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Enhanced energy recovery from lignin-rich cattle manure: impact of coupling mesophilic anaerobic digestion with hyperthermophilic hydrolysis

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ABSTRACT

The recalcitrance of lignin in cattle manure (CM) significantly limits energy recovery during anaerobic digestion. Mesophilic anaerobic digestion of CM was investigated in continuously stirred tank reactors (CSTRs). In phase 1, the control mother reactor (MR) was operated at an SRT of 30 d, while in phase 2 the MR was followed by a hyper-thermophilic hydrolysis reactor (HTH) at 75 °C and SRT of 2 d (HTH2), with recirculation back to the MR which operated at an SRT of 22.4 d. The average steady-state biodegradability based on methane yields, in the MR, after recirculation, was 46 % ± 3 % compared to 42 % ± 5 %, without recirculation, primarily due to enhanced lignin removal of 20 % (12 % without recirculation). HTH1 (1d SRT) was tested at 75 °C to investigate the impact of SRT on solubilization of digested cattle manure (DCM). Biomethanation potential tests (BMP) conducted at 37 °C on DCM, HTH1 and HTH2 achieved biodegradabilities of 17 %, 19 %, and 26 %, respectively. Specific methanogenic activity tests (SMA) at mesophilic conditions for DCM and HTH2 showed comparable maximum specific methane production rate (MSMPR) of 14.6 and 14.1 mL CH_4/g VSS.d for DCM and HTH2, respectively. However, at 55 °C, the MSMPR for HTH2 was roughly three times higher than at 37 °C but it was comparable for DCM at both temperatures. *Firmicutes* and *Bacteroidota* were the main phyla in MR effluent, HTH2 and all SMA tests at different temperatures (37 °C and 55 °C). *Methanosarcina* was the most abundant methanogen at mesophilic and thermophilic temperatures. The predominant mechanism for the enhancement of methane production by the HTH recirculation was not solubilization but the enhanced biodegradation kinetics of particulate organics, including lignin, cellulose, and hemicellulose.

Abbreviations: AD, anaerobic digestion; ADS, anaerobic digester sludge; AMPTS, automatic methane potential test system; AW, agricultural waste; BMP, biomethanation potential test; CH_4, methane; CM, cattle manure; COD, chemical oxygen demand; CSTR, continuously stirred tank reactor; d, day; DCM, digested cattle manure; DCM control, DCM used as inoculum and substrate in BMP without ADS; FW, food waste; h, hour; HRT, hydraulic retention time; HTH, hyperthermophilic hydrolysis; HTH1, hyperthermophilic hydrolysis process operated at 1d SRT; HTH2, hyperthermophilic hydrolysis process operated at 2d SRT; HTH control, HTH effluent used as inoculum and substrate without ADS; ISR, inoculum-to-substrate ratio; LCH, lignin, cellulose, and hemicellulose; MR, mother reactor; MSMPR, maximum specific methane production rate; ND, not detected; NREL, National Renewable Energy Laboratory; OLR, organic loading rate; PCOD, particulate chemical oxygen demand; R^2, squared correlation coefficient; R_{max}, maximum methane production rate; SCOD, soluble chemical oxygen demand; SMA, specific methanogenic activity; SN, soluble nitrogen; SRT, sludge retention time; STD, standard deviation; STP, standard temperature and pressure; TCOD, total chemical oxygen demand; THP, thermal hydrolysis processes; TN, total nitrogen; TS, total solids; TSS, total suspended solids; VFA, volatile fatty acids; VS, volatile solids; VSS, volatile suspended solids; λ, lag phase period; °C, degree Celsius.

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1. Introduction

Recently, climate change has necessitated the transition to renewable energy sources. Among these, waste biomass represents a particularly interesting option due to its carbon neutrality and availability worldwide [1]. Cow manure is especially abundant, with global production reaching approximately 15.5 million tons per day (dry weight) [2]. Effective manure management is crucial, as it is a significant source of methane emissions contributing to global warming and may contain pathogens capable of contaminating soil and water [3]. Anaerobic digestion (AD) has been identified as a promising approach for converting the biodegradable components of manure into methane (CH_4), thereby mitigating its environmental impact [4]. Although cow manure possesses a relatively high calorific value (8.7–18.7 MJ/kg dry basis), its complete degradation is hindered by the presence of recalcitrant lignocellulosic compounds. This structural resistance limits its biodegradability, allowing only 40 %–50 % conversion [5]. The initial hydrolysis step of AD represents the rate-limiting phase [6], as the cross-linking structure of lignin, cellulose, and hemicellulose hinders enzymatic breakdown, thereby impeding carbohydrate fermentation [7].

Various chemical, biological, and physical pretreatment strategies have been explored to increase microbial accessibility to biodegradable compounds by overcoming the recalcitrance of lignocellulosic structures [5]. Thermal pretreatment has gained attention due to its relatively simple operational requirements, low capital investment, and high efficiency [8]. Hyperthermophilic (75 °C) hydrolysis (HTH) pre-treatment could be an alternative approach for specific wastes. However, the hydrolysis of lignocellulosic biomass typically requires thermal hydrolysis processes (THP) at temperatures around 160 °C and pressures of approximately 6.1 atm, leading to high operational costs [9]. Additionally, since the digestion substrate often contains biodegradable matter, pretreatment may result in unnecessary energy consumption. As an alternative, post-digestion treatment has gained interest due to its lower volume requirements, reducing overall costs [10]. This approach is particularly promising for farm-scale biogas systems, as it enhances both biogas quality and nutrient content of fertilizers [11]. Batch anaerobic digestion of post-treated lignocellulosic digestates has shown mixed results. For example, Sambusiti et al. [12] observed a 10 % and 20 % decrease in methane yield after thermal (80 °C for 1 h) and thermochemical (1 g NaOH/100 g TS, 40 °C for 24 h) post-treatment of digested animal manure and lignocellulosic waste mixtures, respectively, attributing this reduction potentially to the formation of inhibitory compounds from the lignocellulosic biomass rather than ammonia inhibition. Similarly, Kaparaju et al. [11] who evaluated the impact of various post-treatments, including maceration, thermal treatment (80 °C for 3 h), freeze-thaw cycling, sodium hydroxide incubation (40 g NaOH/kg VS) and combining thermal and chemical treatments, on the solid fraction of digested cow manure, reported that increased solubilization did not necessarily improve the methane yields.

A hyperthermophilic biological post-treatment may offer comparable benefits to the THP with potentially reduced energy input, owing to its lower operating temperatures and pressures. Acclimatizing mesophilic cultures to hyperthermophilic temperatures increases both extracellular enzyme activity and biomass hydrolysis rates. Hyperthermophilic conditions not only transform the microbiome structure but also stimulate the activity of key extracellular enzymes: proteolytic, cellulolytic, and hemicellulolytic [13]. For example, *Caldicellulosiruptor bescii* is a hyperthermophilic, fermentative bacterium exhibiting optimal growth at pH 7–8 and 70–80 °C, capable of degrading crystalline cellulose and lignocellulosic biomass with maximum growth rates of 4.5 d^{-1} on cellulose and 13.7 d^{-1} on cellobiose [14]. Hyperthermophilic hydrolysis processes can be operated at low HRT between 1d-5d [15,16]. For example, Romero-Güiza et al. [16] who examined the impact of hyperthermophilic hydrolysis at 65 °C and HRT of 3 d as a pretreatment for the mesophilic anaerobic digestibility of a mixture of sewage sludge and crude glycerin at HRT of 12 d and reported a 14 % increase in methane yield to 0.32 L CH_4/g VS in the integrated hyperthermophilic pretreatment treatment with anaerobic digestion compared to the control system at 36 °C and HRT of 15d. Bolzonella et al. [15] employed anaerobic digestate (AD) from plant processing manure and straw as a substrate for hyper-thermophilic post-treatment at 65 °C at an HRT of 3 d, and reported 33 % increase in methane Boe et al. [17] examined the impact of thermal post-treatment at 55 °C, 37 °C and 15 °C on a mixture of digested cow and pig manure in a CSTR with a HRT of 15 days followed by three CSTRs with HRTs of 5.3 days in parallel at temperatures of 55 °C, 37 °C, and 15 °C, and reported increases in biogas production of 11.7 % and 8.4 % for the 55 °C and 37 °C treatments, respectively. On the contrary, Axelsson Bjerg et al. [18] demonstrated that although digestates from food waste (FW), agricultural waste (AW), and a mixture of AW and manure digesters exhibited improved solubilization following thermal treatment at 70 °C for 1 h, methane yields in mesophilic CSTRs at HRTs of 7–15 d were mixed; i.e. FW and AW digestates produced 20 % and 9 % more methane as a result of post-treatment, respectively but the AW-manure mixture exhibited instability due to volatile fatty acid (VFA) accumulation and a decrease of 16 % in the daily methane production. The accumulation observed highlights that moderate thermal treatment is not an optimal approach for digested manure. Most of the literature studies mentioned above focused on the treatment of digestate originating from existing biogas plants, with post-treatment typically conducted in batch mode and digestion assessed in BMPs, with very limited information on the fate of lignin, cellulose, and hemicellulose. Integrating post-treatment with hyperthermophilic digestate recirculation can significantly reduce capital costs compared to two-stage digestion. Studies have demonstrated that the recirculation of digestate not only enhances methane yield but also contributes to improved process stability, making it a valuable approach for optimizing anaerobic digestion performance. In the study conducted by Dohdoh [19], sludge recirculation at 33 %–50 % of the influent significantly influenced performance, increasing methane and VS removal rates. The sludge recirculation contributed to greater buffering capacity, helping to maintain the volatile fatty acids (VFA) to alkalinity ratio within the recommended range, thereby stabilizing digester performance. To the best of the author's knowledge, few publications focused on the coupling of digested sludge recirculation and thermal post-digestion treatment.

In the study conducted by Takashima [20], raw sewage sludge was subjected to anaerobic digestion in a continuous-flow system at an HRT of 20 days, followed by thermal post-treatment at 120 °C for 1 h, once per week, and recirculation back to the digester. The results demonstrated that the post-treatment of digested sewage sludge led to a 21 % increase in methane production.

From a process scale-up perspective, moderate-temperature treatment is preferable due to the higher costs associated with more extreme conditions. While some studies in the literature report negative effects of thermal post-treatment, others have demonstrated its beneficial impact [21]. However, there is a notable lack of research on continuous-flow anaerobic digestion systems that integrate hyperthermophilic post-digestion treatment, particularly for lignocellulosic feedstocks, with the recirculation of treated biomass to the main digester. Also, the impact of hyperthermophilic hydrolysis on both acidogenic bacteria and methanogens warrants further investigation, particularly since there is a wide body of literature indicating suppression of methanogens by treatment at 70 °C [22–24]. However, the challenges and potential inhibitory effects associated with the recirculation of hyperthermophilic-treated digestate, characterized by elevated volatile fatty acid and readily biodegradable COD concentrations, alongside lignin-derived inhibitors, on acetoclastic methanogens remain unknown.

