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Harnessing Oil-Contaminated Soil Microbiomes for Improved Anaerobic Digestion of Cow and Pig Manure

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Abstract

Anaerobic digestion (AD) of animal manures for biogas production faces challenges including nutritional imbalance, foaming, and process instability. This study evaluated bioaugmentation with surfactant-degrading microbial consortia and cell-free extracts derived from well-characterized oil-contaminated soils during cow and pig manure digestion. These previously analyzed soils contained distinct microbial communities dominated by *Pseudomonas* in acidic, high-PAH soils and *Bacillus* in neutral-pH soils with genetic potential for hydrocarbon degradation. Over 30 days, six treatments were assessed using the Automatic Methane Potential Test System (AMPTS II), with pH monitoring, foaming analysis, and 16S rRNA sequencing coupled with PICRUSt2 functional prediction. Supplementation with microbial consortia and extract markedly increased cumulative biogas outputs (cow manure: 407.76 to 603.28 mL/gVS and pig manure: 403.82 to 627.5 mL/gVS), biomethane by 30–50%, reduced digestion time by 5–6 days, and improved pH stability. Foaming reduction was substrate-specific: extracts reduced foam by up to 60% in pig manure, while consortia reduced it by up to 65% in cow manure. Microbial analysis revealed enrichment of fermentative and syntrophic taxa (*Clostridium sensu stricto* and *Paludibacter*) and upregulation of methanogenesis pathways (tetrahydromethanopterin S-methyltransferase). This study illustrates that tailored bioaugmentation utilizing consortia from hydrocarbon-contaminated soils provides an environmentally sustainable method to enhance methane yields, improve stability, and control foaming in manure AD, with outcomes significantly affected by the type of manure and amendment strategy employed.

Keywords: animal waste; foaming; biogas; microbial extract; microbial consortia; surfactant degradation; anaerobic digestion

Check for updates

Received: 18 February 2026
Revised: 26 March 2026
Accepted: 31 March 2026
Published: 3 April 2026

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1. Introduction

Anaerobic digestion (AD) is known as an efficient technique for transforming organic waste into renewable biogas while concurrently alleviating environmental pollution, diminishing greenhouse gas emissions, and facilitating resource recovery [1,2]. Livestock production produces significant amounts of manure worldwide, particularly in South

Africa, where intensified agriculture is augmenting organic waste streams amenable to energy valorization [3,4]. Cattle and pig manure are excellent materials for anaerobic digestion, but their physical and chemical characteristics often create challenges that affect the process pH and cause foaming, which ultimately lowers methane production yields and kinetics [5,6]. The ideal carbon-to-nitrogen ratio for a perfectly working AD is usually around 20–30, often near 25 to balance the risks associated with nitrogen deficiency and ammonia inhibition. However, cow manure has high C/N ratios (20–28) and lignocellulosic fractions, which may limit nitrogen availability for microbial metabolism and impede hydrolysis. In contrast, pig manure has higher protein content and lower C/N ratios (~6–7), which can lead to excessive ammonia, rapid acid production, and reduced methane yields [7,8].

The release of surface-active agents, including proteins, lipids, and biosurfactants, during digestion renders both substrates prone to foaming. Elevated foam activity can reduce effective digester volume, disrupt mixing, and increase operational expenses due to supplementary control measures [9,10]. These limitations highlight the need for solutions that enhance microbial efficiency, improve substrate biodegradability, and alleviate operational issues such as excessive foaming in manure-based anaerobic digestion systems. Bioaugmentation, which is the deliberate introduction of specific microbial strains or consortia into a bioreactor to improve functions like pollutant degradation or substrate hydrolysis, has emerged as a viable solution to these challenges [11]. Dadić et al. [12] isolated a lignocellulose-degrading consortium for Miscanthus digestion, comprising *Enterobacter cloacae* and *Klebsiella pneumoniae*, immobilized on natural carriers for the bioaugmentation of anaerobic digesters; this consortium is specifically characterized as a biomass (lignocellulose/cellulose) degrader that provides intermediates for methanogens. Goswami et al. [13] found that various cellulolytic *Enterobacteriaceae*, including *Enterobacter ludwigii*, optimized their cellulase synthesis and demonstrated that this increased biogas generation from lignocellulosic material in anaerobic digestion. Biological pretreatment reviews highlight microbe-produced extracellular cellulases, hemicellulases, proteases, and other hydrolases to degrade complicated polymers before AD, enhancing substrate accessibility without growing microorganisms in the digester [14,15].

Our prior research on oil-contaminated soils showed different microbial communities, predominantly comprising *Pseudomonas* and *Bacillus* species [16]. Metagenome-assembled genomes (MAGs) validated the existence of genes that encode oxygenases, dioxygenases, and biosurfactant synthesis pathways crucial for the breakdown of hydrocarbons and surfactants. A follow-up investigation revealed that microbial consortia derived from these soils efficiently decomposed several surfactants (SDS, SLS, rhamnolipids, Triton X-100, and their combinations) within a 14-day period [17]. These studies demonstrated the functional capacity of these consortia for surfactant transformation and hydrolysis, justifying their use in anaerobic digestion systems for manure, where surfactant contributes to problematic foaming.

Therefore, the primary hypothesis of this study is that bioaugmentation with surfactant-degrading microbial consortia and their cell-free extracts, derived from oil-contaminated soils, will variably augment methane production, enhance process stability (pH), and diminish foaming in a substrate-specific manner during the anaerobic digestion of cow and pig manure. To our knowledge, no previous research has systematically compared the effectiveness of live consortia and cell-free extracts, both sourced from oil-contaminated environments and characterized by prior metagenomic analysis, in simultaneously enhancing methane yield, controlling foaming, and general system stability. This comprehensive methodology, merging performance measures with thorough microbiological and functional analysis, underscores the originality of this research. The current study thus aimed to: (1) Assess the impact of microbial consortia and extracts on total

methane generation and reaction kinetics in cow and pig manure AD systems; (2) Evaluate substrate-specific effects on pH stability; (3) Quantify foaming dynamics, encompassing foam height and stability, across different bioaugmentation treatments; and (4) Characterize microbial community shifts and predicted functional profiles linked to enhanced anaerobic digestion performance.

2. Materials and Methods

2.1. Sample Collection

The fresh samples of cow and pig manures were collected from a farm in Vlakfontein, Gauteng, South Africa (26°11′43″ S, 27°48′27″ E). To ensure biological replicates, samples were collected from different locations within the farm. All manure samples were put in sterile plastic containers and stored in a portable icebox, then transported to the laboratory at the University of South Africa (UNISA), Johannesburg, where they were temporarily stored at 4 °C to preserve their original structural quality and microbial diversity [18,19]. Within 2 days after collection, the samples were processed for further experimental work [20]. Digestate (D) obtained from a working digester situated at (25°54′ 47″ S, 22°36′ 22″ E) was used as inoculum for the laboratory-based anaerobic digestion (AD) experiments. This operating digester was processing a mixture of cow manure and other organic waste under mesophilic conditions. All chemicals used in the experiments were sourced from Merck, Johannesburg, South Africa, and were of analytical grade.

2.2. Site Description for Microbial Consortia Source

The surfactant-degrading microbial consortia were sourced from oil-contaminated soil collected from two locations in Gauteng, South Africa: Roodepoort (26°9′32″ S, 27°52′15″ E) and Midrand (25°59′57″ S, 28°7′46″ E). As thoroughly detailed in our prior research, these locations have a history of prolonged petroleum hydrocarbon contamination resulting from industrial activity, particularly vehicle repair facilities. At the time of sampling, both areas exhibited visible oil sheen, a hydrocarbon odor, and diminished plant cover. The physicochemical characteristics of these soils, encompassing PAH profiles (predominance of 5–6 ring compounds in Midrand and 4-ring compounds in Roodepoort) and heavy metal concentrations (Zn exceeding 1000 mg/kg in Midrand, Cr surpassing 150 mg/kg in Roodepoort) [16]. A shotgun metagenomic study of the soils indicated that Midrand was predominantly characterized by *Pseudomonas* species (up to 40% relative abundance), whereas Roodepoort was primarily characterized by *Bacillus* species (up to 60% relative abundance). Metagenome-assembled genomes (MAGs) obtained from these locations indicated the existence of genes that encode oxygenases, dioxygenases, and biosurfactant synthesis pathways crucial for the breakdown of hydrocarbons and surfactants. The surfactant-degrading ability of consortia enriched from these soils was subsequently evidenced in our companion investigation, which exhibited excellent biodegradation of SDS (≥99% purity, Merck, Darmstadt, Germany), SLS (≥99% purity, Merck, Darmstadt, Germany), rhamnolipids (≥95%, Merck, Darmstadt, Germany), Triton X-100 (≥99% purity, Sigma-Aldrich, Darmstadt, Germany), and surfactant mixtures at 4% (w/v) concentrations over a period of 14 days [17].

2.3. Preparation of Microbial Consortia and Microbial Extract

2.3.1. Preparation of Microbial Consortia

The surfactant-degrading microbial consortia, initially obtained from the oil-contaminated soils outlined in Section 2.2 and characterized in prior research, were preserved in glycerol at −80 °C. As outlined in our previous research, these consortia were enriched via sequential sub-culturing in mineral salt medium (MSM) supplemented with specific surfactants

(SDS, SLS, rhamnolipids, and Triton X-100) at a concentration of 2% (*w*/*v*) as the only carbon source, incubated at 30 °C with agitation at 160 rpm for a duration of 14–21 days. Successful enrichment was validated through shotgun metagenomics, which demonstrated surfactant-specific selection of degraders: *Pseudomonas* predominated in SLS and SDS enrichments (60.2% and 40.5%, respectively), whereas *Achromobacter* (20.1%) and *Stutzerimonas* (15.3%) were enriched in rhamnolipid and Triton X-100 treatments, respectively.

To prepare for seed culture, 20 µL of the culture was used to inoculate 50 mL of Luria–Bertani (LB) broth and incubated overnight at 37 °C and 160 rpm. The LB-incubated surfactant-degrading microbial consortia (2%, *v*/*v*) were subsequently inoculated into 250 mL Erlenmeyer flasks containing 100 mL of mineral salt medium (MSM) supplemented with 0.5% (*w*/*v*) (Rhamnolipids, Triton X-100, SDS, and SLS). The surfactant mixture functioned as the exclusive carbon and energy source for the enrichment and culture of surfactant-degrading microorganisms. The MSM was prepared by dissolving 4.0 g NH_4NO_3, 1.0 g KCl, 0.5 g KH_2PO_4, 1.0 g K_2HPO_4, 0.01 g $CaCl_2$, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.01 g $FeSO_4 \cdot 7H_2O$ in 1 L distilled water, supplemented with 10 mL trace element solution (0.26 g/L H_3BO_3, 0.5 g/L $CuSO_4 \cdot 5H_2O$, 0.5 g/L $MnSO_4 \cdot 7H_2O$, 0.06 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.7 g/L $ZnSO_4 \cdot 7H_2O$) [21,22]. The pH was adjusted to 7.0 using 1 M NaOH or HCl, and the medium was autoclaved at 121 °C for 15 min. After cooling, the filter-sterilized surfactant mixture (0.22 µm membrane) was aseptically incorporated to attain a final concentration of 0.5% (*w*/*v*). The flasks were incubated at 37 °C, 160 rpm. The operational temperature of 37 °C was employed to align with the circumstances of the subsequent mesophilic anaerobic digestion tests, so as to guarantee that the consortia were pre-adapted to the thermal environment they would face in the digesters. During the exponential growth phase (OD600 of 0.6–0.8, about 24 h), cells were harvested by centrifuging at 4000 rpm for 5 min at 4 °C using a centrifuge (Eins Sci EC684, 100CPR, Eins Sci Instruments, Shanghai, China). The cell pellet was washed thrice with ice-cold 20 mL sterile phosphate-buffered saline (100 mM PBS, pH 7.4; composition: 8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) to remove residual media and metabolites, followed by centrifugation at 4000 rpm for 5 min after each wash. The final washed pellet was resuspended in 5 mL PBS for downstream applications [23].

2.3.2. Preparation of Microbial Extract

To obtain the intracellular fraction (cell-free extract) of the surfactant-degrading consortia, the cell suspension from above was transferred to 15 mL centrifuge tubes and submerged in an ultrasonic bath (Eins Sci SK3300H, Eins Sci Instruments, Shanghai, China; 40 kHz frequency, 150 W power) in a floating tube rack. Sonication was carried out continuously for 15 min at 100% amplitude, while the temperature was kept between 4 and 8 °C. To guarantee consistent cavitation, the tubes were gently shaken every two minutes. After sonication, the soluble lysate was obtained by centrifuging at 16,000× *g* for 20 min at 4 °C in a refrigerated (Eins Sci EC684, 100CPR, Eins Sci Instruments, Shanghai, China) centrifuge. The supernatant was then filtered through a 0.45 µm syringe filter to obtain a crude extract [24]. This cell-free extract aimed to separate the effects of immediate enzymatic catalysis from those necessitating live, metabolically active microorganisms. Live consortia can develop and adapt to digester conditions, whereas the extract delivers a concentrated, single-dose pulse of intracellular enzymes and cofactors, allowing for the assessment of whether the observed enhancements necessitate ongoing microbial activity or can be accomplished through initial biochemical intervention alone [25].

