Masa), were collected at their source of generation within Dutse metropolis. After collection, the food wastes were immediately taken to the experimental site, where they were pooled and blended together (with the help of an electric mixer) to form the homogenized food waste that was used for the biogas production process. After collecting the substrates, both anaerobic and facultative anaerobic bacteria were isolated, characterized, and identified using classical microbiological techniques.

Design of the Experiment

Food waste and rumen content were subjected to anaerobic mono-digestion and co-digestion using mixture design (Combined I-optimal) within Design

Expert (version 13) environment, which generated a total of 100 experimental runs. The anaerobic digestion processes were conducted using a range of parameters such as food waste (0 – 1 kg), rumen content (0 – 1 kg), water content (0 – 1 kg), temperature (28 – 45^{o}C), pH (5 - 9), number digester agitation/day (0 – 6 times/day) and retention time (15 – 40 days).

Anaerobic Digester Specification and Set-up

The study used a batch-type, 2L-capacity plastic anaerobic digester in each of the experimental runs. The digester was equipped with a biogas cleaning system consisting of 0.3L-capacity CO_2, H_2S, and H_2O vapor removal units, each with a 300mL-capacity gas measuring syringe to determine the volume of biogas generated at each stage of the biogas cleaning process (Smith et al., 2020).

Operation of the Anaerobic Digesters

The temperature of the bio-digester was monitored and regulated using a digital thermostat with a temperature probe inserted in a water bath in which the bio-digester was placed (Sulaiman et al., 2020), while the pH was measured and monitored using a digital pH meter with a probe and adjusted using either hydrochloric acid or potassium hydroxide to maintain its stability for a given experimental run (Montalvo-Rodriguez, et al., 2022). The bio-digesters were manually agitated a number of times per day to stimulate the mixing of their contents (Zhang et al., 2021). After the bio-digestion process, the digestate generated inside the 100 bio-digesters was pooled together to form composite digestates. Samples of the composite digestates were then collected to determine the presence and distribution of strict anaerobic and facultative anaerobic bacterial species in the digestates.

Isolation of Bacteria Species

To isolate both strict anaerobic and facultative anaerobic bacteria from the food waste, rumen content, and composite digestates, one (1g) from each of them was suspended in 9mL of sterile distilled water contained in three separate test tubes, with thorough mixing. After this, facultative and anaerobic bacterial species were isolated from the food waste, rumen content, and composite digestates via the agar roll-tube spreading technique and incubated at 30 °C for 2 - 7 days (Singh et al., 2015). After incubation, the isolated colonies were sub-cultured in pre-reduced agar slants and incubated for 2 - 7 days at 30 °C for short-term preservation at 4 °C.

Preparation of Culture Media

The culture media employed to isolate both strict anaerobic and facultative anaerobic bacteria from the substrates and composite digestates samples were adapted from Ogbonna et al. (2018). The culture medium for strict anaerobic bacteria was composed of the following in 1L-capacity Erlenmeyer flasks with butyl rubber corks: 1L of sterile distilled water, 1.0g of NH_4Cl, 2.0g of NaCl, 5.0g of $NaHCO_3$, 0.3g of KH_2PO_4, 0.3g of K_2HPO_4, 0.16g of $MgCl_2.6H_2O$, 0.01g of $CaCl_2.2H_2O$, 12.5mL of oligo element solution, 2.0g of yeast extract, 1.0mL of resazurin solution (1% w/v), 5.5g of D-glucose, 3.0g of sodium acetate, 0.5g of sodium thioglycolate and 15g of agar, final pH was 7.2. The same medium was used to isolate the facultative anaerobes except for the use 6g of agar instead of the 15g that was used the strict anaerobic culture medium (Ogbonna et al., 2018).

Characterization and Identification of Bacteria Species

The bacterial isolates were identified using morphological and metabolic/biochemical tests based on Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and Bergey's Manual of Systemics of Archaea and Bacteria (Whitman et al., 2012). These bacteriological characterization tests conducted include Gram staining, acid-fast staining, motility test, oxygen requirement test, oxidase test, catalase test, coagulase test, citrate test, indole test, urease test, hydrogen sulfide production, nitrate reduction, Methyl red test, Voges Proskauer test, ornithine decarboxylase test, as well as fermentation tests involving glucose, mannitol, sucrose, lactose, maltose, xylose, arabinose, salicin, cellobiose, mannose, melezitose, raffinose, sorbitol, trehalose, glycerol, and cellulose hydrolysis, starch hydrolysis, gelatin hydrolysis, and esculin hydrolysis (Barrow and Feltham, 1993).

