**JOURNAL OF ENVIRONMENTAL SCIENCES AND BUILT ENVIRONMENT RESEARCH (JESBER)**
**2026, Vol. 1, No. 2, pp. 12–26**
**Published by Faculty of Environmental Sciences, University of Calabar, Nigeria**
**Print ISSN: 3141-5547 | OPEN ACCESS**

***Research article***

# TEMPORAL SUCCESSION OF CULTIVABLE BACTERIA, FUNGI AND PRESUMPTIVE METHANOGENS DURING MESOPHILIC ANAEROBIC DIGESTION OF FRUIT PEELS AND COW DUNG IN CALABAR, NIGERIA

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**ABSTRACT**

Anaerobic digestion can recover energy and nutrients from organic wastes, but the temporal behaviour of cultivable microorganisms determines both process performance and digestate safety. This study characterised bacterial, fungal and presumptive methanogenic succession during 45-day mesophilic batch digestion of banana, paw-paw and watermelon peels, cow dung, and their combinations in Calabar, Nigeria. Twelve 60-L digesters were monitored, and slurry samples were cultured at five-day intervals. Isolate-occurrence richness and Shannon diversity were calculated from the archived culture records. Bacterial occurrences declined from 67 on day 5 to one on day 45, while richness fell from 11 to one and Shannon diversity from 2.33 to 0.00. A Clostridium perfringens-like morphotype represented 26.70% of bacterial occurrences and remained detectable through day 45; enteric and opportunistic morphotypes, including Escherichia coli, Enterobacter aerogenes and Pseudomonas aeruginosa, were not detected after day 25. Fungal occurrences declined from 48 to zero by day 35, although yeast persisted through day 30. Presumptive methanogen occurrence peaked on day 30. The findings show strong ecological succession but incomplete hygienisation under mesophilic conditions. Digestate intended for food-crop production should therefore receive microbiological verification and, where necessary, post-treatment before field application.

**KEYWORDS**

anaerobic digestion; biofertiliser; cow dung; digestate biosafety; fruit waste; microbial succession; methanogens; pathogen persistence



## Introduction

Rapid urbanisation and population growth have increased the volume of biodegradable wastes generated in markets, households, food-processing centres and livestock facilities. In many Nigerian cities, fruit peels and animal manures are still discarded in open dumps, drains or uncontrolled heaps, where they create odour, attract vectors, obstruct drainage and contribute to greenhouse-gas emissions. Anaerobic digestion offers a circular alternative because it converts organic residues into biogas and a nutrient-containing digestate that may be returned to soil. The technology is particularly relevant to tropical cities where organic waste is abundant, commercial fertiliser is costly and energy access remains uneven.

Anaerobic digestion is not a single microbial reaction. It is a coordinated ecological process involving hydrolytic, fermentative, acidogenic and acetogenic bacteria, followed by methanogenic archaea. The organisms that dominate each phase depend on substrate composition, pH, temperature, retention time, inoculum history and the accumulation or removal of metabolic intermediates. Co-digestion is often used to balance nutrients and buffering capacity, dilute inhibitors and widen the range of biodegradable carbon sources. Fruit wastes supply readily fermentable carbohydrates, whereas cow dung contributes buffering compounds, minerals and an

established anaerobic inoculum. A well-balanced mixture can therefore support more stable conversion than a single acidic fruit substrate (Karki et al., 2021; Wang et al., 2022a; Zhang et al., 2024).

The microbial composition of digesters has practical importance beyond methane generation. Digestate is increasingly promoted as a biofertiliser, yet raw feedstocks may contain enteric, opportunistic or spore-forming microorganisms. Anaerobic digestion usually reduces many vegetative pathogens, but performance varies substantially across systems. Temperature is a major determinant of hygienisation, and mesophilic treatment may not eliminate resistant or spore-forming organisms. Recent evidence shows that Clostridiaceae can persist during digestion and that digestate safety cannot be inferred solely from stable pH, gas production or nutrient enrichment (Álvarez-Fraga et al., 2025; Franchitti et al., 2024; Pourcher et al., 2023).

The doctoral investigation from which the present analysis was developed evaluated banana peel, paw-paw peel, watermelon peel and cow dung as feedstocks for biogas and biofertiliser production. Reactor performance, nutrient transformation and the agronomic response of hot pepper from the broader experiment have already been reported (Akpan et al., 2019, 2020). However, the time-resolved culture records describing bacterial, fungal and presumptive methanogenic succession were not the focus of those publications. Re-analysing these records provides a useful tropical baseline for understanding the transition from a diverse feedstock-associated community to a narrower late-stage anaerobic community.

Regional research from southern Nigeria further shows that soil microbial status, nutrient balance and crop response are shaped by rhizosphere processes, parent material and organic or biological amendments. Studies of rhizosphere management, mycorrhizal inoculation, petroleum-affected soils, integrated nutrient management, algal biofertilisers and contrasting parent materials collectively demonstrate the need to verify amendment performance under local soil conditions (Akpan, Etukudoh, et al., 2013; Akpan, Udo, et al., 2013; Etukudo et al., 2014; Wemedo et al., 2015; Nwanyeze et al., 2019; Akpan, Njoku, et al., 2020; Akpan, Ubi, et al., 2020; Uko et al., 2020). This regional evidence supports evaluating digestate as both a microbial material and a soil amendment rather than assuming that waste conversion alone guarantees agronomic suitability.