2.4. Proximate and Ultimate Analysis

The total solids (TS) and volatile solids (VS) content of the manures and inoculum were determined using British standards EN ISO 18134-3:2023 (E) [26] and EN ISO 18123:2023 (E) [27], respectively, while the standard EN ISO 16948:2015 (E) [28] was followed for ultimate analysis. Briefly, for TS determination, a measured amount (mwet) of each animal manure sample was placed in a preheated oven set at 105 °C for 24 h and then reweighed after the heating (mdried) to determine the total solids (TS) content using Equation (1).

$$\text{TS}\ (\%) = \frac{m\ (dried)}{m\ (wet)} \times 100 \tag{1}$$

Volatile solids content (VS) in the manures was determined by the loss-on-ignition (LOI) method through heating of the dried material (taken from Equation (1)) from a weight (mdried) to a final solids weight (mburned). The heating took place in a crucible for about 2 h at 550 °C in a preheated muffle furnace. After cooling in a desiccator, the remaining solids in the crucible were weighed to give (mburned). Using these values, VS on a wet basis was calculated through Equation (2).

$$\text{VS}\ (\%) = \frac{m\ (dried) - m(burned)}{m\ (wet)} \times 100 \tag{2}$$

Briefly, for ultimate analysis, a blender was used to finely grind fresh samples of animal manure to a smaller particle size of 2 mm in diameter. Samples were dried in a preheated oven at 55 °C for 24 h in preparation for elemental analysis. The characterization for carbon, hydrogen, nitrogen, and sulphur (CHNS) composition of the samples was performed at the University of Witwatersrand (South Africa) using the CHNS Vario EL Cube instrument (Elementar, Langenselbold, Germany) with sulphanilamide as the standard. Approximately [(1–1.5) ± 0.01 mg] of each sample was weighed using a Labotec precision balance (YP series, Labotec, Midrand, South Africa), packed into a tin capsule, and then placed in the automated sampler of the device. The device's output data were converted from weight to atomic percentage [29].

2.5. Determination of Biomethane Potential Based on Ultimate Analysis

The biodegradation is envisaged to proceed through a microbially catalyzed chemical reaction (Equation (3)), and for an organic compound of empirical formula $C_aH_bO_cN_dS_e$ under anaerobic conditions, the stoichiometric (theoretical) methane production potential (TBMP) is given by Equation (4). This Buswell equation, initially formulated by Buswell and Mueller, continues to serve as the primary method for determining theoretical methane yields based on elemental composition [30].

$$\begin{aligned} C_aH_bO_cN_dS_e + 1/4(4a - b - 2c + 3d + 2e)H_2O \rightarrow 1/8(4a - b + 2c + 3d + 2e)CO_2 + \\ 1/8(4a + b - 2c - 3d - 2e)CH_4 + dNH_3 + eH_2S, \end{aligned} \tag{3}$$

$$\text{TBMP} = \left(\left\{ \frac{(a/2 + b/8 - c/4 - 3d/8 - e/4)22400}{12a + b + 16c + 14d + 32e} \right\} \right) \tag{4}$$

The Dulong method was used in addition to the Buswell methodology to estimate TBMP. The Dulong energetics approach for TBMP determination is predicated on the idea that, in the case of complete biomethanation, methane is responsible for the substrate's energy value, E^0 expressed in MJ/t, which can be computed from the modified Dulong Equation (5) [31].

$$E^0 = 337\text{C} + 1419(\text{H} - 1/8\text{O}) + 93\text{S} + 23.26\text{N} \tag{5}$$

If all of this energy is exclusively assigned to the methane produced, then TBMP can be estimated by Equation (6).

$$\text{TBMP} = E^0/38, \quad (6)$$

2.6. Bio-Methane Production Experiments

The AD experimental evaluation of cow and piggery manure was conducted using an Automatic Methane Potential Testing System (AMPTS® II) of Bioprocess Control, Lund, Sweden (Figure 1). The manufacturer's operating manual instructions [32] and other recommended procedures [33,34] were followed in setting up the experiment. Holliger et al. [33] created standardized protocols for BMP tests, encompassing substrate-to-inoculum ratios, mixing procedures, and termination criteria, which were rigorously followed in our work. The AMPTS II system has been verified in multiple studies as a dependable platform for high-throughput methane potential assessment. The AD experiments included different combinations of materials as follows: manure only [cow (C) or pig (P)], manure combined with inoculum and microbial extract (E) [CDE & PDE], manure in combination with inoculum and microbial consortia (CDC & PDC), and then manure combined with inoculum only (CD & PD). All samples were prepared in triplicate, maintaining a consistent substrate-to-inoculum volatile solids (VS) ratio of 1:2 in each combination. Digester slurry mixtures were prepared by mixing animal manures, digestates, and distilled water to attain a target VS ratio of 1:2 in each reactor for substrate: inoculum. For example, VS contributions from each material were cow (2.5 g), piggery manure (2.5 g), and inoculum (5.01 g). All reactors were topped up with distilled water (10 mL extract and 10 mL consortia suspension) to make 400 mL of sample slurry. In accordance with the AMPTSII standard operating procedure, triplicate samples were prepared and loaded into 500 mL reactor bottles, which were then purged with a low N_2 gas flow (2.5 L/min) for 30 s. The digesters containing the samples were placed in a water bath that operated at 37 °C. The reactors were agitated for 60 min at 60 rev/min after every 2 h over an incubation period of 30 days. The total volume of biogas produced in the reactor was measured in the gas endeavor unit. A wet gas flow measuring device with a 15-flow cell arrangement (a cell dedicated to each separate reactor) uses liquid displacement and buoyancy to measure gas flow. For every 2 mL of gas that flows, this creates and logs a digital pulse. In the CO_2 absorbing unit, CO_2 and H_2S were absorbed using a 3 M NaOH solution combined with 0.4% thymolphthalein pH indicator. Following the absorption unit, the volume of methane (CH_4) remaining in the originally produced biogas would be measured using the same buoyancy and liquid displacement principles as previously described for the gas endeavor unit. Data is expressed as mean ± standard deviation (SD) from triplicate experiments.

2.7. Foaming Activity Measurement

Foaming activity was assessed in parallel with biogas and biomethane production to evaluate the impact of microbial consortia and microbial extract supplementation on digestate foaming and the overall biogas production. Measurements were taken on foam height post-agitation (indicating initial foam formation) and foam stability after 30 min (indicating persistence). The setup used the same material mixtures as those from the AMPTS experiment to ensure consistency. The 500 mL glass bottles (Duran®, DWK Life Sciences GmbH, Mainz, Germany) containing 400 mL slurry mixture (manure combined with inoculum and microbial consortia or microbial extracts, as described in the AMPTS setup) were sealed with red screw caps and fitted with gas collection. To promote anaerobic conditions, the bottles were flushed with high-purity nitrogen gas (99.99%) and sealed.

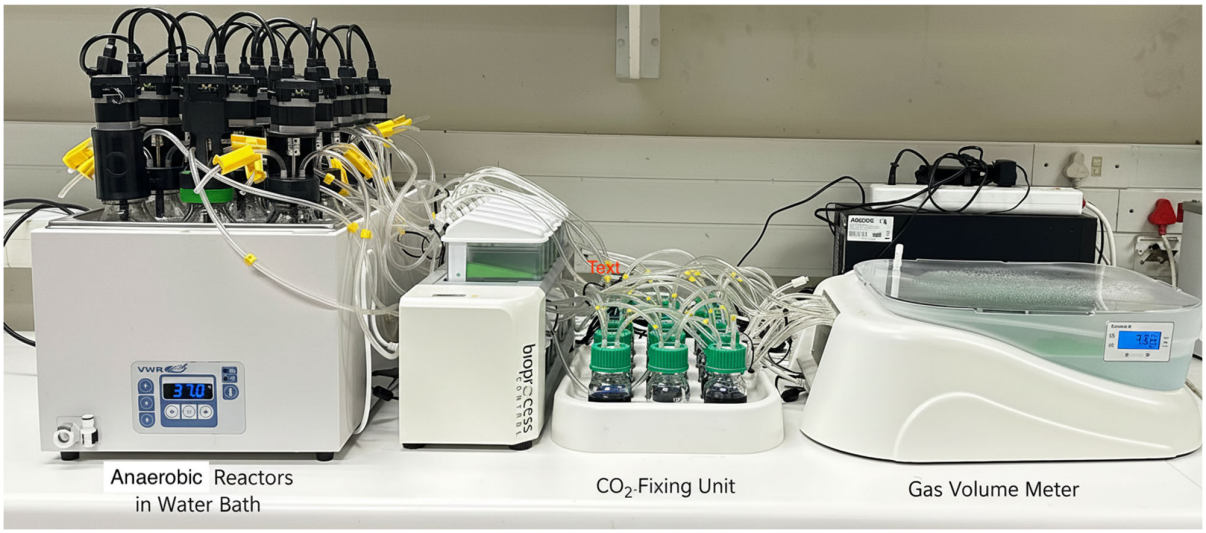

Figure 1. An illustration of the AMPTS setup used in this study.

For foaming experiments (Figure 2), digestion bottles were then placed in a Labotec Inco Shake (Labotec, Midrand, South Africa), model 353, temperature-controlled shaking incubator set to 37 °C and 50 rpm. The shaking speed was chosen to mimic the AMPTS II system's gentle and intermittent mixing. On days 1, 3, 5, 7, 9, 11, 13, 15, 18, 21, 24, 27, and 30, the shaking was paused and bottles were vigorously agitated for 1 min at a time. Using a transparent ruler placed against the bottle wall, the initial foam height was immediately measured as a vertical distance (mm) from the surface of the digestate liquid and the top of the foam layer. Each bottle was measured three times (front, left, and right positions), and the average values for the results were calculated. After 30 min of incubation at 37 °C, the same technique was used to measure the residual foam height.

Figure 2. Experimental setup for foaming activity in manure digestions.

Stability was calculated as the percentage retention:

$$\text{Foam stability} = \frac{\textit{Foam height after } 30 \text{ min}}{\textit{Initial height}} \times 100 \quad (7)$$

Data is expressed as mean ± standard deviation (SD) from triplicate experiments.

2.8. Microbial Community Structure Analysis

Slurry samples from the AMPTS reactors were collected every third day from the start of the digestion process and throughout the incubation period. To ensure that anaerobic conditions in the bottles or reactors were maintained during sampling, the syringe nozzle was placed into the liquid sampling tube prior to the tube being opened by releasing the clamp. After drawing 1 mL of the sample with the syringe and tightening the clamp, the syringe was removed from the sampling tube. From the collected slurry sample, 100 µL was put aside and kept in a cryotube at −20 °C for DNA extraction. DNA was extracted from the digestate using the Faecal/Soil Total DNA™ extraction kit (Zymo Research Corporation, Tustin, CA, USA). The extracted genomic DNA was quantified using the Qubit 2.0 fluorometer (Thermo Fisher, Waltham, MA, USA) and stored in cryotubes at −80 °C until it was sent to Inqaba Biotechnical Industries Pty (Ltd.) in Pretoria, South Africa, for 16S rRNA sequencing using the PacBio sequencing platform (Menlo Park, CA, USA).

2.9. Bioinformatic Analysis of 16S rRNA Sequencing Data

All bioinformatic analyses were conducted in R (version 4.3.0) with the following main packages: phyloseq (version 1.44.0), vegan (version 2.6.4), DESeq2 (version 1.40.0), and ggplot2 (version 3.4.2). Tab-separated text files were used to import Operational Taxonomic Unit (OTU) tables, taxonomic classifications, sample metadata, and phylogenetic trees for Data Import and Phyloseq Object Creation. Merging the OTU table (otu_table), taxonomic assignments (tax_table), sample metadata (sample_data), and phylogenetic tree (phy_tree), a phyloseq object was generated. Samples with fewer than 1000 reads were excluded from the dataset for quality filtering and normalization to guarantee reliable downstream analyses. Taxa exhibiting 0 counts in all samples were subsequently eliminated. For alpha diversity studies, data were rarefied to a uniform depth utilizing rarefy_even_depth with a random seed of 123 to mitigate sequencing depth variance. For the studies of beta diversity and taxonomic composition, numbers were transformed into relative abundances by dividing by the total sample counts [35,36].