RESULTS AND DISCUSSION

Biochemical Characteristics of Bacterial Isolates

According to the result of biochemical characterization, a total of sixteen (16) bacterial species classified under twelve (12) generae were isolated and identified in the food waste, rumen

content, and composite digestates. These bacterial species include *Acetobacterium* sp., *Bacteroides* sp., *two Clostridium* sp., *Enterobacter* sp., *Escherichia coli, two Lactobacillus* sp., *two Pseudomonas* sp., *Ruminococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Syntrophomonas* sp. and *Syntrophobacter* sp. as shown in Table 1, Table 2 and Table 3 respectively.

Table 1. Biochemical characteristics of bacteria isolated during Lab-scale AD study

Biochemical Tests	ISO 1	ISO 2	ISO 3	ISO 4	ISO 5	ISO 6
Gram stain	-	-	+	+	-	-
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Arrangement	Single	Single	Single	Single	Single	Single
Spore	+	-	+	+	-	-
Acid fast	-	-	-	-	-	-
Motility	+	+	+	-	+	+
O_2 requirement	FA	OA	OA	OA	FA	FA
Oxidase	+	-	-	-	-	-
Coagulase	-	-	-	-	-	-
Citrate	+	-	-	-	-	+
Catalase	+	-	+	-	+	+
Indole	+	-	-	-	+	-
Urease	+	+	-	-	-	-
H_2S Production	+	+	-	-	-	-
Nitrate red.	+	-	-	-	+	+
Methyl red	-	+	+	+	+	-
Voges Proskauer	-	-	-	-	-	+
Ornithinedecarboxylase	-	-	-	-	+	+
D-glucose	+/+	+/+	+/+	+/+	+/+	+/+
D-mannitol	+/+	+/+	-	+/+	+/+	+/+
D-sucrose	+/+	+/+	+/+	+/+	+/+	+/+
Lactose	-	+/+	+/+	+/+	+/+	+/+
D-maltose	+/+	+/+	+/+	+/+	+/+	+/+
D-xylose	-	+/+	+/+	+/+	+/+	-
L-arabinose	-	+/+	+/+	+/+	+/+	+/+
Salicin	-	+/+	+/+	+/+	+/+	+/+
Cellulose	-	-	+/+	+/+	-	-
Starch	+/+	+/+	+/-	+/-	-	-
Gelatin	+	-	-	+/-	-	-
Esculin	-	+/-	+/-	-	+/-	-
Glycerol		-	-	+/+	+/+	-
D-cellobiose	-	-	+/+	+/+	-	+/+
D-mannose	-	+/+	+/+	+/+	+/+	-
D-melezitose	-	-	-	-	-	-
D-raffinose	-	+/+	-	+/+	-	+/+
D-sorbitol	-	-	-	+/+	-	-
L-rhamnose	-	+/+	-	+/+	+/+	+/+
D-trehalose	-	-	-	+/+	-	-
Probably identify	*Acetobacterium* sp.	*Bacteroides* sp.	*Clostridium* sp.	*Clostridium* sp.	*Escherichia coli*	*Enterobacter* sp.

OA = Obligate anaerobe, FA = Facultative anaerobe, +/+ = Acid and gas production; +/- = Acid production without gas production, - = No fermentation

Table 2. Biochemical characteristics of bacteria isolated during Lab-scale AD study

Biochemical Tests	ISO 7	ISO 8	ISO 9	ISO 10	ISO 11	ISO 12
Gram stain	+	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Cocci	Cocci
Arrangement	Chain	Chain	Single	Single	Pair	Pair
Spore	-	-	-	-	-	-
Acid fast	-	-	-	-	-	-
Motility	+	+	+	+	-	-
O_2 requirement	OA	OA	FA	A	OA	OA
Oxidase	-	-	+	+	-	-
Coagulase	-	-	+	-	-	-
Citrate	+	-	+	+	-	-
Catalase	-	-	+	+	-	-
Indole	-	-	-	-	-	-
Urease	+	-	+	+	+	-
H_2S Production	-	+	-	-	+	-
Nitrate red.	-	-	+	-	-	-
Methyl red	-	-	-	-	-	-
Voges Proskauer	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-
D-glucose	+/+	+/+	-	+/-	+/+	+/+
D-mannitol	+/+	+/+	+/-	+/+	+/+	-
D-sucrose	+/+	+/+	-	-	+/+	+/+
Lactose	+/+	+/+	-	+/+	+/+	+/+
D-maltose	+/+	+/+	-	+/+	+/+	+/+
D-xylose	+/+	-	-	-	+/+	+/+
L-arabinose	+/+	+/+	-	-	+/+	+/+
Salicin	+/+	-	-	-	+/+	+/+
Cellulose	-	-	-	-	+/+	+/+
Starch	-	-	-	-	-	+/-
Gelatin	-	-	+/-	+/-	-	-
Esculin	-	-	+/-	-	+/-	+/-
Glycerol	-	-	+/+	-	-	-
D-cellobiose	+/+	+/+	-	-	+/+	+/+
D-mannose	-	+/+	-	-	+/+	+/+
D-melezitose	-	-	-	-	+/+	-
D-raffinose	-	-	-	-	+/+	+/+
D-sorbitol	+/+	-	-	-	+/-	+/+
L-rhamnose	-	+/+	-	-	+/+	+/+
D-trehalose	-	-	-	-	+/+	+/+
Probably identify	*Lactobacillus* sp.	*Lactobacillus* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	*Ruminococcus* sp.	*Ruminococcus* sp.

