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Volume 4
Number 2
February 2018
Pages 101-338

Environmental
Science
Water Research & Technology

rsc.li/es-water

ISSN 2053-1400

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PAPER
Jeyong Yoon *et al.*
Electrochemical lithium recovery and organic pollutant removal from industrial wastewater of a battery recycling plant

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Pit filling rate continues posing challenges in developing countries This study provides evidence that faecal sludge–derived biochar accelerated anaerobic degradation without enhancing overall solids breakdown. These findings conclude that faecal sludge-derived biochar may not necessarily reduce pit filling rates and highlight the importance of evaluating different types of biochar as pit additives.

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DOI: 10.1039/D6EW00095A

The impact of faecal sludge-derived biochar as an additive on anaerobic degradation of synthetic human excreta

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Keywords: biochar, faecal sludge, anaerobic digestion, pit latrines, sanitation

Abstract

Pit latrine filling poses a major challenge in developing countries. This study investigated the effect of faecal sludge-derived biochar (FSB) on anaerobic degradation, the dominant process in pit latrines, using synthetic human excreta under laboratory conditions simulating wet pit environments. Results show that FSB addition significantly increased hydrolysis rate and shortened lag phase by up to 1.5 days. However, this enhancement did not translate into improved overall degradation, as volatile solids (VS) reduction decreased under FSB addition, while only minor changes were observed in methane and total gas production This decoupling is attributed to redistribution of organic carbon from gaseous products toward microbial biomass and extracellular matrices, as reflected by elevated extracellular polymeric substance (EPS) carbohydrate concentrations. Microbial community analysis revealed a targeted restructuring toward biofilm-associated hydrolytic and methanogenic populations under FSB amendment, characterised by enrichment of metabolically versatile methanogens (*Methanosarcina*) and proteolytic fermenters. Overall, these findings indicate that FSB promotes microbial aggregation and stabilised anaerobic processes but does not enhance net solids degradation, and therefore the findings do not support the recommendation of FSB as a pit latrine additive for reducing pit filling.

1. Introduction

Pit latrines are sanitation technology used by over 1.8 billion people worldwide and are important to eliminate open defecation, which is still practiced by more than 400 million people globally [1,2]. As countries work towards the Sustainable Development Goal of access to adequate and equitable sanitation for all by 2030, reliance on pit latrines is expected to increase [3]. However, the long-term effectiveness of pit latrines is fundamentally limited by pit filling, which is often associated with unsafe emptying practices, increases household costs, and undermines their long-term public health benefits [4,5].

The persistence of solids within pit latrines reflects the inherently slow degradation of faecal organic matter in situ. Although pit environments are largely anaerobic, the conversion of complex particulate substrates into soluble intermediates is inefficient, with hydrolysis widely identified as the dominant rate-limiting step [6].

Attempts to enhance pit sludge degradation have traditionally relied on commercially available additives which mainly contain biologically active materials, e.g. enzymes or microorganisms. However, both laboratory and field evaluations have repeatedly demonstrated negligible impacts on pit performance [7–10]. These outcomes are generally attributed to fundamental ecological limitations, including dilution of added biomass relative to indigenous communities and poor persistence of introduced organisms in competitive, nutrient-limited environments [11]. As a result, it could be argued that attention should be shifted away from biological inoculation towards alternative, readily-available additives, such as biochar, which have the potential to modify the biochemical conditions in anaerobic degradation.

Rather than introducing new microorganisms, biochar influences the degradation process by altering local pH, substrate availability, and the accumulation of inhibitory intermediates, thereby indirectly supporting anaerobic metabolic activity [12–16]. These properties have

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contributed to growing interest in biochar for faecal sludge management (FSM), where it has been considered for soil improvement, heavy metal (HM) immobilisation, water and nutrient retention, and carbon sequestration [17–20]. For instance, faecal-sludge-derived biochar (FSB) has been reported to reduce the leaching of heavy metals from faecal sludge and surrounding soil because of its adsorption capacity [17]. Previous research on the use of biochar for faecal sludge degradation enhancement, however, has focused on dry composting toilets. For example, it has been reported that biochar (from rice husk) was able to help degrade total organic carbon (TOC) from composting of the source-separated faeces up to 40%, twice higher compared to other traditional additives, and similar effects were found for non-source separated faecal sludge degradation [21]. This beneficial effects have been attributed to the higher C/N ratio, provision of microbial attachment sites via its high surface area and porous structure and nutrients supplementation by biochar [12]. Although rice husk biochar is among the few feedstocks reported directly for faecal sludge degradation-related applications, biochar derived from other materials should present the similar mechanisms, including wood residues, crop straw, manure, sewage sludge, and faecal sludge itself [19,22–24]. Nevertheless, the above findings are largely derived from aerobic or semi-aerobic dry composting systems, leaving the role and potential benefits of biochar in wet pit latrines unrevealed, where anaerobic degradation dominates.

In parallel, the use of additives to enhance anaerobic digestion (AD) performance of organic matters has attracted increasing research interest, including the investigation of biochar addition effects. For instance, under mesophilic AD conditions, the addition of biochar has been reported to enhance methane production across a range of substrates, including animal manure, waste activated sludge, and food waste [25–28,15,29]. Beyond improved methane yields, biochar has also been shown to shorten the lag phase of AD and to facilitate direct interspecies electron transfer (DIET) between syntrophic bacteria and methanogenic archaea, thereby accelerating electron flow and stabilising microbial metabolism [30,31]. However, contrasting

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outcomes have also been reported, with inhibitory or negligible effects attributed to variations in biochar physicochemical properties arising from differences in feedstock type, pyrolysis temperature, and retention time [32,33]. In particular, biochar with elevated heavy metal contents may adversely affect AD performance, as metal ions can disrupt enzymatic structure and function through interactions with protein functional groups [30].

Despite extensive investigation of biochar as an additive for AD, very little is known about using FSB, even though faecal sludge pyrolysis has already been used in developing countries such as Uganda and Rwanda [34–36]. Such biochar exhibits distinct physicochemical characteristics, including high ash content and enriched inorganic fractions [24,18,36], which may alter anaerobic microbial processes relative to conventional biomass-derived biochar. Furthermore, biochar production from faecal sludge involves high-temperature thermochemical treatment, which enables effective pathogen inactivation and thereby makes it safe to handle [37]. In addition, the reuse of FSB as a pit additive represents a circular sanitation approach, whereby a treated faecal sludge product is returned to the system to improve degradation processes

Therefore, this study, for the first time, aimed to investigate the potential of using FSB as an additive to facilitate degradation in wet pit latrine conditions by assessing AD performance of human excreta under laboratory conditions, specifically by: 1) monitoring gas yield and its kinetics, digestate properties and particularly, volatile solid (VS) reduction, and 2) exploring the microbial communities shifts due to biochar addition and DIET performance.

2. Materials and methods

2.1 Simulation of human faeces and urine

To provide controlled and reproducible substrate characteristics suitable for repetitive

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experiments and avoid health and safety risks from the use of real human excreta in the laboratory, this research uses synthetic human excreta that is adopted from previous research [38,39]. The recipe and preparation process are summarised in Table S1. The physicochemical properties of the synthetic faeces and urine were comparable to those of published simulants and real human excreta (Table 1), indicating that the simulant provides a valid representation for the present study. According to the reported daily excreta production per capita, a ratio of faeces to urine simulants of 120g:300ml was used to simulate human excreta [38]. The characteristics of the synthetic human excreta can be found in Table 2.