Various alpha diversity indices were computed from the rarefied dataset utilizing the estimate richness function, encompassing: Estimates of richness: Observed OTUs, Chao1 index. Diversity metrics: Shannon diversity index, Simpson diversity index. Statistical disparities among experimental groups were evaluated using Kruskal–Wallis tests for each diversity index, with pairwise comparisons conducted as necessary. Beta diversity analysis assessed community composition differences utilizing three dissimilarity metrics: Bray–Curtis dissimilarity, which is based on relative abundances. Weighted UniFrac distance (integrating phylogenetic data and abundance). Principal Coordinates Analysis (PCoA) was conducted on each distance matrix utilizing the ordinate tool. The statistical significance of group separation was evaluated using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations through the adonis2 function [37,38]. Relative abundances were computed at the phylum and genus taxonomic levels for the analysis of taxonomic composition, utilizing tax_glom to aggregate OTUs. To facilitate visualization, the ten most prevalent phyla and the fifteen most prevalent genera from all samples were preserved, while the other taxa were consolidated into an "other" category. Bar graphs illustrating mean relative abundances were created for each taxonomic level [39].

To undertake a genus-level differential abundance analysis, DESeq2 (v1.42) in R (v4.3.1) was utilized. The phyloseq software (v1.42)'s tax_glom was used to aggregate the raw counts from 16S rRNA amplicon sequencing to the genus level. Before analysis, genera with extremely low numbers (total counts < 500 or present in fewer than 5 samples) were eliminated. We eliminated samples with library sizes less than 1000 reads. Differential abundance testing between treatment groups specified in the metadata (Description col-

umn) was carried out using DESeq2. The DESeq function was used with default parameters, and size factors were computed using the "poscounts" method to account for sparse count data. For every contrast, $\log_2$ fold changes and corrected *p*-values (Benjamini–Hochberg correction) were calculated [40].

2.9.1. Differential Abundance Analysis

Volcano plots were created to illustrate differential abundance. For each contrast, the $\log_2$ fold change was graphed against the negative $\log_{10}$ of the adjusted *p*-value. Points were color-coded based on enrichment: genera exhibiting a positive $\log_2$ fold change ($\log_2FC > 0$) and an adjusted *p*-value < 0.05 were classified as enriched in the first group of the contrast, whereas genera displaying a negative $\log_2$ fold change ($\log_2FC < 0$) and an adjusted *p*-value < 0.05 were classified as enriched in the second group. All notably enriched genera were annotated on the plots to denote the direction of enrichment. Gray points denote taxa that are not statistically significant. Volcano plots were created utilizing ggplot2 (v3.4) and ggrepel (v0.9.2) in R.

2.9.2. Functional Prediction and Data Processing

Functional profiles were inferred from 16S rRNA gene sequencing data utilizing PICRUSt2. The unstratified predicted metagenome output, annotated with functional descriptions, was utilized for subsequent analysis. All data processing and visualization were conducted in RStudio (vR 3.6.0) utilizing the tidyverse package (v2.0.0) for data manipulation and reshaping. Using stringr's (tidyverse) keyword-based filtering of functional descriptions, predicted functions related to anaerobic digestion and biosurfactant metabolism were chosen. The sample variance as implemented in the matrixStats package (v1.5.0) (rowVars) was used to compute variation across treatments for each function and mean functional abundances per treatment were determined. For visualization, the functions with the highest variance (top N) were chosen. The pheatmap software (v1.0.13) was used to create the heatmaps. Euclidean distance and full linkage were used to cluster rows and columns hierarchically. Functional classes were included as row annotations, and functional abundance values were displayed as row-wise Z-score normalized values. RColorBrewer (1.1-3) was used to create color gradients [41,42].

3. Results and Discussion

3.1. Compositional Analysis of the Feedstocks

The organic elements compositional analysis data of cow manure, pig manure, and the digestate (inoculum) are presented in Table 1 below. These elemental analyses of feedstocks for anaerobic digestion play a critical role in highlighting the nutrient variations that influence the biomethane production potential [43,44]. The highest carbon concentration was found in cow manure (40.74%), followed by pig manure (36.80%), with the lowest level being detected in the digestate (31.73%). The average hydrogen content was comparable across the samples at 5.12% for cow manure, 5.00% for pig, and 4.42% for digestate. Cow manure exhibited the lowest nitrogen concentrations (1.24%), whereas pig manure had more nitrogen content (2.73%), and the digestate had the highest (3.65%); this in turn influenced the C/N ratios to 32.86%, 13.48% and 8.69%, respectively. Sulphur (S) concentrations of 1.00% and 1.06% were detected in pig manure and digestate samples, respectively, while no sulphur was detected in cow manure. These C/N ratios are a crucial measure of nutrient balancing essential for anaerobic bacteria and archaea to produce biogas/biomethane. Since this ratio is important towards promoting microbial growth and preventing ammonia inhibition or carbon limitation, the literature identifies that a C/N ratio range of 20–30 is effective for biomethane production [45]. The C/N ratios above 30 have been associated

with reduced microbial activity and subsequent lower biomethane production as a result of nutritional stress (insufficient nitrogen content) in the feedstock [46]. The cow manure's C/N ratio, which is higher than the optimal range, means that the feedstock would likely constrain the growth of methanogenic archaea [47,48]. Therefore, unless nitrogen is supplemented, biomethane production from cow manure may not be as good as it could be. On the other hand, pig manure had a below-optimal C/N ratio range; this nitrogen-rich profile contributes to possible ammonia buildup, which, if left unchecked, could limit the proliferation of methanogens [49]. Therefore, pig manure would benefit from co-digestion with carbon-rich feedstock to increase the C/N ratio. Comparable volatile solid content between pig and cow suggests that pigs' nutritional content, rather than organic loading, is likely to support higher yields of biomethane [50]. The digestate's low C/N ratio, which is associated with nitrogen-rich substrates, indicates that it cannot be used as a standalone feedstock [7]. However, both cow and pig manure would benefit from co-digestion with the digestate to achieve the optimal 20–30 C/N range [51]. The extracts and consortia exhibited negligible VS contributions, suggesting that their role will likely be catalytic rather than substantial, without many alterations to the bulk nutritional profile of the substrates.

Table 1. Characterization of inoculum, cow, and pig manure. ± represent mean ± SD of three independent replicates (n = 3).

Sample	%C	%H	%N	%S	C/N	TS (%)	VS (%)
Cow manure	40.74 ± 2.45	5.12 ± 0.77	1.24 ± 0.02	-	32.86 ± 2.45	90.42 ± 0.24	18.12 ± 0.98
Pig manure	36.80 ± 2.02	5.00 ± 0.23	2.73 ± 0.08	1.00 ± 0.02	13.48 ± 1.08	81.22 ± 2.11	18.26 ± 1.05
Inoculum	31.73 ± 0.57	4.42 ± 1.52	3.65 ± 0.65	1.06 ± 0.01	8.69 ± 0.42	56.88 ± 1.34	3.34 ± 0.02
Extract	-	-	-	-	-	0.04	0.03
Consortia	-	-	-	-	-	0.07 ± 0.006	0.06

Note that values for organic elements stated in Table 1 are expressed on a dry basis, while volatile solids are expressed on a wet basis.

The sulphur content was only found in pig manure and digestate at concentrations of 1.00% and 1.06%. Sulphur and nitrogen contents within the feedstock are often associated with hazardous and poisonous gases like ammonia and sulphur oxide, which could negatively affect the environment upon their release [52,53]. However, 1% sulphur content is relatively small, indicating that they are suitable for biogas production [54,55]. The total solids (TS) were highest in cow manure (90.42%), slightly lower in pig manure (81.22%), followed by digestate (56.88%), and the smallest content was for microbial extract (0.04%) and microbial consortia (0.07%). While consortia and extract exhibited negligible volatile solids (VS), cow and pig manure had comparable VS content (18.12% and 18.26%, respectively), and the inoculum had 3.34%. These findings highlight that manures and digestate are nutrient-rich, with microbial extract and consortia contributing very little to the solid and nutritional content and overall volatile solid concentrations.

In order to evaluate the maximum methane yield attainable from our feedstock under ideal anaerobic conditions, we calculated the theoretical biomethane potentials (TBMPs) using Buswell and Dulong prediction models. The results are illustrated in Figure 3. The Buswell method, which is based on the stoichiometric conversion of elemental composition (C, H, O, N, S), assuming complete digestion to methane and carbon dioxide, was 150 mL/g VS for digestate/inoculum, 270 mL/g VS for cow manure, and 240 mL/g VS for pig manure. Dulong's method, which is modified from heating value calculations to predict energy-equivalent methane yield, predicted lower estimates of 255 mL/g VS for cow manure, 225 mL/g VS for pig manure, and 120 mL/g VS for digestate/inoculum. The differences

or gaps between these methods demonstrate methodological variations, with Buswell assuming optimal biochemical efficiency, while Dulong considers combustion-like energy release, which underestimates metabolic pathways [56,57]. Integrating these predicted yields with C/N ratios provides a deeper understanding of practical performance. Cow manure's high TBMP indicates a higher potential yield, but its C/N ratio exceeds the optimal range of 20–30, posing a risk of nitrogen deficit, which may decrease microbial activity and limit the amount of biomethane produced. Pig manure's lower-than-optimal C/N ratio increases its susceptibility to ammonia inhibition and could benefit more from co-digestion with the digestate/inoculum to balance its nutritional composition and promote stable digestion and higher yields. Under ideal conditions, pig manure may create more biomethane even though cow manure has a greater theoretical ceiling. To this effect, the automatic methane potential test system (AMPTS) was used to evaluate actual performance in bioaugmentation treatments involving microbial consortia and extracts.

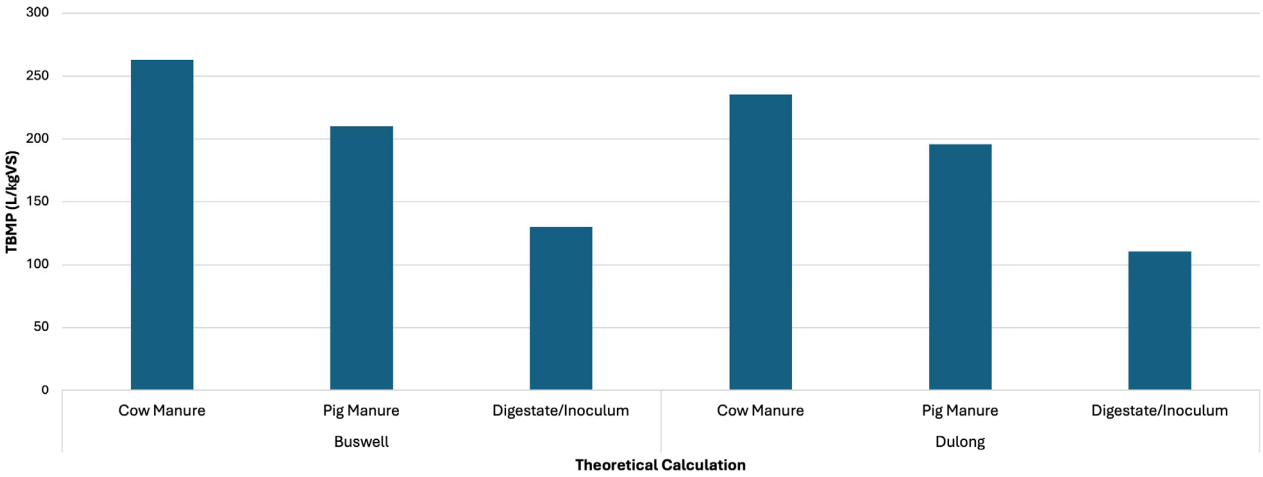

Figure 3. Theoretical biomethane potentials (TBMPs) of manures and inoculum.

3.2. Cumulative Biogas and Biomethane Production

The cumulative biogas and bio-methane curves for cow manure treatments (CDE, CDC, and CD) and pig manure treatments (PDE, PDC, and PD) are shown in Figures 3 and 4. Figure 4 specifically illustrates the paired cumulative biogas and biomethane profiles on identical axes, facilitating a straightforward comparison of total gas volume against methane proportion over the 30-day duration. For cow manure treatments, CDC reached the highest total of approximately 603.28 mL/gVS (67% biomethane), followed by CDE at 508.2 mL/gVS biogas (65% biomethane) and CD at 407.76 mL/gVS biogas (61.5% biomethane). In pig manure treatments, biogas production by PDC was the highest (627.5 mL/gVS biogas, 64.5% biomethane), followed by PDE (439.52 mL/gVS biogas, 63% biomethane) and lastly PD (403.82 mL/gVS biogas, 62% biomethane). The variations in biogas and biomethane concentration reflected the influence of consortia and extract additives on biogas yield. The near-parallel biogas and biomethane curve trajectories (Figure 4) demonstrate that improved methanogenesis, not higher CO_2 production, was the main driver of the yield increases. Across all digestions, biomethane accounted for 61.5–67% of the total biogas, indicating an effective methanogenesis as per the generally understood optimum biomethane content in biogas (more than 60%) from anaerobic digestion of animal waste [58,59]. However, the relative biomethane volumes were higher in treatments. Therefore, the higher percentage increase in biomethane in consortia and extract-supplemented treatments highlights the positive benefit of hydrocarbon- and surfactant-degrading microbial consortia from oil-contaminated soils.