Accordingly, this study aimed to: (i) describe temporal changes in cultivable bacterial and fungal occurrence during 45 days of mesophilic digestion; (ii) determine the persistence of selected morphotypes with potential functional or biosafety significance; (iii) describe the emergence of presumptive methanogenic morphotypes; and (iv) interpret the implications of the observed succession for low-cost digesters and agricultural reuse of digestate. The study contributes a culture-based, time-resolved perspective from locally fabricated batch digesters in a humid tropical environment, while clearly defining the limitations of phenotype-based identification.

## Literature review

### Anaerobic digestion as a microbial food web

Organic matter conversion in anaerobic digesters depends on metabolic cooperation among microorganisms with different physiological roles. Hydrolytic organisms release extracellular enzymes that convert polymers such as cellulose, hemicellulose, proteins and lipids into soluble monomers. Fermentative and acidogenic organisms transform these products into volatile fatty acids, alcohols, hydrogen and carbon dioxide. Syntrophic acetogens then oxidise reduced intermediates under low hydrogen partial pressure, while methanogenic archaea consume acetate, hydrogen, carbon dioxide or methylated compounds to form methane. The final stages are thermodynamically possible only when products are continuously removed by partner organisms, making interspecies exchange central to reactor stability (Wang et al., 2022b; Zhang et al., 2024). Culture-independent studies now reveal substantial functional redundancy and complex cross-feeding within this food web. Nevertheless, culture-based observations remain valuable in settings where sequencing infrastructure is unavailable, particularly for tracking viable indicator organisms and isolating strains for further testing.

**Influence of feedstock composition and co-digestion**

Fruit peels are rich in soluble sugars, pectin and structural carbohydrates, but their rapid acidification can depress pH and inhibit methanogenesis when used alone. Animal manure generally has a lower readily degradable carbon fraction, but it provides alkalinity, ammonia, micronutrients and an indigenous anaerobic community. Combining plant residues with manure can improve the carbon-to-nitrogen balance and buffer volatile fatty acids. Reviews of anaerobic co-digestion show that complementary feedstocks frequently improve methane yield and process stability, although optimal ratios remain substrate-specific (Karki et al., 2021). The broader Calabar experiment similarly showed that the four-component watermelon-cow dung-paw-paw-banana mixture produced the highest biogas volume and a nutrient-rich digestate (Akpan et al., 2019). The present paper does not republish those reactor-performance results; instead, it uses them as contextual evidence that the recorded microbial succession occurred within an active digestion process.

**Microbial succession during digestion**

Microbial communities are expected to change as oxygen is depleted and available substrates shift from simple sugars to fermentation products. Early communities may contain aerobes, facultative anaerobes, yeasts and filamentous fungi introduced with feedstocks. As reducing conditions develop, obligate anaerobes and syntrophic organisms become increasingly important. In a Nigerian study of cow dung and corn cob, culture-based monitoring demonstrated stage-dependent bacterial succession during fermentation (Ogunnusi & Salaudeen, 2022). More recent metagenomic work confirms that food-waste digestion commonly begins with fermentative bacterial dominance and progresses towards stronger representation of methanogenic archaea and methane-associated pathways (Akinsola et al., 2025). These studies support the ecological expectation that temporal patterns, rather than endpoint counts alone, are needed to interpret digester function.

**Digestate hygienisation and pathogen persistence**

Digestate reuse creates a direct connection between waste treatment and food production. Anaerobic digestion can reduce pathogen loads through nutrient depletion, antagonistic interactions, volatile fatty acids, ammonia, temperature exposure and retention time. However, the extent of reduction is highly variable. Burch et al. (2018) found that manure-borne pathogen responses depended on organism and treatment stage. Seruga et al. (2020) likewise showed that anaerobic treatment of food and municipal organic wastes had pathogen-reduction potential but did not justify a universal assumption of complete sanitisation. A one-year survey of agricultural digesters found that indicator and pathogenic bacteria could remain detectable in digestates, demonstrating the need for system-specific monitoring (Pourcher et al., 2023).

Spore-forming organisms deserve particular attention. A comprehensive meta-analysis concluded that temperature, pH and batch duration influence pathogen reduction, but Clostridiaceae, including Clostridium perfringens, are often minimally affected and may even be favoured under some conditions (Álvarez-Fraga et al., 2025). Franchitti et al. (2024) also detected resistant Bacillus and Clostridium species after sludge digestion. Consequently, the disappearance of E. coli or Salmonella does not prove complete hygienisation, because spore-forming indicators can respond differently. Where digestate will contact edible crops or be handled manually, a multiple-barrier approach may be warranted, including controlled storage, composting, pasteurisation, alkaline treatment or validated drying.

Environmental-health investigations conducted in Calabar also illustrate why organic origin or acceptable appearance cannot be treated as evidence of safety. Research on fungal contamination and genotoxic risk in deteriorated food, as well as cytogenetic responses to plant-derived extracts, demonstrates the value of hazard-specific laboratory assessment (Ubi et al., 2023, 2025). Evidence standards must likewise remain appropriate to the question being examined; biomedical treatment-development reviews, for example, employ endpoints that differ from those required for environmental microbial risk assessment (Henshaw et al., 2016).