This study aims to evaluate the effects of hyper-thermophilic post-treatment at 75 °C on manure rich in lignocellulosic compounds. Prior research on hyperthermophilic post-treatment has predominantly explored its application as a pretreatment for single-stage digestion

[12,25] or as an integral part of two-stage AD systems [15,16]. Given the limited data on anaerobic digestion systems that incorporate post-treatment strategies and recirculation, the key objectives of this research are: (1) to assess the degree of solubilization and acidification of hyper-thermophilic post-treatment of digested cattle manure, (2) to evaluate the consequences of recirculating post-treated hyperthermophilic digestate to the original reactor on the overall biodegradability of lignin, cellulose, and hemicellulose contents and determine biodegradation kinetics. (3) to investigate the impact of hyperthermophilic post-treatment on both mesophilic and thermophilic acetolactic methanogens, (4) to delineate microbial community changes at different temperatures. Thus, unlike all the previous work on incorporating hyperthermophilic post-treatment either as a pretreatment to a single-stage digestion system or an integral component of a two-stage AD system, this work focuses on the challenges associated with recirculation of hyperthermophilic hydrolysates of lignin-rich digestates. Collectively, achieving the aforementioned four objectives will provide scientific insight into the interaction between mesophilic anaerobic cultures and hyperthermophilic cultures in two-stage anaerobic digestion systems, shed light on the mechanisms of potential transformation of refractory particulates in the hyper-thermophilic digester, as well as information in the potential toxicity of lignin solubilization products. The novelty of this work stems directly from the detailed solubilization data on LCH in anaerobic hyper-thermophilic biological reactors, and the determination of LCH degradation kinetics after post-treatment. Contrarily to literature data [15,16,18] where pre and post treatment attributed enhanced methane production to solubilization, in this work we proved that for lignin-rich feedstocks, solubilization is not the primary contributor to biogas enhancement. Post treatment by hyperthermophilic hydrolysis process was demonstrated to affect structural changes in the recalcitrant particulate fraction of digested cattle manure as evidenced by higher first order degradation coefficients for the various particulate fractions of the hyperthermophilic hydrolysate.

2. Methodology

To investigate the effect of hyperthermophilic post-treatment of DCM on biogas production, an experiment was designed comparing two systems. The first system used as a conventional mesophilic digester fed with cattle manure (CM) at an SRT of 30 d, based on the raw CM flow, for 240 d as described elsewhere [26], while the second operated for 300 d at a reduced SRT of 24.4 d, based on the raw CM flow, to optimize loadings, and incorporated recycling of 0.2 L/d of hyperthermophilic hydrolysis of DCM at 2d SRT donated henceforth as HTH2 at an HTH2: feed CM ratio of 1:4.5. The research focused on comparing biogas yields and performance between these two approaches to define the effectiveness of hyperthermophilic post-treatment in solubilization and acidification of refractory particulate organics, more specifically lignin, and the enhanced biogas production in continuous-flow systems. The activity of the mesophilic and hyperthermophilic acetolactic methanogens in DCM was investigated. As discussed later, BMP tests were conducted with CM, DCM, HTH as substrate and anaerobic digester sludge (ADS) from the Stratford municipal wastewater treatment plant, Ontario, Canada as inoculum, at an inoculum-to-substrate ratio (ISR) of 2 gVS substrate /gVSS inoculum. BMP tests were also conducted using CM, DCM, and HTH without ADS. CM, DCM, and HTH were used as both substrate (due to their high organic content) and the inoculum (because of their rich microbial population). This eliminated the need for an additional anaerobic digestate.

2.1. Cattle manure feed and inoculum

CM was collected from a cow farm located in Lindsay, Ontario, Canada. After collection, the CM was diluted to a range of 6 % - 7 % solids, was processed through a Kady mill (Kady International, Scarborough, ME, USA) at 60 Hz for 10 min, and then stored at 4 °C to be fed to the MR. In phase 1, the inoculum was ADS collected from Stratford wastewater treatment plant, Ontario, Canada and CM was fed to the lab scale conventional digester at mesophilic conditions and SRT of 30 d as described elsewhere [26], while in phase 2, the inoculum from phase 1 was used and the feed was a mixture of CM and HTH at a ratio of 4.5:1. A 0.4 L working volume HTH reactor was filled initially with 0.4 L of DCM and operated at 75 °C and 2d SRT. The feed to the HTH reactor was the digested CM effluent in phase 2.

2.2. Set up of the mother reactor (MR) integrated with the hyperthermophilic hydrolysis process

The reactors were designed for semi-continuous operation, featuring ports for feeding, wasting, and gas sampling. A circulating water jacket and water bath were used to maintain a constant mesophilic temperature of 37 ± 1 °C. Mixing was achieved via a mechanical stirrer with a tiger shark impeller, operating intermittently at 3000 rpm (1 min on, 2 min off). Anaerobic conditions were established by purging the headspace with nitrogen gas at a rate of 1 L/min for 3 min. In phase 1, a 12-L lab-scale continuous stirred tank reactor (CSTR) (henceforth referred as the mother reactor, control MR, with working volume of 10 L) was operated for 240 d (from day 1–240) at mesophilic conditions, SRT of 30 d and OLR of 2.9 kg COD/m3/d while in phase 2, a 30-L lab-scale CSTR (MR), with working volume of 22 L was operated for over 300 (from day 241–540) at mesophilic conditions and SRT of 24.4 d SRT, based on influent raw cattle manure flow of 0.9 L/d, integrated with hyperthermophilic post-treatment at 75 °C, an SRT of 2 d, and a daily flow of 0.2 L/d.

Daily biogas production was measured using a wet-tip gas meter (Rebel Wet-Tip Gas Meter Company, Nashville, TN, USA) while biogas composition including methane and carbon dioxide was characterized using a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (Mole sieve 5 A, mesh 80/100, 6 ft. 1/8 in). Argon was used as a carrier gas at a flow rate of 30 mL/min and the temperature of the column and thermal conductivity detector (TCD) detector were 90 °C and 105 °C, respectively Lignin, cellulose, and hemicellulose were characterized weekly for raw CM, DCM, and HTH2 effluent according to NREL methods [27]. Cellulose and hemicellulose were measured using a Refractive Index Detector (RID) (Perkin Elmer Series 200, PerkinElmer Instruments Inc., USA) connected to a gradient pump (GP50 Gradient pump) and chromatography oven (LC 25 Chromatography Oven) of a Dionex Ion Chromatogram. The oven was fitted with an Aminex® HPX-87H column (BIO-RAD lab- oratories, USA) which separated the components. The following parameters were used: pump flow rate: 0.6 mL/min; mobile phase: 9 mMH_2SO_4, column temperature: 30 °C and injection volume of 0.5 mL. Data was processed using ONLINE Chromatostation software. TCOD, SCOD, ammonia, VFAs, TN, and SN were analyzed using HACH methods and test kits (HACH Odyssey DR/2500 spectrophotometer manual). TSS, VSS, TS, and VS were analyzed according to Standard Methods for the Examination of Water and Wastewater [28]. CM, MR effluent, and HTH effluent samples were diluted 40 times and 10 times before TCOD and SCOD analysis, respectively but used as is for TS, VS, TSS, and VSS analysis. Phenol was measured using Hach barcoded kits.

2.3. Hyper-thermophilic hydrolysis reactor (HTH) set up

Two 0.4 L HTH reactors were operated at SRTs of 1d (for 70 days) (henceforth referred to as HTH1) and 2d for 204 days (HTH2), 75 °C, fed daily with 0.4 L and 0.2 L of DCM, respectively, collected from the MR to evaluate the effect of SRT on the degree of solubilization and acidification of DCM. A 0.2 L of HTH2 was recycled with 0.9 L of CM to the MR in phase 2 to assess the impact of hyperthermal post-treatment on post-AD of DCM.

2.4. Off-line batch activity tests

2.4.1. Specific methanogenic activity (SMA) tests at mesophilic and thermophilic conditions using DCM and HTH as inoculum

Specific methanogenic activity (SMA) tests were conducted using DCM and HTH2 effluent as inoculum and acetate as a substrate at 2.2 g COD/L to test the activity of acetoclastic methanogens at 37 °C and 55 °C. After the methane production had plateaued, the reactors at 55 °C were transferred to a 70 °C water bath to investigate the effectiveness of hyperthermophilic conditions on DCM and HTH2 for 15 d to degrade the residual acetate. Subsequently, the temperature decreased to 55 °C to study the impact of temperature change on the biodegradability.

2.4.2. Biomethanation potential tests (BMP) at mesophilic conditions using DCM, HTH1, and HTH2 as both substrate and inoculum

Automated methane potential test system (AMPTS) was used to test the biodegradability of DCM, HTH1, HTH2, as substrates using ADS from the Stratford wastewater treatment plant as inoculum at an ISR of 2 g VSS/g VS substrate. Additionally, DCM, HTH1 and HTH2 were tested without adding ADS to assess the methanogenic activity of the hyperthermophilically treated DCM. After the methane production plateaued, samples were collected for final analysis and confirmation of the COD mass balance. For all batch tests, the modified Gompertz equation (Eq. 1) was applied to the average net methane production to estimate the maximum biomass-specific methane production rate (MSMPR) (mL CH_4/gVSS.d). The VSS of the seed sludge was used to normalize the methane rates in the cases where ADS was used, while the VSS of the DCM, HTH1, and HTH2 controls were used to normalize the methane rates, as they constituted the source of active biomass.

$$P\,(t) = P_{max}\; exp\left\{ - exp\left[\frac{R_m e}{P_{max}}\,(\lambda - t) + 1\right]\right\} \quad (1)$$

where, P(t) is the cumulative biogas production at time t, P_{max} is the maximum cumulative methane production (mL CH_4), R_m is the maximum methane production rate (mL CH_4/d), λ is the lag phase period (d), e is the base of the natural logarithm (Euler's number which approximately equals to 2.71828). The Pmax, Rm, and λ were predicted using Eq. (1) with the aid of the Solver function of the Microsoft Excel Tool Pak. The squared correlation coefficient (R^2) was used to evaluate the precision of the model fit.

2.5. Microbial community analysis

The microbial community analyses were conducted for DCM from the mother reactor, HTH2 from the hypethermophilic hydrolysis reactor, DCM after SMA test at 37 °C, HTH2 after SMA test at 37 °C, DCM after SMA test at 55 °C, and HTH2 after SMA test at 55 °C. The analyses were conducted at IRDA lab (Research and Development Institute for the Agri-environment, Quebec, QC, Canada). DCM (MR effluent) and HTH (Effluent) samples were collected once at the end of the steady-state operation, after maintaining stable performance for at least three consecutive HRTs. Samples from the SMA tests were collected at the end of the experiments.

Sample preparation and DNA extraction were conducted as described by Haroun et al. [26]. Illumina MiSeq 2 × 300 bp sequencing was performed by the Genomic Analysis Platform of the Institute for Integrative Biology and Systems (IBIS) at Laval University (Quebec, QC, Canada). DNA extraction from samples was conducted using the Fast DNA Spin Kit for Soil. Genomic DNA quality and quantity were assessed spectrophotometrically via A260/A280 ratios. The V4 regions of archaeal and bacterial 16S rRNA genes were amplified using a double-indexed two-step PCR method optimized for Illumina MiSeq sequencing (IRDA, Québec). Paired-end sequencing (2 × 300 bp) was performed on an Illumina MiSeq platform at the IBIS genomic analysis platform (Université Laval). Microbial abundance was visualized using a heatmap generated with OriginPro software.

2.6. Statistical analysis

The significance of the observed differences in methane yields and biodegradability, as well as LCH removal efficiencies in both phases (control MR without HTH recirculation and MR with HTH recirculation) was evaluated using *t*-test approach at the 95 % confidence level.

3. Results and discussion

3.1. CM and DCM characteristics and mesophilic digester performance

The performance of the 10 L control MR in this study until day 240 was identical to the performance of the MR described by (Haroun et al. 2025). The control MR in phase 1 showed a biodegradability and methane production yield of CM of 42 % ± 3 % and 0.166 ± 0.01 L CH_4/g COD substrate, respectively, with lignin, cellulose, and hemicellulose removal efficiencies of 11.9 %, 54.5 %, and 55.4 %. The data presented in this study will be focused on phase 2 with hyperthermophilic hydrolysis process application to DCM in a CSTR at 2d SRT and recirculation to the MR. Table 1 presents the steady-state physicochemical characterization of CM feed, DCM effluent, and HTH2 after 3 turnover SRTs. The average concentrations of TS and VS of the CM feed were 66,300 mg/L and 55,100 mg/L, respectively, while the average TCOD and SCOD concentrations were 77,700 and 19,150 mg/L, respectively. Statistical correlations for particulate COD (PCOD)-to-VSS of CM feed of 1.189 ($R^2 = 0.98$, $p < 0.05$) and TCOD-to-VS of 1.45 ($R^2 = 0.98$, $p < 0.05$) are presented in the supplementary information (Fig. S1a–b). The initial pH and alkalinity of the CM feed were 7.2 and 10.6 g $CaCO_3$/L, respectively, which confirms that the feed had adequate buffering capacity.