Table 1 Comparison of parameters of faeces and urine simulant from this study and literature, as well as the real human excreta.

	Parameters	Simulant in this study	Simulant from previous studies [38,39]	Real human excreta
Faeces	TS (%)	16.0±0.2	18.4	15-35 [40]
	VS (% of TS)	86.8±0.2	88.5	87 [41]
	pH	6.6	5.2	5.0-8.0 [42]
	TN (%/TS)	5.3±0.3	4.1±0.2	4.7±0.7 [43]
	$N-NH_4$ (mg/l N)	660.1±14.9	-	719±8.7 [44]
	TP (mg/l P)	1711.3±59.3	-	-
	C/N	7.8	17.3	9.6 [43]
Urine	pH	6.0	6.0	6-8.2 [45]
	TS (%)	1.8±0.1	2.4	2.5-3.7 [45]
	VS (% of TS)	44.0±0.3	49.5	60-75 [45]
	pH	6.0	6.0	6-8.2 [45]

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TN (mg/l N)	6751.0±293.5	5200	5000-8000 [46]
N-NH_4 (mg/ N)	202.0±11.7	197	126-603 [47,48]
TP (mg/l P)	462.5±7.4	400	800-2500 [40]
C/N	1.4	0.6	-

2.2 Inoculum and FSB

Centrifuged sludge cake was collected from the anaerobic digester in a sewage treatment plant near London as inoculum for the AD experiments. Fresh inoculum was obtained before each batch experiment and pre-incubated for 7 days to degas and degrade any residual organic matter. Although this inoculum does not perfectly replicate the microbial community of pit latrines, microbial characterisation indicates that it provides a reasonable community-level representation, as it shares dominant phyla commonly reported in pit latrines and taxa associated with human gut and faecal microbiota, as discussed in 3.6.2. Moreover, microbial communities in pit latrines are known to vary substantially due to differences in local environmental conditions, pit design, usage patterns, and user behaviours [49–52], making it inherently challenging to define or reproduce a single “representative” pit latrine microbiome. In this context, the use of a well-established anaerobic digester inoculum offers a controlled and reproducible microbial consortium suitable for mechanistic evaluation of biochar effects, while maintaining relevance to faecal sludge degradation processes.

The faecal-sludge-derived biochar (FSB) used in this study was produced from dried faecal sludge collected at Lubigi Sewage Treatment Plant in Kampala, Uganda, as described in [53]. Carbonisation (i.e. slow pyrolysis intended for solid char production) was conducted using traditional batch-operated retort kilns consisting of modified air-locked metallic drums [53]. The process was initiated using available solid start-up fuels (e.g. wood, charcoal, briquettes),

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which were ignited prior to loading the dried faecal sludge. Once loaded, the kiln was operated under air-locked conditions and left to carbonise overnight (highest heating temperature recorded via thermometer readings: 400 °C), after which the charred material was collected the following day. The char samples were then ground, sieved (<2mm) and homogenised, before being shipped to Imperial College London for storage, analysis and experimental use.

The main characteristics of the feedstock materials relevant to AD are presented in Table 2 and the proximate and ultimate analysis results of FSB are shown in Table S2. Total concentrations of the inorganic elements (i.e. macronutrients, trace elements, heavy metals) of all the materials are shown in Table S3.

Table 2. Characteristics of synthetic human excreta, biochar and inoculum used in this study.

Characteristics	**Synthetic human excreta**	**FSB**	**Inoculum**
TS (%)	5.7±0.1	75.38±0.0	22.3±0.3
VS (%)	4.4±0.1	20.2±0.3	14.0±0.3
VS/TS (%)	76.3±0.5	26.8±0.4	62.8±0.6
pH	6.3	7.9	9.3
C/N	5.3	9.02	N/A

2.3 Design of the AD experiments

Batch AD experiments were conducted at mesophilic temperature (35°C) using 500 mL serum bottles with working volume of 400 mL, equipped with airtight caps. 1 L Tedlar bags were connected to the bottles for gas collection, from which a gas-tight syringe was used to measure the gas volume every other day except for the weekends. N_2 was flushed by a tube connecting to a N_2 cylinder for at least 5 minutes in each bottle before digestion to create anaerobic conditions, where the flushing time was tested to be long enough to flush out air. A detailed equipment set up is included in Fig.S1. The Inoculum to Substrate Ratio (ISR) was set to 4:1

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gVS and Total Solids (TS) of the mixture was adjusted to approximately 4%. This condition was found to be the optimal condition for degradation from the preliminary experiment [56] and represent the average moisture content for wet pit latrines [57]. Based on previous studies [54,29,55], FSB was added to the synthetic human excreta at doses of 0, 3, 6, 9, and 12 g/L and labelled as B0 (control), B3, B6, B9 and B12, respectively. Higher dose implicitly assumes an unlimited supply of FSB, which is unrealistic for on-site sanitation systems. The TS content after FSB addition ranged from 4.38 – 5.19% (Table S4). The AD experiments were finished when the daily methane production for three consecutive days is less than 1% of the cumulative methane production according to the Biomethane Potential Tests (BMP) standard [58], which was 18 days in the presented study. All treatments were in triplicated, and the batch AD experiment was also repeated for three times to reduce variability introduced from the inoculum.

2.4 Analytical analysis

2.4.1 FSB characteristics

Proximate analysis was conducted for the determination of volatile matter, fixed carbon and ash content, following standard method ASTM D7582-15[59] as adapted by [60] for implementation by TGA, on a simultaneous thermal analyser (STA) 449 F5 Jupiter (NETZSCH, Germany). The pH of biochar was measured at a 1:20 ratio (biochar: H_2O [g:mL]) after mechanical shaking for 1h [61].

Carbon and nitrogen content that is reported for C/N ratio were analysed using a Flash Smart CHNS/O Elemental analyser (Thermo Fisher Scientific, Germany).

2.4.2 Gas composition

Gas produced from the AD bottles was sampled from the septum equipped on the cap, and analysed via a gas chromatography (Clarus 600, PerkinElmer, USA) with thermal conductive detector (TCD). Injector and detector temperatures were set at 200 °C and 230 °C, respectively,

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with He as the carrier gas. The oven temperature was initially set at 35 °C and held for 8 minutes, increased to 120 °C at 24 °C/min and held for 3 minutes, and finally increased to 220 °C at 24 °C/min and held for 1 minute. Standard temperature and pressure (STP) were used for gas volume expression as Nml/gVS$_{added}$.

2.4.3 Digestate properties

pH was measured via a benchtop pH meter (Jenway 3540). TS and VS were determined according to Standard Methods [62]. VS reduction was calculated using the Van Kleeck mass-corrected method (Eq. 1) [63]. Notably, the calculation was corrected for the different biochar dosages, as biochar contributes to the initial VS of the system. Accordingly, a biochar-specific $\boldsymbol{VSf_{initial}}$ was used for each treatment to ensure an accurate comparison across biochar addition levels.

$$\boldsymbol{VS\ Reduction}\ (\%) = \frac{\boldsymbol{VSf_{initial} - VSf_{final}}}{\boldsymbol{VSf_{initial} - (VSf_{initial} \times VSf_{final})}} \times$$

Where *VFf* refers to VS/TS ratio.