**Culture-based identification and contemporary molecular standards**

Classical cultivation provides evidence that an organism or morphotype was viable under the selected media and incubation conditions. It also supports isolation for biochemical testing. However, it captures only the cultivable fraction and can misclassify organisms when identification relies mainly on colony morphology, Gram reaction and limited biochemical traits. These limitations are especially important for methanogens, which are archaea with specialised growth requirements and taxonomies that have changed over time. Modern studies increasingly combine 16S rRNA gene sequencing, archaeal markers such as mcrA, metagenomics, metatranscriptomics and metabolomics to link community composition with function (Wang et al., 2022b; Zhang et al., 2024). The culture-derived methanogen names in the present archived dataset are therefore treated as presumptive historical labels rather than definitive species identifications. This distinction allows the records to inform ecological interpretation without overstating taxonomic certainty.

**Digestate quality within a circular bioeconomy**

The value of anaerobic digestion is often assessed through methane yield, yet the environmental outcome also depends on how the residual digestate is managed. Digestate contains water, partially stabilised organic matter, mineral nitrogen, phosphorus, potassium and microbial biomass. Returning these materials to land may close nutrient loops and reduce demand for synthetic fertilisers, but beneficial reuse requires an integrated quality assessment. Agronomic indicators such as nutrient concentration, pH and organic matter should be considered together with stability, trace contaminants and sanitary indicators. This distinction is especially important in decentralised systems, where feedstocks are heterogeneous and process monitoring may be limited. The same treatment that conserves nutrients may not provide the temperature exposure needed to eliminate resistant organisms. Consequently, circularity should not be defined only by diversion from disposal; it should also include safe recovery, transparent quality control and an end use matched to the risk profile of the digestate. The recent pathogen-reduction literature supports this approach because reductions differ markedly among bacterial groups and operating conditions (Álvarez-Fraga et al., 2025; Franchitti et al., 2024). In practical terms, digestate from a documented, well-controlled process can be a resource, whereas an unverified effluent applied directly to edible crops may transfer rather than resolve an environmental-health risk.

Recent Nigerian studies reinforce this multidimensional interpretation of soil-amendment quality. Research on biological nitrogen fixation, multivariate soil-productivity indicators, soil organic carbon and nutrient ratios, organic and inorganic amendments, decomposed oil-palm litter, microbial biomass, enzyme activity and mycorrhizal-rhizobial inoculation shows that fertility outcomes depend on interacting biological, chemical and spatial controls (Omeke et al., 2022a, 2022b; Akpan et al., 2023; Aberagi et al., 2025; Akpan, Afangide, & Peter, 2026; Akpan, Peter, et al., 2026; Peter et al., 2026a, 2026b). Digestate assessment should therefore connect biosafety evidence with microbial-function, nutrient-balance and crop-response indicators rather than relying on nutrient concentration as a stand-alone measure of quality.

**Operational drivers in tropical batch digesters**

Low-cost batch digesters in tropical environments may benefit from ambient warmth, but ambient conditions do not guarantee a stable internal process. Hydrolysis of fruit residues can release fermentable sugars rapidly, producing organic acids faster than methanogens can consume their precursors. Cow dung may moderate this response through buffering and inoculation, although excessive manure nitrogen can also increase free-ammonia stress. Temperature fluctuations, incomplete mixing, leakage, uneven particle size and short-circuiting of the slurry further influence the microenvironments in which organisms survive. Retention time is therefore meaningful only when considered alongside actual temperature and pH histories. A nominal 45-day batch period may include an early adaptation interval, a period of rapid fermentation and a later methanogenic phase, each with different implications for microbial reduction. Reviews of co-digestion and digester microbiomes emphasise that stable performance arises from the interaction of feedstock balance, physicochemical control and

microbial functional redundancy rather than from a single universal recipe (Karki et al., 2021; Wang et al., 2022b; Zhang et al., 2024). For decentralised Nigerian systems, simple monitoring records of loading ratio, temperature, pH, retention time and endpoint indicators could substantially improve reproducibility and provide a defensible basis for deciding whether digestate requires additional treatment.

## Methodology

### Study area and experimental origin

The experiment was conducted at the Faculty of Agriculture Teaching and Research Farm, University of Calabar, Cross River State, Nigeria. Calabar lies within the humid tropical rainforest zone at approximately 5°00′-5°40′ N and 8°04′-8°62′ E. The region has high annual rainfall, warm temperatures and high relative humidity. The archived records originated from doctoral research undertaken during 2015-2016. This manuscript reports a previously unpublished analysis of the time-resolved microbial component of that broader study.

### Feedstocks and batch digesters

Banana (Musa acuminata) peel, paw-paw (Carica papaya) peel and watermelon (Citrullus lanatus) peel were collected from fruit retailers in Calabar metropolis. Cow dung was obtained from the Atimbo abattoir. Fruit peels were sorted and pounded into pulp. Locally fabricated galvanised-zinc batch digesters had a nominal capacity of 60 L and were connected to gasometric chambers. The reactor construction and gas-measurement arrangement have been described in the related process paper (Akpan et al., 2019).

### Feedstock configurations and digestion conditions

Twelve digesters were used: four single-feedstock treatments, six binary combinations, one four-component combination and a water control. For single-feedstock treatments, 13 kg of substrate was mixed with 39 kg of water. For binary mixtures, 6.5 kg of each component was used to maintain a total substrate mass of 13 kg. The four-component treatment contained 3.25 kg each of watermelon peel, cow dung, paw-paw peel and banana peel. Each mixture was diluted at a substrate-to-water mass ratio of 1:3 and digested for 45 days. Temperature and pH were monitored at five-day intervals. Recorded temperatures were predominantly within the mesophilic range, and the pH generally moved towards near-neutral conditions as digestion progressed (Akpan et al., 2019).