Conversely, the average concentrations of TS and VS of the DCM were 49,400 mg/L and 37,940 mg/L, respectively. The steady state TS and VS removal efficiencies in the MR were 31.6 % and 36.8 %, respectively. Average concentrations of TCOD and SCOD in DCM were 50,600 mg/L and 7960 mg/L, respectively, while the average concentration of PCOD was 44,200 mg/L, with TCOD, SCOD, and PCOD removal efficiencies of 36.2 %, 68 %, and 25.7 %, respectively.

The average concentrations of TSS and VSS of DCM were 49,400 mg/L and 37,940 mg/L, respectively. Statistical correlations for TCOD/VS of DCM showed similar results to CM of 1.37 ($R^2 = 0.991$, $p < 0.05$), however, statistical correlations for PCOD/VSS for DCM of 1.279 ($R^2 = 0.991$, $p < 0.05$) in phase 2 with HTH recycling was 8 % higher than in phase 1 of 1.178 (supplementary information, Fig. S2a–b). One possible explanation is that the HTH process hydrolyzes particulate matter with a lower COD value. Theoretically, the COD values of cellulose, hemicellulose, lignin, and biomass are 1.067, 1.067, 1.3, and 1.42 g COD/g substrate, respectively. This suggests that the HTH process is favorable to the solubilization of lignocellulosic compounds over biomass. This is confirmed by the absence of any increase in soluble nitrogen in the HTH reactor (Table 1).

The average pH and alkalinity of DCM were 7.8 and 11.7 g $CaCO_3$/L, respectively. VFAs in the DCM ranged from 1190 mg/L to 3100 mg/L, with an average of 1830 mg/L, with a VFAs-to-alkalinity ratio of 0.156 ± 0.04, confirming stable anaerobic digestion below the inhibition threshold of 0.4 reported by Elazhar et al. [29]. Since phenolics are typical inhibitory lignin hydrolysis byproducts [30], total phenol for CM, DCM, and HTH were 148 mg/L, 117 mg/L, and 147 mg/L, respectively, which is below the inhibition threshold levels of 1250 mg/L [31]. This suggests that partial removal of phenol occurred in the AD of CM. The effluent phenol concentration in the digested CM is in line with the results obtained by Sambusiti et al. [12] who tested the anaerobic biodegradability of thermal post treated anaerobic digestate mixture of maize silage (25 %), sorghum silage (11 %), olive waste (11 %), cow manure (8 %), pig manure (18 %), and turkey poultry manure on

Table 1
Characterization of CM feed, DCM, and HTH2.

Parameter	Average ± STD			No. of samples
	CM	DCM	HTH2	
TCOD (mg/L)	77,700 ± 3430	50,600 ± 2620	50,600 ± 5640	30
SCOD (mg/L)	19,150 ± 2260	6000 ± 520	8900 ± 1190	30
PCOD (mg/L)	59,500 ± 6320	44,200 ± 5100	41,060 ± 5260	30
TS (mg/L)	66,300 ± 5660	49,400 ± 5040	49,300 ± 5780	30
VS (mg/L)	55,100 ± 5600	37,940 ± 4280	38,300 ± 5190	30
TSS (mg/L)	51,000 ± 5560	43,750 ± 6730	40,600 ± 5200	30
VSS (mg/L)	42,200 ± 6830	35,370 ± 5100	31,900 ± 5200	30
TN (mg/L)	2320 ± 250	2610 ± 220	2490 ± 260	30
SN (mg/L)	1120 ± 260	1540 ± 140	1550 ± 280	30
VFA (mg/L)	4680 ± 250	1720 ± 260	2420 ± 500	30
Ammonia (mg/L)	680 ± 87	1120 ± 105	1117 ± 280	30
phenol (mg/L)	148 ± 21	117 ± 23	147 ± 8	18
pH	7.2 ± 0.3	7.6 ± 0.2	7.8 ± 0.2	30
Alkalinity (g $CaCO_3$/L)	10.6 ± 1.3	11.7 ± 1.4	12.0 ± 1.0	30

coconut chips (26 %) (VS basis) at 80 °C for 1 h and reported final phenol concentrations of 110 mg/L in the batch. However, phenol concentration increased by 30 mg/L because of hyperthermophilic hydrolysis at 75 °C and 2 d SRT due to lignin solubilization.

3.2. HTH performance

The physicochemical characteristics of DCM effluent, HTH1, and HTH2 are presented in Tables S1-S2 while the degree of solubilization and acidification for HTH1 and HTH2 are presented in Table 2. The degrees of solubilization of DCM were 106 and 131 mg SCOD/g VSS, and 69 and 91 mg COD/g PCOD for HTH1 and HTH2, with percentage SCOD increase of 82 % and 78 %, respectively. Similarly, the degrees of acidification of DCM were 32 and 40 mg VFAs/g VSS, and 21 and 28 mg VFAs/g PCOD for HTH1 and HTH2, with percentage VFAs increase of 83 % and 84 %, respectively. VFAs accounted for 31 % of SCOD as a result of hyperthermophilic hydrolysis at 75 °C, regardless of the retention time. The results of this study suggest that HTH2 exhibited a 23 % increase in solubilization, and acidification compared to HTH1, suggesting a significant performance advantage. Both HTH1 and HTH2 were connected to gas bags to collect and characterize the gas composition. An average of 0.22 L of biogas was collected daily, with only 4 % hydrogen. Interestingly, no methane gas was detected in the biogas. This observation aligns with the COD analysis, which showed negligible differences (0.7 %–2 %) between the influent DCM and HTH1 and HTH2 effluent samples (Tables S1-S2). Consistent with the findings of the current study, Lin et al. [32] also reported no methane production in a hyperthermophilic reactor treating pig manure at 70 °C with an SRT of 5 d.

Statistical correlations for TCOD/VS of both HTH1 and HTH2 showed comparable results to DCM of 1.461 ($R^2 = 0.988$, $p < 0.05$). However, statistical analysis revealed a significantly higher PCOD/VSS ratio of 1.333 ($R^2 = 0.985$, $p < 0.05$) for HTH-treated samples compared to the 1.279 observed for DCM (supplementary information, Fig. S3a–b), suggesting preferential hydrolysis of components with lower COD values, likely cellulose and hemicellulose, by HTH relative to lignin and biomass.

Table 2
Solubilization and acidification in hyperthermophilic hydrolysis reactors at 1d and 2 d SRT.

Parameter	HTH1	HTH2
Degree of solubilization (mg COD/g VSS)	106 ± 24	131 ± 21
Degree of acidification (mg VFAs/g VSS)	32 ± 9	40 ± 8
Degree of solubilization (mg COD/g PCOD)	69 ± 14	91 ± 15
Degree of acidification (mg VFAs/g PCOD)	21 ± 3	28 ± 4
Number of samples	13	27

3.3. Fate of lignin, cellulose, and hemicellulose during anaerobic digestion and hyperthermophilic hydrolysis

The statistical correlations between the lignocellulosic components (LCH) of CM and their PCOD and VSS are demonstrated in Fig. S4a and b. Similar correlations for DCM are shown in Fig. S5a and b, and for HTH in Fig. S6a and b.

Assuming 1.3 g COD/g lignin and 1.067 g COD/g cellulose and hemicellulose as described by Haroun et al. (2025), LCH represents 81.1 % of PCOD, with 59 % of the LCH COD as lignin and 41 % of the COD as cellulose and hemicellulose. Variations of lignin, cellulose, and hemicellulose in the CM feed and DCM are presented in Fig. 1a and b, with the steady-state data summarized in Table 3. Paired *t*-tests revealed statistically significant differences in lignin, cellulose, and hemicellulose content between DCM and HTH treatments at the 95 % confidence level. The average steady-state removal efficiencies of lignin, cellulose, and hemicellulose in the MR calculated after 3 turnovers of the mean SRTs in phase 1 were 11.9 %, 54.5 %, and 55.4 %. In comparison, lignin, cellulose, and hemicellulose removal efficiencies in the MR in phase 2 were 12.3 %, 51 %, and 54 %, respectively while lignin, cellulose, and hemicellulose concentrations after HTH were 8.1 %, 12 %, and 14.9 % lower than the DCM, respectively (Table 3), with the differences statistically significant at the 95 % confidence level. Furthermore, the overall removal efficiency of lignin, cellulose, and hemicellulose in the MR at 24.4 d SRT coupled with HTH recycling at 2 d SRT was 20 %, 57 %, and 63 %, respectively. Lignocellulosic matter was 78.3 % of the DCM VSS (Fig. S5b), with lignin alone representing 69 % of the LCH (19.8 g/L out of 28.5 g/L combined lignin, cellulose, and hemicellulose). In this current study, as shown in Table 3, a total of 15.13 g LCH/L (12.36 g/L in the MR and 2.75 g/L in HTH) were removed out of the 40.98 g LCH/L in the CM, representing a 36.9 % removal efficiency. Hemicellulose demonstrated the highest percentage removal of 63 %. Given that LCH comprised 81.1 % of the PCOD, the MR achieved a 28 % removal efficiency of LCH as PCOD.

3.4. Methane production in the MR coupled with HTH

Average methane content in the biogas over the entire operational period was 61.8 % ± 3 %. Biodegradability and methane yields, both based on the influent cattle manure only, from the MR are presented in Fig. 2a and b. Steady-state data, collected over the last 158 days in each phase, indicated average COD biodegradability for the MR at mesophilic condition in phases 1 and 2 were 42 % ± 3 % and 46 % ± 3 % (Fig. 2a), respectively, while methane yield of CM in the MR in phases 1 and 2 were 0.166 ± 0.02 and 0.184 ± 0.012 L CH_4/g COD substrate added corresponding to daily methane production rates of 11.9 L/d and 13.68 L/d, respectively (Fig. 2b), reflecting an 11 % increase with HTH recirculation. This clearly demonstrates that the HTH reactor was

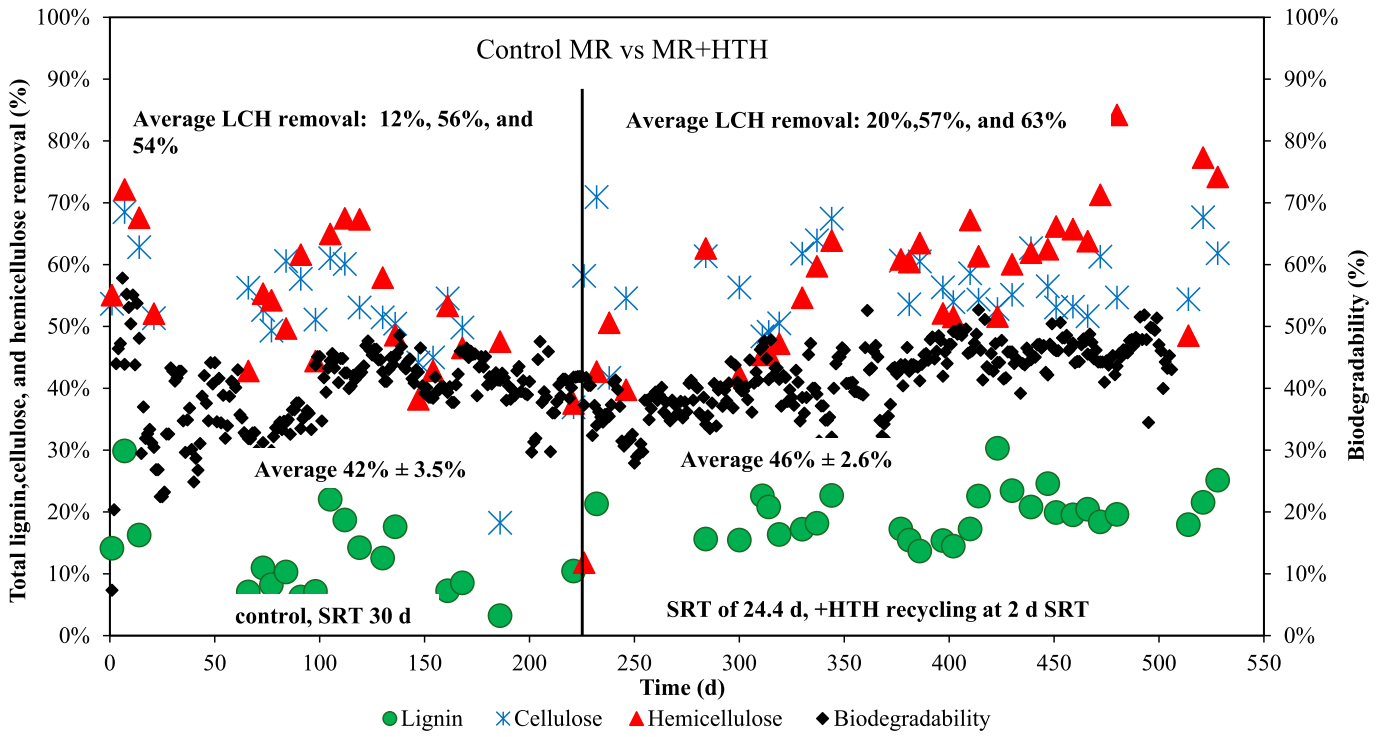

Fig. 1. Lignin, cellulose, and hemicellulose percentage removal in control MR (phase1) and MR + HTH2 (phase 2).

Table 3
Steady-state lignin, cellulose, and hemicellulose removals in phases 1 and 2.

Phase 1				
Parameter	Unit	CM	DCM	%R
	LCH			
Lignin	mg/L	21,700 ± 1930	19,120 ± 790	11.9 %
Cellulose	mg/L	15,800 ± 1930	7190 ± 630	54.5 %
Hemicellulose	mg/L	9950 ± 1200	4440 ± 620	55.4 %
Sum LCH	mg/L	47,450	30,750	35.2 %

Phase 2							
Parameter	Unit	CM	DCM	%R	HTH2	%R	Overall %
LCH							
Lignin	mg/L	22,500 ± 2220	19,750 ± 2180	12.3 %	18,140 ± 1950	8.1 %	20 %
Cellulose	mg/L	12,250 ± 1820	6000 ± 920	51 %	5290 ± 940	12 %	57 %
Hemicellulose	mg/L	6220 ± 1760	2860 ± 600	54 %	2430 ± 500	14.9 %	63 %
Sum LCH	mg/L	40,970	28,610	30.2 %	25,860	9.6 %	36.9 %
LCH as PCOD							
Lignin	mg as PCOD/L	29,250	25,675	12.3 %	23,582	8.1 %	20 %
Cellulose	mg as PCOD/L	13,070	6402	51 %	5644	12 %	57 %
Hemicellulose	mg as PCOD/L	6640	3052	54 %	2593	14.9 %	63 %
Sum LCH	mg as PCOD/L	48,960	35,129	28 %	31,819	9.5 %	35 %

effective in converting the recalcitrant organics in the DCM to more readily biodegradable organics, as the actual HRT with recirculation was 20d, compared to 30d without recirculation. SCOD removal was 68 % as reported in Table 1, while LCH as PCOD removal was 28 %, and therefore non-LCH PCOD degradation with HTH recirculation was estimated (supplementary information) to be 100 %, compared to 43 % without HTH recirculation in phase 1. This indicates that integrating the HTH process with anaerobic digestion effectively degrades both LCH and non-LCH particulates.

To compare the performance of the MR in the two phases at different SRT, a first-order kinetic model for a CSTR was used to calculate k at 30d SRT and estimate the COD removal efficiency at an SRT of 24.4 d. The data from the CSTR, in phase 1 without HTH recycling, suggests a first-order degradation rate of 0.024 d^{-1} (see the calculation in the supplementary information), which will translate to 37 % biodegradability at an SRT of 24.4 d in phase 2. Based on the aforementioned calculations, recycling hyperthermophilic hydrolyzed DCM at 2 d SRT and a ratio of 1:4.5 (HTH: CM) improved the digestibility of CM by 24.3 %. At steady-state, a paired *t*-test was performed to compare methane yields and biodegradability between the control MR without HTH recirculation and the MR with HTH recirculation. The analysis revealed statistically significant differences at a 95 % confidence level for both parameters.

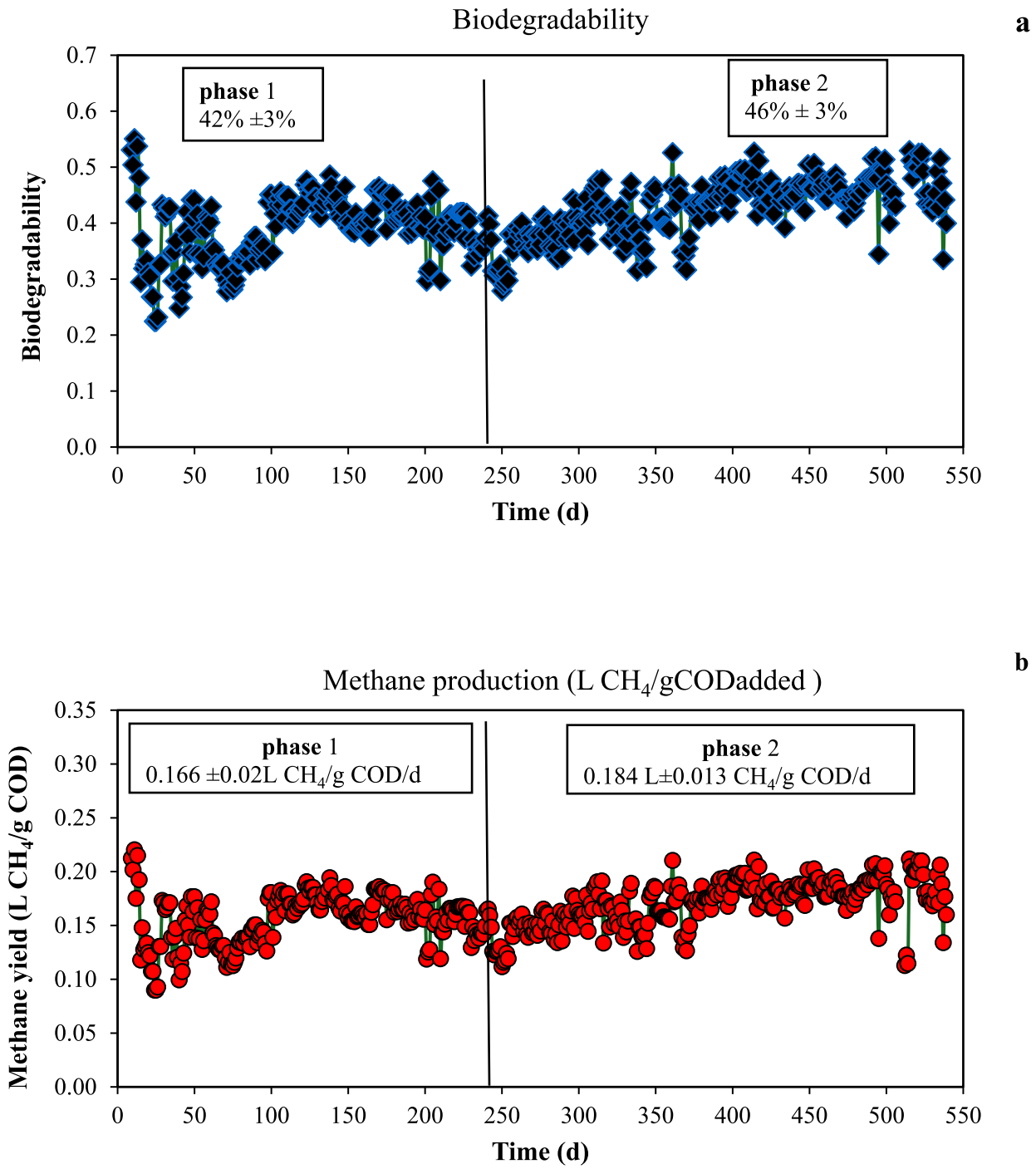

Fig. 2. Temporal variation of biodegradability of CM (a) and methane production yield (b) in the control MR (phase 1) and MR + HTH2 (phase 2).

SCOD of the recycled HTH accounted for only additional daily methane rate of 0.16 L/d (9 % of the total increase) compared to the experimentally observed daily methane of 1.76 L CH_4/d. This suggests that the HTH process significantly altered PCOD structure, rendering it more biodegradable. This will be elaborated upon later in Section 3.8.

The findings of the current study are consistent with Bolzonella et al. [15] who explored integrating post-hyperthermophilic hydrolysis (65 °C, HRT 2–5 days) of digested agro-waste with anaerobic digestion (CSTR, SRT 16 d) and observed a 20 %–38 % increase in methane yield for thermally post-treated digestate compared to nontreated digested agro-waste, with the HRT of hyperthermophilic hydrolysis being a key factor.

3.5. BMP tests at mesophilic conditions using DCM, HTH1, and HTH2

AMPTS was employed to test the post biodegradability of DCM, HTH1, HTH2, using ADS as inoculum at an ISR of 2 g VSS/g VS substrate. Additionally, DCM, HTH1 and HTH2 were tested without adding ADS to assess the methanogenic activity of the hyper-thermophilically treated DCM. Table S3 shows the characterization of the initial BMP samples, while Table S4 shows the characterization of the final BMP samples. Three replicates were used for the sample analysis, and the reported data in Tables S3 and S4 are the averages and standard deviations for the 3 replicates. Fig. S7 shows the net cumulative methane production for all samples at standard temperature and pressure (P = 1

atm, and T = 273 K), including cellulose as a control to confirm the activity of the digester sludge used in this study. In the case of DCM, HTH1, and HTH2 controls, ADS was not used, but rather the samples were used as both substrate and inocula to test the methanogenic activity of DCM after thermal hydrolysis at 75 °C in semicontinuous flow systems at HRTs of 1d and 2d. The term "controls" was used to express the dual purpose of CM, DCM, and HTH, as the second BMP tests were conducted in the absence of ADS. The average net cumulative methane production at STP, i.e. after deducting the average methane produced by the inoculum for DCM + ADS, HTH1 + ADS, HTH2 + ADS, and cellulose + ADS were 212 mL, 266 mL, 319 mL, and 803 mL, respectively while DCM, HTH1, and HTH2 controls produced 1659, 1064, and 1682 mL, respectively (Fig. S7 and Table 4).

Fig. S8 shows the temporal variation of the average net cumulative methane yield per g VS substrate, with ultimate yields of 88, 86, 137, 104, 86, 107, 324 mL CH_4/g VS substrate for DCM + ADS, HTH1 + ADS, HTH2 + ADS, DCM control, HTH1 control, HTH2 control, and cellulose, respectively (Fig. S8 and Table 4). Fig. S9 shows the temporal variations of the average net cumulative methane yield per g COD substrate with ultimate yields of 59, 68, 91, 72, 54, 70, and 309 mL CH_4/g COD substrate added corresponding to biodegradability of 16 %, 19 %, 26 %, 20 %, 15 %, 20 %, and 87 % for DCM + ADS, HTH1 + ADS, HTH2 + ADS, DCM control, HTH1 control, HTH2 control, and cellulose, respectively. The data reported in these experiments is the average of three triplicates with maximum discrepancies not exceeding 7 %, confirming the reproducibility of the replicates. The higher biodegradability of 26 % observed for HTH2 + ADS compared to 19 % for HTH1 + ADS could be due to the higher solubilization of HTH2 of 91 mg SCOD/g PCOD vs 69 mg SCOD/g PCOD for HTH1.

The biodegradability of HTH 2 and DCM controls were comparable at 20 %, which was 25 % higher than the biodegradability of the HTH1 control. One possible explanation is that the recirculation of the HTH2 to the MR improved the activity of mesophilic methanogens. The difference in the performance between HTH1and HTH2 in semi-continuous flow systems in addition to the SRT was the recirculation of 0.2 L of HTH2 daily to the MR, which was not the case for HTH1. This suggests that the HTH2 culture is acclimatized to mesophilic conditions. Despite extensive literature studies indicating methanogen suppression at 70 °C for 30 min [33,34], the results of the current study demonstrate their survival, as evidenced by the methane production in HTH1 and HTH2 (Table 4).

The Gompertz model fits the data perfectly with R^2 ranged from 0.959 to 0.999, and the model parameters are summarized in Table 4. Scrutiny of the DCM data in Table 4 indicates that the maximum specific methane production for the DCM alone was about twice the DCM with Stratford ADS, clearly reflecting the better acclimatization of the DCM to lignocellulosic substrates relative to the municipal ADS. The independent operation of the HTH1 control without recirculation resulted in an approximate 8.9 d lag, indicating the heat-driven inactivation of methanogens, a result supported by Haroun et al. [34] who applied heat to suppress methanogenic activity and enrich biohydrogen cultures for fermentation of glucose and xylose. The maximum specific methane production rates for HTH2 were superior to DCM and HTH1 in both scenarios with ADS and without ADS, clearly indicating that not only as a substrate HTH2 is more readily biodegradable, but also that even though the methanogens are slightly inhibited relative to the DCM as evidenced by the 2-d delay, the methanogenic population is significantly more active. This confirms the findings of the current study, where the biodegradability of HTH2 was the highest due to the recirculation of HTH2 to the MR and acclimatization of mesophilic methanogens. Interestingly, the lag phase was 4.5 times longer in HTH1 control compared to HTH2 control. This suggests that the recirculation of HTH2 is the key factor in reducing the lag to 2 days, likely by promoting microbial acclimation and regrowth. Similarly, the methane production rates observed by the Gompertz model of HTH2 were higher than HTH1 and DCM. Additionally, MSMPRs for all controls were higher than with ADS, indicating better acclimatization of the DCM, as reported elsewhere [26].

3.6. Specific methanogenic activity (SMA) tests at mesophilic and thermophilic conditions using DCM and HTH2 as inoculum

Specific methanogenic activity (SMA) tests were conducted at 37 °C and 55 °C using DCM and HTH2 effluent as inoculum and acetate as substrate at 2.5 g/L to test the activity of acetoclastic methanogens. At mesophilic temperature (37 °C), acetate was completely degraded after 4–5 days (Fig. 3a) with no lag, with both inoculum (DCM and HTH2 effluent), which confirms the comparable activity of mesophilic acetoclastic methanogens as reflected by close maximum specific methane production rates (MSMPR) of 14.6 and 14.1 mL CH_4/g VSS.d, respectively (Table 5).

At 55 °C, 57 % and 59 % biodegradability of acetate were observed for DCM and HTH effluent (Fig. 3b) at methane production rates of 13.6 and 36.5 mL CH_4/g VSS.d, respectively (Table 5). Although the HTH process at 75 °C did not produce any methane gas as discussed above, it has more active thermophilic acetoclastic methanogens as evidenced by the high MSMPR at 55 °C compared to MR (36.5 vs 13.6 mL CH_4/g VSS.d). The biodegradability of acetate under mesophilic conditions was superior to thermophilic conditions using both inocula (DCM and HTH2). This is likely because DCM was acclimatized at 37 °C and HTH2 at 75 °C, neither of which was at 55 °C. This supports the observation that HTH2, with its active biomass, achieved an additional 20 % degradation without the addition of municipal digested sludge, as discussed in Section 3.5. No lag phase was detected in the mesophilic SMA experiment using both DCM and HTH2, as it was activated in the previous mesophilic BMP tests (where the lag phase was 2 days). However,

Table 4
Experimental and Gompertz model results in BMP using DCM, HTH1, and HTH2 with and without ADS.

Parameter	DCM + ADS	HTH 1 + ADS	HTH 2 + ADS	DCM control	HTH1 control	HTH2 control	cellulose
g VS substrate added	2.4	3.1	2.3	15.9[a]	12.3[a]	15.8[a]	2.5
g COD added	3.61	3.93	3.49	23.2[a]	19.80[a]	24.00[a]	2.60
Methane yield (mL)	212	266	319	1659	1064	1682	803
Methane yield (mL/g COD)	59	68	91	72	54	70	309
Methane yield (mL/g VS)	88	86	137	104	86	107	324
biodegradability	0.17	0.19	0.26	0.2	0.15	0.2	0.87
R (mL CH_4/d)	18.5	24	26.9	160.5	133	187.8	336
P (mL)	202	261	320	1460	1098	1641	785
L (d)	ND	0.1	ND[b]	0	8.9	2	0.9
R^2	0.98	0.959	0.96	0.99	0.978	0.99	0.959
g VSS inoculum	4.8	4.94	4.8	13.46	14	12.68	4.94
MSMPR (mL/gVSS/d)	3.9	4.9	5.6	11.9	9.5	14.8	68.0

MSMPR is the maximum specific methane production rate (mL/gVSS/d).
[a] In the control reactors (without ADS), the g VS and g COD of substrate added represent the total VS and COD of the samples.
[b] ND: Not detected

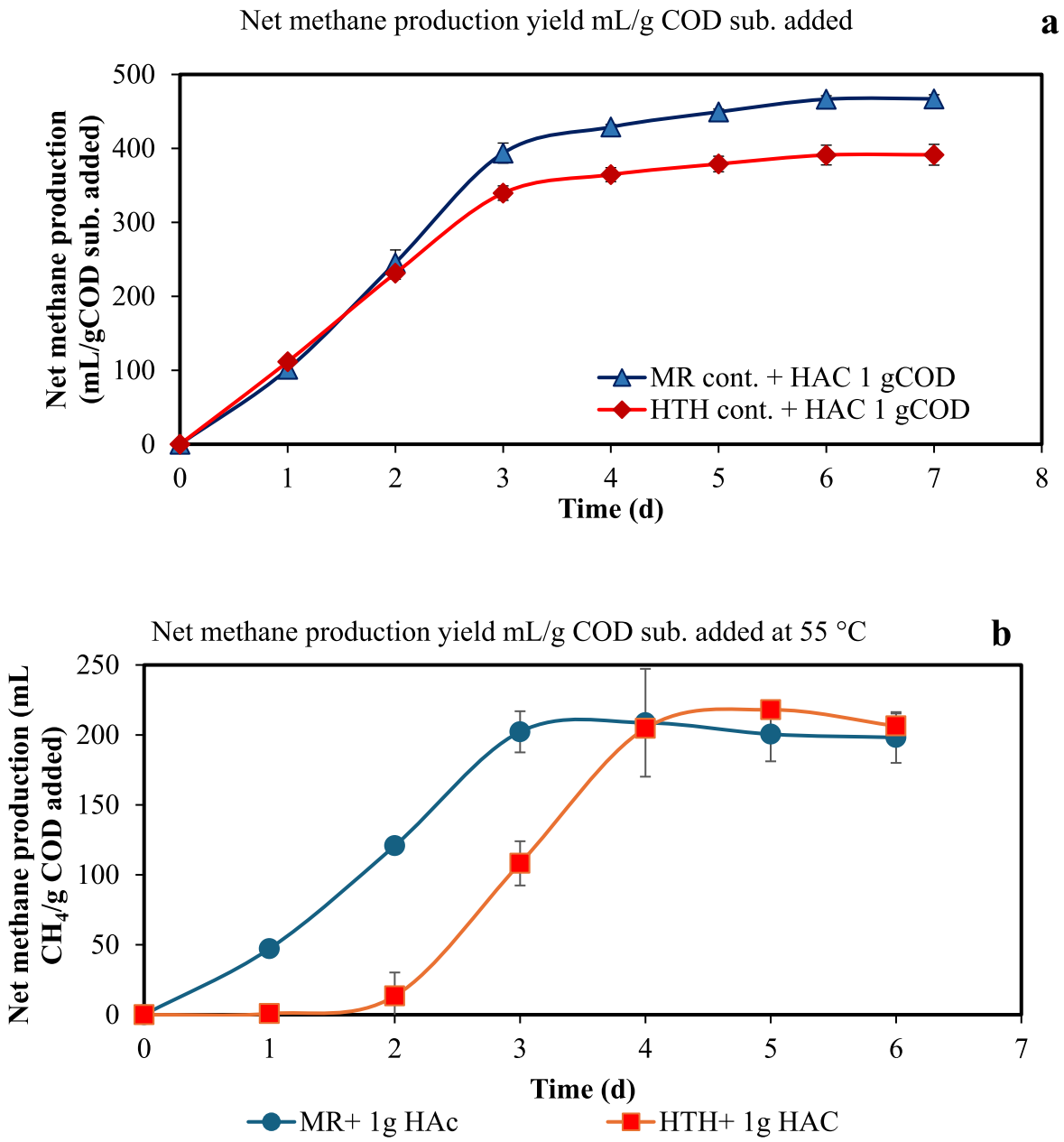

Fig. 3. SMA results for DCM and HTH effluent at (a) mesophilic temperature (37 °C) and (b) thermophilic temperature (55 °C).

Table 5
Biodegradability and Gompertz results from the SMA test of MR and HTH effluent at 37 °C and 55 °C.

Data	Parameters	37 °C		55 °C	
		MR slurry	HTH2 slurry	MR slurry	HTH2 slurry
Experimental results	Methane yield (mL/g COD)	364	367	198	206
	% biodegradability	100	100	57	59
Gompertz data	R (mL CH_4/d)	232	196	108	253
	P (mL)	395	391	222	226
	λ (d)	0.5	0.3	0.6	2.5
	R^2	0.997	0.997	0.98	0.996
	MSMPR (mL CH_4/g VSS.d)	14.6	14.1	13.6	36.5

a lag phase of 2.5 days was observed with HTH2 at 55 °C. The findings of the current study suggest that hyperthermophilic post-treatment of digested cattle manure can be incorporated with recirculation back to the mesophilic digester to enhance manure digestion biogas, and reduce disposed solids, more specifically, when the digester runs at an SRT longer than the lag phase observed in the BMP test of 8.9 days. After 7 d of operation, 20 mL samples were taken for wet and microbial analysis. The reactors were then sealed, purged with nitrogen to stabilize anaerobic conditions, before being placed in a 70 °C water bath connected to an automated methane potential test system for 15 d to investigate the potential of hyperthermophilic digestion of the residual acetate from 55 °C using DCM and HTH2 as inocula. Interestingly, no methane gas was detected. Furthermore, no biogas was produced even after reducing the incubation temperature to 55 °C, clearly confirming that incubation for 15 days at 70 °C has irreversibly inhibited methanogens. This lack of methane is in line with the observation reported with HTH1 and HTH2. Lin et al. [33] investigated hyperthermophilic hydrolysis of pig manure at an initial VS concentration of 77 g/L and TCOD of 100 g/L at an HRT

of 5 days for one year and reported no methane until day 100. Subsequently, from day 200, the methane yield was only 9 mL/g VS, corresponding to a low biodegradability of 1.7 %. Romero-Güiza et al. [16] reported a marginal methane gas of 0.001 L/g VS in a hyperthermophilic hydrolysis process at 3d SRT and 65 °C treating a mixture of sewage sludge and crude glycerine. Microbial analysis was performed on six samples, comprising three from DCM and three from HTH, which were collected after SMA tests at 37 °C, 55 °C, and 70 °C to delineate the shifts in microbial community structure in response to temperature changes. Consequently, the temperature was lowered to 55 °C to attempt thermophilic culture recovery; however, no gas production was observed, indicating a lack of recovery.

3.7. Microbial community analysis

The microbial community analysis was performed to distinguish between microbial variants by analyzing 16S rRNA gene amplicon sequencing reads from Six samples namely, MR effluent, HTH effluent, specific methanogenic activity (SMA) test of MR effluent vs. HTH effluent at 37 °C and 55 °C. A total of 1682 amplicon sequence variants were identified, of which archaea represented a relative abundance of 2.83 % to 28 %, with the rest bacteria (Table S5). Archaea accounted for 4 % and 3 % of the total relative abundance at the Kingdom level in the MR and HTH effluents, respectively, with the remainder primarily consisting of bacteria. It should be emphasized that despite the presence of archaea, which includes methanogens, in the HTH reactor, there was no methane production as presented above. However, relative

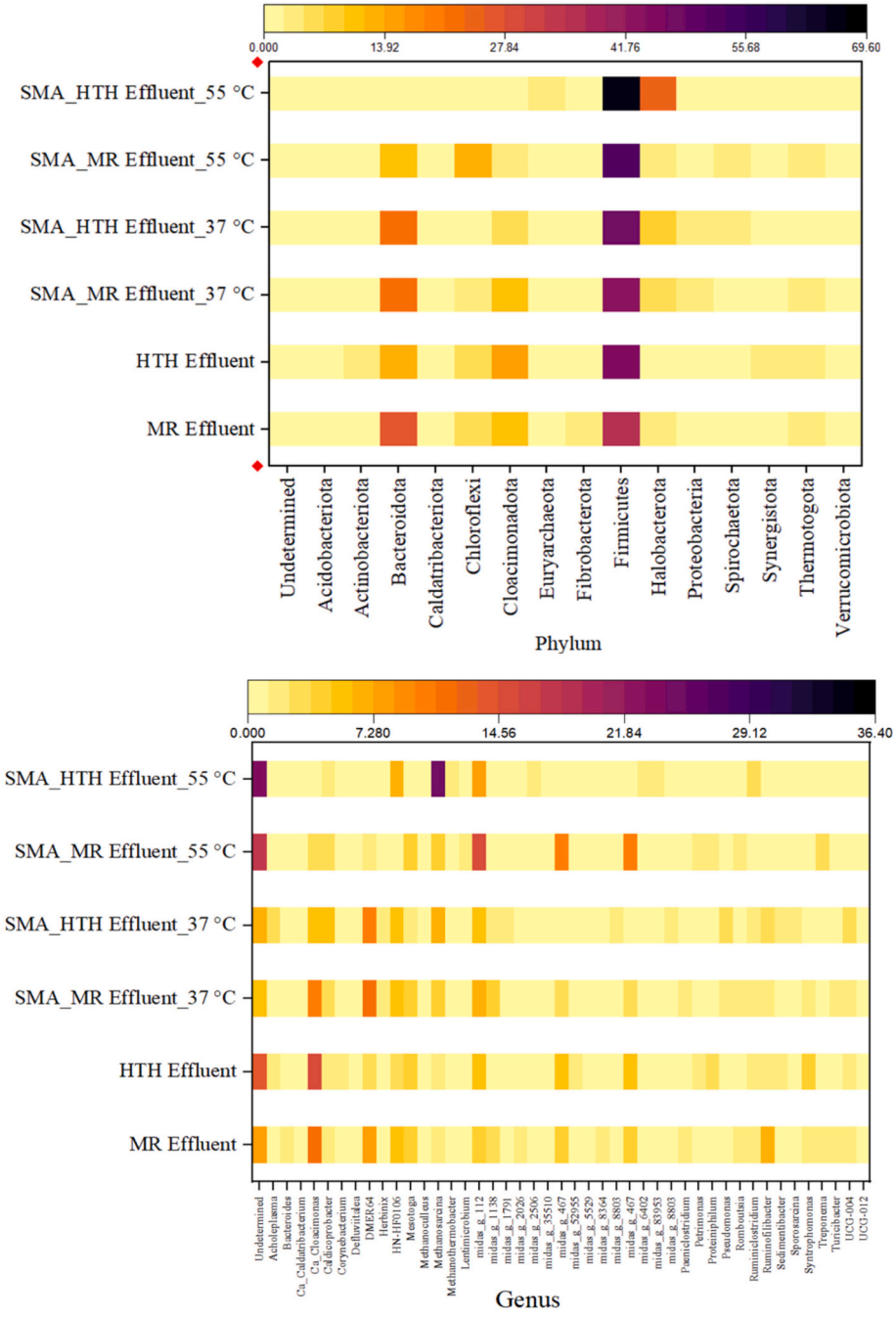

Fig. 4. Relative microbial abundancies using a heatmap at a) phylum level and b) genus level (Color scale on the top of the heatmap allows the identification of the relative abundancy levels for the reported microorganism).

abundance using 16S rRNA sequencing reflects DNA presence, not necessarily viability or activity. In our study, we observed a clear shift in microbial community composition with increasing incubation temperature, where archaeal populations became more predominant from mesophilic to thermophilic [35]. It is also worth mentioning that the observed significant variation in relative abundance of archaea between the MR and HTH effluents on one hand, and the SMA batch tests on the other hand, is closely related to the incubation temperature and availability of acetate, where a direct relation could be built between increase in the incubation temperature (from 37 °C to 55 °C) of the SMA test in the HTH and the increase of the relative abundance of archaea (from 7.83 % at 37 °C to 28 % at 55 °C). Many archaea are thermophiles or hyperthermophiles, meaning they thrive at higher temperatures compared to other microorganisms [36,37]. This trend suggests that higher temperatures selectively favor heat-tolerant archaea. Such dominance can be explained by the inherent thermotolerance and metabolic adaptability of archaea under elevated temperatures, as reported in previous studies [35,38]. While many bacterial and fungal groups exhibit reduced viability or activity at higher temperatures, archaea—particularly thermophilic—are better equipped to thrive, leading to their increased relative abundance in these conditions [38]. The discussion below focuses more on the differences in microbial communities between the MR and the HTH, as these reflect long-term shifts as well as the short-term shifts induced by acetate addition in the SMA tests. Furthermore, this discussion is aimed at relating the functionality of specific microbial groups to observations, rather than addressing microbial diversity and richness.

3.7.1. Comparison of microbial communities in the MR and HTH

Sequences corresponding to major phyla and major genera with relative abundance of greater than 1 % were retained for further analysis, while minor ones were excluded (relative abundance of less than 1 %). At the phylum level, 15 major phyla were identified, while at the genera level, 45 genera were discussed (Tables S6, S7). Dynamic phylum and genus differences between the MR and HTH reactors are discussed.

3.7.1.1. Hydrolyzing microorganisms. The microbial community analysis of the MR and HTH effluents revealed that *Firmicutes* was the most dominant phylum, making up 35.1 % and 45.6 % of the total phylum abundance, respectively (Fig. 4a – Table 6). *Firmicutes* play a fundamental role in anaerobic digestion, particularly in the initial stages of organic matter hydrolysis, which was expressed in its higher relative abundance at HTH effluent (45.6 %) compared to the MR effluent (35.1 %). *Firmicutes* include species known for their cellulolytic activity and are commonly found in bioreactors processing lignocellulosic waste [39]. This finding is supported by the high degree of solubilization in the HTH of 131 mgCOD/g VSS (Table 2) and the LCH solubilization (Table 3).

Chloroflexi, which is known for degradation of halogenated organics to acetate and hydrogen [40], had higher relative abundance in the HTH effluent (6.05 %) vs. MR effluent (4.81 %), which is consistent with the results of previous studies [41], which reported that the abundances of *Chloroflexi* was 16.5 % in thermophilic AD, but only 0.9 % in mesophilic AD. *Fibrobacter* is a key hydrolyzing phylum detected in the MR effluent at a relative abundance of 2.52 %. It has been reported to play an important role in cellulose degradation in the rumen [42,43]. However, its relative abundance decreased significantly in the HTH reactor (0.27 %), likely due to its mesophilic nature, as the hyperthermophilic conditions of the HTH reactor exceed its thermal tolerance. The genus *Caldicoprobacter* is a group of thermophilic, anaerobic, and cellulolytic bacteria known for their role in the hydrolysis and fermentation of lignocellulosic biomass in thermophilic AD systems [13]. The slightly higher relative abundance of *Caldicoprobacter* of 2.2 % in the HTH digester relative to the MR digester of 1.9 % may explain the enhanced removal of LCH as described in Section 3.3 above.

Ruminofilibacter is another hydrolyzing genus found in the MR with a relative abundance of 6.50 %, which is four times the relative abundance at HTH effluent (1.43 %), due to its strict mesophilic environment. This mesophilic anaerobic genus belongs to the phylum *Bacteroidota and* is specialized in the hydrolysis and fermentation of complex plant polysaccharides such as cellulose and hemicellulose, breaking them down into simpler sugars and volatile fatty acids like acetate, propionate, and butyrate (Table 6). Through these processes, *Ruminofilibacter* plays a vital role in the primary degradation of lignocellulosic biomass [44]. Their activity contributes significantly to the conversion of fibrous plant material into bioavailable energy sources in anaerobic digesters.

3.7.1.2. Fermenters. Bacteroidota is the most abundant carbohydrates and proteins fermenting phylum [45] to volatile fatty acids, with a relative abundance of 25.8 % and 12.8 % in the MR and HTH effluents, respectively, reflecting their sensitivity to thermal stress (Fig. 4a and Table 6). *Thermotogota* is another fermentative thermophilic phylum detected in MR (3.88 %) and HTH (3.69 %) effluents. *Thermotogota,* which performs an efficient obligatory sugar oxidation through a syntrophic association with a hydrogenotrophic microbial partner [46]. The relatively similar abundance of *Thermotogota* in both the MR and HTH effluents is noteworthy, given that members of this phylum are typically associated with thermophilic environments. This observation can be primarily attributed to the recirculation of effluent from the HTH reactor to the MR reactor, which likely introduced *Thermotogota* populations adapted to high temperatures into the mesophilic environment. Although *Thermotogota* are not typically active in mesophilic conditions, their genetic material may still be detected through 16S rRNA sequencing, reflecting their presence rather than metabolic activity.

Table 6
Relative abundance and metabolic pathways for key genera of long-term MR vs. HTH experiment.

Kingdom	Phylum	Genus	Relative abundance (%)		Metabolic pathway
			MR	HTH	
Bacteria	*Bacteroidota*	*Bacteroides*	1.55	0.36	Hydrolysis and fermentation
	Firmicutes	*Ruminiclostridium*	2.24	1.55	Hydrolysis and fermentation
	Bacteroidota	*Ruminofilibacter*	6.50	1.43	Hydrolysis and fermentation
	Firmicutes	*Candidatus Caldicoprobacter*	1.9	2.2	Hydrolysis and fermentation
	Proteobacteria	*Pseudomonas*	0.28	0.12	Hydrolysis and fermentation
	Bacteroidota	*Proteiniphilum*	1.05	3.01	Hydrolysis and fermentation
	Spirochaetota	*Treponema*	1.42	0.33	Hydrolysis and fermentation
	Thermotogota	*Mesotoga*	3.88	3.69	Hydrolysis and fermentation
	Bacteroidota	*Lentimicrobium*	0.16	0.06	Fermentation
	Cloacimonadota	*Candidatus Cloacimonas*	11.33	15.70	Syntrophic reactions
	Firmicutes	*DMER64*	7.82	3.22	Syntrophic reactions
	Firmicutes	*Syntrophomonas*	1.76	3.70	Syntrophic reactions
Archaea	*Euryarchaeota*	*Methanosarcina*	2.38	1.78	Mixotrophic methanogenesis
	Euryarchaeota	*Methanoculleus*	0.32	0.00	Hydrogenotrophic methanogenesis

Mixotrophic methanogenic: capable of acetoclastic, hydrogenotrophic, and methylotrophic methanogenesis.

Additionally, the potential for some *Thermotogota* species to exhibit broader temperature tolerance or to survive in inactive states under less favorable conditions [47], may also contribute to their sustained relative abundance in the MR effluent. Thus, the comparable levels of *Thermotogota* across both systems are likely the result of operational recirculation and possibly the resilience or dormancy of thermophilic microorganisms under mesophilic conditions.

The higher relative abundance of *Caldatribacteriota* in the HTH (1.28 %) compared to the MR (0.71 %) can be attributed to the thermophilic or thermotolerant nature of many members within this phylum. *Caldatribacteriota* comprises heterotrophic anaerobic bacteria involved in the fermentation of carbohydrates or fatty acids, which has been associated with thermal environments [48]. The elevated temperature in the HTH reactor likely provided more favorable conditions for their metabolic activity and growth, allowing them to outcompete mesophilic bacteria that are less tolerant to such temperatures.

Proteiniphilum is another fermenter that was expressed in higher relative abundance in the HTH effluent (3.01 %) compared to the MR effluent (1.05 %), which suggests that this genus is well adapted to high-temperature conditions and plays a significant role in the hydrolysis phase of anaerobic digestion (Table 6). *Proteiniphilum* demonstrated the ability to form mono- and disaccharides from complex proteinaceous substrates under extreme thermal conditions [49]. The HTH reactor provides an environment that favors thermotolerant or thermophilic microorganisms capable of accelerating the degradation of substrates into simpler compounds.

3.7.1.3. Syntrophs. The higher relative abundance of *Syntrophomonas,* a key syntrophic fatty acid-oxidizing genus, especially active in degrading butyrate and other long-chain fatty acids [50], in the HTH effluent (3.7 %) compared to the MR effluent (1.76 %) could be attributed to the enhanced fatty acid generation in the HTH reactor (Table 1). This observation is supported by the high degree of solubilization (131 mg COD/g VSS) and acidification (40 mg VFA/g VSS) in the HTH reactor and the higher 40 % VFA concentration than the MR (Table 1). High temperatures can enhance the solubilization and availability of these substrates, possibly supporting higher syntrophic activity.

The genus *DMER64* plays a syntrophic role in anaerobic digestion by oxidizing volatile fatty acids and producing hydrogen or formate, which are then utilized by hydrogenotrophic methanogens via interspecies hydrogen transfer [51]. It was found in both MR effluent (7.82 %) and HTH effluent (3.22 %). The low expression of this genus in the HTH effluent confirms its strict mesophilic properties [52]. It is thus possible that the significantly higher VFA concentrations in the HTH reactor, relative to the MR, resulted in the aforementioned decrease of the relative abundance of *DMER64*. Furthermore, since *DMER64* showed a strong positive correlation with hydrogen-consuming species, indicating a cooperative relationship in methane production [53], the absence of any biogas from the HTH suggests suppression of both hydrogenotrophic and acetoclastic methanogens. *Candidatus Cloacimonas,* which facilitates propionate oxidation [26], was identified in high relative abundance in the MR and HTH effluent, giving 11.33 % and 15.70 %, respectively. Members of the genus *Cloacimonas* have been observed in many mesophilic anaerobic digesters [54], and have been reported to participate in cellulose degradation in anaerobic digesters [55].

3.7.1.4. Methanogens. Methanogenic archaea represented 4 % and 3 % of the total microbial community at the Kingdom level in the MR and HTH effluents, respectively. Notably, although methanogenic archaea were present in the HTH reactor, no methane production was observed, as previously reported at such high temperature. This could be attributed to the methanogenic archaea in the HTH reactor being acclimatized to 75 °C, and their recirculation to the MR enriched, rather than inhibited, these communities.

As presented in Table 6, *Methanosarcina* was detected in both MR and HTH effluents at a comparable relative abundance of 2.38 % and 1.78 %, respectively, thus rationalizing the comparable maximum specific methane production rates observed in the SMA tests at 37 °C (Table 5) and 55 °C (Fig. 3b). *Methanosarcina* is a mixotrophic methanogen capable of acetoclastic, hydrogenotrophic, and methylotrophic methanogenesis [45,56], capable of operating at both mesophilic and thermophilic conditions [57]. This is particularly important as it emphasizes that upon recirculation of the HTH digestate back to the MR, its activity is revived.

Methanoculleus is the only hydrogenotrophic methanogenic archaea that was detected only in the MR effluent (0.32 %). *Methanoculleus* is a thermotolerant to thermophilic genus with many communities thriving at moderate to high temperatures (around 37–55 °C). It is commonly found in anaerobic digesters, particularly those treating manure, sludge, and industrial wastewaters [58].

3.7.2. Short-term changes during SMA experiments induced by acetate addition

Short-term SMA test was conducted using the MR and HTH effluent as inocula using 2.0 g/L of acetate to test their acetoclastic methanogenic activity.

3.7.2.1. Hydrolyzing microorganisms. Microbial community shifts during the short-term SMA test using MR and HTH effluents revealed that *Firmicutes* was the most dominant phylum, accounting for 44.0 %, 47.3 %, 52.8 %, and 65.2 % of the communities, respectively. When comparing the relative abundance of *Firmicutes* in SMA tests at different incubation temperatures, they were higher in the SMA of HTH effluent compared to the SMA of MR effluent at both 37 °C and 55 °C (Fig. 4a – Table 7). The higher abundance of *Firmicutes* in SMA tests using HTH effluent can be attributed to the prior thermal enrichment at 75 °C, which selected heat-tolerant *Firmicutes* and reduced overall community diversity, allowing *Firmicutes* to dominate under thermophilic conditions, potentially contributing to syntrophic pathways rather than direct acetoclastic methanogenesis.

One of the key hydrolytic genera which was detected in the SMA test is *Caldicoprobacter*, at relative abundances of 3.47 %, 5.95 %, 3.10 %, and 2.23 % in the MR and HTH SMA tests at 37 °C and 55 °C, respectively (Table 7). The higher abundance of *Caldicoprobacter* in the HTH at 37 °C (5.95 %) may reflect the carryover of heat-adapted microbes from the original hyperthermophilic environment, which remained active or viable during the SMA incubation.

3.7.2.2. Fermenters. Thermotogota was detected in SMA tests of the MR effluent at 37 °C (3.96 %) and 55 °C (4.35 %) but was inhibited in the SMA tests of the HTH effluent at 37 °C (1.25 %) and 55 °C (0.72 %). *Thermotogota* is a fermentative thermophilic bacterium that performs an efficient obligatory sugar oxidation through a syntrophic association with a hydrogenotrophic microbial partner [46]. Since Thermotogota are fermentative bacteria, their relative abundance declined after acetate addition in the SMA tests of the HTH at all incubation temperatures. This decrease is likely due to acetate overloading, as the HTH initially contained 40 % more VFAs (3360 mg/L) than the MR (2380 mg/L), in addition to the 2000 mg/L of acetate introduced during the SMA test.

3.7.2.3. Syntrophs. The presence of *Cloacimonadota* was notable in the SMA tests using MR and HTH effluents at 37 °C, with relative abundances of 11.0 % and 6.47 %, respectively, but declined significantly at 55 °C to 2.81 % and was undetectable in the HTH-derived inoculum (Table 7). Although *Cloacimonadota* were initially present in the HTH effluent—operated at 75 °C with a short HRT of 2 days—the extended incubation during the 15-d SMA test at 55 °C likely imposed sustained thermal stress that led to their complete disappearance, suggesting possible irreversible thermal inhibition. *DMER64* was found in higher abundance in the mesophilic SMA test of both MR effluent (11.94 %) and

Table 7
Relative abundance and metabolic pathways for key genera of short-term SMA of MR vs. HTH experiment at 37 °C and 55 °C.

Kingdom	Phylum	Genus	Relative abundance (%)				Metabolic pathway
			SMA at 37 °C		SMA at 55 °C		
			MR	HTH	MR	HTH	
Bacteria	*Bacteroidota*	*Bacteroides*	0.02	0	0.08	0	Hydrolysis and fermentation
	Firmicutes	*Ruminiclostridium*	2.04	1.39	0.84	2.46	Hydrolysis and fermentation
	Bacteroidota	*Ruminofilibacter*	1.69	2.43	1.21	0.1	Hydrolysis and fermentation
	Firmicutes	*Candidatus Caldicoprobacter*	3.47	5.95	3.1	2.23	Hydrolysis and fermentation
	Proteobacteria	*Pseudomonas*	2.39	3.36	0.25	0	Hydrolysis and fermentation
	Bacteroidota	*Proteiniphilum*	0.66	1.0	2.31	0.1	Hydrolysis and fermentation
	Spirochaetota	*Treponema*	0.21	0.44	3.4	0.02	Hydrolysis and fermentation
	Thermotogota	*Mesotoga*	3.96	1.25	4.35	0.7	Hydrolysis and fermentation
	Bacteroidota	*Lentimicrobium*	0.18	0.39	1.53	0.69	Fermentation
	Cloacimonadota	*Candidatus Cloacimonas*	10.74	5.45	2.76	0	Syntrophic reaction
	Firmicutes	*DMER64*	11.94	10	1.64	0.02	Syntrophic reaction
	Firmicutes	*Syntrophomonas*	1.69	0.91	0.17	0.33	Syntrophic reaction
Archaea	*Euryarchaeota*	*Methanosarcina*	0.37	0.73	0.26	1.1	Mixotrophic methanogenesis
	Euryarchaeota	*Methanoculleus*	4.03	6.56	3.72	24.35	Hydrogenotrophic methanogenesis
	Euryarchaeota	*Methanothermobacter*	0	0	0	1.81	Hydrogenotrophic methanogenesis

HTH effluent (10 %), than in the MR and HTH (Table 7), but decreased significantly at higher temperatures due to its strict mesophilic nature.

Mesotoga is a mesophilic syntrophic bacterium that oxidizes pentose and hexose sugars from lignocellulosic material hydrolysis and the products (acetate) [59]. These findings are clear from the higher relative abundance of this genus in the SMA of the MR at both 37 °C (3.96 %) and 55 °C (4.35 %), compared to the SMA of the HTH at 37 °C (1.25 %) and 55 °C (0.7 %).

3.7.2.4. Methanogens. Methanosarcina was the major methanogen detected in the SMA tests of MR and HTH effluents at 37 °C and 55 °C (Table 7) at much higher relative abundances than both the MR and HTH. Its highest relative abundance was observed in the SMA test of the HTH effluent at 55 °C (24.4 %), which was 6.5 times higher than its abundance in the SMA test of the MR effluent (3.72 %). The relative abundance of *Methanosarcina* in the SMA test at 37 °C was 6.6 % in the HTH vs 4 % in the MR. The significantly higher relative abundance of the *Methanosarcina* in the SMA of HTH at 55 °C aligns with the highest MSMPR results, which were 2.5 times (36.5 mL CH_4/g VSS.d) higher than the rate of SMA of both MR and HTH at 37 °C and 55 °C (Table 5).

Methanoculleus is another hydrogenotrophic methanogenic archaea which was detected in the SMA test of both MR effluent (0.37 % and 0.26 %) and HTH effluent (0.73 % and 1.1 %) at 37 °C and 55 °C, respectively (Table 7). *Methanoculleus* is thermotolerant to thermophilic methanogens, therefore, its relative abundance was expressed in higher abundances in the SMA of HTH at 37 °C and 55 °C.