For total ammonia nitrogen, soluble chemical oxygen demand (sCOD), and volatile fatty acid (VFA) measurements, samples were filtered by 0.45um membrane to obtain the liquid form. NH_4-N and total nitrogen were determined by Skalar San++ (Skalar Analytical B.V., The Netherlands), where the digestion method for the latter is described in the Supplementary Material. sCOD was measured according to [64] and VFA were determined by HACH test kit (LCK365) and a spectrophotometer (UV-2401PC). Extracellular polymeric substances (EPS) were extracted from homogenized digestate following the formaldehyde–NaOH method [65]. Briefly, 5 mL of sample was treated with 0.03 mL of 38 % (w/w) formaldehyde at 4 °C for 1 h, followed by the addition of 2 mL of 1 N NaOH and incubation at 4 °C for 3 h. The mixture was then centrifuged at 20,000 × g and 4 °C for 20 min, and the supernatant was collected as the EPS extract. Protein and carbohydrate concentrations in the EPS were quantified using the

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Pierce™ BCA Protein Assay Kit (Thermo Scientific™) and the phenol–sulfuric acid method [66], respectively.

The inorganic composition of feedstock material and the AD digestate were quantified using an inductively coupled plasma optical emission spectrometer (ICP-OES) (AVIO 500, Perkin Elmer, USA). Prior to the measurement, 6ml hydrochloric acid and 2ml nitric acid were into 0.4g samples and the mixture was heated for 2h at 95 °C and diluted using 2% nitric acid [67].

2.5 Kinetics model

Of the various anaerobic digestion models, the first-order model and the modified Gompertz model are the most widely recognised and have been extensively applied in previous AD research to describe the kinetic behaviour of the system [68]. The first-order model (Eq.2) is based on the assumption that hydrolysis is the rate-limiting step in AD under pit latrine conditions.

$$B(t) = B_{max}[1 - e^{-kt}](2)$$

where $B(t)$ is the cumulative methane yield at time (t), NmL/g VS_{added}; B_{max} is the methane production potential calculated from the first-order model, NmL/g VS_{added}; k is the hydrolysis rate constant, d^{-1}; and t is the digestion time, d.

The modified Gompertz model (Eq.3) can be used to represent bacterial growth with the assumption that the methane production rate is corresponding to the growth rate of methanogenic microorganisms.

$$B(t) = P \cdot exp\left\{-\exp\left[\frac{R_m \cdot e}{P} \cdot (\lambda - t) + 1\right]\right\} \quad (3)$$

where P is the methane production potential calculated from the modified Gompertz model; R_m is the maximum methane production rate, mL/g VS_{added}/d; λ is the lag phase duration, days; and e is a mathematical constant, 2.71828.

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2.6 Microbial analysis

Microbial community analysis was conducted at the end of the experiment for the control (B0) and the 12 g/L biochar-amended reactors (B12) from two of the three batches, all are in triplicated. DNA extraction was performed on the digestate samples using DNeasy PowerSoil Pro Kits according to the manufacturer's protocol and the DNA quality and concentration were assessed via Agarose Gel Electrophoresis and NanoDrop2000. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified by PCR using the primer pair 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GACTACNNGGGTATCTAAT-3'). PCR amplicons were purified and quantified prior to paired-end sequencing on an Illumina MiSeq platform (PE250). Sequence processing and taxonomic assignment were performed using QIIME2. Alpha and beta diversity metrics were calculated based on the QIIME2 outputs.

2.7 Data analysis

To account for potential batch-to-batch variability in inoculum characteristics, gas and methane yields, as well as other digestate parameters, were normalised to their respective control reactors. Relative values were derived from three independent experimental batches, each consisting of triplicate treatment reactors, and are reported as mean values. Prior to statistical analysis, data quality was assessed through outlier screening. For gas yield measurements, outliers were identified and removed based on a relative standard deviation threshold of <10%, in accordance with the BMP test standard [69]. For other physicochemical parameters, Grubbs' test was applied (two-sided, $\alpha = 0.05$), as the datasets were normally distributed and comprised nine observations. Due to inherent batch-to-batch variability in AD microbiomes driven by operational and inoculum differences, microbial metrics were analysed within each batch rather than pooled across all samples.

Differences in alpha-diversity indices between two independent groups were assessed using the Wilcoxon rank-sum test due to the small sample size and non-normal distribution, while two-sample t-tests were used for other measured parameters. Statistical significance was defined at $P < 0.05$. All statistical analyses were conducted in R (v4.5.2).

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3. Results and discussion

3.1 Gas and methane yield

The relative cumulative methane and gas yield compared with the control (B0) for AD of synthetic human excreta with different doses of FSB added are presented in Fig.1 and the absolute values expressed at Nml/gVS$_{added}$ from each batch are shown in Fig.S2. Across all the FSB doses, FSB addition showed a distinct early-stage enhancement compared to the control, as the relative values are all above 1. Peak methane and biogas production stimulation ranged from 1.48-1.60 and 1.32-1.68-fold, respectively, on day 3. This early response indicates that FSB primarily promotes upstream processes, particularly hydrolysis and acidogenesis, thereby accelerating the solubilisation of particulate organic matter and the formation of gaseous intermediates (e.g. CO_2) and methanogenic precursors [70]. This can be attributed to the macroporous structure (as is produced at low temperature) and mineral-rich surface of FSB that promote microbial attachment, improve enzyme–substrate contact, and stabilise local microenvironments during rapid acidogenesis [13,71]. Similar enhancement has been reported during AD of lipid-rich substrates with sewage-sludge-derived biochar (5 g/L) and during anaerobic co-digestion of activated sludge and food waste amended with sawdust-derived biochar (2-15 g/L), which both applied similar doses as in this study [14,72].

Cumulative methane and gas production showed only minor differences between FSB-added reactors and the control as relative methane and gas production in all treatments gradually converged toward the control by the end of digestion on day 18. Conversely, biochar addition has been shown to increase methane yields when using biochar derived from bagasse biomass, wood, digested sludge, etc. [28,29,73]. One of the reasons for the observations in this study could be the lack of additional bioavailable carbon source compared to the other types of biochar. Unlike plant-derived biochar, which typically contains higher carbon content (40-71% [74]), FSB has been reported to contain less than 45% carbon [17] or even lower as 8-24% [75]. The FSB used

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in this study contains only 17.28±0.21% carbon (Table S2), which could be due to the extensive biological degradation and the high ash content of faecal sludge prior to pyrolysis [75]. Consequently, FSB is unlikely to act as a supplementary carbon source for methanogenesis.

Furthermore, since the preliminary experiments helped build the optimal condition by adjusting ISR and TS content, the system likely operated close to its biochemical methane potential and FSB addition would not promote additional gas production. A similar conclusion was also reported by [76] who found that a oak sawdust-derived biochar addition failed to affect methane production form AD of dog food under the optimal ISR (2:1), while it enhanced methane production when the substrate was overloaded (ISR 1 and 0.5). In contrast, real pit latrine conditions are characterised by continuous faecal input and consequently lower effective ISR, under which FSB addition may be more likely to improve gas production. Therefore, further studies are needed to investigate the FSB performance under lower ISR and higher TS content conditions.

Some studies also suggest that the high surface area of biochar generated by high pyrolysis temperature (>500 °C) could enhance absorption of the produced CH_4 [77,78], which is less likely to be the main reason for the unchanged methane and biogas yields observed here, as the present FSB was produced at 400 °C which normally has a surface area low to 2.1 m^2/g [79], and there was no decrease in gas yield as FSB dose increased.