***Table 1. Experimental feedstock configurations***

| Feedstock treatment | Code | Substrate mass |
|---|---|---|
| Banana peel | B | 13 kg B |
| Paw-paw peel | P | 13 kg P |
| Watermelon peel | W | 13 kg W |
| Cow dung | C | 13 kg C |
| Watermelon + banana | W+B | 6.5 kg each |
| Watermelon + paw-paw | W+P | 6.5 kg each |
| Cow dung + paw-paw | C+P | 6.5 kg each |
| Watermelon + cow dung | W+C | 6.5 kg each |
| Paw-paw + banana | P+B | 6.5 kg each |
| Cow dung + banana | C+B | 6.5 kg each |
| Watermelon + cow dung + paw-paw + banana | W+C+P+B | 3.25 kg each |
| Water control | Control | No organic substrate |

*Note. All organic treatments were diluted with water at a substrate-to-water mass ratio of 1:3 and digested for 45 days. The treatment design was previously described by Akpan et al. (2019).*

### Sampling and microbial enumeration

Slurry was withdrawn at five-day intervals. Serial dilutions were prepared using sterile diluent. Total viable bacteria were enumerated by surface plating appropriate dilutions on nutrient agar and MacConkey agar, followed by incubation under the conditions documented in the original laboratory protocol. Fungi were enumerated on Sabouraud dextrose agar supplemented with antibacterial agents and incubated for 48-72 hours. Anaerobic cultures were incubated in sealed anaerobic jars with commercial oxygen-removal packs. Methanogenic enrichment used basal carbonate-yeast-tryptone medium supplemented with acetate, methanol, formate or hydrogen as energy sources. The original methods followed standard culture and enumeration procedures described by Zuberer (1994), Balch et al. (1979) and Holt et al. (1994).

### Phenotypic characterisation

Bacterial colonies were differentiated using colonial appearance, Gram reaction, cell morphology, motility and biochemical tests including catalase, oxidase, carbohydrate utilisation, indole, urease, citrate, methyl red and Voges-Proskauer reactions. Fungi were assessed using colony colour, aerial growth, hyphal characteristics, spores and lactophenol cotton blue microscopy. Presumptive methanogens were assigned historical phenotype-based labels from cell morphology, motility, gas production, substrate use, pH and temperature responses. Because molecular confirmation was not performed, bacterial and fungal species names are interpreted as culture-based morphotypes, and methanogen names are reported as presumptive labels.

### Data reconstruction and analysis

Archived occurrence tables recorded the number of times each morphotype was recovered at each sampling day across the batch-digester experiment. These occurrence counts were reconstructed into day-by-taxon matrices. For each day, total isolate occurrences and observed richness were calculated. Shannon diversity was calculated as $H' = -\Sigma pi \ln(pi)$, where pi is the proportion of occurrences assigned to each morphotype at that sampling time. Pielou evenness was calculated when richness exceeded one. Relative frequency for each morphotype was calculated as its cumulative occurrences divided by the cumulative occurrences of all morphotypes in the corresponding microbial group. Last detection day was used as a simple measure of persistence. Because the digestion phase was not documented as a replicated factorial microbial experiment and the occurrence records were pooled across reactors, analyses were descriptive; no inferential tests comparing individual substrate formulations were performed.

### Ethical and publication considerations

The study used agricultural wastes and did not involve human participants or live experimental animals. The feedstock preparation, nutrient transformation, biogas production and pepper-response components of the doctoral project have been published previously (Akpan et al., 2019, 2020). The present manuscript is restricted to the time-resolved microbial succession records and associated biosafety interpretation. The related publications are cited to disclose the shared experimental origin and prevent duplicate presentation of previously reported results.

## Results and discussion

### Overall contraction of the cultivable community

The culture records showed a pronounced contraction of the cultivable community during the 45-day retention period. Total bacterial isolate occurrences fell from 67 on day 5 to 45 on day 10, 34 on day 15, 23 on day 20 and 17 on day 25. Only two bacterial occurrences were recorded on day 30, followed by one occurrence at each of days 35, 40 and 45. This represents a 74.6% reduction by day 25 and a 98.5% reduction by day 45 relative to day 5. Bacterial richness declined from 11 morphotypes on day 5 to ten on day 10, eight on day 15 and six on

days 20 and 25. From day 30 onwards, only one morphotype remained detectable. Shannon diversity decreased from 2.33 on day 5 to 1.32 on day 25 and reached zero when the late community became taxonomically singular.

The fungal transition was faster. Total fungal occurrences were 48 and 45 on days 5 and 10, respectively, but declined to nine by day 15. Only two to three occurrences were recorded from days 20 to 30, and no fungal morphotype was recovered on days 35, 40 or 45. Fungal richness fell from eight morphotypes on days 5 and 10 to three on day 15 and one from days 20 to 30. Shannon diversity declined from 1.95 to 1.88 during the first ten days, fell to 0.85 on day 15 and became zero thereafter. Figure 1 and Table 2 illustrate this progressive narrowing.