OA = Obligate anaerobe, A = Aerobe, FA = Facultative anaerobe, +/+ = Acid and gas production; +/- = Acid production without gas production, - = No fermentation

Table 3. Biochemical characteristics of bacteria isolated during Lab-scale AD study

Biochemical Tests	ISO 13	ISO 14	ISO 15	ISO 16
Gram stain	+	+	-	-
Shape	Cocci	Cocci	Rod	Rod
Arrangement	Cluster	Chain	Single	Single
Spore	-	-	-	-
Acid fast	-	-	-	-
Motility	+	-	+	-
O_2 requirement	FA	FA	OA	OA
Oxidase	+	+	-	-
Coagulase	+	+	-	-
Citrate	-	-	-	-
Catalase	+	-	-	-
Indole	-	-	-	-
Urease	+	+	-	-
H_2S Production	-	-	+	+
Nitrate red.	-	-	+	-
Methyl red	-	+	-	-
Voges Proskauer	-	+	-	-
Ornithine decarboxylase	-	-	-	-
D-glucose	+/+	+/+	+/-	+/+
D-mannitol	+/-	+/-	+/-	-
D-sucrose	+/+	+/+	+/-	-
Lactose	+/+	+/+	+/-	-
D-maltose	+/+	+/+	+/-	-
D-xylose	-	-	+/-	-
L-arabinose	-	+/+	-	-
Salicin	-	-	-	-
Cellulose	-	-	-	-
Starch	-	+/-	+/-	-
Gelatin	+/-	-	-	-
Esculin	+/-	+/-	-	-
Glycerol	-	-	-	-
D-cellobiose	-	-	+/-	-
D-mannose	-	+/+	-	-
D-melezitose	-	-	-	-
D-raffinose	-	+/+	-	-
D-sorbitol	-	-	-	-
L-rhamnose	-	-	-	-
D-trehalose	-	+/+	+/-	-
Probably identify	*Staphylococcus s*p.	*Streptococcus* sp.	*Syntrophomonas* sp.	*Syntrophobacter* sp.

OA = Obligate aerobe, OAN = Obligate anaerobe, FA = Facultative anaerobe, +/+ = Acid and gas production; +/- = Acid production without gas production, - = No fermentation

Bacterial Composition of the Food waste and Rumen content

Five (5) bacterial species belonging to four (4) generae were isolated from the food waste while eleven (11) bacterial species belonging to nine (9) generae were isolated from the rumen content as shown in Figure 1.