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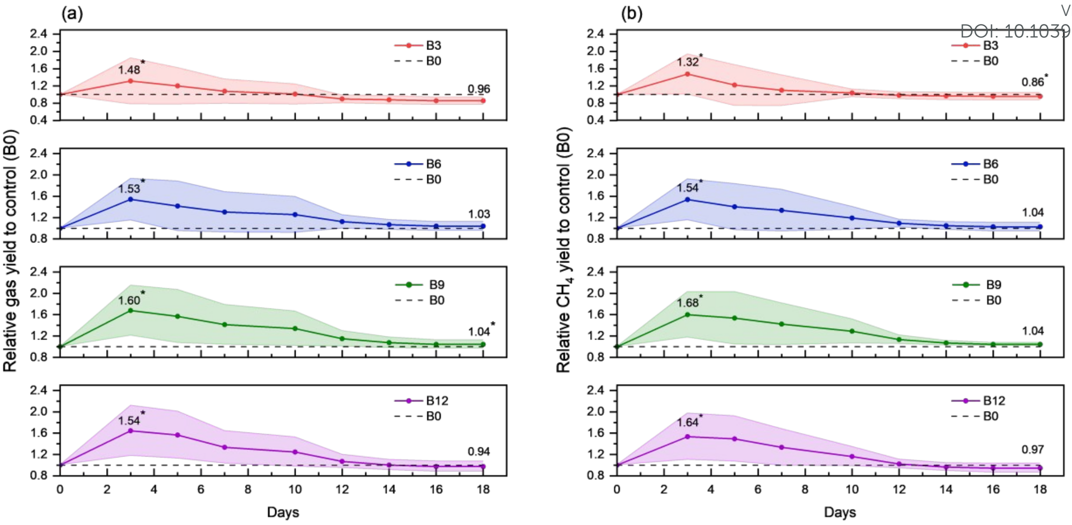

Figure 1. Relative (a) cumulative gas and (b) methane production from AD of synthetic human excreta added with biochar compared with control (B0) (n=9). Area represents standard deviation. *Stands for significance level <0.05.

3.2 Kinetic models analysis

Unlike qualitative curve comparisons, kinetic simulations provide process-specific parameters that allow direct assessment of hydrolysis and methane production kinetics, offering a more mechanistic and transferable interpretation of biochar effects on AD performance. Model-derived kinetic parameters showed batch-to-batch variability (e.g. first-order rate *k* ranging from ~0.04 to ~0.14 d^{-1}), reflecting differences in inoculum activity between experimental batches. In contrast to gas yields, kinetic parameters represent fitted process rates and are therefore not directly normalized across batches. Accordingly, model outputs are interpreted based on consistent within-batch trends for comparison with literature in Table 3. A fitted model for each batch is presented in Fig. S3.

In all three batches, FSB-amended reactors generally exhibit higher hydrolysis rate constant *k* and higher maximum methane production rates R_m compared to the corresponding batch controls, further indicating enhanced early-stage digestion kinetics. This is consistent with a

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previous study [28] which reported that biochar addition resulted in 1.2–1.4-fold increases in the hydrolysis efficiencies of major organic components. In parallel, lag phase λ was reduced by up to 1.5 days in FSB-amended treatments, particularly in B3–B9, which aligns with the experimental observation of accelerated early methane and gas production. This shortened lag phase was also consistent with the higher first-order hydrolysis rate constant (k) observed in the FSB treatments, suggesting faster initial substrate conversion. This could be resulting from the enhanced biofilm manufacture [80], which is further confirmed by the increased EPS carbohydrate content following FSB addition (see 3.3). A previous study [81] also observed shortened lag phase by 10% and 27% with 1 and 10 g/L, respectively, of dairy manure-derived biochar added to AD of the same feedstock, providing a relatively relevant comparison with another manure-based substrate.. These results suggest that, from a practical sanitation perspective, FSB addition may be able to accelerate the pit latrine content degradation and reduce solids accumulation rates, since pit latrines are operated under continuous feeding mode.

Moreover, the methane production potential parameters B_{max} and P fitted by the two models are both reduced by FSB addition, which reflects changes in methane production dynamics rather than a reduction in actual biodegradation potential. This could be because by increasing hydrolysis rate and shortening the lag phase, FSB accelerated early methane production, causing earlier curve saturation and a lower fitted methane potential due to parameter coupling.

Table 3. Results of first-order model and modified Gompertz model analysis for methane production from three AD batches of synthetic human excreta with biochar added at different doses.

	First-order model			Modified Gompertz model			
	B_{max}	K	R^2	P	R_m	λ	R^2

Batch 1	B0	329.40	0.04	0.954	194.94	14.21	2.52	0.993
	B3	150.85	0.10	0.982	122.10	13.45	0.98	0.998
	B6	220.70	0.10	0.986	177.86	17.95	0.73	0.996
	B9	248.66	0.09	0.976	197.96	21.20	1.04	0.994
	B12	199.47	0.11	0.978	165.88	18.77	0.94	0.995
Batch 2	B0	243.22	0.04	0.927	125.37	17.52	3.33	0.999
	B3	206.03	0.05	0.924	116.88	17.12	3.14	0.997
	B6	269.04	0.04	0.940	140.24	17.46	2.96	0.998
	B9	175.40	0.07	0.929	113.63	17.00	2.86	0.997
	B12	129.53	0.09	0.947	95.87	13.93	2.21	0.998
Batch 3	B0	196.12	0.14	0.990	177.13	19.07	0.04	0.986
	B3	206.52	0.13	0.981	184.08	18.23	0.00	0.977
	B6	196.36	0.14	0.988	179.10	19.54	0.00	0.983
	B9	203.84	0.15	0.985	187.00	21.20	0.05	0.982
	B12	200.29	0.14	0.988	181.52	20.59	0.18	0.987

3.3 VS reduction and digestate properties

VS, VFAs, NH_4-N, sCOD and EPS (protein and carbohydrates) were measured at the end of the AD of synthetic human excreta, as shown in Fig.2. VS reduction decreased following FSB addition lower to 0.6-fold with the highest dose of FSB, even using the Van Kleeck mass-corrected approach which accounts for the solid contribution of biochar (Fig.2a). This confirms that although FSB increased the first-order rate constant and reduced the lag phase, it did not enhance the overall extent of organic matter mineralisation in this study. Instead, by providing higher surface area and more favourable attachment sites than the control, biochar promotes

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biofilm formation and stimulates EPS production, diverting a fraction of degradable carbon into biomass-associated VS rather than CH_4 and CO_2 [82]. This reflects in the elevated carbon - rich carbohydrates in EPS with FSB added (Fig. 2f). Protein content in EPS showed insignificant changes among all treatment, consistent with the absence of significant changes in NH_4-N concentration (Fig. 2c). Evidence from the literature indicates that biochar mitigates the ammonia inhibition during AD, particularly in ammonia-stressed systems. For example, fruitwood-derived biochar reduced NH_4-N by approximately 25% during AD of chicken manure, while corn stover and pine wood derived biochar reduced NH_4-N concentration by up to 13% and 11% in sewage sludge digestion [83,32]. In the present study, although NH_4–N concentrations across three batches did not differ significantly with FSB addition, modest reductions (up to 22%) were observed in individual batches (Fig. S4). These results suggest that ammonia mitigation by FSB was not a consistent effect under the tested conditions.