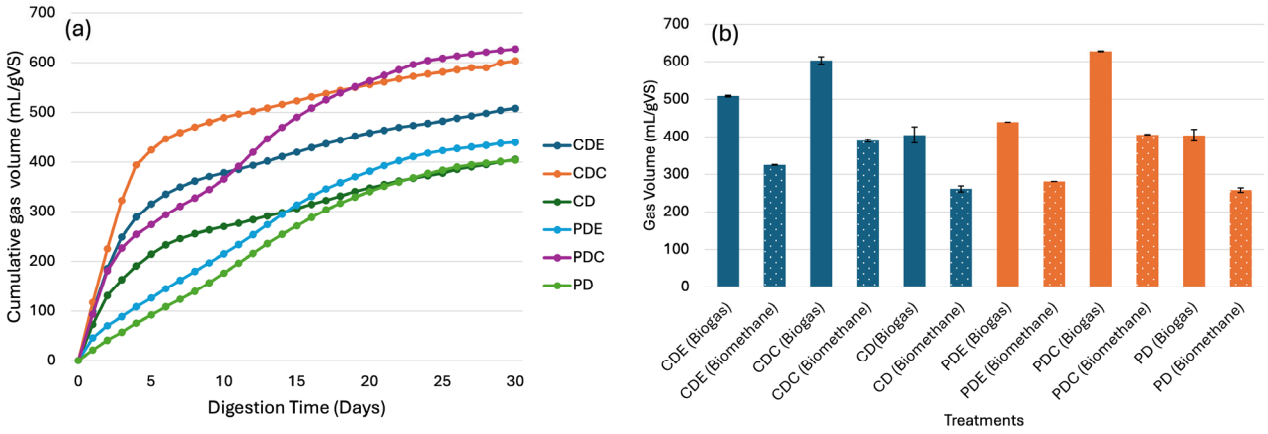

Figure 4. (**a**) Cumulative biogas for different substrate combinations. (**b**) Cumulative biogas and bio-methane for different substrate combinations.

Compared to both CD and PD, microbial consortia in CDC and PDC enhanced yields in biomethane by 30–50%. This enhancement is likely due to improved hydrolysis of complex organic substrates such as lignocellulosic cow manure (C/N = 32.86) and proteinaceous pig manure (C/N = 13.48) by a combination of biosurfactants, protease, lipases, cellulase, etc., to increase substrate bioavailability [57,60]. Moderate increases of up to 25% were due to the microbial extract addition, presumably through both intra- and extracellular enzymes and compounds that contributed to the initial breakdown but lacked the overall sustained microbial synergy [61,62]. Pig manure treatments outperformed cow manure treatments in the overall yields due to pig manure's higher digestibility despite its lower C/N ratio, which may have been improved by the inoculum (digestate) and consortia addition [20,63]. By introducing microbial consortia previously adapted to hydrocarbon and surfactant degradation, it probably improved the hydrolysis and methanogenesis of anaerobic digestion. Furthermore, the supplementation could have helped mitigate inhibition from ammonia or other inhibitory substances. In comparison, the slight difference in yield between PDC and CDC (627.5 mL/gVS vs. 603.28 mL/gVS) may reflect pig manure's protein-rich profile being more susceptible to consortia-driven degradation, which could account for PDC's minor advantage over CDC, which requires further optimization. The untreated CD and PD, which produced only 407.76 mL/gVS and 403.82 mL/gVS cumulative gas, respectively, show baseline digestion, which is limited by slower hydrolysis due to carbon excess in cow dung and nitrogen surplus in pig manure in accordance with their C/N ratios.

The reported increase in methane yields by about 30–70% in comparison to untreated substrates is consistent with the degree of improvement seen in the best-performing treatments [64,65]. Customized microbial consortia and process additives can reduce inhibitory conditions, enhance hydrolysis and acidogenesis, and ultimately boost methane yield and process stability, according to reviews of manure pretreatment and additive use [64–66]. According to Sihlangu et al. [59], the higher methane fractions obtained in CDC and PDC (up to 67%) are comparable to or slightly above typical values for well-optimized manure digestion processes, suggesting that the consortia and microbial extract not only increased gas volume but also improved gas quality. Overall, the order of PDC > PDE > PD in pig manure and CDC > CDE > CD in cow manure shows that the interaction between microbial consortia and extract additives is critical to improving anaerobic digestion performance. Consistent with broader evidence that consortia engineering and additive supplementation are promising tools for optimizing biogas production from livestock manures, the observed

variations in total biogas yield and biomethane concentration show how these interventions can shift the metabolic balance toward more efficient methanogenesis [65–67].

A kinetic study demonstrated that consortium supplementation expedited digestive kinetics. The CDC and PDC reached 90% of total methane generation by day 12–15, whereas untreated controls (CD, PD) achieved this by day 18–21, resulting in a 33% decrease in necessary retention time. The highest initial production rates were observed in consortia-treated digesters (CDC: 116–120 mL/gVS per day; PDC: 96–100 mL/gVS per day), followed by extract treatments (CDE: 96–100 mL/gVS per day; PDE: 44–48 mL/gVS per day), while control groups had the lowest rates (CD: 72–76 mL/gVS per day; PD: 20–24 mL/gVS per day). The pronounced initial slopes in consortia-amended systems indicate expedited hydrolysis and acidogenesis, aligning with their concentration of fermentative species. The 5–6 day decrease in productive phase time indicates that bioaugmentation enhances both overall yields and digester throughput capacity. Similar kinetic benefits (shorter lag phase) have been reported when biological pretreatments or bioaugmentation consortia are applied to manure or manure–residue mixtures, where effective methane production time was shortened by ~15–20% compared with untreated controls [68,69].

3.3. pH Trends During 30-Day Anaerobic Digestion

The pH was observed during a 30-day period as a measure of process stability, indicating the potential equilibrium between volatile fatty acid (VFA) production and the general pH buffering capability of the system (Figure 5). The pH of all the samples ranged between 7.5 and 8.0, indicating neutral to slightly alkaline starting conditions, a state common before the accumulation of VFAs. For cow manure samples, the pH dropped within the first three to six days, likely due to the quick build-up of VFAs as organic matter started to hydrolyze and acidify. This decline suggests a mild VFA buildup, as shown by pH declining to roughly 6.5–7.0. By day 30, the pH had steadily recovered to between 6.8 and 7.2, suggesting that methanogens had successfully utilized VFAs. In both cow and pig manure digesters, the brief pH drops into ~6.0–7.0 are consistent with VFA accumulation during early hydrolysis/acidogenesis, followed by gradual VFA consumption and pH recovery as methanogenesis takes over [70,71].

Among the samples, CDC had a more stable and consistent pH trajectory, which peaked and stabilized at 7.2. This suggests a minimal VFA accumulation and stronger buffering capacity. CDE showed moderate fluctuations around pH 7.0, representing a balanced yet slightly variable VFA accumulation. The pH of CD (untreated) mostly stayed the lowest (at 6.8), suggesting VFA buildup or less buffering capacity of the system. According to Wang et al. [66] and Sun et al. [72], cow treatments did not fall into the strongly inhibitory zone (6.0) that is associated with overloaded or destabilized systems. Similarly to stable manure and co-digestion systems, where the final pH settles around neutral or slightly alkaline, recovery to 6.8–7.2 by day 30 suggests that methanogens successfully used VFAs and restored near-neutral conditions [73,74].

For piggery manure samples, the initial pH decline was sharper, dropping to 6.5 by day 3, potentially due to a quicker and more intense VFA accumulation brought by piggery waste's higher organic content or microbial activity. The drop in pH was subsequently followed by a more marked recovery phase with fluctuations, suggesting dynamic VFA generation and utilization. PDC dropped gently from 7.8 to 6.2 by day 6 and subsequently recovered to a constant pH of 7.0 before increasing to around pH 8.0 by day 30, indicating the best VFA utilization or pH buffering capacity. PDE declined from pH 7.9 to 6.0 on day 9 and reached 7.5 by day 30, displaying a possibility of a moderate residual VFA profile. According to Wang et al. [66] and Vanegas et al. [70], pig manure frequently has a larger organic content and can acidify more quickly, which corresponds with the faster decline to

~6.5 by day 3 in all pig treatments. Sustained low pH is associated with VFA formation and instability in many pig-manure systems, necessitating the use of exogenous alkali to restore neutrality [70,72]. PD went from pH 8.0 to 5.8 by day 9 and recovered to 7.2 by day 30. In comparison to microbial extract-treated samples (CDE and PDE) and untreated (PD and CD), the consortia supplemented (CDC and PDC) generally maintained higher or more stable pH levels, suggesting better VFA management and utilization. Higher transient VFA stress is suggested by sharper pH dips and slower recovery in PD and CD, which is consistent with studies where VFA accumulation and decreased methanation efficiency were caused by inadequate buffering or overloaded circumstances [66,72]. This is likely from the influence of microbial consortia added in shaping anaerobic digestion performance. Overall, this data indicates that bioaugmentation, especially with live consortia, enhances process robustness to substrate-induced acid stress. Improved VFA utilization is suggested by observed pH stabilization, and trends in pH closely mirrored the kinetics of methane generation; however, direct VFA measurements were not carried out, which is a limitation and a crucial area for further research.

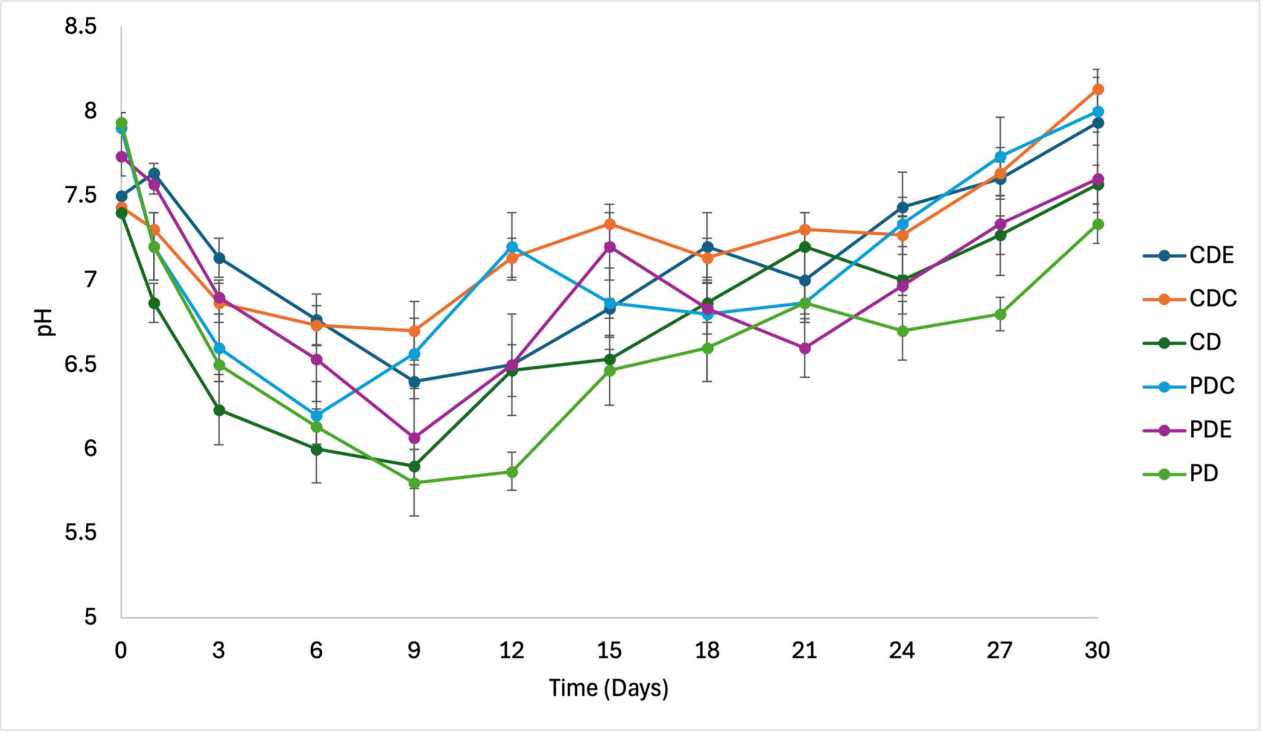

Figure 5. The pH dynamics during anaerobic digestion over a 30-day period. Cow manure is represented by CDE, CDC, and CD, and piggery manure is represented by PDE, PDC, and PD.