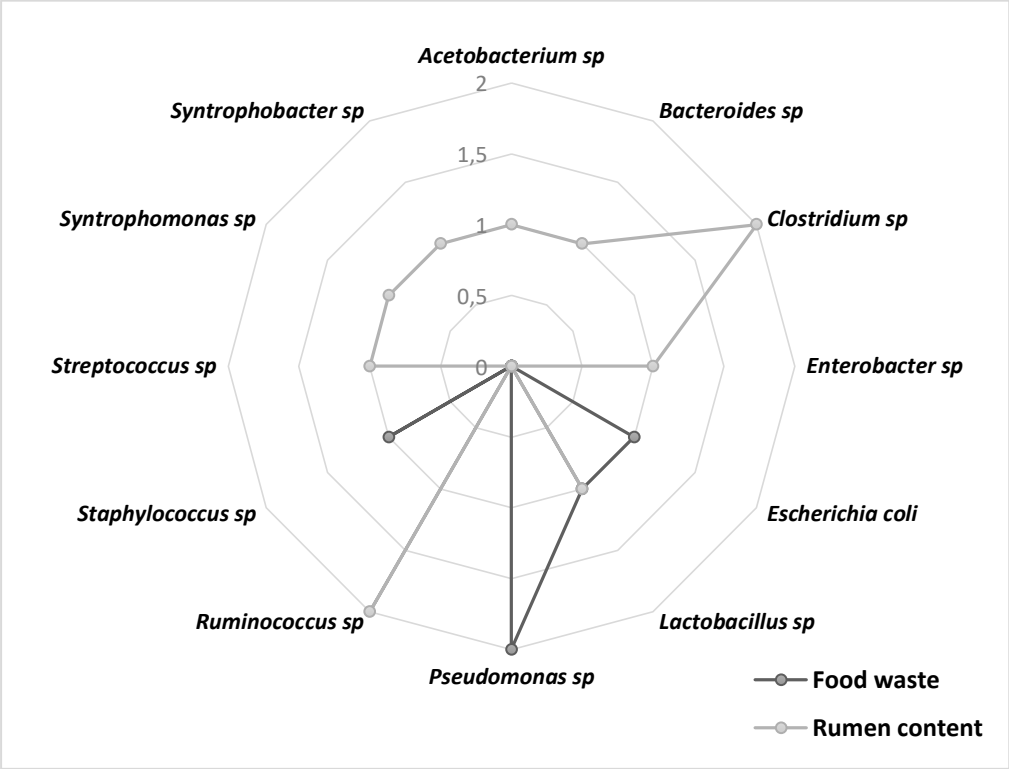

Figure 1. Bacterial species isolated from the food waste and rumen content

Bacterial Composition of the Composite Digestates

After the anaerobic digestion process inside the 100 bio-digesters, about fourteen (14) facultative anaerobic and strict anaerobic bacterial species were isolated and identified in samples of the composite digestate generated inside the bio-digesters as shown in Figure 2.

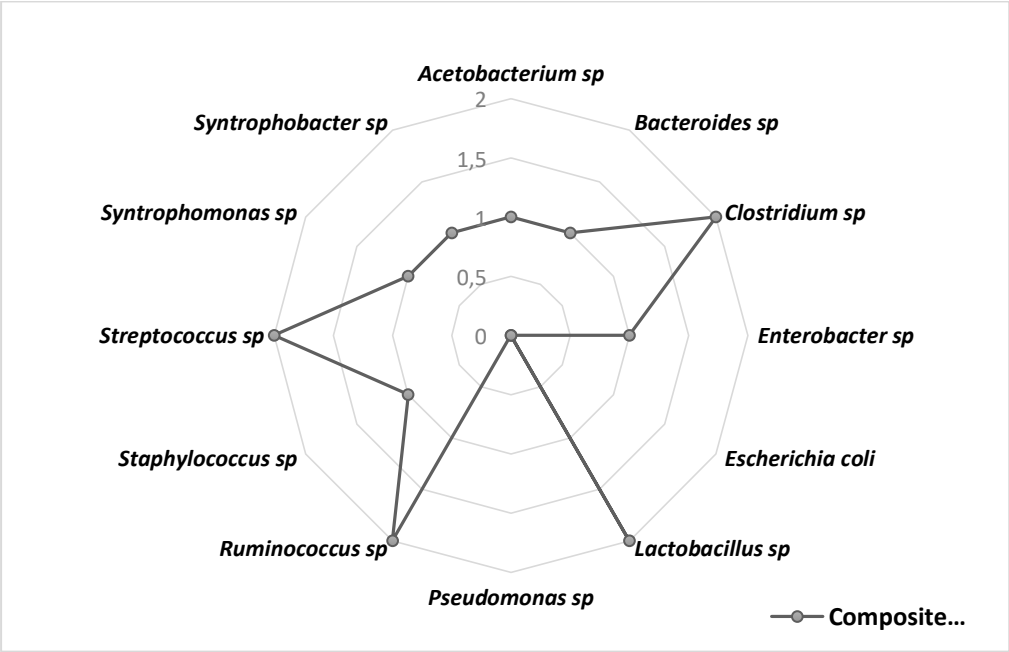

Figure 2. Bacterial species isolated from the composite digestates in the 100 bio-digesters

Table 4. Microorganisms involved in biogas production

Stage	Microorganisms
Hydrolysis	*Bacteriodes* sp.
Acidogenesis	*Enterobacter* sp.
	Escherichia coli
	Lacttobacillus sp.
	Pseudomonas sp.
	Ruminococcus sp.
	Staphylococcus sp.
	Streptococcus sp.
Acetogenesis	*Acetobacterum* sp.
	Clostridium sp.
	Enterobacter sp.
Methanogenesis	*Syntrophomonas* sp.
	Syntrophobacter sp.

A total of five (5), eleven (11), and fourteen (14) bacterial species were isolated and identified in the food waste, rumen content, and the composite digestates respectively. With the exception of *Escherichia coli* and two of the *Pseudomonas sp*, all other bacterial species identified in the substrates (such as the food waste and rumen content) were also found in samples of the composite digestates, suggesting that they may have played important roles in the anaerobic digestion process inside the 100 bio-digesters.

Acetobacterium sp. is involved in the production of biogas by converting alcohols and sugars to acetic acid, which is later converted to methane and CO_2 by methanogens (Zhang et al., 2019). *Bacteroides sp.* play a crucial role in the hydrolysis stage of anaerobic digestion, where they break down complex organic compounds into simpler compounds, such as proteins and polysaccharides, into simpler compounds, such as amino acids and sugars, which can be further degraded by other microbes in the system (Lu et al., 2021). *Clostridium sp.* is involved in the acetogenic stage of anaerobic digestion, converting acetate, H_2, and CO_2 into acetic acid and other volatile fatty acids, releasing additional H_2 and CO_2 (Chen et al., 2019). Methanogens then convert these acids into methane and CO_2. *Enterobacter sp.* is a facultative anaerobic bacteria that can play a role in acidogenic stage of anaerobic digestion, where it ferments simple organic compounds to produce organic acids, which are then converted by other bacteria such as *Clostridium sp*., and methanogens to biogas (Naveena et al., 2020). *Escherichia coli* can be present in biogas systems and can play a role in the acidogenic stage of anaerobic digestion (Mao et al., 2019). However, it is also a potential source of disease and foodborne illnesses, so it is important to monitor and control its populations in biogas systems (Kougias et al., 2020). *Lactobacillus sp.* play a role in the acidogenic stage of biogas production, where they ferment sugars and other organic compounds into lactic acid, which is then used by other bacteria, such as *Clostridium sp.* and methanogens, to produce biogas (Li et al., 2020).

Furthermore, *Pseudomonas sp.* is a bacteria commonly found in different environments. In biogas production, it can ferment sugars and simple organic compounds to produce acetic acid (Liu et al., 2019). *Ruminococcus sp.* play a role in the acidogenic stage of anaerobic digestion, where they ferment complex carbohydrates and other organic compounds into volatile fatty acids, such as acetic acid and propionic acid, which are then used by other bacteria, such as *Clostridium sp* and methanoges, to produce biogas (Yang et al., 2019). *Staphylococcus sp.* can play a role in the acidogenic stage of anaerobic digestion, where they can ferment sugars and other simple organic compounds into organic acids (Li et al., 2020). However, *Staphylococcus sp*. can also be a problem in biogas production as they are known to produce toxic compounds, such as hydrogen sulfide, which can be harmful to other bacteria in the system and reduce the overall efficiency of the biogas production process (Hao et al., 2019). *Streptococcus sp*. ferments sugars into lactic acid in the acidogenic stage of anaerobic digestion for biogas production,

but can also produce acetic acid that inhibits the methanogenic stage, reducing efficiency (Zheng et al., 2020). *Syntrophomonas sp*. breaks down complex organic compounds produced in the acidogenic stage into acetic acid and hydrogen, which methanogens use as a substrate for methane production, improving the overall efficiency of biogas production (Chen et al., 2021). Finally, *Syntrophobacter sp*. play a vital role in biogas production by breaking down organic compounds such as fatty acids and long-chain volatile fatty acids that are produced in the acidogenic stage into acetic acid and hydrogen, which are then used by methanogens, thereby increasing the efficiency of the process (Wang et al., 2021).

CONCLUSION

This study isolated and identified some bacterial species associated with biogas production from food waste and rumen content. The study found a total of 16 bacterial species belonging to 12 different generae, with the bacterial composition varying among the food waste, rumen content, and composite digestate generated after the bio-digestion process. The identified bacterial species play critical roles in different stages of anaerobic digestion and understanding their distribution and interactions is crucial for optimizing biogas production processes. The study also highlighted potential issues with certain bacterial species, such as *Escherichia coli*, *Staphylococcus sp*., and *Streptococcus sp*. that could negatively impact biogas production efficiency. Overall, the findings of this study provide valuable insights into the microbial community structure and function during biogas production and could contribute to the development of more efficient and sustainable biogas production processes.

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