Fig. 2b shows a significant decrease in relative total VFA concentration with FSB added, achieving approximately 0.75-fold for B3-B12 compared to the control. A similar reduction in VFA accumulation has been reported by a previous study [84] which applied dairy manure-derived biochar into AD of dairy manure at different temperatures. Biochar has been widely reported to enhance VFA turnover during anaerobic digestion by stimulating early-stage hydrolysis and acidogenesis while accelerating downstream VFA consumption through improved syntrophic interactions and methanogenic activity [16,72]. This indicates faster intermediate conversion rather than suppressed acidogenesis in this study. Despite this, sCOD remained stable in FSB-amended reactors, indicating that faster VFA consumption was balanced by the formation and release of other soluble components, such as soluble microbial products and EPS-derived carbohydrates associated with biochar-enhanced biofilm activity.

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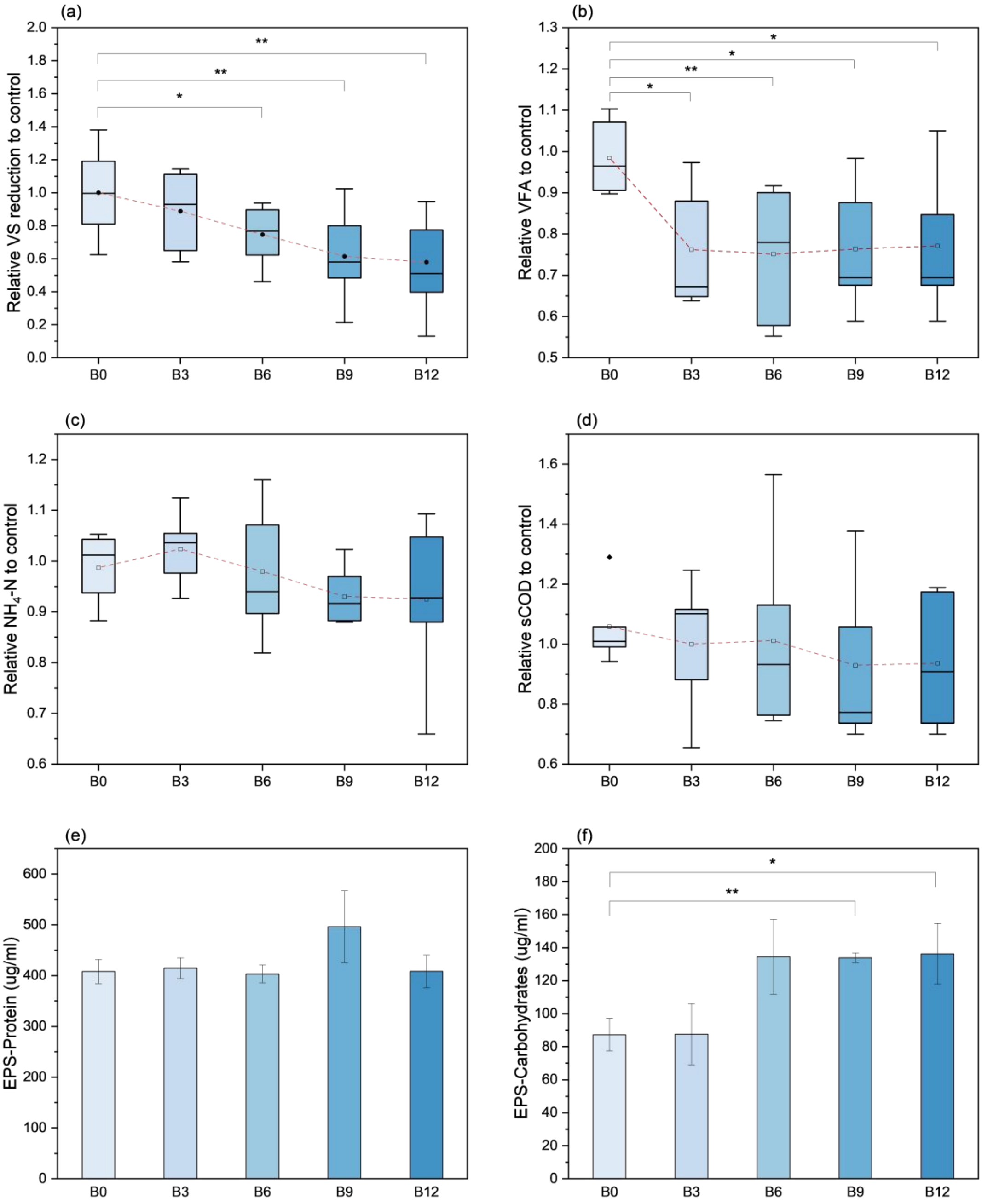

Figure 2. Relative VS reduction, VFAs concentration, NH_4-N concentration, and sCOD concentration compared to control and EPS content in the AD digestate of synthetic human excreta with FSB added. Boxplots are plotted on median, first quartile, third quartile and minimum/maximum values. * and **stands for significance level <0.05 and 0.01.

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3.4 Heavy metals concentrations and environmental implications

Heavy metals are among the key parameters considered in evaluating the environmental safety of pit latrine contents, particularly with respect to soil and groundwater contamination [85]. Fig.3 shows the total heavy metal concentrations in the AD digestate from one of the batches (Batch 2), with higher values observed in the FSB-amended reactors, attributable to the addition of biochar (see Table S3). Several reference limits were used to contextualise the measured heavy metal concentrations and labelled as lines in Fig.3: 1) regulatory thresholds for agricultural land application in South Africa and EU [85], to assess potential environmental and public health risks, and 2) IC_{50} values for AD processes [86,87], representing a 50% reduction in biological activity, to evaluate possible impacts on AD performance, given the sensitivity of key microbial processes to heavy metals [88,87]. The heavy metal concentrations ranged between 2.9-18.8 mg/L (Cd), 6.3-39.6 mg/L (Cr), 17.5-158.8 mg/L (Pb), and 103.3-147.5 mg/L (Zn), 26.9-67.5 mg/L (Cu), and 8.1-22.5 mg/L (Ni). Most metals complied with the regulatory limits for agricultural land application, except for Cd and Cu in the B3 and B6 treatments. Lower total heavy metal concentrations were observed in Batch 1 (Table S5), in which all measured metals were below the corresponding limits.

Generally, the solubility and mobility of heavy metals in aqueous environments are strongly pH dependent. Since digestate produced by AD typically exhibits a neutral to alkaline pH (approximately 8–8.5) [89] and biochar addition increases soil pH [90], conditions under which the solubility of many heavy metals is substantially reduced. For example, Cu exhibits its highest leachability under acidic conditions (pH 4–5) and decreases progressively as pH increases [91]. This limits metal dissolution when digestate is in contact with water and consequently lowers the potential for leaching and soil contamination. Furthermore, heavy metals associated with FSB are predominantly retained within the internal structure of biochar by adsorption and formation as complex with biochar functional groups, further constraining their release during

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percolation or leaching processes [30,92]. These results indicate that, despite elevated total metal concentrations in some FSB-amended treatments, the resulting digestate generally remains suitable for land application under existing regulatory frameworks.

On the other hand, Fig.3 shows that heavy metal concentrations in FSB-added reactors have exceeded the theoretical inhibitory limits except for Ni, while the absence of delayed methane onset or reduced production rates suggests that metal-related inhibition was unlikely to be responsible for the lack of enhanced cumulative methane production. This discrepancy can be attributed to the fact that the heavy metal concentrations reported here represent total metal contents, including both dissolved and solid-bound (inert) fractions, whereas inhibitory limits are primarily defined based on the bioavailable dissolved fraction that directly influences microbial activity. Finally, the reported heavy metal values should not be applied indiscriminately, as heavy metal behaviour and trace element requirements are highly dependent on process conditions, substrate composition, biochar properties, and site-specific soil and hydrogeological characteristics [93].

