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Enhanced Methane Productivity and Process Stability Through the Co-Digestion of High-Yield Sorghum With Swine Manure.

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Abstract

Sorghum is a promising substrate for biogas production; however, previous studies with the biomass sorghum cultivar BRS716 showed that mono-digestion was limited by volatile fatty acid (VFA) accumulation at high organic loading rates, compromising process stability. Co-digestion with organic residues such as swine manure is an effective strategy to mitigate this limitation by providing buffering capacity and essential nutrients to methanogenic microorganisms. Therefore, the co-digestion of sorghum silage (BRS716 cultivar) and swine manure was evaluated in a continuous stirred tank reactor (CSTR) operated for 260 days, with progressive increases in organic loading rate (OLR) of 0.5 $g_{VS}\ L^{-1}_{reactor}\ d^{-1}$, derived exclusively from sorghum. Process performance was monitored through biogas production and composition, pH, intermediate-to-partial alkalinity ratio (IA/PA), VFA concentration, and solids removal. The system reached a maximum OLR of 6.21 $g_{VS}\ L^{-1}\ d^{-1}$, resulting in a volumetric methane productivity of 1.11 $L_{NCH4}\ L^{-1}_{reactor}\ d^{-1}$, and a specific methane yield of 179.2 $L_{N\ CH4}\ kg_{VS}^{-1}$. No VFA inhibition was observed, confirming process stability. At the shortest HRT evaluated, VS removal decreased, indicating that further HRT optimization may improve solids conversion under high OLR operation. Microbial analysis revealed enrichment of bacteria associated with lignocellulosic degradation and acidogenesis, whereas *Methanosaeta* dominated the archaeal community, supporting the predominance of acetoclastic methanogenesis. Overall, these findings demonstrate that co-digestion with swine manure enables stable operation at high organic loading rates and increased methane productivity, representing a promising strategy for integrated crop–livestock biogas systems.

1. Introduction

The use of biomass for energy production is a key alternative to reduce dependence on fossil fuels in the global energy system, due to its versatility and contribution to greenhouse gas mitigation [1]. Among the main biofuels generated from biomass conversion, biogas stands out as an energy carrier produced through anaerobic digestion (AD), a well-established biological process that converts organic waste into methane (CH_4) and carbon dioxide (CO_2).

Energy crops such as maize, barley, beet, potato, sunflower, and sorghum have been used as substrates for biogas production due to their high biomass productivity and methane yields [2]. Among these crops, sorghum is particularly promising because it does not compete with food crops for arable land, can thrive in low-fertility soils, and shows high tolerance to climatic stress and drought conditions [3].

Despite its agronomic advantages, the mono-digestion of sorghum is often limited by the accumulation of volatile fatty acids (VFAs), particularly propionic acid [4]. This phenomenon has been associated with the low nitrogen content and limited availability of essential micronutrients in lignocellulosic substrates, can disrupt the balance between intermediate and partial alkalinity (IA/PA), decrease pH, and inhibit the activity of methanogenic archaea [5]. As a result, the achievable organic loading rates (OLRs) are restricted, limiting overall process efficiency and economic viability. Although the addition of buffering agents such as sodium hydroxide [6] and biochar [7] has proven effective in mitigating these effects, their application increases operational costs and may hinder scalability.

Co-digestion with residues possessing alkalinizing potential may represent a cost-effective strategy. According to Marchetti and Vasmara [8], the use of swine manure in co-digestion with substrates susceptible to VFA accumulation enhances process stability by supplying alkalinity as well as essential macro and micronutrients for the anaerobic microbiota, in addition to enabling higher OLRs.

Conversely, biogas plants treating swine manure as the sole substrate also face operational limitations. Due to the high water content and consequently low organic matter concentration of swine manure, these systems typically operate at relatively low organic loading rates (of 0.7 to 1.9 $g_{VS}\ L^{-1}_{reactor}\ d^{-1}$) resulting in limited methane productivity [9, 10]. An alternative is to carry out co-digestion with external substrates, such as the high-yielding sorghum hybrid BRS716.

Previous studies evaluating the mono-digestion of BRS716 sorghum silage [11] demonstrated stable operation up to an OLR of 2.5 $g_{VS}\ L^{-1}_{reactor}\ d^{-1}$, whereas higher loading rates led to propionic acid accumulation and process instability, limiting methane

productivity. These findings motivated the present study, in which co-digestion with swine manure was proposed as a strategy to overcome the limitations observed during sorghum mono-digestion.

Therefore, the present study investigated the anaerobic co-digestion of BRS716 sorghum silage and swine manure under continuous operation and progressively increasing OLRs. Specifically, this study evaluated (i) whether co-digestion prevents the propionic acid accumulation observed during sorghum mono-digestion, (ii) the maximum stable OLR achievable under co-digestion, and (iii) the resulting gains in methane productivity. Temporal variations in the microbial community were also investigated to provide mechanistic insights into the enhanced performance of the co-digestion system. From an applied perspective, this approach represents a promising strategy for full-scale biogas plants, as swine manure is continuously generated throughout the year, whereas sorghum biomass can be preserved as silage, ensuring a stable and reliable substrate supply.

2. Materials and methods

2.1. Substrates sampling and storage

The sorghum cultivar used was BRS 716, a biomass hybrid sorghum known for its high biomass yield, ranging from 120 to 150 t_{FM} ha^{-1} [12], which enables high energy production per unit area. The BRS 716 sorghum used in this study originated from the same cultivation batch described by Lazaroto et al. [9], harvested 150 days after planting at Embrapa Maize and Sorghum (Sete Lagoas, MG, Brazil) [11]. After silo opening, the silage was divided into daily portions according to the volatile solids required for each organic loading rate, vacuum-packed, and stored at -24°C until use to prevent biological degradation.

Swine manure was collected from a finishing pig farm in Toledo, Paraná, Brazil, where pigs are reared from 23 kg until slaughter. Sampling was conducted at two distinct time points, identified in the study as SM1 and SM2, during which the volatile solids content varied among samples (Table 1), likely due to differences in water management practices at the facility. After collection, the manure was divided into daily portions and stored at -24°C until use.

2.2. Experimental Setup

To evaluate the performance of the co-digestion of sorghum and swine manure, a continuous stirred tank reactor (CSTR) was operated at lab scale conditions (Embrapa Swine and Poultry) under progressive increases in OLR. The reactor had a working volume of 12 L and a headspace of 6 L. It was maintained under mesophilic conditions (37°C), with temperature control achieved by circulating preheated water in a thermostatic bath. Mechanical mixing was performed intermittently, consisting of 5 min of agitation followed by 20 min of rest at a constant speed of 60 rpm using an IKA® RW 20 digital mechanical stirrer.

The reactor was inoculated with 12 L of digestate obtained from a biogas plant located in Toledo, Paraná, Brazil. This mesophilic full-scale digester (900 m³) receives a daily input of 30 m³ of swine manure.