3.7.3. Impact of HTH recirculation on microbial community structure and its role in anaerobic digestion stability and efficiency

System stability and efficiency in anaerobic digestion are strongly influenced by the structure, diversity, and functionality of the microbial community. In the current study, the implementation of a HTH recirculation selectively enriched thermotolerant microbial communities, which supported improved degradation of recalcitrant lignocellulosic fractions and overall metabolic synergy. The community analysis showed an increased abundance of *Firmicutes* and *Thermotogota*, both of which are associated with hydrolysis and fermentation at high-temperature conditions. These groups can break down complex organic polymers such as cellulose and lignin into VFAs, which are then converted into methane by syntrophic and methanogenic partners. In addition, recirculation promoted the establishment of functionally robust and complementary communities including thermophilic hydrolyzing communities such as *Caldicoprobacter* (thermophilic and cellulolytic bacteria known for their role in the hydrolysis and fermentation of lignocellulosic biomass) [13] and *Proteiniphilum* (hydrolysis of complex proteinaceous substrates under extreme thermal conditions) [49] which were enriched in the HTH effluent, helping accelerate the breakdown of complex lignocellulose. Syntrophs, especially *Syntrophomonas*, which degrade long-chain fatty acids in cooperation with hydrogenotrophic methanogens, increased in both relative and absolute abundance (up to 3.7 % in HTH vs. 1.76 % in MR), and methanogens, particularly *Methanosarcina* and *Methanoculleus*, appeared in the recirculated system, showing versatility under mesophilic and thermophilic conditions. The 20 % lignin degradation and 10 % increase in biodegradability observed in the MR after HTH recirculation are clear outcomes of these enriched microbial groups. Furthermore, the ability of *Methanosarcina* to perform both acetoclastic and hydrogenotrophic methanogenesis [60,61] makes the system more resilient to organic overloading and higher VFA concentrations.

3.8. Mechanism of enhanced methane production with HTH recirculation

Given the relatively long digestion time at mesophilic conditions of 24–30 days, only refractory organics would persist in the digestate alongside microorganisms. As mentioned above, the lack of soluble nitrogen increase in the HTH reactor suggests the lack of solubilization of proteinaceous material and microorganisms. The recycled soluble COD in the HTH reactor, from the solubilization of LCH, was estimated to contribute only 9 % of the observed increase in methane production, based on the 69 % biodegradability of soluble organics discussed above (Section 3.4). The other potential mechanism involves changing the structure of particulate organics and LCH through lignin cleavage. This key process enhances biodegradability by disrupting the protective lignin matrix, making internal polysaccharides more accessible [62,63]. Fan et al. [59] reported that various pretreatment of lignocellulosic biomass induce substantial structural modifications, notably facilitating lignin removal, increasing cellulose and hemicellulose surface area, and decreasing cellulose crystallinity, which collectively enhance accessibility for subsequent biodegradation. To evaluate this hypothesis, we estimated the first-order degradation coefficient of the particulate COD in the raw cattle manure in the completely-mixed digester using the data without recirculation. Subsequently, assuming that there are no synergistic effects arising from the recirculation of the HTH we determined the corresponding first-order rate for the particulate COD in the recycled HTH. A simple model including the solubilization of PCOD in the HTH reactor, and the biodegradability of the incremental SCOD, as well as the PCOD degradation kinetics for both the CM and the HTH was developed to predict the increase in methane and mesophilic digester particulate COD as a function of recirculation rate. The model accounts for PCOD solubilization in the HTH reactor, the biodegradability of the resulting SCOD, and the degradation kinetics of particulate COD in both the CM

and HTH systems Fig. 5 below shows this relationship. It is evident that the PCOD in the mesophilic digester increases sharply above a recirculation rate of 0.4 L/d and would result in almost no reduction of PCOD in the mesophilic digester compared with the influent. Since wasting is from the mesophilic digester, there would be no reduction in disposed solids. Furthermore, we used the above approach to estimate the biodegradation kinetics of lignin, cellulose, and hemicellulose without the HTH and with HTH recirculation. Table 8 presents the first-order degradation rates for PCOD, lignin, cellulose, and hemicellulose in both control MR and MR with HTH recirculation. The rate of PCOD degradation in the case of control MR without HTH recirculation was 0.01116 d^{-1} while the rate of PCOD degradation with HTH recirculation was 0.0282 d^{-1} confirming the increment of daily methane per day of 1.78 L CH_4/d observed in the current experiment (11.9 LCH_4/d for the control MR without HTH recirculation vs 13.68 LCH_4/d) was likely due to enhancing the biodegradability of particulate organics and LCH through structural changes. Similarly, the degradation rates of lignin, cellulose, and hemicellulose were all higher with HTH recirculation compared to the control MR without HTH recirculation. Specifically, lignin degradation rate increased from 0.0045 d^{-1} to 0.0076 d^{-1}, cellulose from 0.0399 d^{-1} to 0.0414 d^{-1}, and hemicellulose significantly from 0.0414 d^{-1} to 0.235 d^{-1}. These accelerated rates further corroborate the proposed mechanism of structural modification rendering these components more biodegradable.

The aforementioned hypotheses are supported by the BMP data for DCM and HTH2 with ADS (Table 4), which showed that the maximum specific methane production rate of HTH2 was 44 % higher than DCM (5.6 mL CH_4/gVSS/d vs 3.9 mL CH_4/gVSS/d). This confirms the aforementioned hydrolysis rate constant calculations for PCOD of DCM and HTH2 (0.01116 d^{-1} vs 0.0282 d^{-1}) in which HTH process changes PCOD structure.

Additionally, microbial community analysis revealed that the integration of the HTH recirculation resulted in substantial shifts in functional microbial species that aligned with the enhanced lignin degradation and biodegradability observed in the mother reactor. Table S8 compares the relative abundance and bacterial count for the current study with the mesophilic digester only at an SRT of 30 days. These community-level enhancements, particularly the presence and enrichment of lignocellulolytic and syntrophic guilds, provide strong evidence for the improved biochemical conversion efficiency achieved under HTH recirculation.

Notably, the HTH recirculation almost doubled the total microbial cell density in the main reactor from 3.09 × 1012 to 5.02 × 1012 AU/mL, and selectively enriched functional microbial species that support the observed 67 % increase in lignin breakdown and 10 % increase in biodegradability as compared to the MR performance without recirculation in our published article [26]. As apparent from Table S8, the concentrations of most species increased with HTH recirculation, with the exception of *Methanothrix* and *Corynebacterium*. For instance, the relative abundance of *Syntrophomonas*, a key syntrophic fatty acid degrader, reached 1.76 % (1.14 × 1011 AU/mL) whereas it was undetectable in the non-recirculated system. Methanogenic archaea *Methanoculleus*, involved in hydrogenotrophic methanogenesis, also appeared (0.32 %, 2.06 × 1010 AU/mL) only after implementing HTH recirculation. More importantly, the concentration of the high growth rate acetoclastic methanogen, *Methanosarcina,* increased by about 25 %. Hydrolytic genera such as *Ruminiclostridium, Herbinix,* and *Ruminofilibacter* were also enriched exclusively or significantly in the current study, supporting enhanced lignocellulose degradation. Notably, although the relative abundance of Ca. *Cloacimonas*, *DMER64, and HN-HF0106* decreased compared to the non-recirculating system, their absolute cell numbers increased (e.g., Ca. *Cloacimonas*: 7.37 × 1011 AU/mL vs. 6.06 × 1011 AU/mL), suggesting that functional syntrophic interactions remained stable or improved. These community-level enhancements, particularly the presence and enrichment of lignocellulolytic and syntrophic guilds, provide strong evidence for the improved biochemical conversion efficiency achieved under HTH recirculation.

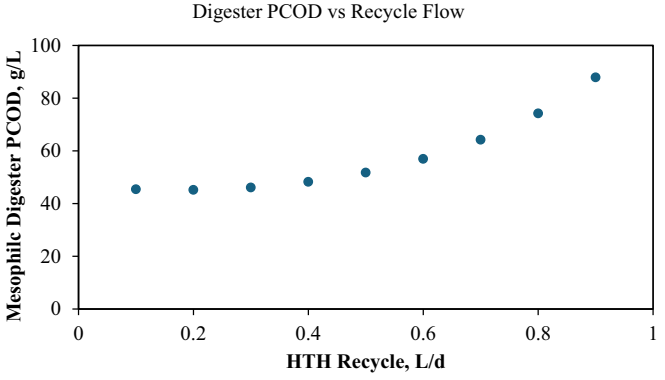

Fig. 5. Correlation between mesophilic digester PCOD and HTH recirculation rate.

Table 8
First-order degradation rates for PCOD, lignin, cellulose, and hemicellulose in both control MR and MR with HTH recirculation.

Parameter	k (d^{-1}) control MR	k (d^{-1}) MR with HTH recirculation
PCOD	0.01116	0.0282
Lignin	0.0045	0.0076
Cellulose	0.0399	0.0414
Hemicellulose	0.0414	0.235

3.9. Energy balance

While the HTH process increased the system's energy demand, preliminary energy balance calculations suggest the strategy can remain energetically favorable when integrated with heat recovery. Table S9 presents the energy balance comparison between the control system (no HTH recirculation) and the system with HTH recirculation for a 100 m^3/d cattle manure flow, using measured methane yields, estimated heat losses, mixing demands, and sludge heating energy based on digester geometry and insulation specifications. Assuming 80 % recovery of the heat required to preheat the sludge from 35 °C to 75 °C, a realistic estimate for industrial systems, the net energy output increased to 9960 kWh/d, surpassing the control's 9656 kWh/d. Although a full techno-economic assessment is outside the scope of this study, our results support the technical feasibility of the approach, especially when scaled to utilize the significant annual volume of cattle manure generated.

4. Conclusions

The outcome of this study demonstrates that hyperthermophilic hydrolysis is an effective approach for enhancing the anerobic digestion of lignin-rich digested cattle manure. Integrating hyperthermophilic hydrolysis with anaerobic digestion significantly improved the biodegradability of lignin-rich cattle manure to 46 % at an SRT of 24.4 d in a continuous flow system compared to an estimated 37 % (corresponding to 42 % at an SRT of 30 d). The hyperthermophilic hydrolysis process, which did not produce biogas, exhibited enhanced solubilization performance at a 2-day SRT compared to a 1-day SRT. Although the SCOD fraction of the recycled HTH accounted for only 9 % of the incremental gas observed by the experimental of 1.76 L CH_4/d, the higher PCOD degradation rate of 0.0282 d^{-1} for HTH compared to 0.01116 d^{-1} for PCOD degradation rate of DCM confirmed that the enhanced methane production rate with HTH recirculation is attributed to rendering particulate organics more readily biodegradable. This was supported by 69 %, 4 %, and 468 % increase in lignin, cellulose, and hemic-cellulose

degradation rates, respectively. SMA testing showed comparable maximum specific methane production rates for HTH2 and DCM at 37 °C, indicating that the inhibition of methanogens in the HTH reactor was reversible. Interestingly, in the 55 °C SMA, the HTH's maximum specific methane production rate was 2.7 times higher than MR's, a result consistent with the microbial community analysis. HTH recirculation to the MR has not only increased the concentration of various microbial groups but also enriched thermophilic, syntrophic, and high-growth rate acetoclastic and hydrogenotrophic communities in the MR and adapted the HTH microbial community to grow at both mesophilic and thermophilic. Although the relative abundance of archaea was higher in the MR effluent than in the HTH effluent, the mesophilic and thermophilic SMA tests showed an opposite pattern, with higher relative abundance in the HTH than the MR. *Methanosarcina* was the predominant methanogen in the MR and HTH, with its relative abundance significantly increasing in the short-term SMA tests at mesophilic and thermophilic conditions, as expected.

CRediT authorship contribution statement

Basem Haroun: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mohamed El-Qelish:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mina Nayebi Shahabi:** Writing – original draft, Methodology, Formal analysis. **Gaia Mazzanti:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Christopher Muller:** Writing – review & editing. **Embrey Bronstad:** Writing – review & editing. **Shubhashini Oza:** Writing – review & editing. **Farokh Kakar:** Writing – review & editing, Conceptualization. **Katherine Y. Bell:** Writing – review & editing, Conceptualization. **Tonia Tommasi:** Writing – review & editing, Funding acquisition. **Francesca Demichelis:** Writing – review & editing, Funding acquisition. **George Nakhla:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2025.167965.

Data availability

Data will be made available on request.

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