The pattern is consistent with ecological selection during oxygen depletion, acidogenesis and subsequent stabilisation. Early samples contained organisms introduced with fresh feedstocks and organisms capable of exploiting soluble carbohydrates. As digestion progressed, depletion of oxygen, accumulation and later conversion of volatile intermediates, and competition under reducing conditions removed many aerobic and facultative populations. The persistence of a small subset therefore represents selective survival rather than an absence of microbial activity. Modern sequencing studies similarly report marked community turnover as fermentative functions give way to syntrophic and methanogenic processes (Akinsola et al., 2025; Wang et al., 2022a).

***Table 2. Temporal changes in isolate-occurrence diversity***

| Day | Bacterial occurrences | Bacterial richness | Bacterial H′ | Fungal occurrences | Fungal richness | Fungal H′ |
|---|---|---|---|---|---|---|
| 5 | 67 | 11 | 2.33 | 48 | 8 | 1.95 |
| 10 | 45 | 10 | 2.23 | 45 | 8 | 1.88 |
| 15 | 34 | 8 | 1.85 | 9 | 3 | 0.85 |
| 20 | 23 | 6 | 1.45 | 2 | 1 | 0.0 |
| 25 | 17 | 6 | 1.32 | 3 | 1 | 0.0 |
| 30 | 2 | 1 | 0.0 | 3 | 1 | 0.0 |
| 35 | 1 | 1 | 0.0 | 0 | 0 | 0.0 |
| 40 | 1 | 1 | 0.0 | 0 | 0 | 0.0 |
| 45 | 1 | 1 | 0.0 | 0 | 0 | 0.0 |

*Note. H′ is Shannon diversity calculated from culture-positive isolate-occurrence counts. A value of zero indicates that either no morphotype or only one morphotype was detected.*

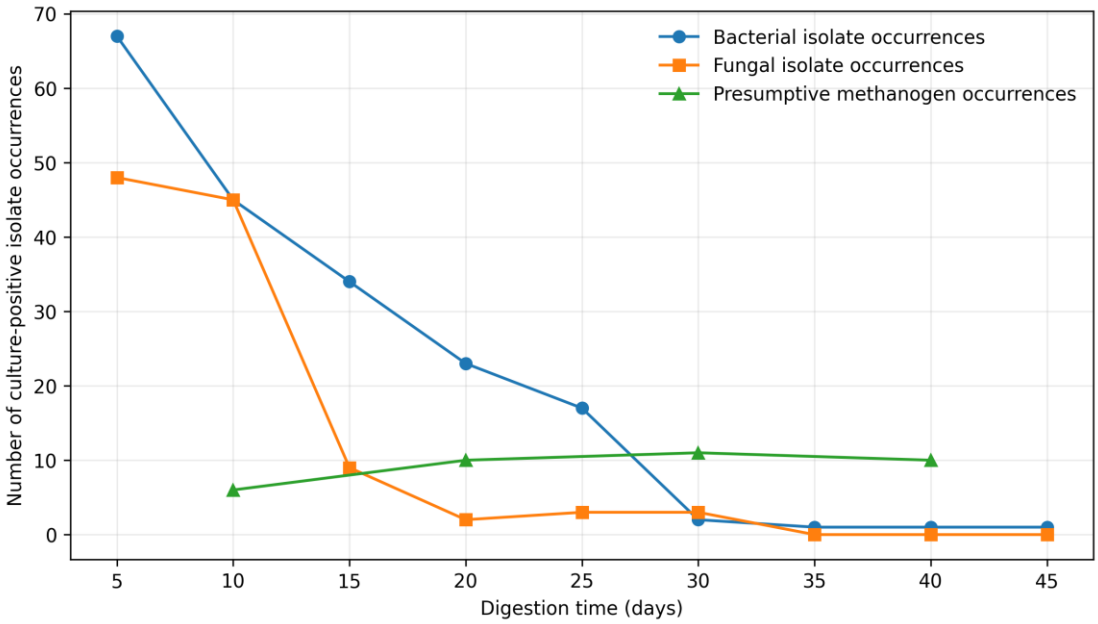


***Figure 1. Temporal change in bacterial, fungal and presumptive methanogen isolate occurrences during batch anaerobic digestion.***

**Diversity evenness and the timing of ecological selection**

The diversity indices provide additional information beyond the reduction in total occurrences. Bacterial evenness was high during the earliest observations: Pielou evenness was approximately 0.97 on days 5 and 10, indicating that the initially recovered morphotypes were comparatively well distributed. Evenness then declined to 0.89 on day 15, 0.81 on day 20 and 0.74 on day 25. Thus, the bacterial community did not merely lose taxa; it also became progressively concentrated in the few morphotypes able to tolerate the developing anaerobic environment. Once only the clostridial morphotype was recovered, evenness was no longer informative and Shannon diversity was zero. The fungal community followed a sharper pattern. Fungal evenness declined from approximately 0.94 on day 5 to 0.90 on day 10 and 0.77 on day 15 before only yeast remained. These values distinguish two linked processes: disappearance of sensitive taxa and increasing dominance by tolerant groups.

The timing of the decline also identifies a critical transition between days 10 and 20. Bacterial occurrences decreased by 48.9% over that interval, while fungal occurrences decreased by 95.6%. This interval likely captured the strongest selection against aerobic feedstock-associated organisms as oxygen became depleted and reduced metabolites accumulated. Between days 20 and 30, bacterial occurrences fell by a further 91.3%, but the presumptive methanogen record rose from ten occurrences on day 20 to 11 on day 30. Although these pooled culture records cannot establish a direct causal relationship, the opposing trajectories are consistent with replacement of an early mixed cultivable community by organisms adapted to late anaerobic conversion. The result demonstrates why a single endpoint sample would have been insufficient: it would show persistence of one bacterial morphotype but would conceal the substantial early diversity and the period during which enteric and fungal records disappeared.

**Bacterial succession and persistence**

A Clostridium perfringens-like morphotype was the dominant and most persistent bacterial record. It accounted for 51 of 191 cumulative bacterial occurrences (26.70%). Its occurrence increased from four on day 5 to eight on day 10 and 12 on both days 15 and 20. It remained prominent on day 25 and was the only bacterial morphotype detected from day 30 through day 45. This trajectory is biologically plausible because clostridia are obligate anaerobes and many form environmentally resistant endospores. However, the phenotype-based method cannot

distinguish toxigenic C. perfringens from closely related clostridial organisms. The result is therefore best interpreted as persistence of a C. perfringens-like clostridial morphotype requiring confirmation by species-specific or toxin-gene assays.

Enterobacter aerogenes-like isolates represented 12.57% of cumulative occurrences and remained detectable through day 25. Pseudomonas aeruginosa-like isolates represented 11.00%, while Bacillus subtilis-like, Bacillus cereus-like and Escherichia coli-like isolates each contributed approximately 9.94-9.95%. These morphotypes also disappeared after day 25. Staphylococcus aureus-like and Streptococcus pyogenes-like records were last observed on day 15. Salmonella typhimurium-like and Corynebacterium pyogenes-like morphotypes were last observed on day 10, while Micrococcus luteus-like isolates occurred only on day 5.

The disappearance of the enteric morphotypes is encouraging, but culture non-detection should not be equated with absolute absence. Counts may fall below detection limits, cells may become viable but non-culturable, or the selected media may not recover injured organisms. Nevertheless, the temporal pattern agrees with evidence that anaerobic digestion can substantially reduce vegetative enteric bacteria. Burch et al. (2018), Seruga et al. (2020) and Pourcher et al. (2023) each reported organism-specific reductions during digestion. The persistence of the clostridial morphotype also agrees with recent evidence that spore-forming Clostridiaceae respond less predictably than E. coli and other vegetative indicators (Álvarez-Fraga et al., 2025; Franchitti et al., 2024).

***Table 3. Persistence and cumulative frequency of bacterial morphotypes***

| Culture-based morphotype | Cumulative occurrences | Relative frequency (%) | Last detection day |
|---|---|---|---|
| Clostridium perfringens-like | 51 | 26.7 | 45 |
| Enterobacter aerogenes-like | 24 | 12.57 | 25 |
| Pseudomonas aeruginosa-like | 21 | 11.0 | 25 |
| Bacillus subtilis-like | 19 | 9.95 | 25 |
| Bacillus cereus-like | 19 | 9.94 | 25 |
| Escherichia coli-like | 19 | 9.94 | 25 |
| Streptococcus pyogenes-like | 13 | 6.81 | 15 |
| Staphylococcus aureus-like | 12 | 6.28 | 15 |
| Salmonella typhimurium-like | 6 | 3.14 | 10 |
| Corynebacterium pyogenes-like | 6 | 3.14 | 10 |
| Micrococcus luteus-like | 2 | 1.05 | 5 |

*Note. Names are phenotype-based assignments reconstructed from the archived records and are not molecularly confirmed. "Last detection day" denotes the final sampling point at which the morphotype was cultured.*

### Fungal succession

Penicillium-like isolates were the most frequently recovered fungal morphotype, accounting for 23.64% of all fungal occurrences. They were recorded on days 5, 10 and 15 but not thereafter. Aspergillus niger-like isolates accounted for 15.45% and were also last observed on day 15. Mucor-like and Rhizopus-like isolates represented 14.55% and 12.73%, respectively, and were not detected after day 10. Fusarium oxysporum-like, Verticillium-like and Trichoderma-like morphotypes disappeared by days 10-15.

Yeast was less frequent overall (11.82%) but was the only fungal group that persisted beyond day 15. Its occurrence increased gradually and remained detectable through day 30. Yeast persistence may reflect tolerance of acidic early conditions and the capacity of some yeasts to ferment soluble sugars under oxygen-limited conditions. Their disappearance after day 30 coincided with the late methanogenic phase and exhaustion of readily fermentable substrates. The rapid loss of filamentous fungi is expected because most are primarily

aerobic, although fungal enzymes released early in digestion may still contribute to polymer breakdown. The culture records therefore suggest a short early fungal role followed by bacterial-archaeal dominance.

***Table 4. Persistence and cumulative frequency of fungal morphotypes***

| Culture-based morphotype | Cumulative occurrences | Relative frequency (%) | Last detection day |
|---|---|---|---|
| Penicillium-like | 26 | 23.64 | 15 |
| Aspergillus niger-like | 17 | 15.45 | 15 |
| Mucor-like | 16 | 14.55 | 10 |
| Rhizopus-like | 14 | 12.73 | 10 |
| Yeast | 13 | 11.82 | 30 |
| Fusarium oxysporum-like | 12 | 10.91 | 10 |
| Verticillium-like | 6 | 5.45 | 10 |
| Trichoderma-like | 6 | 5.45 | 10 |

*Note. Fungal labels are based on culture and microscopy in the archived records; molecular confirmation was not performed.*

## Emergence of presumptive methanogens

Presumptive methanogenic morphotypes were documented at days 10, 20, 30 and 40. Total occurrences increased from six on day 10 to ten on day 20 and peaked at 11 on day 30, before remaining high at ten on day 40. The most frequent historical labels were Methanogenium cariaci-like (24.30%) and Methanothrix soehngenii-like (21.62%), followed by Methanogenium organophilum-like and a 'Methanoculleus/Methanosarcina barkeri-like' label (16.22% each). Less frequent labels included Methanoplanus limicola-like, a ruminantium-like methanogen and Methanococcus voltae-like.