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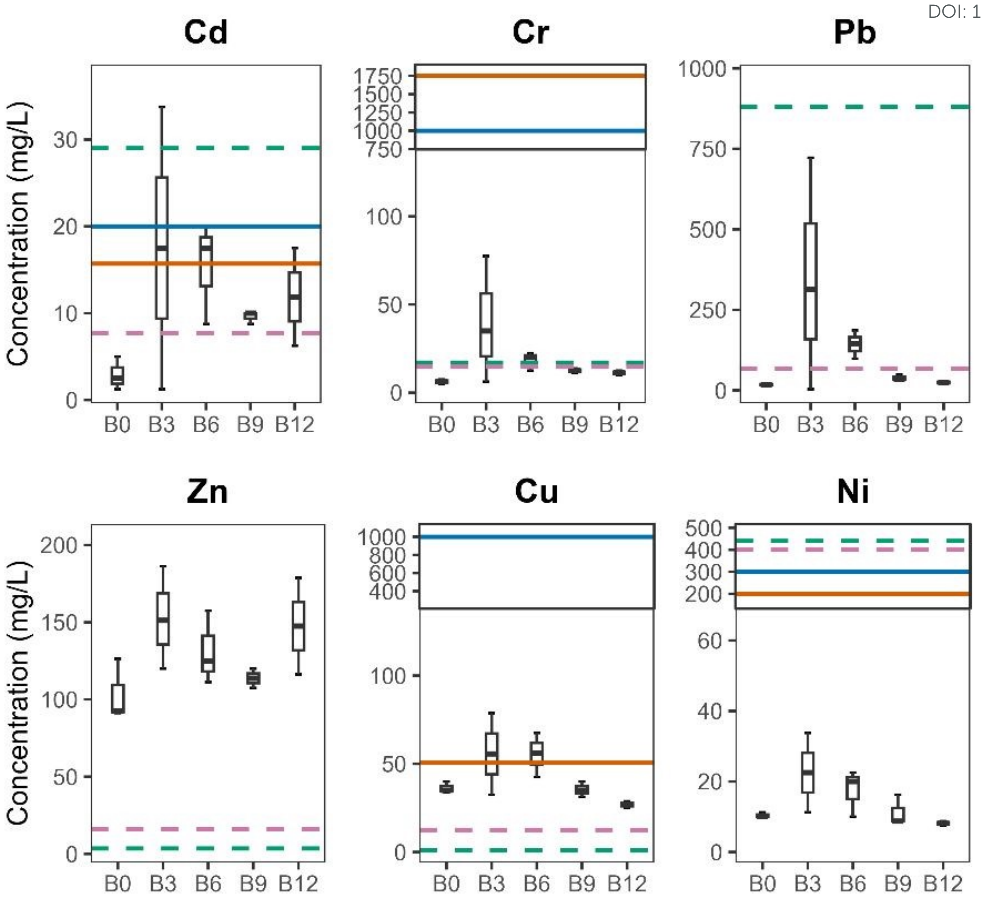

Figure 3. Heavy metal concentrations in AD digestate, limits for spreading for agriculture in South Africa (blue solid line) and the European Union (Red solid line) [85], and the Inhibitory concentrations of HM that reduces biological activities rate by 50% (IC_{50}) for Acidogenesis (green dash line) and Methanogenesis (purple dash line) [86,87]. For Cr, Cu and Ni, the y-axis is displayed with a break to accommodate the large separation between measured concentrations and regulatory or inhibitory threshold values. For Pb and Zn, there is no corresponding limit from the same reference. Boxplots are plotted on median, first quartile, third quartile and minimum/maximum values.

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3.6 Shifts in microbial communities

AD microbial communities are known to vary between experimental batches due to subtle differences in microbial assembly and inoculum state [94]. Therefore, microbial responses were evaluated within each batch and interpreted based on consistent trends, rather than pooled statistical significance alone.

3.6.1 Alpha and beta diversity

Digestate samples from B0 and B12 obtained from two independent AD batches were selected for 16S rRNA sequencing to provide a comprehensive microbial community assessment. Alpha diversity describes within-sample microbial richness and evenness and was evaluated using multiple indices (Fig. 4). No significant differences were observed between B0 and B12 in either batch for Chao1 richness (Fig. 4a). Similarly, Shannon and Simpson indices, which reflect community diversity and evenness with sensitivity to rare and abundant taxa, respectively, showed no statistically significant differences between treatments in either batch (Fig. 4b, c; $p > 0.05$). The Good's coverage values approached 1 for all samples, indicating sufficient sequencing depth to support these observations (Table S6).

In contrast, Wilcoxon test shows a clear difference between batches ($p<0.01$), with higher richness and diversity observed in Batch 1. This suggests a batch effect and reinforces that within-batch analyses are necessary. Same conclusion can be gained from Principal Coordinates Analysis (PCoA) (Fig.4d) that Batch 1 and Batch 2 separated clearly along PC1 (60.44% variance). Regarding FSB effect, B0 and B12 formed distinguishable clusters in Batch 1 whereas overlapped in Batch 2, suggesting that the biochar treatment could influence microbial community structure, though inconsistently between batches.