The reactor was operated for 260 days and divided into 13 operational phases (I–XIII), as summarized in Table 1. During Phase I (37 days), the system was fed exclusively with swine manure (480 mL d^{-1}) to establish a baseline for mono-digestion performance and to ensure process stabilization prior to sorghum addition. Stable operation was defined as variations below 10% in daily biogas production, methane productivity, and methane yield for ten consecutive days.

From Phase II onward, the OLR was progressively increased by 0.5 g_{VS} $L^{-1}_{reactor}$ d^{-1} through the addition of BRS 716 sorghum silage. The contribution of each substrate to the OLR, as well as the corresponding hydraulic retention time (HRT), C/N ratio, and nitrogen concentrations during each operational phase, are presented in Table 1.

Table 1

Contribution of sorghum and swine manure to the OLR (g_{VS} L^{-1} d^{-1}) in each reactor phase and its influence on HRT, C/N ratio, and nitrogen concentrations during each operational phase

	Phases												
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIII**
HRT (d)	25.0	23.0	22.2	22.2	21.4	20.6	20.0	19.3	19.3	18.7	18.2	17.3	16.8
OLR sorghum	-	0.5	1	1.5	2	2.5	3	3.5	3.5	4	4.5	5	5.5
OLR swine manure	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.71	0.71	0.71	0.71	0.71
Total OLR (sorghum+swine manure)	0.57	1.07	1.57	2.07	2.57	3.07	3.57	4.07	4.21	4.71	5.21	5.71	6.21
Operational time (d)	37	16	18	17	18	23	18	35	8	22	13	17	17
C/N ratio	2.6	5.1	7.4	9.6	11.6	13.5	15.3	16.9	18.3	19.9	21.3	22.7	23.9
TAN (mgN L^{-1})	1675	1598	1486	1300	1188	895	870	-	873	933	1004	942	1057
TKN (mgN kg^{-1})	2219	1935	1806	1920	2070	2017	2033	2134	2217	2398	2760	2732	2824

Note: organic loading rate (OLR); hydraulic retention time (HRT); Total Ammoniacal Nitrogen (TAN);

Total Kjeldahl nitrogen (TKN).

The gas volume and composition were continuously monitored for CH_4, CO_2, O_2, H_2S, and H_2, using an AwiFLEX Cool$^+$ automatic gas analyzer (AWITE Bioenergie GmbH).

2.3. Analytical determinations

The substrates were characterized for total solids (TS), fixed solids (FS), volatile solids (VS), and mineral content (N, P, K, Na, Ca, Mg, Cu, Zn, Fe, and Mn), as well as crude fiber. The sorghum biomass was also analyzed for lignin, cellulose, and hemicellulose contents. For the digestate, in addition to these parameters, pH, IA/PA ratio, and VFAs (formic, acetic, propionic, isobutyric, butyric, isovaleric, n-valeric, isocaproic, hexanoic, and heptanoic acids), total Kjeldahl nitrogen (TKN), and total organic carbon (TOC) were also monitored.

To assess the composition of the ensiled BRS 716 biomass, near-infrared spectroscopy (NIRS) models developed by Guimarães et al. [13] were employed. Spectra were acquired in reflectance mode with a NIRFlex 500 spectrometer (Büchi Labortechnik, Flawil, Switzerland). Prior to analysis, the samples were dried at 60°C, ground to pass through a 1-mm sieve, and placed in standard Petri dishes. Spectra were recorded at 25 ± 2°C, averaging 32 scans in the 1,000–2,500 nm range (4 nm resolution). The final spectrum for each sample was the average of three replicates. The NIRS models, developed from 957 samples representing different genotypes and harvest dates, were used to estimate lignin, cellulose, and hemicellulose contents via chemometric analysis of log(1/R) data.

TS, FS, and VS were determined by gravimetric analysis, following the methodology described in the Standard Methods for the Examination of Water and Wastewater [14]. Samples were dried at 105°C to determine TS, incinerated at 550°C for two hours to obtain FS, and VS was calculated by difference.

Intermediate-to-Partial Alkalinity (IA/PA) was determined by titration using a Titrator (848 Titrino plus, Metrohm, Switzerland), according to Standard Methods [14].

VFAs were quantified by gas chromatography with flame ionization detection (GC-FID) using a Shimadzu Nexis GC2030 system (Japan) equipped with a headspace autosampler (HS-20). Separation was performed on an Rtx®-Wax capillary column (30 m × 0.25 mm × 0.50 µm, Restek, USA) using nitrogen as the carrier gas. The headspace conditions employed were an oven

temperature of 85°C, a vial equilibration time of 30 min, a vial pressurization time of 1 min, and a sample injection time of 1 min, with a transfer line temperature of 95°C. The GC oven temperature program was set at 80°C (1 min), increased to 180°C at 8°C min^{-1} and then to 200°C at 20°C min^{-1}, where it was held for 2 min. Digestate samples were centrifuged at 7,000 rpm for 7 min, acidified to pH 2.0 with 40% (v v^{-1}) phosphoric acid, and diluted as necessary to fit the analytical concentration range of the calibration curves. Octanoic acid (200 mg L^{-1}) served as an internal standard. Calibration curves were constructed using certified reference material (CRM46975, Supelco). Limits of detection (LOD) and quantification (LOQ) in mg L^{-1} were: formic (1.55; 4.65), acetic (7.90; 23.75), propionic (8.00; 23.75), isobutyric (8.35; 25.00), butyric (8.20; 24.55), isovaleric (8.40; 25.25), n-valeric (8.30; 24.95), isocaproic (8.55; 25.60), hexanoic (8.50; 25.45), and heptanoic acid (8.00; 25.45) respectively.

Mineral contents (Na, K, P, Ca, Mg, Fe, Zn, Mn, and Cu) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 8300, PerkinElmer, Waltham, USA) after nitric–perchloric acid digestion.

Crude fiber was determined according to AOAC Official Method Ba 6a-05 [15] using an Ankom A2000 fiber analyzer (Ankom Technology, Macedon, NY, USA). Total Kjeldahl Nitrogen (TKN) was determined in both solid and liquid samples according to AOAC [15] procedures. TOC was quantified using a TOC analyzer (TOC-LCSH, Shimadzu®, Japan) equipped with an SSM-5000A solid sample combustion unit. Samples (sorghum and digestate) were dried at 65°C, ground, and weighed into ceramic boats. Combustion was carried out at ~ 900°C in an oxygen-rich environment with a catalyst. The resulting CO_2 was quantified using a non-dispersive infrared (NDIR) detector.

2.4. Biochemical Biogas Potential (BBP) and Biochemical Methane Potential (BMP)

The evaluation of biochemical biogas potential (BBP) and biochemical methane potential (BMP) was conducted in accordance with the methodologies described by Holliger et al. [16]. The samples were incubated separately in triplicate in 250 mL reactors, along with a microbial inoculum prepared as described by Steinmetz et al. [17], maintaining an inoculum-to-substrate VS ratio of 2.0 (2 g of VS sorghum sample with 4 g of VS inoculum).