3.4. Foaming Activity and Stability Profile over 30 Days of Anaerobic Digestion

In anaerobic digestion, foaming activity and stability are crucial factors because excessive foam is capable of disrupting process efficiency by reducing working volume, impairing mixing, etc. Excessive foam can hinder mixing, lower effective volume, and diminish biogas recovery in anaerobic digestion. Foaming behavior is directly related to gas production, surface-active chemicals, and microbial population dynamics [75]. While stability shows how long gas stays trapped because of extracellular polymeric substances (EPS), proteins, lipids, VFAs, and particular microbes that lower surface tension and strengthen bubble films, foam activity (height) primarily reflects gas generation and the presence of surfactants [75,76]. Therefore, the effects of microbial extract and consortia on foaming activity and foaming stability profiles monitored over the 30-day anaerobic digestion are presented in Figures 6 and 7.

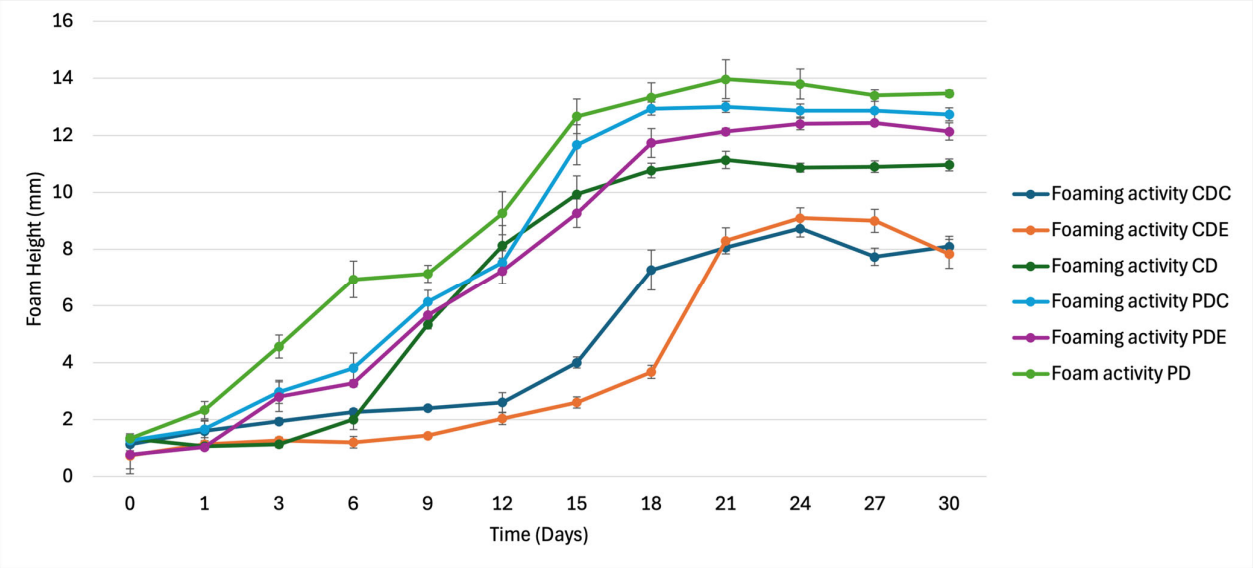

Figure 6. Foaming height measurements during 30-day anaerobic digestion of cow (CDE, CDC, and CD) and piggery manure (PDE, PDC, and PD) treatments.

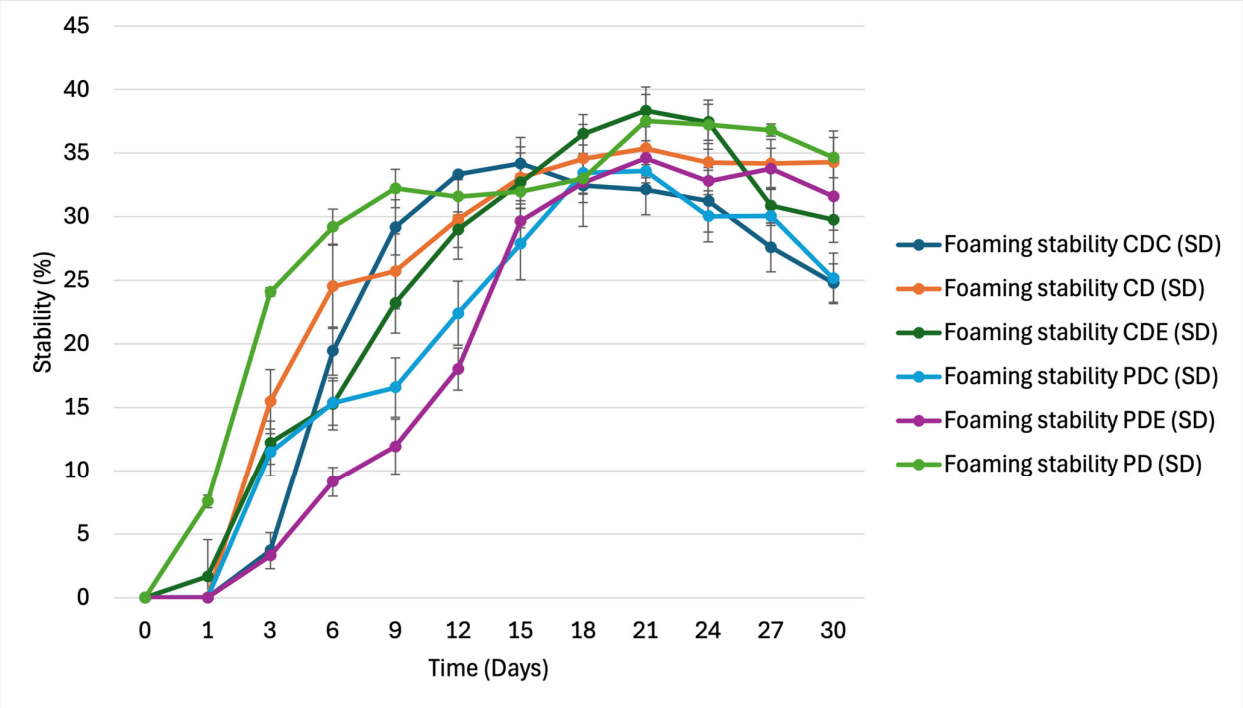

Figure 7. Foam stability measurements during 30-day anaerobic digestion of cow manure treatments (CDE, CDC, and CD) and piggery manure treatments (CDE, CDC, and CD).

The low initial foam height (0–2 mm, days 0–3) in both piggery and cow dung digesters denotes low gas generation and/or still-low accumulation of compounds that cause foam during the lag, hydrolysis, and early acidogenesis stages. There are numerous reports of similar connections between minimum foaming, low biogas, and start-up phases [75]. Foaming activity increased from day 6 and peaked between days 15–30, coinciding with increased acidogenesis, VFA formation, and methanogenesis. This period also saw the highest gas production and surfactant release (from protein, lipid, and EPS breakdown [75,77]. According to Syed Sabeer et al. [76] and Harirchi et al. [78], the higher peaks in pig manure (12–14 mm) compared with cow manure (9–11 mm) are consistent with pig manure's higher

soluble organic and nitrogen content, which promotes rapid VFA and gas generation and has been associated with stronger foaming and ammonia/VFA-related instability. The untreated control (PD) in piggery digesters exhibited the highest foaming activity (peak 14 mm) and stability (37.5%), indicating significant accumulation of surface-active materials (proteins, lipids, VFAs, and EPS) that lower surface tension and stabilize foam, as well as intense gas evolution [79].

Microbial consortia (CDC) and extract (CDE) both decreased foam heights (to ~8.8–9.2 mm) and slightly decreased stability, whereas the control (CD) had the highest activity (11 mm) and stability (36–38%). This suggests that biological additives can moderate foam even in more resistant, fiber-rich substrates, which is consistent with broader evidence on additive-assisted process stabilization [65,77]. The highest foam heights in cow manure digesters were lower, but the stability was slightly higher (up to 38%), suggesting that the foams were less intense but more persistent. This is consistent with the slower hydrolysis and higher lignocellulosic content of cow dung, which can promote more stable foams and EPS-rich flocs while limiting gas peaks [6,77]. According to this study, piggery manure produces foaming that is intense but less lasting. This is likely due to more soluble organic matter that produces VFAs more quickly, thus higher gas production rates, but prevents long stability, likely due to microbial treatment. The higher lignocellulosic content in cow manure, on the other hand, results in lower foaming activity but more stable foam. This could be because of slower degradation kinetics and the presence of organic compounds, natural surfactants, and microbial communities that improve foam elasticity, though this was lessened in treated samples (CDC and CDE).

The need for substrate-specific foam management techniques is shown by the varied responses of cow manure and pig dung. Targeted biological additives (microbial strains, consortia, and enzymes) and operating-parameter management are promising for antifoaming and defoaming, but they must be customized to manure composition, EPS dynamics, and nitrogen content, according to reviews on foaming and livestock manure digestion [75,80]. Microbial extracts and consortia in the current system show promise as biological defoaming agents, concurrently moderating foam and supporting more stable digestion, according to current directions for biologically based control of foaming in anaerobic reactors [66,81].

3.5. Dynamics of Microbial Communities Influencing Process Performance

To elucidate the microbial populations influencing variations in biogas production, pH stability, and foaming, 16S rRNA gene sequences from digestate samples gathered throughout the 30-day incubation period were examined. These analyses included alpha diversity indices, temporal succession patterns, and differential abundance, offering a thorough understanding of how consortia and extract amendments interacted with native microbial communities in a substrate-dependent manner.

3.5.1. Alpha Diversity and Community Richness

Alpha diversity metrics (Observed species, Chao1, Shannon, and Simpson) revealed distinct substrate-driven community structures (Figure 8). Alpha diversity analysis revealed microbial structure dependent on substrate across treatments. Consortia-amended digesters typically demonstrated marginally increased variety and less variability, indicating improved community stability and functional redundancy. The extract supplementation led to increased richness, especially in pig dung systems, possibly indicating nutrient-driven enhancement of metabolically diverse species. Baseline manure-digestate systems had modest diversity but demonstrated increased distribution, signifying a less stable community organization. These data indicate that both microbial augmentation

and extract amendment affect microbial community complexity, potentially impacting anaerobic digestion efficiency and process resilience.

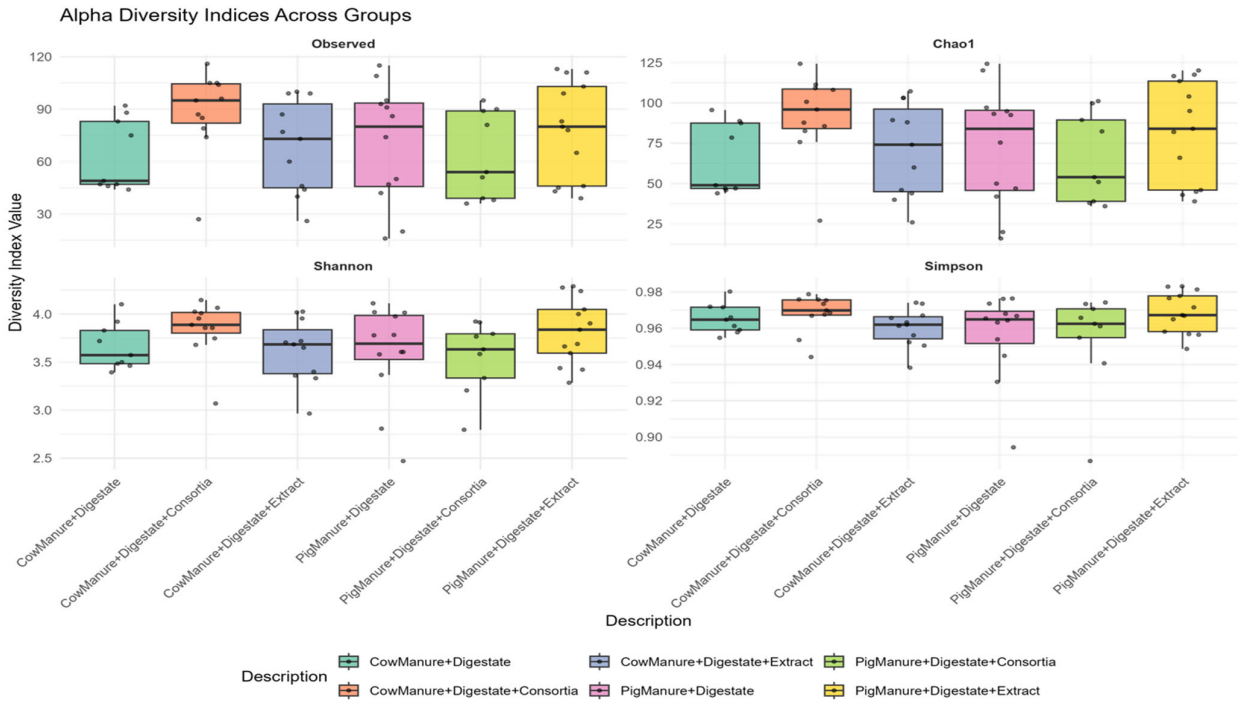

Figure 8. Alpha diversity metrics in cow and pig manure anaerobic digestates under different treatments (microbial community augmentation and microbial extract addition).

When combined, these indices characterize community complexity and can be utilized to deduce redundancy and functional capacity in anaerobic digesters [82,83]. More possible metabolic pathways and functional redundancy, which enhance process stability and resilience to disturbances like ammonia stress, loading shocks, or temperature variations, are frequently indicated by higher alpha diversity and evenness [83,84]. Even when some taxa are lost during anaerobic digestion, functionally redundant communities typically continue to produce methane [83,84]. Studies where bioaugmentation or mixed inocula boosted functional redundancy and improved recovery under inhibition or high load are consistent with consortia-amended digesters exhibiting slightly more variety and reduced variability [85,86]. Increasing richness by extract supplementation is compatible with nutritional or growth-factor inputs that create new ecological niches and sustain a wider variety of metabolically specialized taxa, particularly in pig dung systems [66,87]. However, process performance is determined by the balance between richness, evenness, and the existence of important functional groups; more richness by itself does not ensure stability [82].

Higher alpha diversity and functional redundancy are positively correlated with methane production, process stability, and resistance to disturbances, according to studies on anaerobic digestion and other microbial ecosystems [83,84]. Therefore, the conclusion that microbial augmentation and extract addition reshape community complexity in ways that can affect digestion efficiency and resilience is supported by the observed shifts to higher and more consistent diversity in consortia-amended systems, richness boosts with extract, and more variable baseline communities. According to Wang et al. [66] and Li et al. [84], consortia typically increase variety and decrease between-reactor variability, suggesting improved functional redundancy and stability. Extracts primarily increase richness, especially in pig manure systems, indicating the proliferation of specialized species driven by nutrients or niches [83,87].

3.5.2. Time-Based Microbial Succession and Differential Abundance

The 30-day period's temporal characterization of genus-level abundances revealed notable variations in community succession and stability (Supplementary Figure S3). Both cow and pig dung anaerobic digesters showed treatment-dependent changes and substrate-specific community structure during the 30-day digestion period, according to the temporal profiling of genus-level microbial distribution. The addition of microbial consortia or extracts affected the relative abundance, stability, and succession patterns of several fermentative and syntrophy-associated genera, whereas core anaerobic digestion taxa remained consistent across all treatments, according to samples taken at three-day intervals. Numerous independent investigations across various sizes consistently indicate a preserved core microbiome in anaerobic digestion. Tao et al. [88] identified *Bacillus*, *Clostridium*, *Bacteroides*, and *Syntrophomonas* in all 20 lab-scale reactors studied, with their abundances significantly connected to biogas production. This pattern is also applicable to full-scale installations: Całusińska et al. [89] observed 20 digesters over the course of one year and documented a stable Firmicutes/Bacteroidetes core comprising approximately 70% of the microbial community, irrespective of feedstock or plant configuration. A recent meta-analysis of over 1258 samples from 32 high-rate digesters identified more than 100 bacterial and six archaeal taxa present in at least 50% of the samples [90]. Reviews generally corroborate these findings, designating Firmicutes and Bacteroidetes as principal fermenters and *Methanosaeta*, *Methanosarcina*, and *Methanobacterium* as the predominant methanogens [87,91,92]. This convergence indicates that AD microbiomes are non-stochastic and functionally organized around a predictable core, necessitating more investigation into the metabolic mechanisms that support their survival and performance.

Cow Dung Digestate vs. Consortia

The supplementation of cow manure digestate with external microbial consortia resulted in slight yet noticeable alterations in the local microbial community, as demonstrated by differential abundance analysis in Figure 9. A volcano plot indicated a restricted number of significantly affected taxa, with a peak statistical significance of roughly $-\log_{10}(p\text{-value}) \sim 12$, suggesting a regulated shift rather than a substantial disruptive alteration. Taxa demonstrating notable enrichment subsequent to the addition of consortia comprised *Alkaliflexus* (seq77), *Guopingia* (seq531), *Paludibacter* (seq363), and *Coprobacter* (seq104). In contrast, numerous taxa that were moderately prevalent in the untreated digestate baseline had a notable decline in relative abundance following amendment. The list includes *Proteiniphilum* (seq335), *Oliverpabstia* (seq108), *Soehngenia* (seq121), *Seramator* (seq135), *Thermanaerosceptrum* (seq227), *Anaerovorax* (seq159), *Buttiauxella* (seq588), *Pseudobacteroides* (seq347), and *Chitinispirillum* (seq193). The observed changes presumably indicate community restructuring caused by the competitive integration of new functional populations and subsequent modifications in ecological interactions, rather than direct inhibitory repression.

The differential abundance patterns outlined are characteristic of bioaugmentation, resulting in targeted taxonomic alterations within a predominantly stable core population in anaerobic digestion [93,94]. Research on the digestion of cow manure or lignocellulosic materials frequently indicates an improved performance with minimal alterations in the overall community structure, however evident the enrichment of particular bacterial guilds (cellulolytic, fermentative, syntrophic) following bioaugmentation [93,95]. Communities that partially return to their previous state upon depletion of the additional substrate, signifying regulated adjustment rather than irreversible disruption [95]. The increase in specific fermentative or saccharolytic species following the inclusion of consortia, coupled with the reduction in many moderately common baseline taxa, exemplifies a pattern of competitive integration and niche reallocation rather than direct suppression. Bioaugmentation of

cow-manure systems often enhances cellulolytic and fermentative taxa (e.g., *Clostridium*, *Fibrobacter*, *Bacillus*, *Brevefilum*) that facilitate hydrolysis and methanogenesis [8,95]. Concurrently, additional fermenters or secondary degraders diminish in relative abundance when substrate spectra, cross-feeding, and syntrophic connections are restructured [96,97].

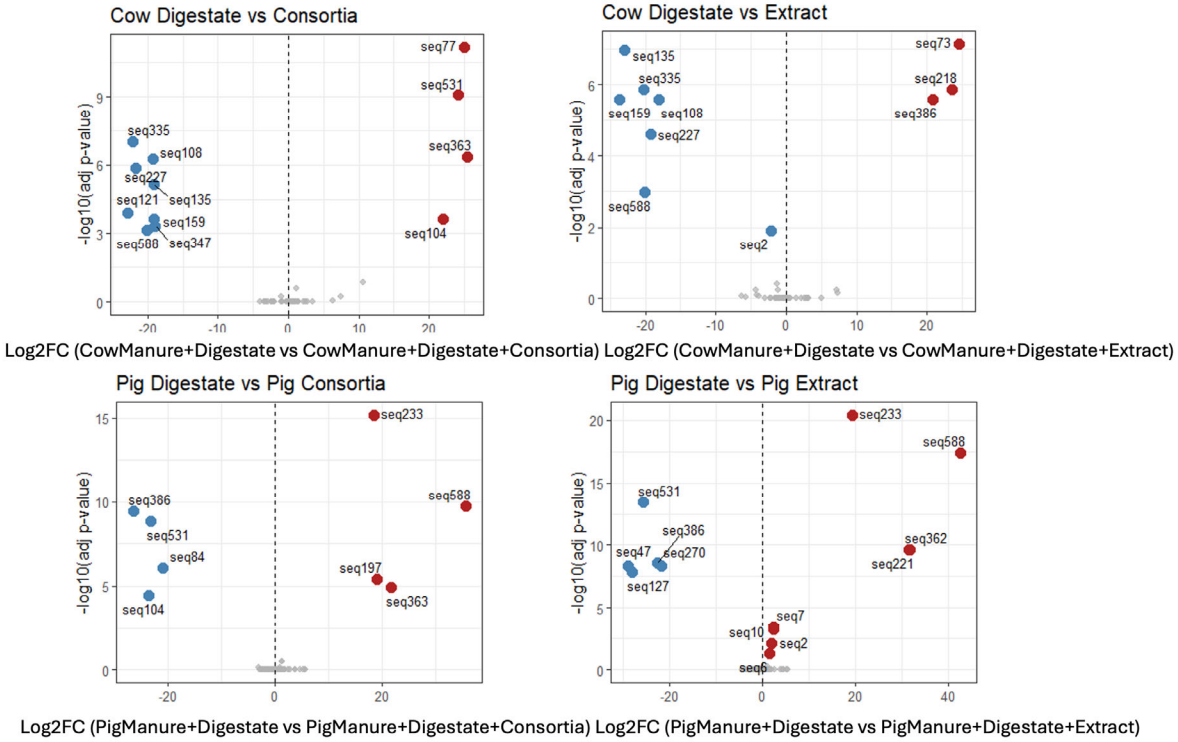

Figure 9. Differential abundance volcano plot highlighting taxa relative abundance enriched and reduced by consortia and extract addition.

Cow Dung Digestate vs. Extract

Unlike the consortia amendment, the addition of extract to cow manure digestate caused negligible disruption to the existing microbial population. The differential abundance analysis, depicted by a volcano plot, demonstrated minimal statistically significant alterations (highest $-\log_{10}(p\text{-value}) \sim 8$) with a sparse data point distribution, suggesting a restricted overall effect. Taxa demonstrating a notable reduction in relative abundance subsequent to extract amendment and consequently enriched in the untreated digestate comprised *Buttiauxella* (seq588), *Seramator* (seq135), *Proteiniphilum* (seq335), *Anaerovorax* (seq159), *Oliverpabstia* (seq108), *Thermanaerosceptrum* (seq227), and *Mucinivorans* (seq2). Conversely, only a limited number of taxa exhibited significant enrichment from the extract, including *Lignipirellula* (seq386), *Millionella* (seq73), and *Keratinibaculum* (seq218). The little community change indicates that the extract amendment affects the digestate ecosystem mostly through indirect biochemical or metabolic mechanisms, rather than through direct competitive displacement or encouragement of certain microbial populations seen with the addition of live consortia.

The depicted volcano plot, featuring a limited number of significant taxa and a maximum $-\log_{10}p$ of approximately 8, aligns with the pronounced community resistance to non-living amendments observed in soils and digestates. The application of cattle manure digestate to soils resulted in minimal alterations to the dominant bacterial and fungal community composition when the indigenous community remained intact, notwithstanding evident functional and respiration responses [98]. Additives like compost-derived biopolymers or minerals can marginally influence certain methanogens or bacterial groups,

while the overall community structure and performance remain only little altered [99,100]. The limited group of taxa diminished following extraction (e.g., *Proteiniphilum, Anaerovorax*) indicates nuanced competitive or resource-related modifications rather than extensive suppression, akin to the manner in which digestate or biochar amendments alter specific functional groups while predominantly influencing respiration, enzyme activity, or electron transfer pathways [98,101]. The literature indicates that extracts primarily function through biochemical and physicochemical modifications, such as electron shuttling and nutrient and toxin buffering, rather than fundamentally altering the microbial network, unlike live consortia [101,102].

Pig Dung Digestate vs. Consortia

The modification of pig dung digestate with external microbial consortia resulted in a more significant community rearrangement than that reported in cow digestate. Differential abundance analysis indicated mild yet clear shifts, with a volcano plot illustrating a higher number of significant alterations (maximum $-\log_{10}(p\text{-value}) \sim 20$). Taxa that exhibited considerable reduction after consortium supplementation and were consequently concentrated in the untreated pig digestate were *Lignipirellula* (seq386), *Guopingia* (seq531), *Romboutsia* (seq84), and *Coprobacter* (seq104). The consortia adjustment markedly enhanced taxa including *Clostridium sensu stricto* (seq233), *Buttiauxella* (seq588), *Macellibacteroides* (seq197), and *Paludibacter* (seq363). The increased magnitude and frequency of substantial changes suggest that the native microbial community in pig digestate is more reactive to biological amendment than that in cow digestate, experiencing more pronounced competitive restructuring where introduced consortia members outcompete specific resident taxa while enhancing others.

Taxa such as *Clostridium sensu stricto*, *Buttiauxella*, *Macellibacteroides*, and *Paludibacter* that proliferated following the addition of consortia are generally consistent with fermentative and acidogenic functions. Clostridiales members (e.g., *Clostridium sensu stricto*) are recognized as primary and secondary fermenters in anaerobic digestion, hydrolyzing complex polymers and converting sugars and amino acids into volatile fatty acids (VFAs), hydrogen (H_2), and carbon dioxide (CO_2), which support downstream syntrophs and methanogens [83,103]. Therefore, enrichment suggests an increased capacity for hydrolysis and primary fermentation, which may raise substrate turnover and biogas precursors. Bacteroidota-like fermenters, such as Macellibacteroides, have been shown to play an important role in breaking complex proteins and polysaccharides. They also frequently produce short-chain fatty acids and add to the digesters' overall carbon flux [51,104]. Their augmentation indicates enhanced ability to break down complex organic substances and sustain VFA generation under modified conditions. *Paludibacter* species, which are commonly found in anaerobic, carbohydrate-rich environments, ferment carbohydrates to acetate, propionate, and succinate and integrate into cross-feeding networks to promote methanogenesis [51,83,96]. Their enrichment suggests that syntrophic fermentation networks are being strengthened. *Buttiauxella*, a facultative anaerobe in the Enterobacteriaceae family, engages in mixed-acid fermentation and exhibits metabolic plasticity by utilizing alternate electron acceptors when they are temporarily accessible [104,105]. The increase in this relative abundance may signify more flexibility in redox balance and intermediate turnover. These groups' combined enrichment indicates that the external consortia are influencing the pig digestate community to become a more functionally redundant, fermentation-competent assemblage, which is a hallmark of stable, effective anaerobic digestion.

Pig Dung Digestate vs. Extract

The differential abundance study of pig dung digestate, with and without extract addition, indicated a more significant community impact compared to the cow digestate trial. The volcano plot revealed a pattern of few to moderate significant alterations (maximum $-\log_{10}$(*p*-value) ~20), marked by an even distribution of both enriched and suppressed taxa. Taxa demonstrating a notable reduction in relative abundance after the addition of extract and consequently enriched in the untreated pig digestate comprised *Guopingia* (seq531), *Lignipirellula* (seq386), *Alistipes* (seq47), *Arcobacter* (seq270), and *Christensenella* (seq127). The extract amendment markedly enhanced a specific group of taxa, comprising *Buttiauxella* (seq588), *Clostridium sensu stricto* (seq233), *Stieleria* (seq362), *Teretinema* (seq221), *Vagococcus* (seq7), and *Mucinivorans* (seq2). The more pronounced community reaction in pig digestate to the extract, in contrast to the little effect observed in cow digestate, indicates a superior intrinsic metabolic or ecological sensitivity to this modification. This conclusion, in conjunction with the consortia data, suggests that the indigenous community structure of the source substrate (pig versus cow) is a pivotal factor in the results of both biological and metabolic processes.

The high responsiveness in pig digestate supplemented with exact aligns with findings indicating that pig gut- and manure-derived communities are typically species-rich, metabolically versatile, and predominantly composed of taxa capable of swiftly utilizing a variety of substrates, unlike the more functionally specialized, fiber-adapted ruminant microbiota found in cattle [106,107]. The rise in the relative abundance of *Buttiauxella*, *Clostridium sensu stricto*, *Stieleria*, *Teretinema*, *Vagococcus*, and *Mucinivorans*, alongside the decrease in *Guopingia*, *Lignipirellula, Alistipes*, *Arcobacter*, and *Christensenella*, indicates that pig-derived microbial communities demonstrate greater intrinsic ecological and metabolic sensitivity to organic extracts compared to cow-derived digestate communities [108,109]. These changes correspond with extensive evidence indicating that modifications can preferentially benefit copiotrophic and fermentative groups while inhibiting taxa adapted to different environments, therefore altering metabolic pathways and end-product profiles in anaerobic digestion [110,111]. These findings, when combined with consortia performance data, provide evidence that cow and pig digestates are not interchangeable inocula/substrates because their different baseline community compositions significantly alter the magnitude and direction of biological and metabolic reactions to extract and consortia amendment in anaerobic digestion processes [78,110].

3.5.3. Predicted Functional Profiles in Anaerobic Digestion Systems

Variance-based heatmap analysis of PICRUSt2-predicted functional profiles indicated substrate-specific and treatment-dependent changes in microbial metabolic pathways related to anaerobic digestion and biosurfactant metabolism (Figure 10). Functions were chosen for their significant variability among treatments to underscore those most reactive to changes, with hierarchical clustering highlighting parallels in patterns of functional abundance. Overall, cow manure-based treatments were distinctly separated from pig manure treatments, highlighting substrate origin as a key determinant of anticipated metagenomic content. In substrates, consortia modifications induced more significant changes in functional Z-scores than extracts, aligning with ecological inoculation effects rather than biochemical modulation.

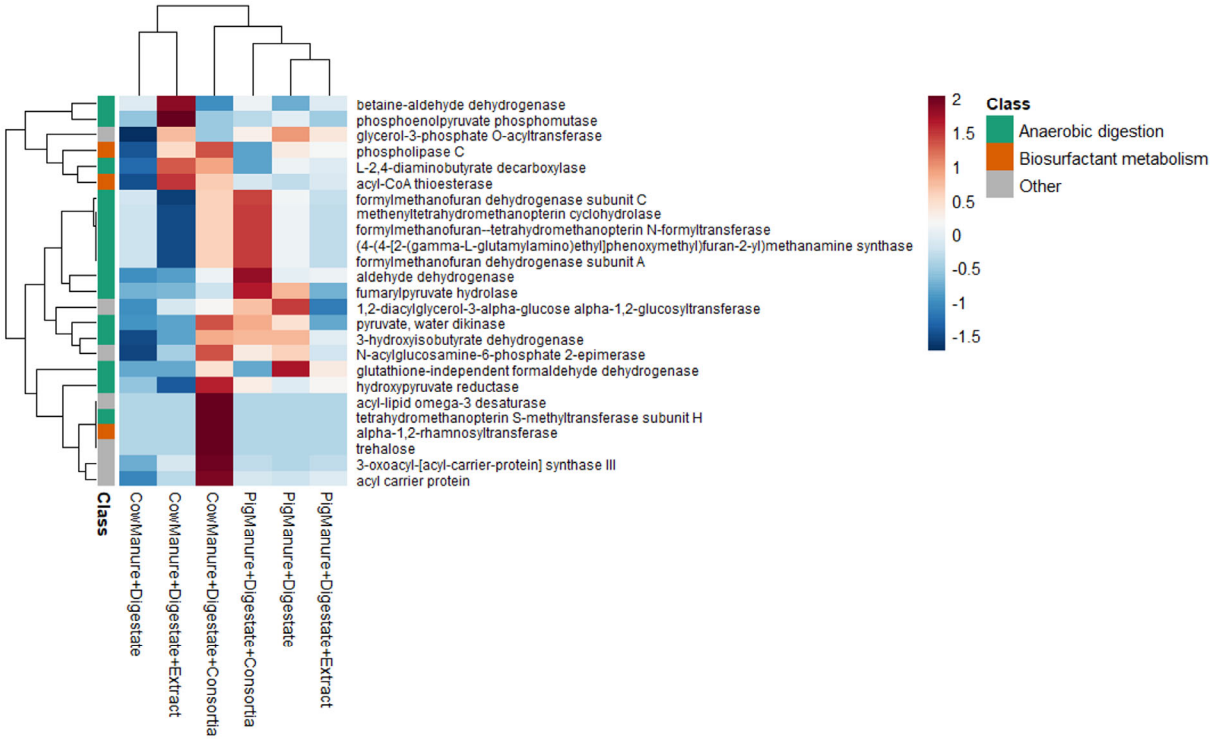

Figure 10. Variance-based heatmap of PICRUSt2-predicted functional profiles from 16S rRNA data, emphasizing anaerobic digestion and biosurfactant metabolism. Rows: High-variance functions clustered (Euclidean distance, complete linkage) and annotated by class (green: anaerobic digestion; orange: biosurfactant metabolism; gray: other). Columns: Treatments (cow/pig digestate ± consortia/extract), clustered.

Cow Manure and Digestate (CD)

In CD, AD functions like L-2,4-diaminobutyrate decarboxylase (Z-score: −1) and 3-hydroxyisobutyrate dehydrogenase (Z-score: −1) showed low relative abundance, while biosurfactant metabolism exhibited negative Z-scores for phospholipase C (Z-score: −1) and acyl-CoA thioesterase (Z-score: −1.5), suggesting baseline suppression of lipid-processing pathways. Low Z-scores indicate less nitrogen and carbon routing through these fermentation branches, which is consistent with AD communities that prioritize core hydrolysis/acidogenesis over specialized amino acid funnels when substrate N-load is modest [78,83]. L-2,4-diaminobutyrate decarboxylase and 3-hydroxyisobutyrate dehydrogenase are involved in amino acid metabolism and branched-chain amino acid metabolism. Phospholipase C and acyl-CoA thioesterase are pivotal in lipid and biosurfactant metabolism; their inhibition correlates with findings indicating that digesters processing less lipid-dense or more fibrous substrates exhibit a diminished presence of lipid/fatty-acid metabolism genes in comparison to carbohydrate/protein metabolic functions [78,112]. Collectively, these patterns suggest that in CD during anaerobic digestion, the fundamental metabolic condition is carbohydrate-centric, exhibiting minimal dependence on specialized amino acid pathways and diminished lipid processing and biosurfactant metabolism. This may restrict the degradation of fat-rich or hydrophobic wastes unless process conditions or inocula are specifically designed to enhance lipid-degrading guilds and their corresponding enzymes [113,114].

Cow Manure Digestate with Microbial Consortia (CDC)

CDC displayed elevated AD functions, including L-2,4-diaminobutyrate decarboxylase (Z-score: 1), pyruvate water dikinase (Z-score: 1.5), hydroxypyruvate reductase (Z-

score: 1.5), and tetrahydromethanopterin S-methyltransferase subunit H (Z-score: 2), alongside biosurfactant enrichments in phospholipase C (Z-score: 1.5) and alpha-1,2-mannosyltransferase (Z-score: 2). Betaine-aldehyde dehydrogenase (Z-score: −1) was suppressed in AD. The elevation of L-2,4-diaminobutyrate decarboxylase, pyruvate water dikinase, and hydroxy-pyruvate reductase indicates an enhancement of amino acid and pyruvate-centered fermentation, aligning with evidence that consortia and bioaugmentation improve amino acid and energy metabolism in stressed or modified anaerobic digestion systems [115–117]. The elevated levels of tetrahydromethanopterin S-methyltransferase (H-subunit) directly indicate enhanced hydrogenotrophic/CO_2-reduction methanogenesis, consistent with metagenomic reconstructions that demonstrate the enhancement of methanogenesis-related enzymes restores or improves anaerobic digestion performance [118,119]. Enrichment of phospholipase C and alpha-1,2-mannosyltransferase suggests enhanced lipid and glyco-/glycolipid processing at interfaces, similar to systems where biosurfactants or surfactant-like amendments stimulate hydrolysis, extracellular electron transfer, and methane metabolism [120,121]. This suggests that consortia inoculation aids in unlocking lipid-associated energy sources, in contrast to the baseline cow dung + digestate inhibition of lipid-processing genes [119,120]. While suppression of betaine-aldehyde dehydrogenase suggests a decreased dependence on betaine-based osmoprotection, positive Z-scores for several AD and biosurfactant enzymes show a functionally reconfigured community that is more energetically geared toward methanogenesis and surface-active metabolism.

Cow Dung Digestate with Microbial Consortia (CDC)

CDE showed strong positive Z-scores in AD pathways, such as betaine-aldehyde dehydrogenase (Z-score: 1.5), phosphoenolpyruvate phosphomutase (Z-score: 2), and L-2,4-diaminobutyrate decarboxylase (Z-score: 1), but negative values for formylmethanofuran dehydrogenase subunit C (Z-score: −1.5), methylene tetrahydro methanopterin cyclohydrolase (Z-score: −1), formylmethanofuran dehydrogenase subunit A (Z-score: −1), (4-[4-[12-(gamma-L-glutamylamino)ethyl]phenoxymethyl]furan-2-yl)methanamine synthase (Z-score: −1), formylmethanofuran-tetrahydromethanopterin N-formyltransferase (Z-score: −1), and hydroxypyruvate reductase (Z-score: −1). Biosurfactant metabolism featured positive Z-scores for acyl-CoA thioesterase (Z-score: 1.5). CDE integrates augmented fermentative and stress-related AD functions with diminished core hydrogenotrophic methanogenesis: Betaine-aldehyde dehydrogenase (1.5) indicates the active synthesis of glycine betaine, facilitating osmoprotection and the utilization of carbon and nitrogen from choline and analogous substrates [122,123]. In line with AD systems, where acidogenesis and VFA production are supported by amino-acid and pyruvate-linked branches, phosphoenolpyruvate phosphomutase (2) and L2,4-diaminobutyrate decarboxylase (1) show reinforced C3/C4 and amino-acid routing into central metabolism [124,125]. The reduction in formylmethanofuran dehydrogenase subunits (A, C), methylene tetrahydromethanopterin cyclohydrolase, and formylmethanofuran–H_4MPT N-formyltransferase suggests a downregulation of the CO_2 → formyl-MFR → H_4MPT pathway, representing the initial stages of hydrogenotrophic methanogenesis [124,125]. Comparable reductions in formylmethanofuran dehydrogenase and associated enzymes correlate with diminished methane recovery under stresses such as toxicants [125,126]. Despite an overall increase in upstream fermentative capacity, the low relative abundance of hydroxypyruvate reductase (−1) suggests a move away from this particular C_2/C_3 redox pathway [124,125]. The enrichment of acyl-CoA thioesterase (1.5) suggests an increased turnover of fatty acyl-CoA, which modulates the pools of free fatty acids and CoA, potentially influencing lipid and biosurfactant dynamics at the cell surface [127,128]. Seemingly, the microbial extract may enhance fer-

mentative/AD and osmoprotective capabilities, as well as lipid-interface metabolism, while functionally dissociating these from the traditional CO_2-reduction methanogenesis pathway, suggesting increased VFA potential but diminished methane-conversion efficiency.

Pig Manure Digestate (PD)

PD exhibited negative Z-scores in AD for betaine-aldehyde dehydrogenase (Z-score: −1), but positive for fumarylpyruvate hydrolase (Z-score: 1), 3-hydroxyisobutyrate dehydrogenase (Z-score: 1), and glutathione-independent formaldehyde dehydrogenase (Z-score: 2). Although osmolyte routes aid AD stability under stress in other systems, suppressed betaine-aldehyde dehydrogenase (−1) indicates decreased glycine–betaine osmoprotection [129,130]. According to Aboudi et al. [131] and Ijoma et al. [129], fumarylpyruvate hydrolase and 3-hydroxyisobutyrate dehydrogenase (+1 each) indicate improved catabolism of aromatic and branched-chain amino acids that feed into central carbon, which is appropriate for pig manure communities that support robust acidogenesis/acetogenesis. The robust glutathione-independent formaldehyde dehydrogenase (+2) indicates active detoxification of C1 intermediates, safeguarding microorganisms from aldehyde stress and preserving AD performance [129]. Overall, PD seems biased towards detoxification and overflow metabolism instead of osmoprotection or stringent methanogenic coupling.

Pig Manure Digestate with Consortia (PDC)

PDC had mixed AD responses, with L-2,4-diaminobutyrate decarboxylase suppressed (Z-score: −1) but formylmethanofuran dehydrogenase subunit C (Z-score: 1.5), methylenetetrahydro methanopterin cyclohydrolase (Z-score: 1.5), formylmethanofuran-tetrahydromethanopterin N-formyltransferase (Z-score: 1.5), and aldehyde dehydrogenase (Z-score: 2) enriched; glutathione-independent formaldehyde dehydrogenase was suppressed (Z-score: −1). Biosurfactant metabolism showed negative Z-scores for phospholipase C (Z-score: −1). A reinforced CO_2-reduction methanogenesis module (formyl-MFR/H_4MPT pathway) is indicated by upregulated formylmethanofuran dehydrogenase (subunit C, +1.5), methylene tetrahydromethanopterin cyclohydrolase (+1.5), and N-formyltransferase (+1.5). This is frequently associated with better methane conversion from pig-manure systems where methanogenesis is otherwise more prone to failure [131,132]. Aldehyde dehydrogenase (+2) indicates enhanced detoxification and redox equilibrium assistance for the methanogenic population. L-2,4-diaminobutyrate decarboxylase (−1) and glutathione-independent formaldehyde dehydrogenase (−1) exhibit diminished amino acid and C1 overflow pathways, suggesting a trade-off: enhanced specialized methanogenesis and less diversified upstream/defensive metabolism. Phospholipase C (−1) in biosurfactant metabolism indicates diminished lipid-interface and membrane remodeling abilities, in contrast to scenarios where lipid-active functions promote hydrolysis and electron transport [129].

Pig Manure Digestate with Microbial Extract (PDE)

PDE displayed negative Z-scores in AD for phosphoenolpyruvate phosphomutase (Z-score: −0.5) and pyruvate water dikinase (Z-score: −1), indicating suppression of carbohydrate metabolism pathways. The negative effects of phosphoenolpyruvate phosphomutase (−0.5) and pyruvate water dikinase (−1) indicate a reduction in carbohydrate/PEP–pyruvate routing, potentially restricting energy-conserving processes and volatile fatty acid availability for methanogens [129]. In summary, cow-based (CD/CDC/CDE) configurations are more effective for high, rapid methane yields when methanogenesis is not inhibited, while pig-based (PD/PDC/PDE) configurations offer stronger detoxification and stability. Consortia (PDC) are required to fully unleash their methanogenic potential.

3.6. Integrated Interpretation, Implications, and Limitations

The literature has established that animal manure anaerobic digestion (AD) has difficulties with foaming, ammonia inhibition, early acidification from VFA accumulation, limited biogas output, and lower biodegradability of fibrous substrates [133,134]. Solutions have been proposed to mitigate these issues that include co-digestion, bioaugmentation, antifoams, etc. [135,136]. However, it has been stressed that in order to prevent ineffectual interventions, solutions must be substrate and microbiology specific or not detrimental to the essential microbial communities [111,135]. The present study shows that cow manure (C/N = 32.86) and pig manure (C/N = 13.48) both achieved high biomethane yields when augmented with digestate and surfactant-degrading consortia or extracts compared to untreated (PD and CD). This demonstrates that AD performance is governed by microbial functioning rather than merely physicochemical characteristics [66,137].

In AD systems, foaming is still a significant operational limitation that frequently results in decreased working capacity, poor mixing, and gas entrapment, which leads to reactor overflow [10,138]. The study's bioaugmentation approach by utilizing surfactant-degrading microbial consortia (CDC and PDC) and extracts (CDE and PDE) derived from oil-contaminated soil directly addresses foaming, which is one of the expensive operational bottlenecks. This is evidenced by the reduction in foaming activity and foaming stability in both pig and cow manure. Unlike the use of chemical defoamers or antifoamers that introduce secondary pollutants and are costly, the strategy in this study works catalytically without changing the nutritional composition of the feedstock. Microbial extract (in PDE) worked best for piggery manure, where foaming activity was intense, by reducing foaming activity, likely via enzymatic degradation of surfactants and VFAs. Despite cow dung having lower foaming activity but a more stable foam, CDC (consortia) showed greater reductions in foam stability. These results highlight substrate-specific outcomes that facilitate foam control, minimizing operational challenges and reducing dependency on chemical defoamers.

Another significant issue addressed was the process instability, which is frequently brought up by VFA buildup and the ensuing pH drop. The observed consortia and extracts' pH stabilization and biomethane increased yields in treated digestion, potentially resolving VFA-induced acidification and methanogenic suppression [139]. For piggery manure, where quick VFA production often puts the system in danger of failure, this strategy has a particularly significant effect. The synergy between the consortia/extract's hydrolytic and methanogenic processes likely speeds up substrate breakdown and VFA uptake, cutting the duration of productive phases by more than 5 days. These data also suggest that the advantages of bioaugmentation are not universal but result from the interaction between the type of amendment and the features of the substrate [140]. Foaming control exhibited substrate specificity. Extract-based treatments have shown notable efficacy in reducing foam intensity in pig dung, likely via the enzymatic degradation of foam-stabilizing chemicals. Consortia treatments demonstrated greater efficacy in cow dung, perhaps indicating prolonged microbial community reorganization that reduces biosurfactant accumulation.

In addition, these results highlight the importance of microbiome optimization for improved AD. By utilizing oil-adapted and surfactant-degrading consortia for both foam mitigation and yield improvement, the study allows for low-cost and scalable bioaugmentation. This positions microbial consortia as a nexus for waste valorization, bioremediation, and the production of renewable energy. However, laboratory-scale batch reactors may inadequately simulate continuous full-scale digesters. Functional predictions based on 16S rRNA datasets indicate an abundance of pathways related to fermentation, syntrophic metabolism, and methanogenesis; however, these predictions must be regarded with caution as they do not directly quantify gene expression (metatranscriptomics) or enzyme

activity analyses. This study illustrates a bidirectional relationship in which hydrocarbon-contaminated soils supply functional consortia for manure digestion, while digestate from anaerobic digestion may improve the bioremediation of contaminated sites, exemplifying the integrated environmental management principles that are increasingly vital to sustainable biotechnology [141].

Future research should concentrate on (1) validating these findings in continuous stirred-tank reactors (CSTRs) over prolonged operational durations (e.g., 6–12 months) to evaluate long-term community stability and process resilience; (2) utilizing shotgun metagenomics and metatranscriptomics to directly quantify gene expression of critical functional pathways (alkyl sulfatases, methanogenesis enzymes) instead of depending on predicted profiles; (3) creating synthetic minimal consortia comprising solely essential functional members (e.g., *Clostridium*, *Syntrophomonas*, *Methanosaeta*) to facilitate systematic exploration of interspecies interactions; (4) fractionating extracts to pinpoint specific foam-reducing enzymes or metabolites for prospective commercial product development; (5) showcasing technology at a pilot scale (1–10 m^3) under authentic farm conditions with fluctuating feedstock composition and operator variability.

4. Conclusions

This study shows that surfactant-degrading microbial consortia and their cell-free extracts from oil-contaminated soils improve substrate-specific anaerobic digestion performance. Supplementation with microbial consortia and extract markedly increased cumulative biogas outputs (cow manure: 407.76 to 603.28 mL/gVS and pig manure: 403.82 to 627.5 mL/gVS) and biomethane by 30–50%. These improvements demonstrate that living consortia give higher benefits through persistent microbial activity and syntrophic establishment, while extracts provide immediate but transient enzymatic effects. Bioaugmentation reduced digestion retention time by 5–6 days, with 90% of cumulative methane production achieved by day 15 in treated digesters compared to day 18–21 in controls (i.e., a 33% reduction). Consortia-supplemented systems maintained a pH of 6.2 compared to 5.8 in PD controls and recovered to pH 8.0 versus 7.2 by day 30, suggesting better syntrophic partnerships between fermentative bacteria and methanogenic archaea for potential volatile fatty acid management. Foaming reduction was substrate-specific: extracts reduced foam by up to 60% in pig manure, while consortia reduced it by up to 65% in cow manure. These conflicting results emphasize the need for manure type- and foaming mechanism-specific enhancement techniques. Consortia supplementation significantly enriched fermentative and syntrophic taxa, including *Clostridium sensu stricto*, *Paludibacter*, and *Macellibacteroides* in pig manure systems while inhibiting suspected foam-formers like *Lignipirellula* and *Guopingia*. Consortia-treated digesters had higher levels of tetrahydromethanopterin S-methyltransferase subunit H (Z-score: +2) and formylmethanofuran dehydrogenase (Z-score: +1.5) according to PICRUSt2 functional predictions, indicating increased hydrogenotrophic methanogenesis. This study therefore shows that bioaugmentation with surfactant-degrading microbial consortia and oil-contaminated soil extracts can increase methane production, process stability, and foaming in manure anaerobic digestion in an eco-friendly way. The substrate-specific responses show that tailored augmentation is needed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr14071165/s1. Figure S1: Beta Diversity of manure treatments; Figure S2: Relative abundance at the phylum level; Figure S3: Stacked bar plots illustrating the relative abundance (%) of microbial genera in anaerobic digestates from cow manure (top row) and pig manure (bottom row) across multiple time points. Treatments include untreated digestate (left),

digestate amended with consortia (middle), and digestate amended with extract (right); Figure S4: An overview of Picrust2 analysis.

Author Contributions: Conceptualization, T.S.M. and M.I.M.; methodology, M.I.M.; software, R.N.; validation, R.N., M.I.M. and T.S.M.; formal analysis, M.I.M. and R.N.; investigation, M.I.M. and C.R.; resources, T.S.M.; data curation, R.N.; writing—original draft preparation, M.I.M.; writing—review and editing, C.R. and T.S.M.; visualization, R.N.; supervision, T.S.M.; project administration, T.S.M.; funding acquisition, T.S.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the Department of Science and Innovation, South Africa (DSI) and the Technology Innovation Agency (TIA) grant number 2022/FUN252/AA, and the National Research Foundation (NRF) grant number SRUG210315589956 awarded to T.S.M.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. The metagenomic datasets generated and analyzed during the current study are available in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession number PRJNA1425521 for public access.

Acknowledgments: The authors would like to thank the Institute of Catalysis and Energy Solutions (ICES) for their support; the National Research Foundation of South Africa (NRF) and the Department of Science and Innovation (DSI, South Africa); and the Technology Innovation Agency (TIA) for their sponsorship. The authors would also like to thank Simla Maharaj for English editing and proofreading the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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