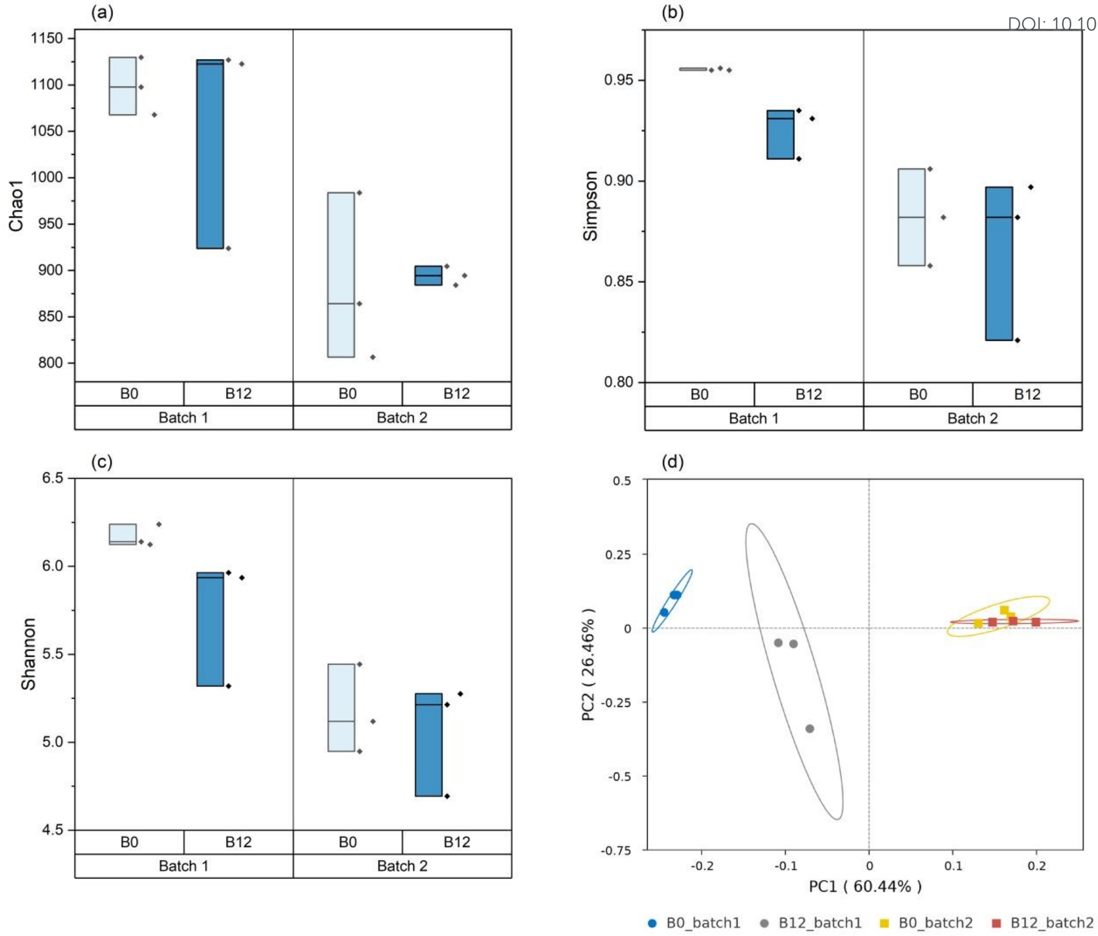

Figure 4. Alpha diversity indices (a) Chao1, (b) Simpson, (c) Sannon and (d) Principal Coordinates Analysis (PCoA) with 95% confidence ellipses of B0 and B12 digestate from two AD batches. Wilcoxon test between B0 and B12 for each batch showed no significant differences (p>0.05).

3.6.2 Microbial community structures

As shown in Fig.5a, *Halobacteriota* (22.66±1.73% and 32.89±1.15), *Bacillota* (also known as *Firmicutes*) (32.07±4.07% and 30.18±0.18), *Coprothermobacterota* (12.61±1.94% and 7.91±0.39%) and *Actinomycetota* (10.52±1.09% and 7.53±0.49%) were the prominent phyla in both batches for B0 and B12. Within the dominated phylum, *Firmicutes*, *Halobacteriota, Bacteroidota, Synergistota,* and *Chlorofexota* have been widely detected to dominate the field faecal sludge in different countries such as Ghana, Zambia, Senegal, Uganda, etc. and AD of

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faecal sludge [49,52,95–97]. This similarity suggests a broad community-level representativeness of this study and real pit latrines despite the use of synthetic feedstock. *Halobacterota* as an archaeon mainly consists of methanogens that use acetic acid as an electron donor and was reported to account for 42 % of the archaeal community in AD of swine manure [98,99] and its relative abundance consistently increased in B12 in both batches. This suggests enhanced methanogenic capacity and improved conversion of fermentation intermediates reflecting the decreased VFA concentration observed in B12. Although *Coprothermobacterota* is a thermophilic phylum, it presented in this study because the inoculum collected from the sewage treatment plant had undergone thermophilic disinfection prior to the mesophilic AD. Other dominated phylum showed slight or inconsistent changes between B0 and B12. Overall, FSB addition had limited impact on restructuring the bacterial communities at phylum level, which is consistent with the findings from AD of food waste with biochar addition [100].

Twenty-two taxa were selected from the top 30 genera based on their functional relevance to anaerobic digestion and are presented in Fig. 5b. The full annotation table can be found in Table S7. At genus level, hydrolysis, acidogenesis and methanogenesis related taxa account for most of the microbial communities while acetogenesis related taxa remain at low relative abundance (<5%) (Fig.5b), which is typical for a stable mesophilic AD system [101]. *Methanosarcina* was the dominant genus detected in both batches, accounting for approximately 20–30% of the total microbial community, with a higher relative abundance observed in B12. As a metabolically versatile methanogen, *Methanosarcina* is capable of acetoclastic, hydrogenotrophic, and methylotrophic methanogenesis, and has been reported to engage in syntrophic interactions with *Syntrophomonas via* DIET [102,103]. The higher relative abundance of *Methanosarcina* was accompanied by a consistent decrease in the hydrogenotrophic methanogen *Methanobacterium*, which was detected at much lower relative abundance following FSB addition. This opposing trend may suggest that FSB amendment

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influenced methanogen-associated community patterns, potentially favoring alternative methanogenic routes beyond strict hydrogen-mediated pathways, including acetoclastic methanogenesis. Similar shifts in methanogen-associated community structure have been reported in biochar-amended anaerobic co-digestion systems treating raw straw and cow manure [104]. In contrast, *Methanothrix,* a strictly acetoclastic methanogen that has also been reported to participate in DIET-based electron uptake [102], remained stable in relative abundance under FSB amendment. Taken together, the enrichment of *Methanosarcina*, the stability of *Methanothrix*, and the decline of hydrogenotrophic *Methanobacterium* indicate a reduced reliance on hydrogen-mediated methanogenesis and are consistent with a possible biochar-mediated modulation of methanogenic electron transfer pathways. Moreover, although *Methanosarcina* increased in relative abundance, this does not necessarily translate into higher methane or biogas production, as methanogenic activity is primarily governed by substrate availability rather than methanogen abundance. However, because the 341F/806R primer set was not specifically optimized for archaeal profiling, these archaeal patterns should be interpreted with caution.

FSB addition consistently enriched the proteolytic genus *Proteiniphilum,* indicating that protein hydrolysis was maintained or enhanced under biochar addition [105]. In contrast, several fast acidogenic and amino-acid-fermenting taxa, including *Romboutsia* and *Intestinibacter*, decreased across both batches, suggesting a restructuring of the acidogenic consortium rather than inhibition of upstream digestion [106,107]. This shift implies that FSB favoured aggregate associated proteolytic fermenters, which is consistent with the observed increase in carbohydrate-rich EPS and altered carbon partitioning. In addition to AD-related microorganisms, *Candidatus_Microthrix* consistently increased in all batches. This genus has been widely associated with sludge bulking in wastewater treatment plants, which are primarily attributed to its filamentous morphology embedded within a carbohydrate-rich EPS matrix

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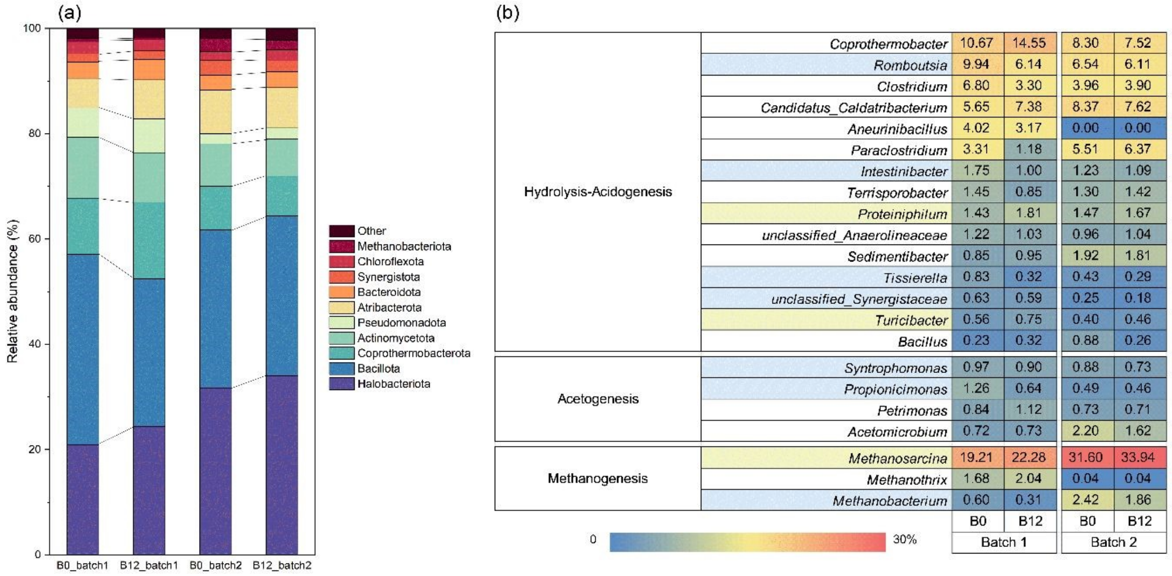
[108,109], which is consistent with the measured increase in EPS carbohydrates. Furthermore, the slight decrease in *Syntrophomonas* and *Propionicimonas* likely reflects reduced demand for H_2-dependent syntrophic oxidation under FSB amendment, consistent with stable VFA profiles and a shift in methanogenic electron transfer pathways.