The gas volume was measured by 500 mL graduated eudiometer tubes for gas collection. Incubation was carried out at under mesophilic conditions (37°C) for 45 days, with daily monitoring of biogas volume. Gas composition was monitored using a GEM5000 gas analyzer (LANDTEC, USA) for CH_4, CO_2, O_2, and H_2S.

Control tests were included to ensure assay reliability: a negative control consisting of inoculum only to quantify endogenous methane production, a positive control comprising inoculum and microcrystalline cellulose (Sigma Aldrich, 20 µm) to validate inoculum activity, and a reference sorghum material from BIOGASFORT® Proficiency Test. The results were validated using the OBA platform [18].

2.5. Microbial community analysis

Microbial community analyses were conducted for the inoculum and samples collected during Phases X and XIII. For each sampling, 50 mL of digestate was collected and centrifuged at 5000 rpm for 10 min. The supernatant was discarded, and the solid fraction was stored at -80°C until further processing.

DNA was extracted using the *DNeasy PowerSoil* Kit (Qiagen) according to the manufacturer's instructions. DNA concentration was determined with a Qubit fluorometer (Thermo Fisher Scientific). Microbial community characterization was performed through next-generation sequencing (NGS) using the *Illumina MiSeq* platform (Illumina Inc., San Diego, CA, USA). Library preparation was carried out by PCR amplification targeting the V3-V4 region of the 16S rRNA gene for both Bacteria and Archaea using primers 341F/806R [19, 20].

Read quality was assessed with FastQC [21]. Sequence processing and analysis were performed in *QIIME2* (Quantitative Insights Into Microbial Ecology) [22]. Amplicon sequence variants (ASVs) in FASTA format and their relative abundance values were taxonomically assigned using the SILVA database (version 138) [23].

Functional prediction analyses were performed using PICRUSt2 [24], with the generated ASVs as input. The resulting gene abundance data were normalized by 16S rRNA gene copy number, and functional inference was conducted using the KEGG Orthology (KO) database [25].

All sequences were deposited in the NCBI database under BioProject accession number PRJNA1217904.

2.6. Statistical analysis

For the statistical analysis of the experimental data, a one-way analysis of variance (ANOVA) was used to determine whether there were significant differences in biogas and methane yields among the reactor operating phases. When ANOVA indicated statistically significant differences (significance level of 5%, $p < 0.05$), Tukey's post-hoc multiple comparison test was employed to identify which pairs of groups differed from each other. All statistical analyses were conducted using Microsoft Excel with the Real Statistics Resource Pack add-in, version 9.5.5 [26]

3. Results and Discussion

3.1. Substrate characterization

The sorghum exhibited a high proportion of VS, accounting for 95.7% of its total solids (TS), whereas in swine manure, this ratio did not exceed 77% (Table 2). This difference can be attributed to the higher inorganic fraction in the manure, resulting from the presence of feces, urine, soil particles, and mineral residues derived from feed and water spillage.

Table 2
Characterization of BRS 716 sorghum and swine manure used in codigestion in a CSTR-type reactor.

Parameters	Swine manure 1	Swine manure 2	Sorghum BRS716
TS (g kg^{-1})	18.60 ± 0.4	23.9 ± 2.0	340.4 ± 4.4
VS (g kg^{-1})	14.3 ± 0.3	17.9 ± 1.8	326.0 ± 0.8
FS (g kg^{-1})	4.3 ± 0.1	6.0 ± 0.2	14.4 ± 0.1
Lignin (%TS)			5.1 ± 0.1
Cellulose (%TS)			28.3 ± 0.0
Hemicellulose (%TS)			32.1 ± 0.2
Crude Fiber (%TS)	0.1 ± 0.0	0.2 ± 0.0	26.5 ± 0.9
C (%TS)	33.8	38.0	44.9 ± 1.1
N (%TS)	12.9	8.6	0.6 ± 0.0
C/N	2.6	4.4	74.8
TKN (mg kg^{-1})	1738 ± 106	2232 ± 64	2169 ± 138
P_{total} (mg kg^{-1})	278 ± 55	426 ± 48	1192 ± 98
K (mg kg^{-1})	998 ± 55	1223 ± 206	11016 ± 820
Ca (mg kg^{-1})	510 ± 84	669 ± 112	2075 ± 150
Mg (mg kg^{-1})	166 ± 31	224 ± 31	1066 ± 80
Fe (mg kg^{-1})	<LOD	<LOD	104 ± 52
Na (mg kg^{-1})	391 ± 89	457 ± 69	<LOD
Zn (mg kg^{-1})	<LOD	<LOD	<LOD
Cu (mg kg^{-1})	<LOD	<LOD	<LOD
Mn	<LOD	<LOD	<LOD
BBP ($L_{Nbiogas}$ $Kg_{vs\ add}^{-1}$)	721 ± 147	857 ± 177	582 ± 12
BMP (L_{NCH4} $Kg_{vs\ add}^{-1}$)	497 ± 102	609 ± 126	298 ± 4

Note: total solids (TS); fixed solids (FS); volatile solids (VS); total Kjeldahl nitrogen (TKN); biochemical methane potential (BMP); biochemical biogas potential (BBP). Below the limit of: detection (< LOD); quantification (< LOQ).

LOD and LOQ (mg kg^{-1}) for Sorghum: Na, K, and Mn (25; 75); P, Cu, Fe, and Zn (50; 150), Ca and Mg (100; 300).

LOD and LOQ (mg kg^{-1}) for Swine manure: Na, K, Ca and Mg (10; 30); Cu, Fe, and Zn (5; 15), Mn (25; 75); P (2; 6)

Although sorghum presented a higher VS content, its lignocellulosic composition limits biomass biodegradability, which resulting in a lower biochemical methane potential (BMP) compared to swine manure (Table 2). In contrast, swine manure presented higher nitrogen concentrations, providing essential nutrients and contributing to a more balanced substrate composition for anaerobic digestion [27].

3.2. OLR progression and its impact on the C/N ratio

In Phase IX, the initial swine manure was replaced with a new batch (swine manure 2), which exhibited a higher VS concentration (Table 2), resulting in an increase in total OLR from 4.07 to 4.21 $g_{VS}\ L^{-1}\ d^{-1}$. To maintain process stability and allow microbial adaptation to the new substrate, the sorghum OLR was kept constant during this phase.

According to Bumharter et al. [28], the carbon-to-nitrogen (C/N) ratio is among the most important parameters affecting methane production and process stability during anaerobic co-digestion. Ratios ranging from 15 to 25 are generally considered favorable because they provide a balanced nutrient supply while minimizing the risks of excessive ammonia accumulation or nutrient limitation. Deviations from this range may negatively affect process performance by promoting VFA accumulation or ammonia-related inhibition.

The physicochemical characteristics of sorghum and swine manure demonstrated a strong complementarity between the two substrates. Sorghum presented high carbon content and limited nitrogen availability (C/N = 74.8), whereas swine manure showed the opposite trend, with very low C/N values (2.6 and 4.4). As a result, the progressive increase in sorghum loading while maintaining constant manure addition gradually balanced the overall C/N ratio in the digester, increasing from 2.6 in Phase I to 23.9 in Phase XIII (Table 1).

The extremely low C/N ratio observed for swine manure alone could potentially increase the risk of ammonia-related inhibition during mono-digestion due to excess nitrogen availability. In contrast, sorghum supplementation not only increased carbon availability but also contributed to lowering nitrogen concentrations within the reactor, as reflected by the reduction in TAN values from 1675 $mg_N\ L^{-1}$ to values close to 900–1000 $mg_N\ L^{-1}$ throughout the operational phases (Table 1). This progressive adjustment of C/N and TAN values coincided with stable reactor operation under increasing OLR conditions.

3.3. Reactor performance

The reactor was successfully operated at an organic loading rate (OLR) of 6.21 $g_{VS}\ L^{-1}\ d^{-1}$, maintaining process stability throughout the digestion period (Fig. 1). During the start-up period (Phase I), fluctuations were observed in biogas and methane yields (Fig. 1a), methane concentration (Fig. 1c), and the IA/PA ratio (Fig. 1e), likely reflecting microbial adaptation to the feedstock.

In the subsequent phases, no signs of acidification or accumulation of VFAs were detected, despite the limitations commonly associated with lignocellulosic digestion. VFA concentrations remained below the limits of quantification throughout the entire experiment. Unlike mono-digestion of BRS716 previously reported by Lazaroto et al. [11], in which propionate accumulation represented the main limiting factor above an OLR of 2.5 $g_{VS}\ L^{-1}\ d^{-1}$, co-digestion completely prevented detectable accumulation of VFAs even at OLRs approximately 2.5-fold higher. These observations suggests that the addition of swine manure effectively mitigated the instability previously observed under mono-digestion conditions.

Supporting this result, the pH remained stable with minimal fluctuations (7.37 ± 0.09), as did the IA/PA ratio (0.12 ± 0.04 g HAc g_{CaCO3}^{-1}) (Fig. 1e), indicating stable performance throughout operation under increasing OLRs.

Reactor performance under increasing OLRs showed distinct trends for methane productivity and methane yield. Methane productivity increased consistently throughout the operational phases, reaching a maximum of 1.11 $L_{NCH4}\ L^{-1}_{\ reactor}\ d^{-1}$ at an OLR of 6.21 $g_{VS}\ L^{-1}\ d^{-1}$ (Table 3).

In contrast, methane yields were higher at lower OLRs (0.57 and 1.07 $g_{VS}\ L^{-1}\ d^{-1}$) when manure predominated in the feed, and gradually decreased with increasing sorghum proportion. A statistically significant reduction was only observed above 4.71 $g_{VS}\ L^{-1}\ d^{-1}$, indicating lower substrate conversion efficiency at higher loading rates. This reduction likely resulted from the progressive increase in lignocellulosic biomass proportion combined with the shorter HRT values applied at higher OLRs, which may have limited the time available for complete substrate degradation. Nevertheless, methane yield remained above 200 $L_N\ Kg_{VS}^{-1}$ up to an OLR of 4.21 $g_{VS}\ L^{-1}\ d^{-1}$, demonstrating stable reactor performance over a wide operating range.

These results indicate that increasing OLR favored volumetric methane production, whereas methane conversion efficiency gradually declined at higher loading rates. Considering both methane productivity and specific methane yield, OLRs between 1.57

and 4.21 g_{VS} L^{-1} d^{-1} represented the most favorable operational range, maintaining methane yields above 200 L_N Kg_{VS}^{-1} while simultaneously providing a substantial increase in volumetric methane production.

Table 3
Biogas and methane yield and productivity of biogas and methane at different organic loading rates (OLR) during codigestion of sorghum and swine manure

OLR	Yield (L_N Kg_{VS} $^{-1}$)		Productivity (L_N $L_{reactor}$ $^{-1}$ d^{-1})	
	Biogas	Methane	Biogas	Methane
0.57	624 ± 21^{A}	432 ± 17^{a}	0.36 ± 0.01^{A}	0.25 ± 0.01^{a}
1.07	439 ± 25^{B}	288 ± 16^{b}	0.47 ± 0.03^{B}	0.31 ± 0.02^{a}
1.57	398 ± 24^{C}	250 ± 16^{c}	0.63 ± 0.04^{C}	0.39 ± 0.03^{b}
2.07	388 ± 20^{CD}	233 ± 12^{cd}	0.80 ± 0.04^{D}	0.48 ± 0.03^{c}
2.57	378 ± 22^{CDE}	214 ± 13^{de}	0.97 ± 0.06^{E}	0.55 ± 0.03^{d}
3.07	396 ± 8^{CDE}	225 ± 5^{de}	1.22 ± 0.03^{F}	0.69 ± 0.02^{e}
3.57	382 ± 7^{CDE}	214 ± 5^{de}	1.36 ± 0.03^{G}	0.76 ± 0.02^{f}
4.07	376 ± 10^{CDEF}	209 ± 3^{e}	1.53 ± 0.04^{H}	0.85 ± 0.01^{g}
4.21	367 ± 10^{CDEF}	206 ± 5^{ef}	1.55 ± 0.04^{H}	0.87 ± 0.02^{g}
4.71	365 ± 16^{CDEF}	201 ± 8^{efg}	1.72 ± 0.08^{I}	0.95 ± 0.04^{h}
5.21	355 ± 20^{DEF}	192 ± 13^{efg}	1.85 ± 0.10^{J}	1.00 ± 0.07^{hi}
5.71	343 ± 8^{EF}	184 ± 3^{fg}	1.96 ± 0.04^{K}	1.05 ± 0.02^{ij}
6.21	333 ± 5^{F}	179 ± 3^{g}	2.07 ± 0.03^{L}	1.11 ± 0.02^{j}

Note: Superscript letters within the same column indicate no significant difference according to ANOVA followed by Tukey's test at $p \leq 0.05$.

Compared with the sorghum mono-digestion previously reported by Lazaroto et al. [11], co-digestion markedly improved reactor performance. Methane productivity increased to more than twice the value obtained during mono-digestion, while the maximum applicable OLR increased from 2.5 to 6.21 g_{VS} L^{-1} d^{-1}. These results demonstrate the benefits of co-digestion, which enabled stable operation under substantially higher loading rates.

3.4. Impact of HRT on the Co-Digestion of Sorghum and Swine Manure

Hydraulic retention time is a critical operational parameter that directly influences the performance of AD systems. Although there is no universally optimal HRT for CSTRs, values typically range between 15 and 20 days, depending on the characteristics of the substrate being digested [29]. A review by Wang et al. [30] reported that optimal biogas yields from co-digestion of swine manure and energy crops were achieved at HRTs longer than 19 days.

In the present study, the HRT remained above 19 days until Phase IX, after which it decreased to 17 days in Phase XIII as the organic loading rate (OLR) increased (Table 1). This change coincided with a decline in VS removal efficiency from 58 ± 2% to 44 ± 6% (Fig. 1f). However, this response should not be attributed exclusively to the reduction in HRT, since the composition of the feedstock also changed throughout reactor operation. Because the organic loading supplied by swine manure remained nearly constant throughout the experiment, all subsequent increases in OLR resulted exclusively from increasing the proportion of sorghum in the feedstock.

This effect is explained by the different biodegradability of the two substrates. The BMP of swine manure ranged from 497 ± 102 to 609 ± 126 L_{NCH4} $Kg_{vs\ add}^{-1}$, whereas sorghum presented a BMP of 298 ± 4 L_{NCH4} $Kg_{vs\ add}^{-1}$. Therefore, as the proportion of sorghum increased, a larger fraction of the volatile solids entering the reactor consisted of less readily biodegradable organic matter, requiring longer retention times for complete conversion. Consequently, the decline in VS removal was likely the combined result of the shorter HRT and the progressively lower overall biodegradability of the feedstock.

The hydrolysis of lignocellulosic biomass is generally considered the rate-limiting step of anaerobic digestion. Therefore, shortening the HRT reduced the time available for hydrolysis and subsequent microbial conversion of structural carbohydrates, limiting the degradation of the additional volatile solids introduced with sorghum.

Despite the lower solids conversion, methane productivity remained stable and no accumulation of VFAs was observed throughout reactor operation. These findings indicate that the decrease in VS removal was not associated with microbial inhibition or process instability but rather with incomplete degradation of the more recalcitrant organic fraction before its withdrawal from the reactor.

3.5. Microbial community analysis

The microbial community was characterized in the inoculum and in samples collected at OLRs of 4.71 g_{VS} L^{-1} d^{-1} (Phase X) and 6.21 g_{VS} L^{-1} d^{-1} (Phase XIII), to assess temporal dynamics at the phylum (Fig. 2) and genus (Fig. 3) levels for bacterial and archaeal populations. Next-generation sequencing of 16S rRNA gene amplicons yielded 31,768 (inoculum), 30,111 (Phase X), and 59,375 (Phase XIII) high-quality, non-chimeric reads.

The bacterial phylum Firmicutes was the most representative in the inoculum (71%) and Phase XIII (45%). However, in Phase X, Firmicutes decreased to 36%, while Bacteroidota predominated (46%) (Fig. 2). This shift is consistent with lignocellulosic co-digestion systems, where changes in substrate composition and degradation dynamics may alter the relative abundance of hydrolytic and fermentative microorganisms over time [31]. Firmicutes and Bacteroidota both contribute to the hydrolysis of complex organic matter, including cellulose and hemicellulose, with Bacteroidota acting as primary fermenters during early digestion, generating VFAs that support subsequent methanogenesis [32, 33]. The phylum Fibrobacterota, also involved in cellulose degradation, was detected in Phases X (8%) and XIII (5%), whereas other phyla commonly associated with lignocellulosic digestion, such as Cloacimonadota, Proteobacteria, and Spirochaetota, were present in lower abundances [34].

Among archaea, Halobacterota was the most abundant phylum (12% inoculum; 1.54% Phase X; 1.83% Phase XIII), while Euryarchaeota appeared at < 0.03%. The relative abundance of methanogens during lignocellulosic AD is typically low, as bacterial hydrolysis is rate-limiting [35].

The taxonomic profile at the genus level showed similarities among the three samples, although changes in relative abundance were observed from the inoculum to Phases X and XIII (Fig. 3). *DMER64* (Bacteroidota) predominated in both co-digestion phases (X: 11.61%; XIII: 23.24%), Members of the *DMER64* group have been associated with degradation of complex organic matter and fermentation processes in anaerobic environments [36].

Other genera, such as *Ruminofilibacter, MBA03* (Firmicutes), *BBMC-4* (Fibrobacterota), *HN-HF0106* (Firmicutes), *Bacteroidetes_vadinHA17* (Bacteroidota), *Ruminiclostridium, Herbinix, Bacteroidales_UCG-001* (Bacteroidota), *Christensenellaceae_R-7_group* (Firmicutes), and *LNR_A2-18* (Cloacimonadota), increased in relative abundance from the inoculum to the co-digestion phases. These microorganisms are involved in fiber breakdown, fermentation, and provision of metabolites for methanogenesis (Fig. 3) [37–39]. The genus *Proteiniphilum* remained relatively stable in both co-digestion phases (X: 7%; XIII: 6%). This genus is responsible for the decomposition of macromolecules, such as proteins and amino acids, found in both sorghum and swine manure [40].

Methanosarcina was detected only in the inoculum (4%), whereas *Methanosaeta* dominated both co-digestion phases (X: 1.53%; XIII: 1.78%), indicating a shift toward acetoclastic methanogenesis and the establishment of a specialized acetate-utilizing community. The predominance of *Methanosaeta* is consistent with efficient acetate consumption and may have contributed to the absence of VFA accumulation and the stable reactor performance observed throughout the experimental period.

The predominance of *Methanosaeta* has been widely reported in the co-digestion of manure with lignocellulosic residues. Studies involving mixtures of swine manure with food waste, garden waste, corn straw, dairy manure, and vegetable residues have consistently identified this genus as the dominant methanogen [41–45]. These findings, together with the persistence of *Methanosaeta* under increasing OLRs in the present study, reinforce the robustness of the microbial consortium and its role in efficient acetate conversion, preventing the accumulation of intermediate compounds and contributing to stable reactor performance.

3.6. Metabolic prediction

Functional metabolic prediction yielded 5,153 KO entries for the inoculum, 4,875 for Phase X, and 5,801 for Phase XIII. Predicted functional profiles were consistent with the observed microbial community shifts and reactor performance.

Several predicted functional markers associated with lignocellulose hydrolysis were identified (Fig. 4). β-glucosidase (K05350), responsible for cleavage of β-1,4-glycosidic bonds in cellobiose during cellulose depolymerization [46], showed higher predicted abundance in Phases X and XIII, consistent with the enrichment of hydrolytic taxa such as Firmicutes and Bacteroidota (Fig. 2).

Similarly, enzymes associated with carbohydrate hydrolysis, including β-galactosidase (K01190), α-glucosidase (K01187), and α-L-fucosidase (K01206) [47], also exhibited higher predicted abundance in Phases X and XIII, suggesting increased functional potential for degradation of complex carbohydrates present in sorghum biomass. Genes associated with VFA metabolism were also highly represented, which is consistent with the absence of detectable VFA accumulation during reactor operation.

The acetoclastic pathway displayed higher abundances of key genes involved in acetate conversion, particularly acetyl-CoA synthetase (K01895), acetate kinase (K00925), phosphate acetyltransferase (K00625), and acetyl-CoA C-acetyltransferase (K00626). This functional profile is consistent with the predominance of Methanosaeta (Fig. 3) and suggest that acetate represented an important intermediate in methane production.

Genes associated with hydrogenotrophic methanogenesis, including formate dehydrogenase (K00123) and formylmethanofuran dehydrogenase (K00200–K00203), were more abundant in the inoculum, whereas their relative abundance decreased during reactor operation. In contrast, genes related to methylotrophic methanogenesis showed the lowest representation [48].

Based on physicochemical and microbial characterizations together with the functional profile inferred from KOs, the acetoclastic pathway was inferred to be the predominant route for methane production during co-digestion of sorghum BRS716 and swine manure.

The increased predicted abundance of hydrolytic and VFA-conversion pathways likely contributed to efficient substrate degradation and methane production, thereby supporting stable reactor performance under high OLRs.

3.7. Full-Scale Scenario Projection

Table 4 presents a projected scenario for the implementation of large-scale methane production through the codigestion of BRS716 sorghum and swine manure in a 1000 m³ reactor. The projection is based on reactor performance at an OLR of 6.21 g_{VS} L^{-1} d^{-1}, with sorghum and swine manure contributing 88.6% and 11.4% of the organic load, respectively.

Table 4
Projected full-scale scenario for methane production through the codigestion of BRS 716 sorghum and swine manure in a 1000 m^3 reactor

Parameter	Unit	Sorghum BRS716	Swine manure	Reactor
Volatile solids	$kg\ t^{-1}$	324	18	-
Organic loading rate	$kg_{VS}\ m^{-3}_{reactor}\ d^{-1}$	5.5	0.7	-
Daily fresh matter input	$t\ d^{-1}$	17	40	-
Daily fresh matter input	$t\ yr^{-1}$	6194	14510	-
Required planting area	ha^{-1}	53	-	-
Reactor volume	m^3	-	-	1000
Biogas productivity	$m^3_{Nbiogas}\ m^{-3}_{reactor}\ d^{-1}$	-	-	2.1
Methane productivity	$m^3_{NCH4}\ m^{-3}_{reactor}\ d^{-1}$	-	-	1.1
Biogas production	$m^3_{Nbiogas}\ d^{-1}$	-	-	2067
Methane Production	$m^3_{NCH4}\ d^{-1}$	-	-	1113

To sustain operation of such a reactor, approximately 6,194 tonnes of sorghum per year would be required, which would demand 53 hectares of land, assuming a fresh matter yield of 117 t ha^{-1} in a single harvest. Given that the sorghum growth cycle is approximately 150 days, the land would remain available for other crops during the rest of the year, enabling a crop rotation system.

In addition to sorghum, 40 $m^3\ day^{-1}$ (or 14,510 $m^3\ year^{-1}$) of swine manure would be needed. According to the BiogásFORT® calculator [49], this volume could be sourced from a finishing pig farm with 8,850 animals, which is representative of medium-scale swine production systems in regions with intensive livestock activity. This scenario suggests the potential applicability of integrating crop and livestock systems for continuous substrate supply and biogas production.

This scenario demonstrates that co-digestion enables the use of complementary substrates with distinct temporal availability, combining the continuous generation of swine manure with the seasonal production of sorghum preserved as silage. Such integration could contribute to maintaining a stable substrate supply and support sustained reactor operation throughout the year.

In addition, the integration of biomethane production with agricultural reuse of digestate may improve the environmental sustainability of the system. Anaerobic treatment of swine manure has the potential to reduce methane and nitrous oxide emissions associated with conventional manure management practices while recycling nutrients back to agricultural soils.

4. Conclusion

This study demonstrated that co-digestion of BRS716 sorghum and swine manure enabled stable anaerobic digestion at organic loading rates of up to 6.21 $g_{VS}\ L^{-1}\ d^{-1}$. The addition of swine manure improved substrate nutritional balance, preventing acidification while maintaining favorable operating conditions throughout reactor operation.

Compared with the previously reported mono-digestion of the same sorghum biomass, co-digestion substantially improved reactor performance by increasing the maximum stable organic loading rate by 2.5-fold while more than doubling methane productivity. These findings demonstrate that co-digestion is an effective strategy for overcoming the operational limitations previously associated with sorghum mono-digestion, enabling process intensification while maintaining stable reactor performance.

The microbial community was dominated by hydrolytic and fermentative taxa, including DMER64, Ruminofilibacter, MBA03, BBMC-4, HN-HF0106, Bacteroidetes_vadinHA17, Ruminiclostridium, and Herbinix, consistent with the enrichment of predicted hydrolytic functions. The archaeal community was dominated by *Methanosaeta*, supporting the predominance of acetoclastic methanogenesis, consistent with the stable reactor performance and the absence of detectable VFA accumulation.

From an applied perspective, the full-scale projection suggests the technical feasibility of integrating BRS716 sorghum silage with swine manure in commercial biogas plants. The complementary characteristics of these substrates—continuous manure generation and seasonal sorghum production preserved as silage—provide a reliable year-round substrate supply while enabling higher methane production. Furthermore, the proposed strategy provides a practical opportunity to increase the organic loading rate of existing swine manure digesters that currently operate below their treatment capacity due to the low solids content of the manure, thereby enhancing biomethane production without requiring additional reactor volume. Overall, this approach represents a promising strategy for intensifying biomethane production while improving the utilization of agricultural residues in integrated crop–livestock systems.

Future research should focus on optimizing hydraulic retention time, evaluating biomass pretreatment strategies, and validating the process at full scale to facilitate large-scale implementation in integrated crop-livestock systems.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Generative AI and AI-assisted technologies in the writing process.

During the preparation of this work, the authors used ChatGPT by OpenAI to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the published article.

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Author Contribution

ACL, RLRS, and AK: Conceptualization. ACL, MCP, JFFS, DCT, DBA, RLRS, FGA, RACP, MLFS, FEM, IVMS, and SP: Methodology. ACL, MCP, JFFS, DCT, DBA, FGA, RACP, MLFS, FEM, IVMS, and SP: Investigation. ACL: Data curation and writing of the original draft. MCP, RLRS, and AK: Supervision. AK: Funding acquisition. All authors: Reviewed and edited the manuscript and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

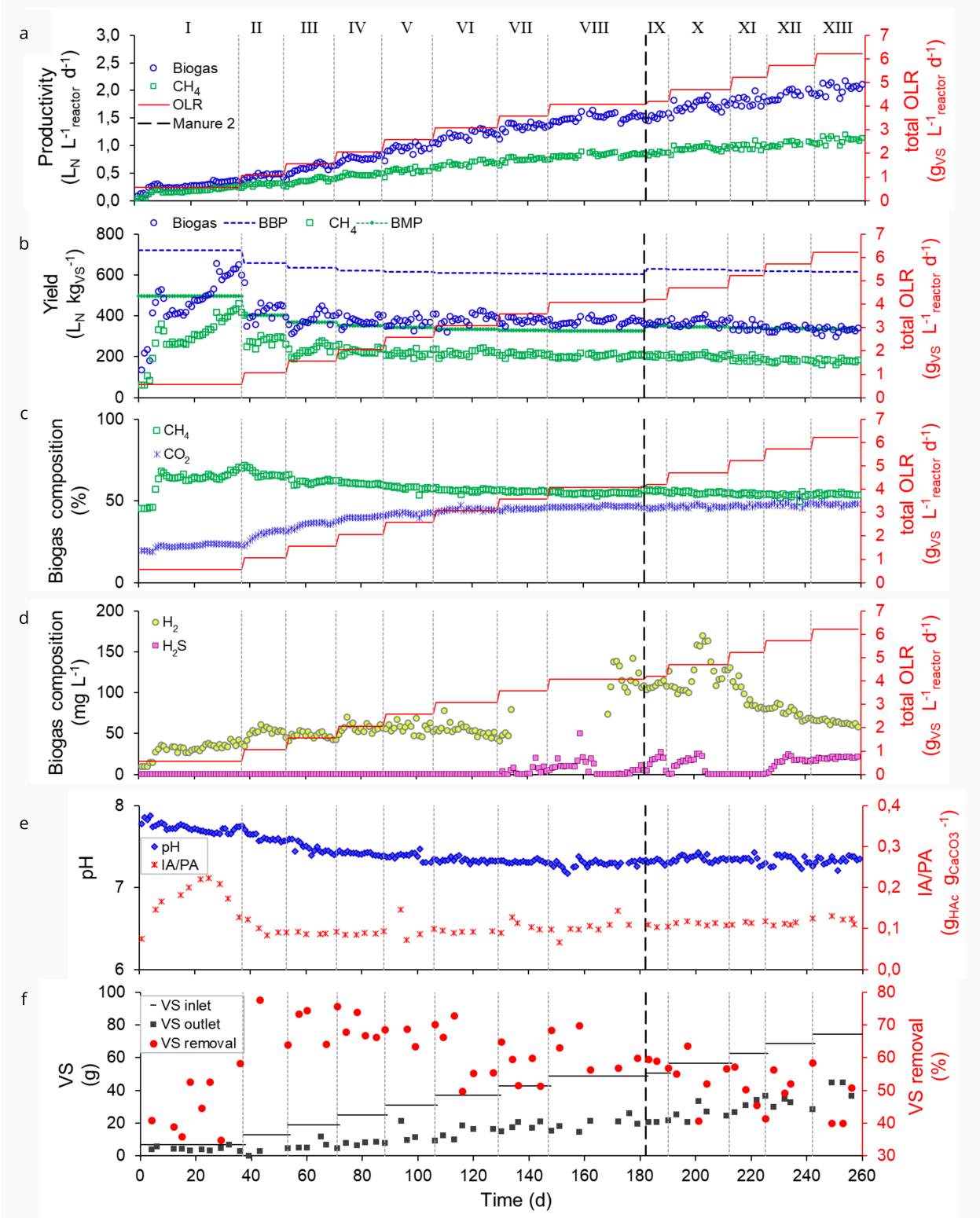


Figure 1

Reactor monitoring parameters during anaerobic co-digestion of sorghum BRS716 and swine manure (a) Biogas and methane productivity; (b) Biogas and methane yield; (c) Biogas composition (CH_4 and CO_2); (d) H_2S and H_2; (e) pH and IA/PA ratio; (f) Effluent volatile solids and removal efficiency.

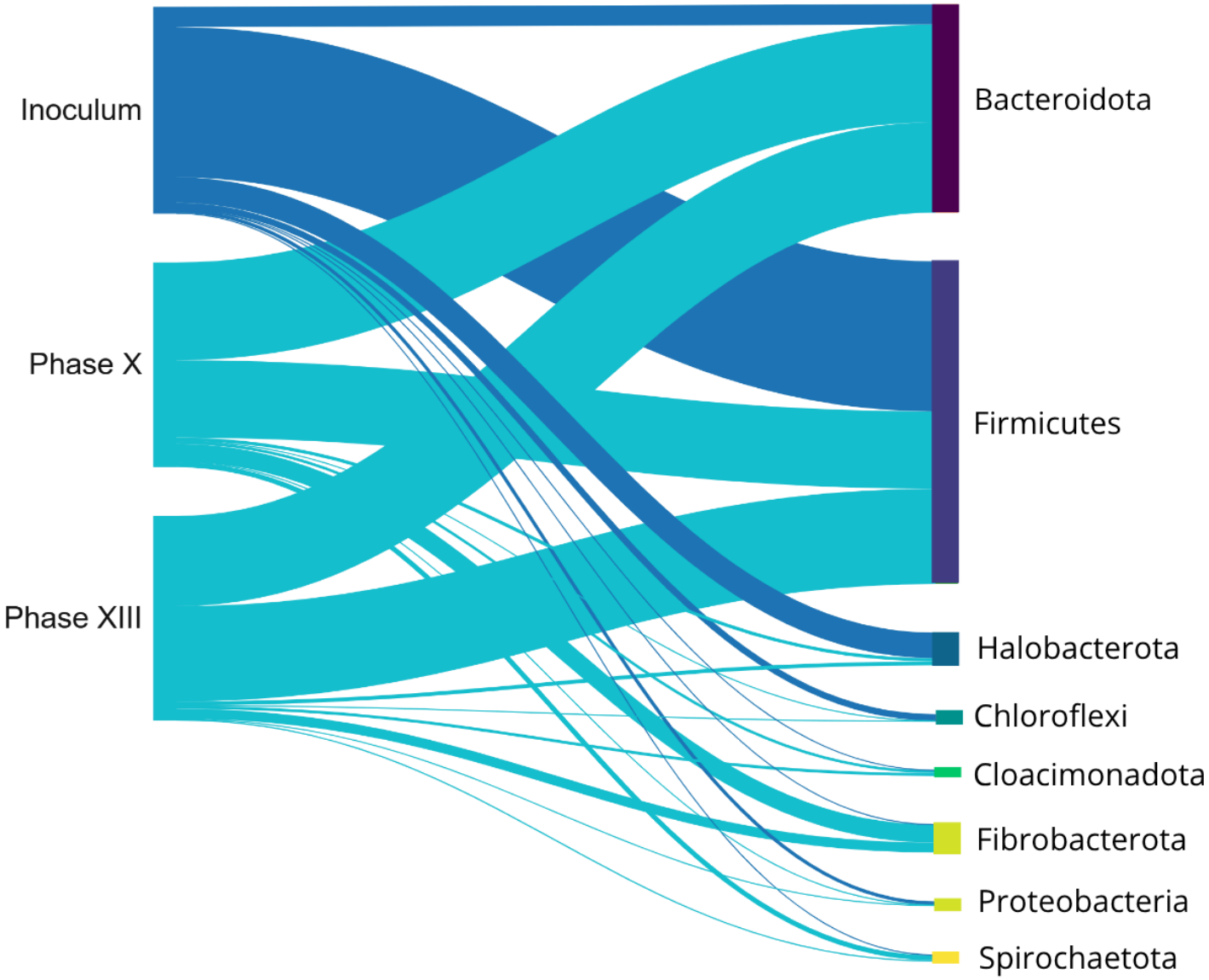


Figure 2

Sankey diagram summarizing the taxonomic profile of the phyla Bacteria and Archaea present in the inoculum and in Phases X and XIII.

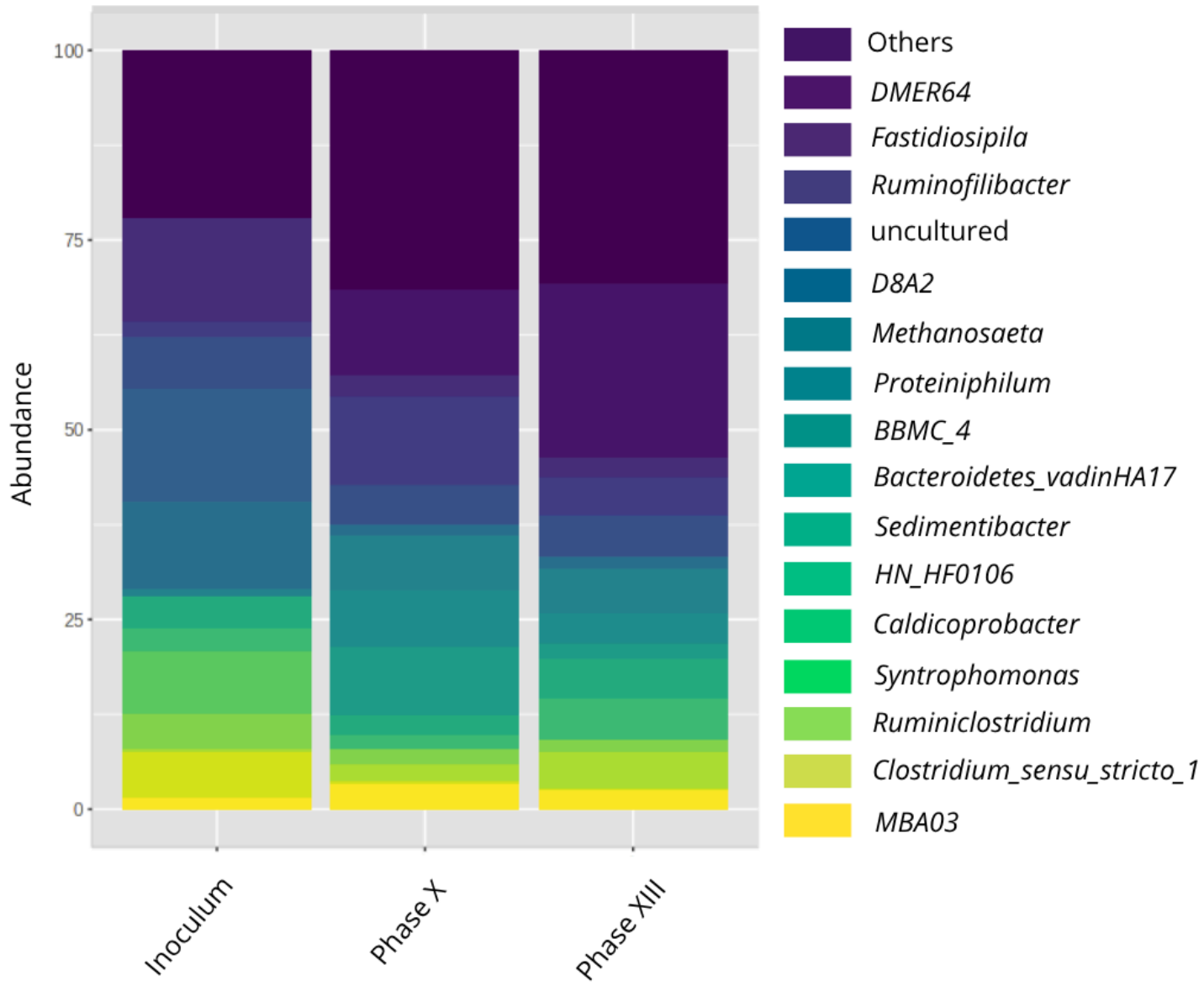


Figure 3

Dynamics of the relative abundance of bacterial and archaeal genera present in the inoculum and in Phases X and XIII.

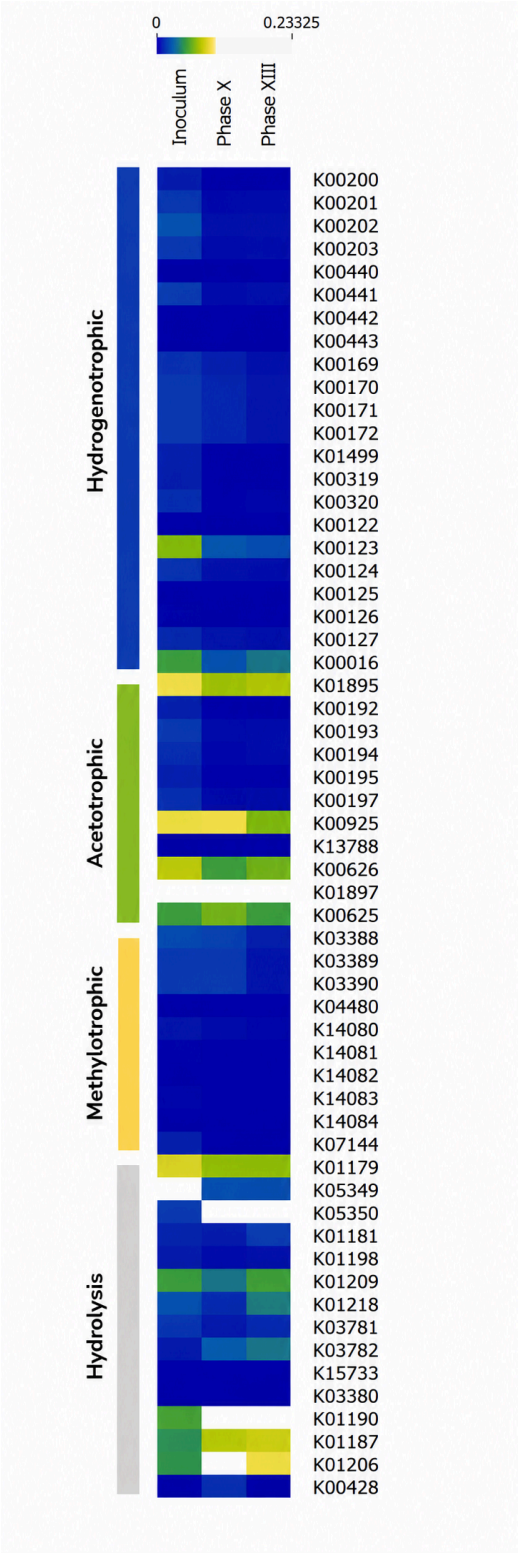


Figure 4

Main KEGG Orthology (KO) entries associated with hydrolysis and methanogenesis pathways predicted by PICRUSt2 based on the KEGG reference database.