The increase between days 10 and 30 broadly coincided with the phase in which the related reactor study recorded increasing biogas production, with the four-component treatment producing the highest late-stage gas volume (Akpan et al., 2019). This temporal agreement supports the interpretation that methanogenic activity strengthened after early hydrolysis and fermentation. However, the archived methane data measured total displaced gas rather than methane composition, and the methanogen assignments were not based on 16S rRNA or mcrA sequencing. The present result should therefore be read as evidence of increasing methanogenic enrichment, not definitive proof of the abundance of particular archaeal species.

***Table 5. Presumptive methanogenic morphotypes recovered during digestion***

| Historical phenotype-based label | Cumulative occurrences | Relative frequency (%) | Detection window (days) |
|---|---|---|---|
| 'Methanogenium cariaci'-like | 9 | 24.3 | 10-40 |
| 'Methanothrix soehngenii'-like | 8 | 21.62 | 10-40 |
| 'Methanogenium organophilum'-like | 6 | 16.22 | 10-40 |
| 'Methanoconpullum/Methanosarcina barkeri'-like | 6 | 16.22 | 10-40 |
| 'Methanoplanus limicola'-like | 4 | 10.81 | 20-30 |
| 'Methanoculleus/Methanobrevibacter ruminantium'-like | 3 | 8.1 | 30-40 |
| 'Methanococcus voltae'-like | 1 | 2.7 | 40 |

*Note. Labels reproduce or cautiously regularise historical phenotype-based assignments in the doctoral records. They should be regarded as presumptive methanogenic morphotypes pending archaeal 16S rRNA or mcrA confirmation.*

## Process interpretation

Three ecological phases can be inferred. The first phase, approximately days 0-10, contained the highest bacterial and fungal richness and reflected feedstock-associated, hydrolytic and facultative organisms. The second phase, approximately days 15-25, showed sharp loss of fungal diversity, continued decline of facultative bacteria and

increasing dominance of the clostridial morphotype. The third phase, approximately days 30-45, was characterised by very low cultivable bacterial richness, no recovered fungi after day 30 and sustained presumptive methanogen occurrence. This phase structure is compatible with the sequential biochemical demands of anaerobic digestion, although the actual microbial network was certainly more diverse than the cultivable fraction suggests.

The four-component feedstock had previously shown the strongest biogas and digestate-nutrient performance (Akpan et al., 2019). Co-digestion likely provided complementary carbon, nitrogen, micronutrients and buffering. Still, the pooled occurrence records do not allow valid statistical attribution of individual morphotypes to a particular feedstock combination. Future experiments should replicate each feedstock ratio and analyse samples separately so that microbial community structure, methane yield and pathogen reduction can be linked quantitatively.

**Digestate biosafety implications**

The endpoint culture profile provides a mixed message. Many enteric, opportunistic and fungal morphotypes were no longer cultured by day 45, indicating substantial biological stabilisation. At the same time, persistence of a clostridial morphotype through the final sampling day demonstrates that mesophilic retention alone did not deliver complete hygienisation. This distinction matters because digestate may be applied to soil, handled without protective equipment or used around vegetable crops that receive minimal cooking.

A practical safety framework should therefore combine process control with endpoint verification. Digesters should maintain documented temperature, pH and retention time; feedstocks should be protected from post-digestion contamination; and digestate should be tested using suitable indicator organisms. Where the intended use involves leafy vegetables, peppers or other food crops, additional treatment should be considered when spore-forming indicators remain detectable. Options include composting with a validated thermophilic phase, pasteurisation, controlled alkaline treatment, solar or mechanical drying under conditions that achieve a documented reduction, or extended storage where locally validated. The choice should account for nutrient preservation, cost and farmer accessibility.

The finding also cautions against describing digestate as pathogen-free merely because gas production has ceased or because odour has declined. Álvarez-Fraga et al. (2025) showed that pathogen reduction depends on organism and operating conditions, with thermophilic treatment and heat-based post-treatment generally providing stronger control. Franchitti et al. (2024) similarly demonstrated that risk reduction during digestion is partial. Low-cost tropical digesters can still provide major environmental benefits, but safe agricultural recycling requires a multiple-barrier approach.

**Methodological limitations and interpretive boundaries**

The study has several limitations that should guide interpretation. First, cultivation recovers only a fraction of the microbial community and favours organisms compatible with the selected media. Second, taxonomic assignments were based on morphology and biochemical characteristics; they should not be treated as molecularly confirmed species. Third, microbial occurrence data were pooled across the experimental digesters, so substrate-specific succession cannot be separated. Fourth, the digestion treatments were not documented as replicated microbial reactors, preventing inferential comparison among feedstock formulations. Fifth, the original records did not include pathogen gene targets, toxin profiles, antibiotic resistance or methane percentage.

These limitations do not invalidate the temporal pattern, but they define its appropriate use. The data provide a viable-culture baseline showing community contraction, differential persistence and late methanogenic enrichment. A modern validation study should combine culture methods with 16S rRNA and ITS amplicon sequencing, mcrA profiling, quantitative PCR for E. coli, Salmonella and C. perfringens markers, toxin-gene screening where relevant, and direct methane measurement. Such work would distinguish active functional organisms from residual DNA and would permit robust risk assessment of digestate intended for crop production.

## Implications and conclusion

### Environmental and policy implications

The results support the use of anaerobic digestion as part of an integrated organic-waste management strategy in Calabar and comparable tropical cities. Fruit markets and abattoirs generate complementary wastes that can be collected separately and processed rather than dumped. Co-digestion can recover biogas, reduce the volume of unstable waste and retain nutrients in a form that may support soil fertility. These benefits align with circular-economy objectives, reduced pressure on landfills and lower dependence on mineral fertilisers.

However, waste-to-fertiliser policies should distinguish nutrient quality from sanitary quality. A digestate may contain useful nitrogen, phosphorus and exchangeable bases while still harbouring resistant microorganisms. Local guidelines should therefore specify minimum process records and microbial criteria for different end uses. Application to non-food crops or incorporation into soil well before planting may present a different risk profile from surface application to vegetables. Extension programmes should train operators in feedstock handling, digester sealing, retention-time control, protective equipment and post-treatment options.

Implementation should also be place-sensitive. Research from Calabar, Uyo and the Cross River Basin indicates that environmental outcomes are influenced by spatial distribution, service access, livelihood dependence, institutional support and community participation (Eneyo & Ekpenyong, 2018; Eneyo et al., 2023; Eneyo & Archibong, 2024; Eneyo, 2025a, 2025b, 2025c, 2026a, 2026b; Eneyo et al., 2026). These insights support locating collection and treatment facilities close to concentrated waste generators, preventing poorly managed discharges into livelihood-dependent aquatic systems, and integrating operator training with environmental and sustainable-development education (Akpan & Eneyo, 2026).

### Research and implementation pathway

A phased implementation pathway would allow municipalities, universities and farmer groups to translate these findings without imposing laboratory requirements that are unrealistic for every small digester. At the operator level, standard log sheets should record feedstock sources, mixture ratios, loading date, daily or periodic temperature, pH, visible leakage, mixing and discharge date. At the cooperative or institutional level, representative digestate batches can be tested for indicator organisms and nutrient quality before recommendations are issued. Where food-crop use is intended, testing should include a vegetative enteric indicator and a resistant spore-forming indicator, because either group alone may give an incomplete picture. These records would create local evidence for safe retention periods and post-treatment combinations rather than relying exclusively on standards developed for different climates and reactor types.

Research should also move from pooled observations to replicated reactor experiments. Each single and combined feedstock treatment should be operated in independent replicates, with matched physicochemical measurements and gas-composition analysis. Amplicon sequencing can describe bacterial, archaeal and fungal succession, while quantitative polymerase chain reaction can track selected pathogen and functional genes. Culture remains important because it demonstrates viability and provides isolates for confirmation, but it should be paired with molecular assays and, where possible, viability-sensitive approaches. Linking these measurements to germination tests, soil application and crop uptake would clarify whether a digestate is simultaneously stable, agronomically useful and microbiologically acceptable. Such an approach would strengthen evidence for decentralised waste valorisation in southern Nigeria and provide regulators with data for end-use-specific guidance.

### Conclusion

Mesophilic anaerobic digestion produced a clear succession from a diverse early bacterial-fungal community to a narrow late-stage community dominated in culture by a C. perfringens-like clostridial morphotype, alongside increasing presumptive methanogenic occurrence. Bacterial isolate occurrences declined by 98.5% between days

5 and 45, while fungi were no longer recovered after day 30. Several enteric and opportunistic morphotypes disappeared during the process, indicating substantial stabilisation, but the persistent clostridial record shows that 45-day mesophilic retention cannot be assumed to sanitise digestate completely.

The study contributes time-resolved evidence from low-cost tropical batch digesters and highlights an operational trade-off: conditions that support anaerobic conversion may also permit persistence of spore-forming organisms. Digestate reuse should therefore be based on both agronomic value and verified biosafety. For food-crop applications, endpoint microbial testing and a validated post-treatment step are recommended when resistant indicators remain detectable. Future replicated studies should integrate molecular community profiling, pathogen-specific assays and methane composition to confirm the functional and sanitary interpretation of the culture-based succession observed here.

## Declarations

**Acknowledgements:** The author acknowledges the technical support of the Soil Science and Microbiology laboratories of the University of Calabar during the original doctoral research.

**Funding:** No external funding was reported for the original study. This manuscript did not receive specific funding for secondary analysis or preparation.

**Conflict of interest:** The author declares no conflict of interest.

**Author contribution:** Joyce Fidelis Akpan conceived the original study, conducted the investigation, curated the archived records and is responsible for verification and approval of the submitted manuscript. Any additional authors must be added only after confirming their substantial contributions in accordance with journal policy.

**Ethical approval:** The study involved organic wastes, laboratory microorganisms and crop-production materials. It did not involve human participants or experimental animals; therefore, human or animal ethical approval was not applicable.

**Data availability:** The analysed data are contained in the doctoral thesis and the tables presented in this manuscript. The underlying laboratory records should be made available by the corresponding author upon reasonable request, subject to institutional approval.

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