Overall, FSB reshaped the microbial community structure toward biofilm-associated hydrolytic and methanogenic populations, supporting stable methanogenesis while promoting EPS-mediated carbon retention.



Hydrolysis-Acidogenesis	*Coprothermobacter*	10.67	14.55	8.30	7.52
	Romboutsia	9.94	6.14	6.54	6.11
	Clostridium	6.80	3.30	3.96	3.90
	Candidatus_Caldatribacterium	5.65	7.38	8.37	7.62
	Aneurinibacillus	4.02	3.17	0.00	0.00
	Paraclostridium	3.31	1.18	5.51	6.37
	Intestinibacter	1.75	1.00	1.23	1.09
	Terrisporobacter	1.45	0.85	1.30	1.42
	Proteiniphilum	1.43	1.81	1.47	1.67
	unclassified_Anaerolineaceae	1.22	1.03	0.96	1.04
	Sedimentibacter	0.85	0.95	1.92	1.81
	Tissierella	0.83	0.32	0.43	0.29
	unclassified_Synergistaceae	0.63	0.59	0.25	0.18
	Turicibacter	0.56	0.75	0.40	0.46
	Bacillus	0.23	0.32	0.88	0.26
Acetogenesis	*Syntrophomonas*	0.97	0.90	0.88	0.73
	Propionicimonas	1.26	0.64	0.49	0.46
	Petrimonas	0.84	1.12	0.73	0.71
	Acetomicrobium	0.72	0.73	2.20	1.62
Methanogenesis	*Methanosarcina*	19.21	22.28	31.60	33.94
	Methanothrix	1.68	2.04	0.04	0.04
	Methanobacterium	0.60	0.31	2.42	1.86
		B0	B12	B0	B12
		Batch 1		Batch 2	

Figure 5. Relative abundance of microbial community of B0 and B12 digestate from two AD batches at (a) phylum level for top 10 phyla and (b) at genus level selected from top 30 genera that are related to AD processes. In b, genera show consistent changing trend in both batches are coloured blue for decreasing and yellow for increasing.

4. Implications, limitations, and future work

According to the findings in this study, the primary role of FSB is more likely associated with accelerating early-stage hydrolysis rather than enhancing overall mineralisation. Under continuously fed pit conditions with low effective ISR ratios, increased hydrolysis may facilitate the breakdown and disintegration of freshly added faecal solids, potentially influencing physical accumulation patterns within the pit. However, the absence of increased VS reduction in this study indicates that such hydrolytic enhancement does not necessarily translate into greater long-term stabilisation or volume reduction of pit contents. Consequently, its application as a pit additive may not, on its own, provide sufficient benefit to justify its use.

A further consideration is that the limited methane response to FSB may reflect FSB-specific properties, including comparatively lower carbon content, lower accessible surface area/porosity, and potentially weaker buffering or adsorption capacity relative to lignocellulosic biochar [17,75,79]. By contrast, lignocellulosic biochar often exhibits higher surface area and alkalinity and generally lower intrinsic HM contents [110,111], which may increase their capacity to adsorb inhibitory intermediates and support syntrophic metabolism, thereby more readily enhancing methane production under stressed or overloaded conditions. Nevertheless, FSB retains a unique advantage in circularity, as it valorises faecal sludge into a reusable pit additive with potential operational and resource-recovery benefits.

Extrapolation of the findings of this study, conducted under controlled, idealised conditions, to real pit latrine systems remains uncertain, as pits operate under non-optimised and highly dynamic conditions characterised by continuous faecal input, lower effective ISR, variable moisture content, and long solids retention times. Under such conditions, the effects of FSB on hydrolysis, solids stabilisation, and gas production may differ substantially from those observed in batch laboratory assays. Future research should therefore prioritise long-term and semi-continuous experiments, field-scale pit simulations, and dissolved-phase metal speciation analyses to better resolve the role of FSB in regulating degradation pathways and pit-filling kinetics.

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5. Conclusions

This study demonstrated that FSB addition to the anaerobic digestion of synthetic human excreta accelerates early-stage solubilisation of particulate organics while decreased the VS reduction. This was accompanied by a significant increase in carbohydrate-rich EPS, indicating a shift in carbon partitioning from mineralisation toward retention within extracellular polymeric matrices and microbial biomass. Microbial community analysis revealed insignificant changes in the diversity and evenness of microorganisms while suggesting targeted restructuring toward aggregate-associated populations, including enrichment of metabolically versatile methanogens (*Methanosarcina*) and proteolytic fermenters (*Proteiniphilum*). Although FSB offers advantages in circularity and sanitation safety, the findings of this study suggest that its application as a pit additive is unlikely to deliver substantial performance benefits.

Author contributions

Mengru Qiu- Conceptualization, Formal analysis, Methodology, Validation, Visualization, Writing – original draft. **Maria E. Koulouri**- Writing – review and editing. **Laure Sioné-** Supervision. **Michael R. Templeton-** Conceptualization, Funding acquisition, Supervision, Writing – review and editing.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The microbial sequencing data were uploaded to NLM-NCBI, see http://www.ncbi.nlm.nih.gov/bioproject/1415342. The supporting data has been provided as

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part of the Supplementary information, including description of human excreta synthesis, proximate and ultimate analysis of FSB, total element concentrations of all materials, digestion method for TN, batch methane, gas, properties data, kinetic model fitting results, HM concentrations in other batches, alpha diversity results, and relative abundance of all top 30 genus. see DOI:

Acknowledgements

The authors would like to thank the Department of Civil and Environmental Engineering of Imperial College London for a Skempton scholarship. This project was supported by the Royal Academy of Engineering under the Research Chairs and Senior Research Fellowships programme. The authors would also like to thank Water For People Uganda team for their help in acquiring the biochar.

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Data availability

The microbial sequencing data were uploaded to NLM-NCBI, see http://www.ncbi.nlm.nih.gov/bioproject/1415342. The supporting data has been provided as part of the Supplementary information, including description of human excreta synthesis, proximate and ultimate analysis of FSB, total element concentrations of all materials, digestion method for TN, batch methane, gas, properties data, kinetic model fitting results, HM concentrations in other batches, alpha diversity results, and relative abundance of all top 30 genus. see DOI: