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Enhancing biogas production from lignocellulosic digestate through priming and post-digestion

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ABSTRACT

The application of priming, the increase of microbial organic matter turnover by the addition of easily degradable carbon, was investigated for improving biogas production and post-digestion efficiency. Continuously stirred tank main- and post-digesters were operated on manure-based agricultural waste. Potato starch was added as the priming substrate to the post-digester. Addition of 0.2 g VS/L starch every other day increased biogas production from 413 ± 97 to 509 ± 58 mL biogas/day. The increased biogas production exceeds the output from the individual substrates confirming the priming effect. Additionally, the specific biomethane production increased from 74 ± 16 to 116 ± 43 mL/g VS. A shift in microbial community was detected, benefiting lignocellulolytic activities, as degradation of cellulose, hemicellulose and total sugar was enhanced. The results indicate successful application of priming which can improve the overall efficiency of biogas production from manure-based feedstock.

1. Introduction

Anaerobic digestion (AD) offers solutions to many sustainability challenges (Feiz and Ammenberg, 2017) including waste management and valorization (Rene et al., 2023), water management (Angelidaki et al., 2011), renewable energy production in the form of biomethane and nutrient recycling (Feiz and Ammenberg, 2017). The initiative REPowerEU, was launched in 2022 by the European Union (EU), with a goal to scale up the annual biomethane production to 350 TWh by 2030 (European commission, 2022). The initiative serves as an important waypoint for increasing European energy security and achieving net-zero emissions by 2050 (Gas for Climate, 2022). Manure has been pointed out as the largest biomass contribution to reach that goal (covering 32 % of the target) (Gas for Climate, 2022). Manure as substrate in AD processes has several advantages, such as sustainable energy recovery (Feiz and Ammenberg, 2017), reduction of methane emissions from agriculture and animal husbandry (Scheftelowitz and Thrän, 2016), and production of biofertilizer with more bioavailable nitrogen compared to undigested manure (Feiz et al., 2022; Kadam et al., 2024). However, AD processes using manure as the main substrate have shown low degradation efficiencies (23–62 %) of influent organic waste (i.e. volatile solids (VS) reduction) and high residual biomethane potential (Ahlberg-Eliasson et al., 2017; Ekstrand et al., 2022) indicating large potentials in improving processes to increase both biomethane production and reduce greenhouse gas emissions from digestates.

Additionally, manure digestion face process challenges such as low biomethane yields (100–300 mL/g VS) (Ahlberg-Eliasson et al., 2017) and requirements for long retention times (Ahlberg-Eliasson et al., 2017; Ruile et al., 2015). Both challenges can be connected to generally large content of lignocellulose in manure. Lignocellulose degradation in AD is complex as the structure can vary largely due to e.g. plant type and growth stage (Shrestha et al., 2017). Additionally, the three main building blocks; cellulose, hemicellulose and lignin have different molecular structures and mechanisms for degradation. Generally, the structure has hydrophobic properties which makes hydrolytic activity problematic (Marriott et al., 2016).

One way to improve the degradation and biomethane yield from manure is the addition of a well-functioning post-digester (PD) which

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also mitigates the emission of greenhouse gas from the digestate (Ekstrand et al., 2022; Gålfalk et al., 2024). A potential challenge with post-digestion is maintaining high microbial activities due to the relatively low organic load in PDs and harder to degrade organic matter (OM) (Perman et al., 2022). One approach to increase microbial activity, and possibly degradability and gas production, is to induce a priming effect in the PD.

Priming is a phenomenon commonly observed in soil where the availability of easily degradable carbon can increase the microbial activity and improve the degradation of recalcitrant organic matter (Insam and Markt, 2016; Kuzyakov et al., 2000). The effect is observed as an increase in the production of CO_2 or NH_4^+, where the total turnover is larger than the sum of its parts, "1 plus 1 equals more than two" as stated by Insam and Markt (2016). In the context of AD, it was first observed in a study where sewage sludge was digested with small amounts of whey and synergistic effects similar to priming were observed in terms of biogas production (Aichinger et al., 2015). Potential priming mechanisms that have been reported for soils are an increased abundance or activity of the microorganisms (Blagodatskaya and Kuzyakov, 2008), especially groups employing a K-strategist lifestyle, i.e. slow-growing microorganisms that feed on recalcitrant and insoluble substrates (Fontaine et al., 2003). Potential mechanisms for priming are still largely an area of research (Bernard et al., 2022; Tao and Liu, 2025). Priming has been classified as apparent or real (Blagodatskaya and Kuzyakov, 2008). Real priming is an actual increase of the turnover of the hard-to-degrade OM and usually a long-term effect, while apparent priming more short-term and related to the fast turnover of microbial biomass and more easily degradable OM (Blagodatskaya and Kuzyakov, 2008; Kuzyakov, 2010; Lin et al., 2022).

Thus, careful consideration needs to be taken when adding easily degradable material as a priming agent, to avoid enrichment of fast-growing microorganisms (r-strategists) that use up e.g. trace elements and nutrients, outcompete the K-strategists and cause a negative effect in the long run (Blagodatskaya and Kuzyakov, 2008; Fontaine et al., 2003; Insam and Markt, 2016; Zeng et al., 2025).

In AD processes, Ahlberg Eliasson et al. (2023) observed a potential priming effect by digesting potato starch together with manure in the main digester (MD), leading to an increased degradation of fibers, most likely connected to an increase in relative abundance of the cellulose degrader *Ruminiclostridium*. Lin et al. (2022) investigated potential priming effects by co-digesting swine manure with rice straw using apple waste and fructose as priming agents. A priming effect, labelled as an apparent priming effect, was achieved and an enrichment of hydrolytic Bacillota (previously Firmicutes) taxa and increased acetoclastic methanogenesis was observed (Lin et al., 2022). Ekstrand et al. (2022) discussed the potential priming effect in relation to the degradation efficiency of proteins, and Ahmed (2017) combined the priming agent (sugar beet silage) with maize- or grass silage in the MD. These studies showed that there is potential for priming effects in AD systems, especially the mechanistic role of priming.

Previous studies related to AD and priming focused on potential effects in the primary digestion step with different substrates, while corresponding studies during post-digestion are still lacking. The gaps in the current literature on priming in AD systems (such as effects on post-digestion and different concentrations of priming agents) call for further explorations. With this as a background, this study highlights the potential for inducing a priming effect in the PD, using starch as a priming agent, to be able to target residual OM, specifically lignocellulosic feedstocks like manure-based digestates. The potential priming effects were investigated on biomethane production, organic degradation efficiency, macromolecule content and the microbial community in a semi-continuous post-digestion using continuously stirred-tank biogas reactors (CSTBRs).

2. Material and methods

2.1. Experimental setup

Two laboratory-scale CSTBRs (Benchtop biogas reactor Dolly©, Belach Bioteknik, Skogås, Sweden) were operated as a MD and PD system. The PD was the experimental focus and target for priming. The MD was operated at an organic loading rate (OLR) of 2.0 g VS/L/day and a working volume of 6 L, while the PD was operated at an OLR of 0.8–1.0 g VS/L/day of digestate from the MD and a working volume of 4 L. Both digesters were operated at 37 °C. The hydraulic retention time (HRT) was 30 days in both MD and PD. During the experimental period, potato starch (Fisher Chemical™, Starch, potato, extra pure, SLR) was added to the PD feed every second day as the intended priming substrate. This addition pattern was used to determine changes when only digestate was fed to the PD and investigate if there was a higher gas production from digestate alone. Starch was added at 0.05 g VS/L (day 386–400), 0.1 g VS/L (day 401–436), and 0.2 g VS/L (day 437–563) corresponding to roughly 5 %, 10 %, and 20 % of ingoing VS from the digestate (average from the control period). Analytical grade starch was selected to increase control over which nutrients are added to the reactor. Concentration of the priming agent is an important factor for priming as high concentrations can cause enrichment of fast-growing microorganisms (r-strategists) that consumes e.g. trace elements and nutrients, thus causing a negative effect in the long run (Fontaine et al., 2003; Zeng et al., 2025).Therefore the selected initial concentration of the priming agent was a relatively low percentage of ingoing VS of the ordinary digestate as substrate. The starting amount of starch was chosen in relation to a previous study (not published) where starch corresponding to 5 % of ingoing VS was added during post-digestion and a priming effect was determined as increased gas rate and biomethane production for a similar digestate. The final level of 20 % of ingoing VS had previously been used by Ahlberg Eliasson et al. (2023) during main digestion and in this case the priming was observed as an increased degradation of cellulose and hemicellulose as well as an enrichment of cellulose degrader Ruminiclostridium. To double the load in each seemed logical as it would provide clear shifts.

The CSTBRs were operated for a total of 563 days. The MD was inoculated using digestate from a full-scale agricultural biogas plant in Sweden, which has been shown to have a large portion of lignocellulose remaining in the digestate (Ekstrand et al., 2022). The substrate used for the MD was primarily a mixture of manure and agricultural residues (Table 1). The substrate was collected upon one occasion from the same full-scale biogas plant as the inoculum. The substrate was mechanically pre-treated using a food-processer to a particle size of <4 mm to homogenize the material and mitigate the risk of clogged reactor outlets. The substrates were initially stored at −20 °C until weekly feed preparation and then stored at 4 °C.

The PD was started on day 21 of operation of the MD by inoculating with one liter of digestate from the MD. The PD was then fed with MD digestate to the final working volume (4 L) which was reached on day 59. Initially, the PD was operated for a stabilization period of 90 days (3 HRT) during which the process could reach steady-state after the start-up. This period was followed by the control period (day 90 to 314 (>7 HRT)) which provided a baseline to compare the processes before and after priming. Finally, followed by the experimental priming period until day 563.

2.2. Process monitoring

An overview of the process monitoring is compiled in Table 2. The gas production and flow were registered daily by a gas meter working on a liquid displacement method connected to the reactors (Belach Bioteknik, Skogås, Sweden). Gas composition was analyzed using a portable gas analyzer (Biogas 5000, Geotech, Chelmsford, UK) measuring, CH_4, CO_2, O_2, H_2 and H_2S once per week. pH was analyzed twice per week

Table 1
The table shows the substrates fractions used in the main digester (MD) (S1–S6), their total solids (TS) and volatile solids (VS) and the percentage used in the reactor on the basis of VS and on weight basis of the substrate portion of the feed. TS and VS for substrates were measured during several occasions of the experiment and the values presented show the averages and standard deviations of the measurements. Additionally, chemically pure substrates used for the batch tests on post-digestates (before and after starch amendment) and their TS and VS are presented, (starch, cellulose and peptone were measured in the lab, while * and ** indicate that the values used are those provided on the analytical grade chemicals used).

Substrates MD	Substrate classification	TS (%)	VS (% of TS)	% of VS in main reactor	% (weight basis)
Waste grains and shells	S1	84.0 ± 0.1	97.6 ± 0.1	23	3.6
Waste products from grains and pellets	S2	72.7 ± 0.2	91.4 ± 0.3	27	5.0
Waste products from peas	S3	86.7 ± 0.1	95.2 ± 0.2	5	0.8
Solid swine/chicken manure + silage	S4	46.1 ± 0.1	88.2 ± 0.1	14	4.3
Animal fat sludge	S5	3.3 ± 0.1	80.6 ± 0.3	1	2.5
Liquid swine/cow manure + slaughterhouse waste	S6	5.4 ± 0.1	79.7 ± 0.3	30	83.8
Total feed		6.8 ± 0.1	89.0 ± 0.1		
Pure substrates used for batch tests					
Starch		85.0 ± 0.1	99.6 ± 0.2		
Cellulose		97.4 ± 0.1	100.0 ± 0.0		
Oleate*		100	100		
Peptone		93.8 ± 0.1	97.0 ± 0.0		
Sucrose**		100	100		
Glucose**		100	100		
Acetate*		100	100		
Filter paper		96.0 ± 0.1	100 ± 0.0		

Table 2
Overview of the analyzed parameters during the experiments.

Analysis	Measurement interval
Ammonium-Nitrogen	Every other week
Enzymatic activity	Samples collected weekly and the selected to determine possible changes connected to priming
Gas production	Measured every day at feeding
Gas concentration	Measured once per week
Macromolecules	Samples taken at the end of the control period and at the end of the priming phase at 0.2 g VS/L addition
Microbial community	Samples collected weekly and the selected to determine changes connected to priming
Organic matter turn-over efficiency	Performed before priming started and at the end of the priming period.
pH	Measured twice per week
Total solids/volatile solids (TS/VS)	Measured once per week
Volatile Fatty Acids (VFA)	Measured twice per week

with a pH meter (InoLab 7310, WTW, Welheim, Germany) according to the European standard method (EN12176:1998). Volatile fatty acids (VFA) were analyzed twice per week according to Jonsson and Borén (2002) on a gas chromatograph with a flame ionizing detector (Agilent 8860 GC system, Agilent Technologies, Santa Clara, CA, USA). The VFAs quantified were acetic, propionic, butyric, iso-butyric, caproic, iso-caproic, valeric and iso-valeric acid. Once per week, the total solids (TS) and VS were analyzed according to the Swedish standard (SS-EN 12880), with the modification that 10–15 g of digestate was used. Ammonium nitrogen (NH_4^+-N) was analyzed every other week (on a few occasions, every third week) using the Hach LCK 302-kit after proper dilution of digestate. Quantification was done using a spectrophotometer (DR 2800, Hach Lange GmbH, Düsseldorf, Germany).

Samples for analysis of macromolecules were collected three times during the control period (days 238, 258 and 300) and two times during the experimental period (days 528 and 566) from the post digester. TS, VS, Kjeldahl Nitrogen, NH_4^+-N, protein, crude fat, lignin, cellulose, hemicellulose, xylose, mannose, glucose, galactose, arabinose were the components analyzed by Eurofins Environment Testing Sweden AB according to Ekstrand et al. (2022) and MoRE Research Örnsköldsvik AB, Sweden, as described by Perman et al. (2022). The results were then recalculated from the original units to g/kg wet weight (WW) for comparable results. Protein was calculated according to Eq. (1).

$$\text{Protein} = (\text{Kjeldahl} - \text{N} - \text{NH}_4{}^+ - \text{N}) \bullet 6.25 \quad (1)$$

2.3. Batch test for organic matter turn-over efficiency

A batch experiment was performed as previously described by Karlsson et al. (2012) to investigate the microbial activity of the digestate, before and after addition of starch, and to evaluate possible changes in the degradation ability of different types of organic substrates. Digestate from the PD, from control period and at the end of the experimental period, was used as inoculum for the batch experiment. 14 substrates were used, six representing the substrates added to the MD and eight chemically pure substrates (Table 1). The tests were performed in triplicates in pressure-safe glass bottles with a total volume of 330 mL where substrate (2 g VS/L) and inoculum (60 g, TS 2.8 ± 0.1 % and VS 70 ± 0.9 % of TS) were added to the bottles with no additional nutrients added. Inoculum alone and cellulose were used as negative and positive controls, respectively.

The bottles were incubated at 37 °C in a water bath for 63 days, corresponding to a little over the total HRT for the MD and the PD combined. Pressure and gas concentration in the bottles were analyzed on days 1, 2, 4, 7, 11, 21, 37 (for the test after priming this sample point was on day 39) and 63. The pressure was measured using a pressure meter (Testo 312–3 0…6000 hPa, West Chester, United States) and the biomethane concentration was analyzed on a gas chromatograph with a thermal conductivity detector (Agilent 990 MicroGC, Agilent Technologies, Santa Clara, CA, USA). The volume of biomethane was calculated and normalized to standard pressure and temperature (1 atm, 0 °C). Using the modified Gompertz model (Eq. (2)) (Nopharatana et al., 2007) kinetic parameters were calculated.

$$M = P \times \exp\left\{ -\exp\left[\frac{R_m \times e}{P}(\lambda - t)\right] \right\} \quad (2)$$

M is the cumulative biomethane production ($NmL^3 \bullet kg$ VS) at a time (t), P is the biomethane potential of the substrate ($NmL^3 \bullet kg$ VS), R_m the maximum biomethane production rate (NmL^3 $CH_4 \bullet g$ g $VS^{-1} \bullet day^{-1}$), λ t is the lag phase time (day), e the value for exp.(1) (approximately 2.718281), and t the anaerobic digestion time (day). The fit of the modelling was evaluated by the coefficient of determination R^2.

2.4. Calculation of net production of biogas and analysis of gas registration time

To evaluate if a higher gas production was acquired from the digestate after priming, a normalized biogas production was calculated for the experimental phase. For this calculation a practical biogas potential of the starch was calculated using the theoretical biomethane potential (393 mL CH_4/g VS) calculated from the Buswell equation (Buswell and Mueller, 1952). Utilization of the substrates was reported between 74 and 88 % (Ekstrand et al., 2022). The practical potential used in the calculation, 320 mL CH_4/g VS starch, corresponded to utilization of 81 % of the theoretical potential which is similar to what is reported previously (Ahlberg Eliasson et al., 2023). The practical potential was subtracted from the daily biogas production providing the adjusted value named the "Net Biogas production", V_{net}, which can be seen in Eq. (3).

$$V_{net} = V_{biogas} - m_{VS_{Starch}} \times \frac{Y_{CH_4/starch}}{c_{CH_4}} \quad (3)$$

V_{biogas} is the daily amount of produced biogas in mL, $m_{VS_{Starch}}$, the added amount of starch in g VS, $Y_{CH_4/starch}$was the practical biomethane yield of the starch (set to 320 mL CH_4/g VS) and c_{CH_4} was the average biomethane concentration from the weekly measurements from the PD (59 %). This estimate assumes that the full practical potential was produced at the day of addition, where the net biogas and net biomethane production were calculated. Additionally, to evaluate if the starch had any effect on the degradation rate and microbial activity, the time when the gas meter first registered gas production (gas volume > 0) was calculated and compared before and after priming.

2.5. Microbial community analysis

Samples for microbial community analysis were collected in triplicates on a weekly basis as whole digestate and saved at −20 °C until DNA extraction according to MiDAS (2016). To avoid possible contaminations the samples were transferred from the sampling bottle to sterile, free from DNA/DNase/RNase 15 mL polypropylene test tubes (article no. 62.554.502, Sarstedt) immediately after sampling. Samples were selected from the inoculum of the MD (day 0), at the end of control period (day 297, 304, 311 and 317) and experimental period (day 437, 521, 528, 535 and 542) from the PD.

Extraction was done using aliquots of 200 µL using the FastDNA Spin Kit for Soil (MP Biomedicals Europe) according to manufacturer's instructions. An additional washing step with guanidine thiocyanate was included for humic acid removal, as previously described by Danielsson et al. (2017). 70 µL of DNase-free water was used for the elution of the DNA. Qubit 3.0 Fluorometer with a Qubit dsDNA BR Assay Kit (Invitrogen, Thermo Fisher Science, Waltham, MA, USA) was used to measure DNA concentrations. 16S rRNA-gene amplicon libraries for bacteria and archaea of the V4 region (515F- 806R) were prepared for Illumina sequencing from the DNA samples as described by Perman et al. (2022). Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Science, Waltham, MA, USA) was used to measure the final PCR product and 20 ng of DNA from each sample were submitted for sequencing on the Illumina platform NovaSeq 6000, carried out by Novogene (Cambridge, UK). For the PD samples, complementary preparation of 16s rRNA-gene amplicon libraries for bacteria and archaea of the V4 region (515F-806R) was performed by Novogene (Cambridge, UK) as the initial sequencing gave results with low reads.

16S rRNA-gene sequences were processed according to the DADA2 pipeline tutorial (version 1.16) (Callahan et al., 2016), using the DADA2 package (version 1.20.0) in R (version 4.1.1). Primer and adapter sequences were removed from the raw data using Cutadapt (Martin, 2011). Sequences were trimmed at base number 140 and 200 for forward and reverse reads, respectively. The PD samples were trimmed at base number 175. The maximum number of expected errors was set to 1. Taxonomy was assigned to the sequences using the Silva reference database training set (version 138.1) (Quast et al., 2013).The package phyloseq (version 1.36.0) was used to organize the data and visualize relative abundances of the sequences (Mcmurdie and Holmes, 2013). Samples with <8000 reads were filtered out before the visualization. To analyze β-diversity in the PD, before and after priming, unweighted and weighted principal co-ordinate analysis (PCoA) was used similar to Perman et al. (2024).

2.6. Enzymatic activity

Samples for extracellular enzymatic activity analysis (cellulase and amylase) were saved weekly. Digestate was centrifuged at 10000 RCF for 10 min (20 °C) in sterile 50 mL polypropylene test tubes (article no. 62.547.254, Sarstedt) and supernatant was saved at −20 °C for further analysis. Samples were analyzed for the control period (day 287, 294, 301, 315), just before priming started (day 385), and at the end of the priming period (day 518, 525, 532, 548, 553, 560) to capture relative changes in enzymatic activity before and after priming. The frozen samples were thawed and centrifuged for 20 min at 7200 RCF and 4 °C. 1 mL aliquots of supernatant were transferred to microcentrifuge tubes and stored at −20 °C. Before analysis, an additional centrifugation step of 10 min at 16000 RCF (20 °C) was performed. Cellulase and amylase activity assays were both performed using a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany) with samples in Corning 3880, 96 well, half area microtiter plates (Corning Inc. NY, USA).

Cellulase activity was assayed in duplicates using resurofin cellobioside (ab189817, Abcam, UK) in accordance with manufacturer's protocol, except for that samples (50 µL) were not diluted with reaction buffer prior to assay. Fluorescence emission was measured at room temperature for 4 h, with excitation at 545 nm and emission at 600 nm.

The amylase activity was assayed in the same manner, but with an amylase activity assay kit (Invitrogen product nr. E33651, Thermo Fisher Scientific Inc.) in accordance with manufacturer's protocol, with excitation at 477 nm and emission at 525 nm.

As a measure of relative enzymatic activity, the ΔRFU (Relative Fluorescence Units) over the 4 h timespan was calculated for each background subtracted sample. Additionally, the ratio between PD and MD was calculated and presented as percentage of activity in the PD compared to MD.

2.7. Statistical evaluation

A paired two-sided *t*-test ($p < 0.05$) was used for comparing the means of the control and experimental periods and for the comparisons between MD and PD assuming normal-distribution of the data. Normal-distribution was verified using a quantile-quantile plot. To determine the priming effect the net daily biogas production during the experimental period was compared to the daily biogas production during the control period. Potential outliers due to errors with gas meters were removed before the analysis. For the process parameters the test was made, comparing the means of the weekly measurements from the control and experimental periods. For the statistical analysis Microsoft® Excel for Microsoft 365 MSO was used as well as Python in Google Colab for the calculations of the data for the gas production and determination of priming effect.

3. Results & discussion

3.1. Effect of starch priming on gas production

A stable process performance in the MD was important for the evaluation of potential priming effects in the PD. Instabilities, such as VFA accumulation, in the main process could create a post-digestion process with fluctuations in e.g. gas production, as reported by

Axelsson Bjerg et al. (2024). After an initial process disturbance during start-up of the reactor (day 23 to day 30), where minor VFA accumulation (1–5 mM) occurred, the process was stable running on 2 g VS/L*day. The gas production in the MD was stable throughout the experiment (see Supplementary Material). Specific biomethane production in the MD was 315 ± 17 mL CH_4/g VS, which corresponded to a daily biogas production of 6683 ± 638 mL and a daily biomethane production of 3783 ± 358 mL CH_4.

The specific biomethane production in the PD during the control period was 74 ± 16 mL/g VS*day corresponding to a daily average biogas production of 413 ± 97 mL (Fig. 1A–B). The 0.05 g VS/L and 0.1 g VS/L additions showed no significant difference with an average net biogas production (gas production from starch subtracted) of 340 ± 56 and 404 ± 69 mL/day, respectively. These levels were only assessed for 14 days and 35 days so it is possible that if they had been running for longer clearer results could have been seen. The third period of 0.20 g VS/L starch addition showed a significant increase in specific biomethane production (*t*-test, $p < 0.05$) by 56 ± 58 % up to 116 ± 43 g VS/L*day, with a biomethane concentration of 57 ± 2 %.

The net biogas production also increased significantly by 23 ± 14 % to an average of 509 ± 58 mL/day. Additionally, on the days where no starch was added, the daily biogas production increased significantly (*t*-test, $p < 0.05$) with 27 ± 14 %, to an average of 526 ± 57 mL/day, compared to the control period. It could be argued that this increased

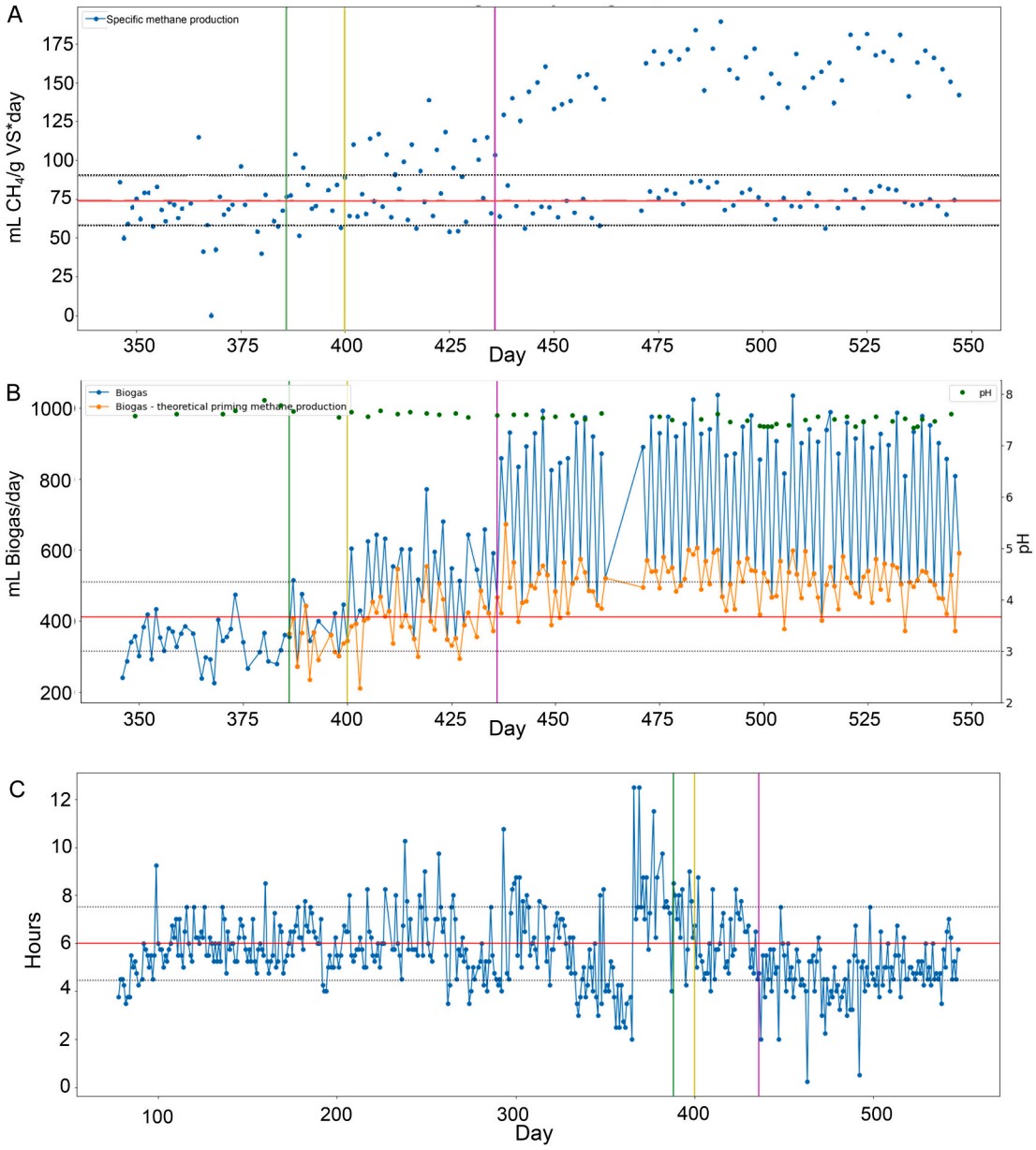

Fig. 1. The figure presents A) A zoom in of the daily specific gas production during the experimental phase, B) the corrected biogas production (orange) where biogas production from the starch addition is subtracted and the total biogas production (blue) as well as pH during the experimental phase (green dots). The lines show the connection between the corrected biogas production and the daily biogas production. C) The time for the first registration of gas in the PD during the control period day 90–314 and experimental period until day 550. In all graphs the green line shows the start of the priming period (0.05 g VS/L), yellow the start of 0.10 g VS/L starch-addition and purple the start of 0.20 g VS/L addition. The red line shows the mean values for each parameter during the control period and the black horizontal lines show the standard deviation during the control period. The specific methane production increased significantly during starch addition (A), as did the net biogas production (B), and the time it took for the gas to register reduced implying increased microbial activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

gas production could partially be a result of the degradation of starch from previous days. However, the average net biogas production from days when priming occurred was 483 ± 57 mL/day, which is significantly higher than then the control period (413 ± 97 mL/day), indicating that a majority of the starch was degraded on the day of starch addition. Starch addition contributed to a decrease in the standard deviation of net daily biogas production from ±97 mL/day to ±59 mL/day, implying a decrease in temporal variations of biogas production. Generally, the variability in biogas and biomethane production in AD systems originates from the inhomogeneity of the substrate, such as grain and straw in the case of agricultural digesters. It is later carried over when the PD operates using the effluent from the MD. Despite careful considerations to ensure equal daily organic loading rates, a certain degree of variability is unavoidable. Additionally, the starch addition every other day contributes to increase the variability in biogas and biomethane production in the PD.

A previous study reported increased transformation efficiencies from manure to CH_4 by 39 ± 13 % and 65 ± 14 % respectively when apple waste and fructose were used as priming substrates in a MD (Lin et al., 2022). Additionally, the biogas yield increased by 44 % when sugar beet silage (14 % of VS) was combined with grass silage in another study (Ahmed, 2017). Both studies reported considerably higher increases compared to what was detected in the present study. Though, but they were also performed in the MD where the organic load was considerably higher and a short term apparent priming effect could be targeting more easily degradable OM (Fontaine et al., 2003).

The time when gas registration was observed decreased significantly (22 %), from 6.0 ± 1.5 to 4.7 ± 1.2 h (Fig. 1C), when starch was added at 0.20 g VS/L. The addition of easily degraded starch affected time until gas registration both on days with and without starch addition (4.6 ± 1.0 and 4.8 ± 1.2 h, respectively). During the first levels of starch addition the time to register gas production was 6.9 ± 1.6 and 5.9 ± 1.2 h, respectively, revealing no significant difference compared to the control period. Ahlberg Eliasson et al. (2023) showed that when cattle manure was co-digested with starch the time needed to reach the same biomethane potential was reduced from 25 to 11 days (56 % decrease). A faster gas production is a desirable goal as it can shorten the residence time in the reactor, specifically relevant for manure substrates where the high-water content make it challenging to keep up both HRT and OLR (Ahlberg-Eliasson et al., 2017).

3.2. *Effect of starch priming on process stability and residual organic matter*

Both the MD and the PD were stable during control and experimental periods of reactor operation, with low or no VFAs (<1 mM) and pH of around 7.4–7.7 (Table 3). The material fed to the PD was relatively recalcitrant (Ekstrand et al., 2022), therefore addition of easily degradable starch could potentially cause a disturbance in the system, especially if inhibitory compounds, such as VFAs, were formed (Kuzyakov et al., 2000). However, both the pH and VFA concentrations remained stable throughout both the control and experimental periods, and the starch was added at levels that posed no risk of overloading the PD. Digestate from the full-scale biogas plant from which the substrate was collected had pH values between 7.7 and 8.4 and periods of VFA accumulation up to 50 mM (Axelsson Bjerg et al., 2024; Ekstrand et al., 2022). The process reproduced in the lab had a lower OLR compared to the full-scale plant, contributing to increasing its overall stability.

The potential effects on OM could give indications on where the extra biogas and biomethane from the priming effects originates from. TS and VS remained stable at around 3.3 ± 0.2 to 3.3 ± 0.3 % TS and 73.9 ± 0.9 to 74.5 ± 1.5 % VS (of TS) for the MD and around 2.7 ± 0.1 to 2.8 ± 0.1 % TS and 69.9 ± 0.7 to 70.1 ± 1 % VS (of TS) for the PD (Table 3). There was no significant difference between the experimental and the control periods for TS and VS (*t*-test, $p < 0.05$). No significant changes in VS-reduction were detected during the experimental period (23 ± 8 % VS reduction at 0.2 g VS/L starch addition) in comparison to the control period (21 ± 6 %). Similar level of starch addition (20 % of on VS basis) to cattle manure in a MD promoted an increase of VS reduction to 38.8 ± 0.3 % compared to 27.9 ± 0.7 % in the control digester (mono-digestion of cattle manure) (Ahlberg Eliasson et al., 2023).

The increase in gas production observed during the experimental period (0.2 g VS/L addition) indicated an enhanced degradation of residual substrate in the digestate, even though no significant changes in VS reduction were observed A possible reason for this could be that the enhanced degradation of OM occurred along with an increase in microbial mass as an anticipated effect of priming (Kuzyakov et al., 2000), especially since both microbial biomass and residual OM are represented in the measurement of VS (Ekstrand et al., 2022).

NH_4^+-N in the PD did not reveal any significant changes due to priming compared to the control period. Though, NH_4^+-N was significantly higher (15 ± 7 %) in the PD throughout the study (Table 3), indicating that the addition of the PD lead to a higher mineralization of nitrogen. This is in line with previous results from Perman et al. (2022) where the addition of several digestion steps showed an increased protein mineralization. The priming did not reveal any significant effect on the NH_4^+-N in the PD compared to the control period.

The PD did not show a significant difference in the residual protein content before and after priming (Fig. 2A), which makes sense, as the total protein is calculated from Eq. (1) which includes Kjeldahl-nitrogen and NH_4^+-N. However, the protein content decreased by 32 ± 9 % from the MD to the PD, from 10 ± 2 g/kg wet weight (WW) to 7.2 ± 0.9 g/kg WW and 6.9 ± 0.9 g/kg WW in PD during the control- and the experimental period respectively (see Supplementary Material) confirming the importance of a PD for improved protein degradation. Additionally, the measured protein content includes proteins in the form of microbial biomass, meaning that even if an improved protein degradation occurred, it might not be detected if the microbial biomass increased simultaneously. It has been previously reported that remaining proteins in digesters could be connected to fractions of microbial biomass which is not targeted (Ekstrand et al., 2022). Similarly to protein, crude fat and lignin did not show any significant changes before and after priming (Fig. 2A).

However, a clear percental reduction of cellulose (28 %), hemicellulose (25 %), and total sugars (22 %) (Fig. 2A–B) was seen in the PD after priming, supporting that a priming effect occurred and that starch priming mainly targets residual carbohydrates. Similar findings were

Table 3
Overview of process parameters of the main digester (MD) and the post-digester (PD). -C presenting the averages and standard deviation of the measurements from the control period (days 90–314), while -Exp shows the averages and standard deviation of the measurements results from the experimental period (days 386 to 563). The process was stable during the experiment and no significant changes occurred for the monitoring parameters due to starch addition.

	MD-C	MD-Exp	PD-C	PD-Exp
TS (%)	3.3 ± 0.3	3.3 ± 0.2	2.7 ± 0.1	2.8 ± 0.1
VS (% of TS)	74.5 ± 1.5	73.9 ± 0.9	69.9 ± 0.7	70.1 ± 1.4
pH	7.6 ± 0.1	7.6 ± 0.1	7.6 ± 0.1	7.5 ± 0.1
VFA (mM)	<1	<1	<1	<1
NH4 + -N (mg/L)	1370 ± 60	1370 ± 60	1580 ± 70	1560 ± 120

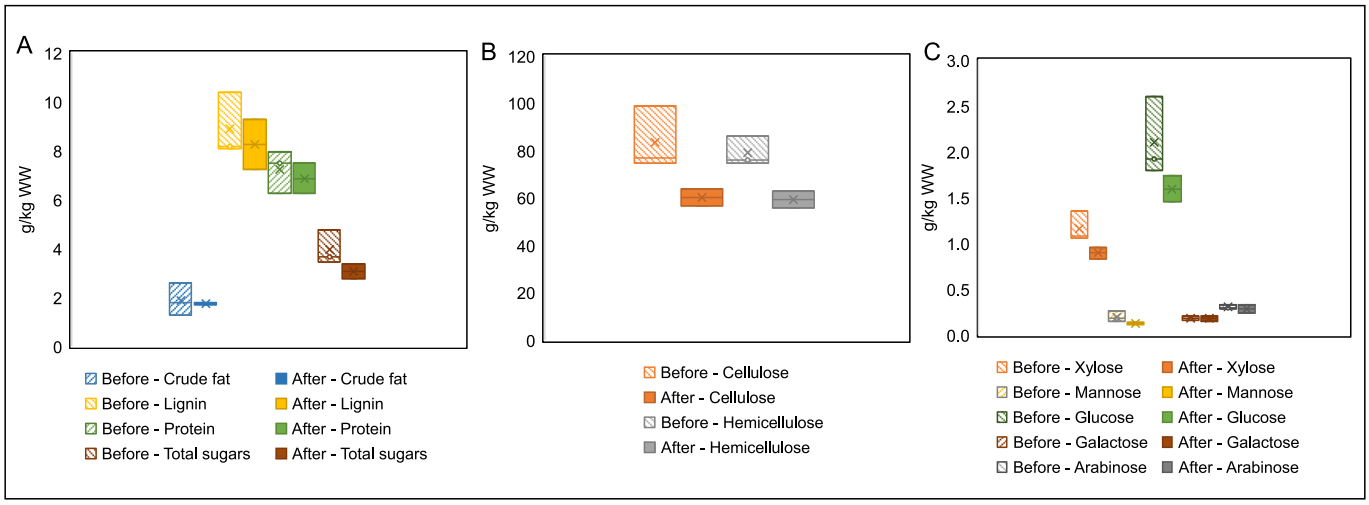

Fig. 2. An overview of the concentration of A) macromolecules, B) sugars and C) hemicellulose and cellulose before and after priming in the PD. The results showed the data samples collected on days 238, 258 and 300 for the control period and the days 528 and 566 from the experimental period. The box plots have been created using measured values from control and experimental period. The × in the box shows the average value of the measurements, the line shows the median of the measurements, and the lower and upper part of the box shows the 25th and 75th percentile of the samples, respectively. A significant decrease was observed for total sugars, more specifically for xylose and glucose. Additionally, the concentrations of cellulose and hemicellulose decreased after priming.

observed by Ahlberg Eliasson et al. (2023) when cow-manure was co-digested with starch at similar ratios as this study (20 % of VS), which resulted in a higher degradation of cellulose and hemicellulose. For specific sugars (Fig. 2C), a significant change could be observed for glucose and xylose. Apparent priming effect is commonly related to increased degradation of more easily degradable OM (Kuzyakov, 2010; Lin et al., 2022) which is something that can be connected to a decrease in the concentration of total sugars after priming. Lin et al. (2022) attributed the witnessed priming effect to apparent priming when using fructose and apple waste as a priming substrate. In the present study, both more complex structures and easily degraded structures were targeted, showing indications of both apparent and real priming effects.

3.3. *Effects on organic matter turn-over efficiency in batch due to starch priming*

Effects on microbial organic matter turn-over efficiency due to starch priming were further evaluated in a batch test using digestate from the PD before and after priming as inoculum. Biomethane production and rates from substrate fractions fed to MD and more chemically pure substrates (Table 1), before and after priming, were analyzed (Table 4). S4 (mix of solid manure and silage) and filter paper were the only ones that showed a significant difference (*t*-test, $p < 0.05$) after priming. S4 had a higher production (255 ± 61 mL CH_4/g VS before priming and 366 ± 94 mL CH_4/g VS after priming) while filter paper had a lower production (484 ± 3 and 350 ± 29 mL CH_4/g VS, respectively).

Despite large variations between replicates, a higher average biomethane production was measured for some of the substrates, specifically S1-S4, cellulose, oleate and sucrose (Table 4 and Supplementary Material). S1–S4 all contained large fractions of carbohydrates or lignocellulosic materials which were in line with what was seen for the degradation of macromolecules. In contrast, S5–S6 (fat sludge, manure, and slaughterhouse waste), starch, acetate, glucose, and filter paper seemed to be negatively affected by the priming. The negative effect on starch after priming was unexpected, as this was the substrate used as priming agent. However, the load of starch added in the batch test was considerably higher compared to what was fed to the PD (2 g VS/L compared to 0.2 g VS/L), which could have contributed to this unexpected result. The biomethane production from starch after priming (320 ± 21 NmL CH_4/g VS) were similar with results from Ahlberg Eliasson et al. (2023) of 321 ± 7 NmL CH_4/g VS.

In the present study, the biomethane production before addition (381 ± 75 NmL CH_4/g VS) showed a larger variation that could mask potential negative effects. Glucose showed lower gas production after priming in the batch. However, in the analysis of the macromolecules in the continuous system a higher degradation after priming was observed. Before priming, glucose addition led to a production of around 500 NmL CH_4/g VS in the batch test, which is higher than usually reported for glucose in BMP tests (Raposo et al., 2012). This could potentially be a short-term priming effect which was not as prominent after the experimental priming period had occurred.

Biomethane potential curves calculated from fitting of the Gompertz-model showed that for many of the applied substrates, the lag-time in biomethane formation was close to zero (Table 4), indicating that there were no immediate inhibitory effects. S3, Oleate, Filter paper and Cellulose were the only substrate fractions with $\lambda > 0$. Oleate had the longest lag-time of 2.3 ± 1.2 days before priming and 4.0 ± 0.4 days after priming. Biomethane production rate (R_m) increased for the majority of the investigated substrates after priming (except for S3 substrate; Table 4), the highest increase could be seen for S4 (105 %).

3.4. *Starch priming's effect on microbial community structure and enzyme activity*

A potential effect of priming is shifts in the microbial community (Ahlberg Eliasson et al., 2023; Blagodatskaya and Kuzyakov, 2008; Lin et al., 2022). The microbial community in the digesters was investigated by targeting the 16S rRNA-gene in samples taken from the PD both before, during and after starch priming (Fig. 3A), as well as from the MD during the same time-periods (see Supplementary Material).

At the start of the control period (day 297), a similar community structure, especially on phylum level, was observed in both the MD and PD (see Supplementary Material and Fig. 3A), with dominance of the phyla Bacillota and Bacteroidota, as well as Cloacimonadota and Euryarchaeota. The sequencing of the samples from the MD varied in terms of sequencing depth, making a comparison between MD and PD somewhat limited. Still, some differences could be observed between the reactors, such as higher relative abundance (RA) of genera *Candidatus* Cloacimonas (phylum Cloacimonadota) and *DMER64* (phylum Bacteroidota) in MD compared with PD. These two genera also exhibited changes in abundance, with Ca. Cloacimonas showing higher levels during the control period (RA up to 27 %). However, after day 388, the

Table 4
The final biomethane production (mL CH_4/g VS) and the standard deviation of the measurement. P (biomethane potential) shows the values from the Gompertz modelling, R_m is the maximum rate according to the modelling λ shows the lag time in days. The R-squared indicating the fit of the model is also shown. S1-S6 are the substrate fractions used in the main digester (MD).

	Measured methane potential		P (Biomethane potential)			R_m (Rate)			λ (lag time)			R-squared	
	Before	After	Before	After	Percentual change	Before	After	Percentual change	Before	After	Percentual change	Before	After
S1	286 ± 68	371 ± 92	274 ± 18	334 ± 17	22 %	9.9 ± 1.8	15.5 ± 2.6	57 %	−1.3 ± 1.8	−0.5 ± 1.3	−60 %	0.97	0.98
S2	264 ± 43	354 ± 106	263 ± 16	335 ± 16	28 %	6.6 ± 0.9	12.8 ± 1.8	92 %	−3.2 ± 1.8	−1.4 ± 1.4	−59 %	0.98	0.98
S3	290 ± 112	461 ± 103	280 ± 14	440 ± 23	57 %	15.6 ± 2.6	15.5 ± 2.1	−1 %	0.4 ± 1.1	0.5 ± 1.4	24 %	0.98	0.99
S4	255 ± 61	366 ± 94	260 ± 15	350 ± 15	35 %	5.9 ± 0.7	12.1 ± 1.4	105 %	−5.8 ± 1.8	2.5 ± 1.2	−57 %	0.99	0.99
S5	752 ± 184	680 ± 151	708 ± 61	632 ± 30	−11 %	21.6 ± 5.0	31.2 ± 5.2	44 %	−4.3 ± 29	−1.9 ± 1.4	−56 %	0.95	0.98
S6	462 ± 56	382 ± 129	547 ± 54	386 ± 19	−29 %	8.3 ± 0.7	9.3 ± 0.8	12 %	−2.9 ± 1.6	−2.2 ± 1.3	−25 %	0.99	0.99
Cellulose	365 ± 142	472 ± 220	355 ± 38	457 ± 20	29 %	9.8 ± 2.3	18.6 ± 2.4	89 %	−1.2 ± 2.9	0.7 ± 1.1	−158 %	0.95	0.99
Starch	381 ± 75	320 ± 21	368 ± 26	331 ± 12	−10 %	10.5 ± 2.0	16.1 ± 2.3	53 %	−6.7 ± 2.7	−4.1 ± 1.3	−39 %	0.97	0.98
Acetate	492 ± 17	465 ± 44	563 ± 49	463 ± 13	−18 %	7.7 ± 0.8	13.4 ± 1.1	74 %	−5.7 ± 2.6	−7.5 ± 1.2	−52 %	0.99	0.99
Peptone	397 ± 68	401 ± 97	404 ± 37	391 ± 16	−3 %	8.2 ± 1.5	13.3 ± 1.7	63 %	−0.6 ± 3.6	−5.1 ± 1.5	−51 %	0.97	0.99
Oleate	961 ± 151	1041 ± 37	929 ± 45	1018 ± 16	10 %	34.7 ± 4.4	49.0 ± 2.5	41 %	2.3 ± 1.2	4.0 ± 0.4	79 %	0.99	0.99
Sucrose	371 ± 73	432 ± 83	351 ± 27	417 ± 16	19 %	11.9 ± 2.6	18.8 ± 2.4	58 %	−3.4 ± 2.4	−1.4 ± 1.1	−58 %	0.96	0.99
Glucose	503 ± 79	337 ± 21	389 ± 31	322 ± 14	−17 %	10.8 ± 2.2	12.0 ± 1.6	12 %	−5.3 ± 2.7	−3.6 ± 1.4	−32 %	0.96	0.99
Filter paper	484 ± 3	350 ± 29	451 ± 36	342 ± 11	−24 %	17.0 ± 3.7	19.5 ± 2.2	15 %	0.8 ± 2.1	1.8 ± 0.8	130 %	0.96	0.99

RA of this genus dropped to <1 %, while *DMER64* increased, over time reaching a RA of 65 %. No alterations in process operation or performance occurred during this period, making the shift unexpected. Both *DMER64* and Ca. Cloacimonas have previously been linked to similar functions, i.e. degradation of lipids and VFAs (i.e. propionate and butyrate) (Lee et al., 2019; Pelletier et al., 2008; Shakeri Yekta et al., 2019).

The genus *Clostridium* sensu stricto *1* (phylum Bacillota) and family *Prolixibacteraceae* (phylum Bacteroidota), were the most abundant groups in the PD and both groups have been linked to degradation of substrates such as sugars and amino acids (Huang et al., 2014; Lawson and Rainey, 2016; Sun et al., 2020). The higher RA of potential VFA degraders in the MD compared with the PD might be attributed to a higher OLR and level of easily degradable OM, likely resulting in relatively high VFA production. During the period of starch addition, an increase in RA of *DMER64* was observed also in the PD. This could be due to the increased sugar load during priming, accelerating VFA turnover, or linked to the high RA of *DMER64* in the MD. In either case, *DMER64* likely helped maintain low VFA concentrations in the PD, preventing acid accumulation during the priming phase.

As illustrated by PCoA plots (Fig. 3B), changes in the microbial community structure were observed from the start of the priming-phase (from day 437). Especially the weighted PCoA showed clear differences between the samples taken before and after priming respectively, indicating that starch addition influenced the microbial population. An increase in RA correlating with the priming phase was observed for the families *Prolixibacteraceae* (RA 9–16 % before and 11–26 % after priming) and *Anaerolineaceae* (phylum Chloroflexi) (RA <1 % before and 1–5 % after priming at 0.2 g VS/L), as well as genera *DMER64* (RA 4–9 % before and 8–20 % after priming) and *Christensenellaceae R-7 group* (phylum Bacillota) (RA 3–6 % before and 4–12 % after priming). The genus *Clostridium* sensu stricto *1* instead showed a decreasing trend after priming (RA 27–36 % before and 18–26 % after priming), though still remaining a dominant group.

The improved degradation of cellulose and hemicellulose observed suggests that the activity of cellulolytic bacteria was boosted during this period. Well-known clostridial cellulose degraders such as *Hungateiclostridiaceae*, *Ruminiclostridium* and *Herbinix* (Koeck et al., 2015; Rettenmaier et al., 2021; Zhang et al., 2018) were observed throughout the experiment, but only at low RA (generally <1 %). The highly abundant sequence belonging to *Clostridium* sensu stricto *1* shared high similarity (>98 %) with a known cellulolytic species (*Clostridium cellulovorans*) (Doi and Tamaru, 2001) indicating that this genus contributed to cellulose degradation in the PD, in line with previous observations of this genus in lignocellulose-degrading AD systems (Varongchayakul et al., 2024). However, since the RA of all the above mentioned clostridial groups remained constant or decreased during the priming period, it is difficult to say if they contributed to the priming effect.

In contrast, another genus within Clostridia, i.e., *Christensenellaceae R-7 group*, increased in RA after priming and similar trends could be observed for the families *Anaerolineaceae* and *Prolixibacteraceae.* These groups all include members known for fermenting various sugars and potentially hydrolyzing cellulose (Huang et al., 2014; Mcilroy et al., 2017; Morotomi et al., 2012; Podosokorskaya et al., 2013; Sun et al., 2020), although *Anaerolineaceae*, in particular, are considered less competitive than cellulolytic species that can attach directly to the substrate (Xia et al., 2016). Potentially, using a priming agent which includes cellulose could be an option to enrich more specialized lignocellulose degraders, rather than predominantly favoring groups focused on fermentation.

Priming could possibly lead to increased enzymatic activity, therefore extracellular cellulase and amylase activity were measured over time in the MD and the PD. Both cellulase and amylase activities (Fig. 4A–B) were significantly higher (*t*-test, $p < 0.05$) in the MD compared to the PD indicating a need for increased enzymatic activity in the PD to target remaining residual OM. Cellulase activity changed over

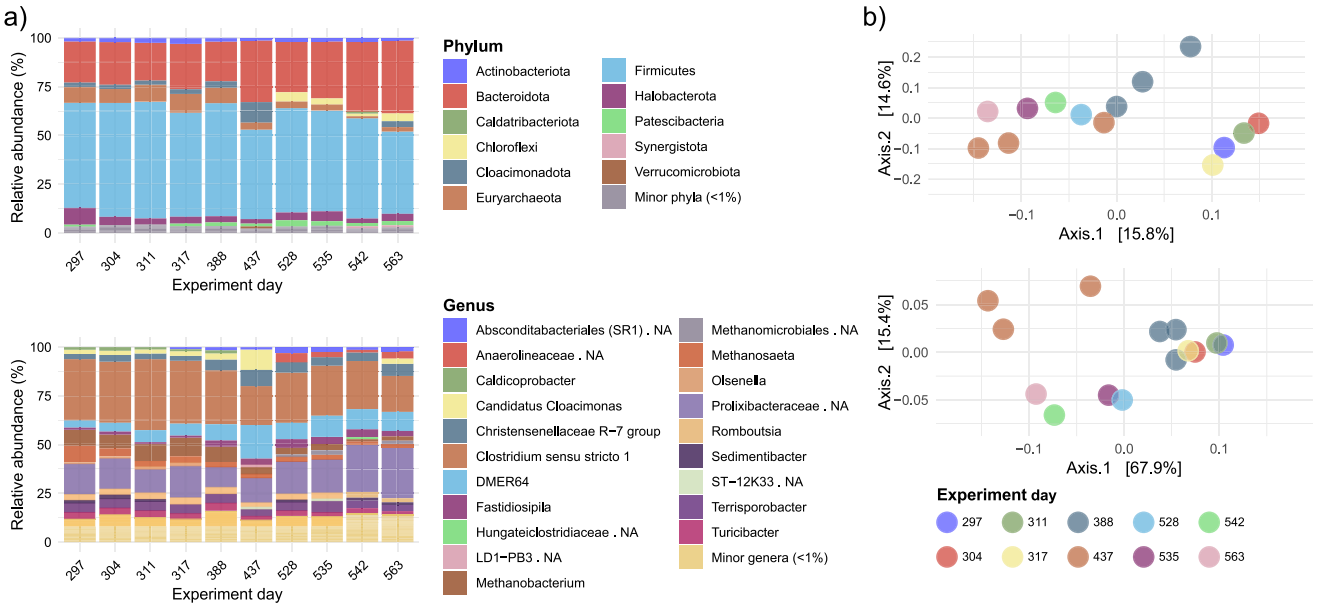

Fig. 3. Analysis of 16S sequences from the PD step, from selected time-points before (day 297–388), during (day 437–542) and after (day 563) starch priming. a) Relative abundances of taxa at phylum (top) and genus (bottom) level. Groups with <1 % relative abundance in all samples are displayed as Minor phyla and Minor genera, respectively. b) PCoA plots illustrating β-diversity in the PD, using unweighted (top) and weighted (bottom) UniFrac distances. The starch addition contributed to a shift in the microbial community.

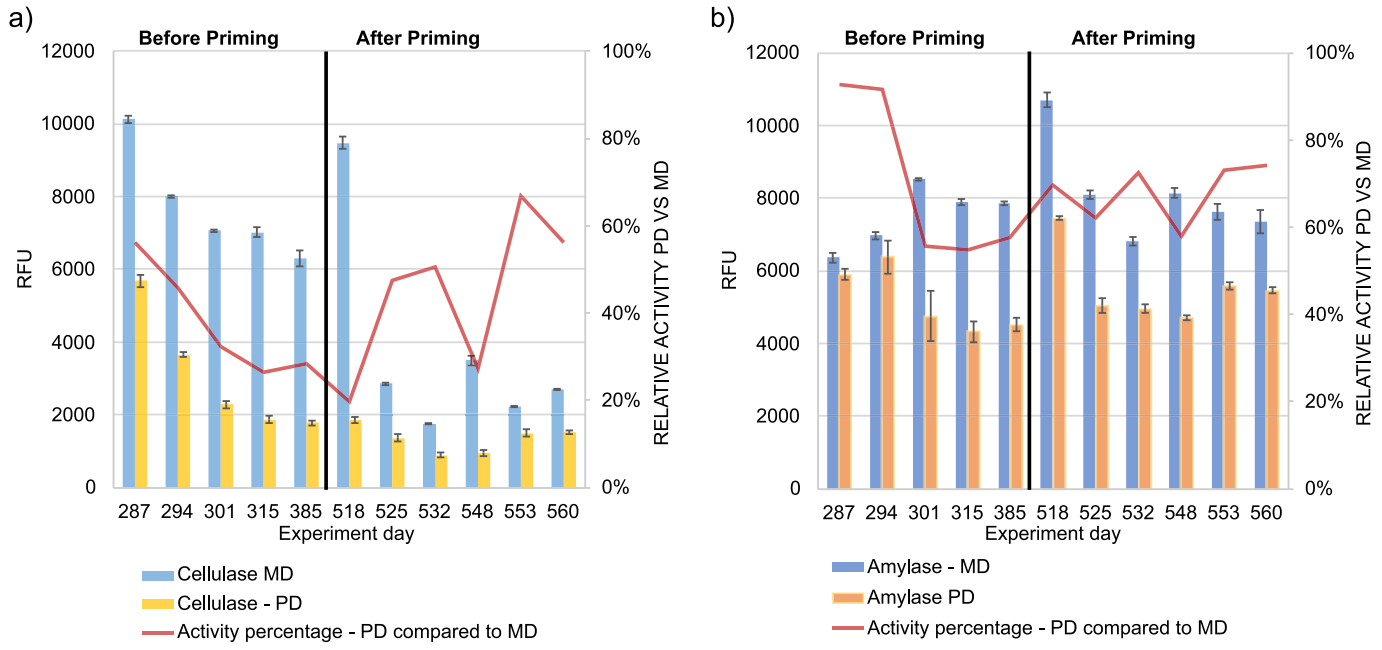

Fig. 4. The results from the analysis of the Cellulase and Amylase activity in the main digester (MD) and post-digester (PD). The enzymatic activity is presented as relative fluorescence units (RFU). The black vertical line divides the measurement between before and after priming. The red line represents the relative microbial activity between the PD and the MD and highlighted that after priming the cellulase activity in the PD was more similar to the MD. The amylase activity did not change after the starch addition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

time in both the MD and the PD, with decreased activities at the end of the experiment. During the control period the cellulase activity was 7700 ± 1400 ΔRFU in the MD and 3050 ± 1560 ΔRFU in the PD. The corresponding values for the experimental period were 2600 ± 620 and 1340 ± 350 ΔRFU. The decrease in activity in the MD most likely affected the activity in the PD as well. The ratio between cellulase activity in the PD and the MD indicated an upgoing trend for cellulase activity during the experimental period (Fig. 4A), making the activity in the PD more similar to the MD. This could be a contributing factor to the reduction of cellulose, hemicellulose, and total sugars in the post-digestate. The amylase activity showed no significant changes between the control and the experimental period. An increase in amylase activity would be expected as starch was used as the priming agent, but that priming did not significantly affect starch degradation, a result in line with the batch tests.

Only extracellular enzymes were measured, but they are most vital in the degradation of the hard-to-degrade OM, while intracellular enzymes are more involved in degradation of easily degradable compounds

(Kuzyakov, 2010). Increases in extracellular enzymes have been connected to the more long-term and real priming effects (Kuzyakov, 2010). Previous studies investigating priming in relation to AD (Ahlberg Eliasson et al., 2023; Ahmed, 2017; Lin et al., 2022) have not specifically focused on the enzymatic activity. Though enrichment of hydrolytic taxa has been observed previously (Ahlberg Eliasson et al., 2023; Lin et al., 2022). In this study though, a clear effect on enzymatic activities was not obvious.

Although the increased hydrolytic activity (e.g. increased degradation of cellulose and hemicellulose) coincided with the increased relative abundances of fermentative species with potential cellulolytic functions, the potential positive effect of priming on hydrolytic activity is not reflected in an increased activity of the cellulase and protease activity measurements.

3.5. *Mechanistic role of starch priming in post-digestion and possibilities for implementation*

AD consists of a cascade of microbial pathways where complex OM is degraded through hydrolysis, fermentation and methanogenesis, with

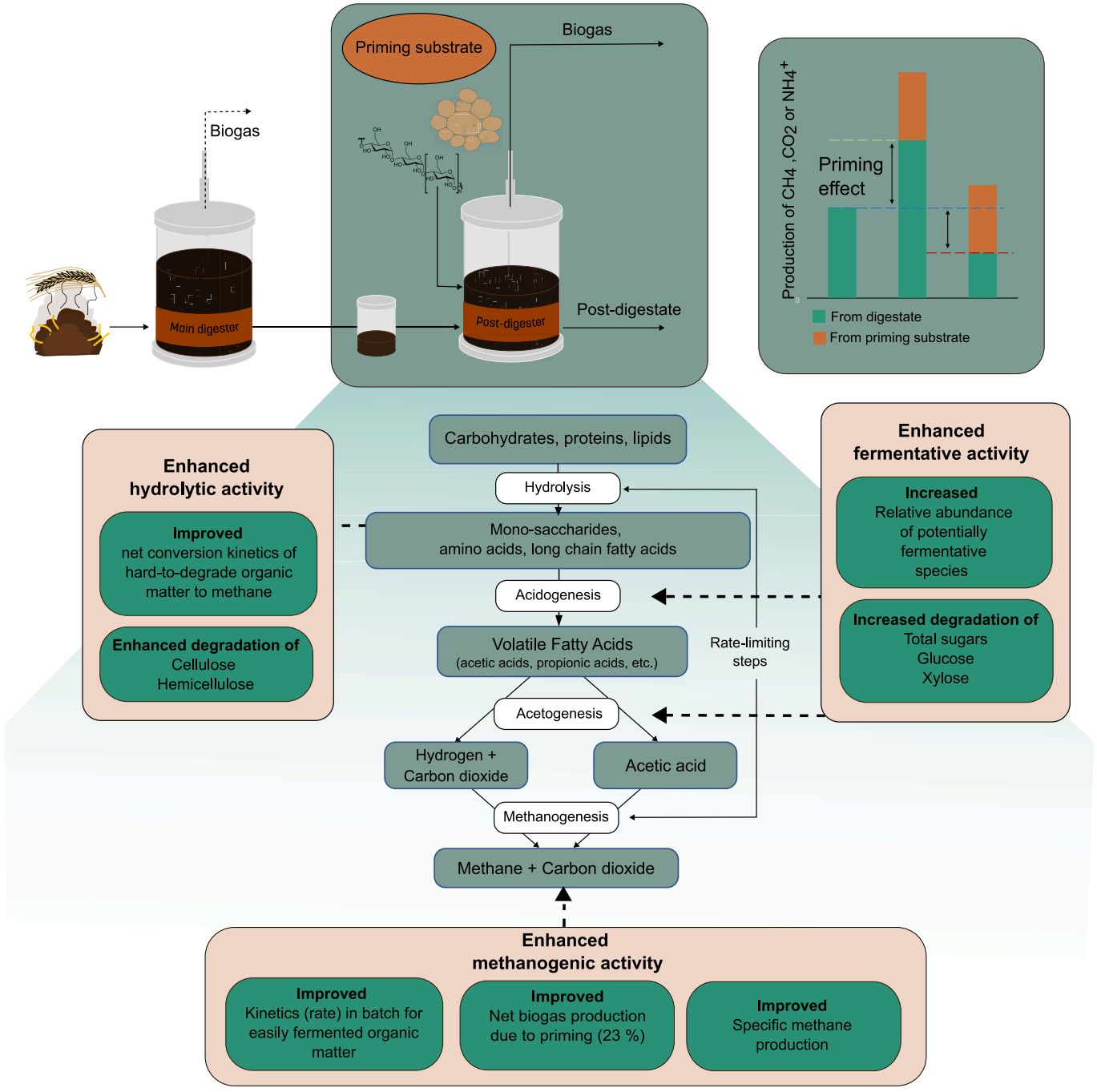

Fig. 5. Shows an overview of the observed priming effects in the current study (in green boxes) while the orange boxes indicate the potential primary mechanism behind the effects; enhanced hydrolytic activity, fermentative activity, and methanogenic activity. The dashed lines highlight which part of the degradation chain is affected by the starch priming during post-digestion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

biomethane and carbon dioxide as main end products (Angelidaki et al., 2011). Previous priming studies have suggested that priming particularly intervenes with hydrolysis (Ahlberg Eliasson et al., 2023; Lin et al., 2022) and acetoclastic methanogenesis (Lin et al., 2022). Hydrolysis is the rate limiting step for hard-to-degrade organic matter and responsible for initial solubilization of complex structures (Angelidaki et al., 2011). Acetoclastic methanogenesis becomes rate limiting upon rapid fermentation and acetogenesis in relation to the rate of acetate utilization by methanogens.

In the present study, the priming effect during post-digestion was primarily observed as an increased net biogas production and increased degradation of cellulose, hemicellulose, and sugars (specifically glucose and xylose). Additionally, the rate of gas production in PD was faster upon daily feeding after initiation of priming even on the days when starch was not added. This indicated that the addition of starch essentially increased hydrolytic activity in the PD as the structures remaining in the digestate were generally hard to degrade. The increase in gas production, gas kinetics, as well as degradation of residual lignocellulosic OM, were connected to increased microbial hydrolysis and fermentation in the PD. Whether the observed changes in the microbial community structure can be considered real or apparent priming effects, as described by Blagodatskaya and Kuzyakov (2008), or if the priming mainly affected r- or K-strategists is difficult to deduce (Fontaine et al., 2003; Zeng et al., 2025). Still, the higher RA of several sugar fermenters, with potential to also hydrolyze polymeric carbohydrates, combined with the improved conversion of lignocellulose, indicates a possible priming effect.

An increased gas production rate was observed for 13 of the 14 tested substrates in the batch experiment, most likely due to increased microbial activity in several of the degradation steps. The increased rate in the batch test affected both more hard-to-degrade OM (i.e., cellulose) and more easily digested OM (sucrose and acetate) which strengthens that an increased activity in the steps of hydrolysis, fermentation and methanogenesis occurred. Also, several batch substrates (S1-S4, cellulose, oleate and sucrose; Tabel 3) indicated higher biomethane production which also support an effect on methanogenesis. The addition of starch provided the generally low energy environment in the PD with easily digestible energy to increase primarily hydrolytic and fermentative activity. The potential mechanistic roles of starch priming in post-digestion are illustrated in Fig. 5.

The possibilities for priming have been highlighted in different processes. Several of the studies which have discussed priming in AD has discussed the potentials for priming in manure processes (Ahlberg Eliasson et al. (2023) and Lin et al. (2022). However, Ahmed (2017) investigated priming in agricultural waste and have shown possibilities for other agricultural waste (grass silage more specifically). Additionally, Insam and Markt (2016) discussed a priming effect could be seen when whey was combined with sewage sludge. This shows that there are possibilities for several different processes. What need to be highlighted is that the type of "priming agent" used might differ for different processes. In the different studies, glucose, whey, starch, and apple waste have been used as priming agents. It is possible that depending on what type of residual organic matter you want to target the priming agent might need to be adapted to the process, e.g. the starch largely seems to help target cellulose and hemicellulose.

In contrast to using a priming agent as a direct substrate in the MD process, it is instead a substrate for microorganisms. However, it is selected as a complement to the main substrate of the AD process to promote specific microbial function, as in this case with a desire to increase the degradation of lignocellulose. Like a normal substrate the amount added is important because of the risk of overload. Another key aspect is to add enough priming agent (as we addressed by assessing different levels of starch addition) to trigger the desired function of the microorganism present in the digester towards an enhanced degradation of hard-to-degrade fraction of influent OM.

Priming with starch-containing organic material is an approach that could be applied easily in large-scale biogas plants if the facility has access to starch-rich substrates and a post-digester installed. For this study, a pure lab-grade potato starch was used as the priming agent to not introduce any additional factors related to impurities of industrially produced starch. However, in a large-scale application other starch sources are most likely to be used. A possible source of starch as priming agent is waste potatoes. In a report from The Swedish Board of Agriculture it was described that 17 % (75,000 tons) of the winter potatoes (potatoes that can be stored and are aimed to be delivered to stores) grown in Sweden in 2021 were not used as food (Strid et al., 2023). 11 % of these was lost already at the farm while almost 7 % was lost at the packing stage and the report estimated that around 1600 tons of these wastes were used for biogas production in Sweden (Strid et al., 2023) which shows possibilities for these for e.g. priming in a post-digester. In addition to potato waste, other easy to degrade organic material could be used as priming agent for the PDs. For example, Lin et al. (2022) used apple waste as the priming agent in addition to glucose, this could potentially be used as a fraction to induce a priming effect. However, for full-scale application it is important to not add to much priming agent to the process as this could lead to negative priming effects, as discussed previously.

4. Conclusions

Application of starch addition (0.2 g VS/L) to the PD processing manure-based digestates promoted priming effects such as increased overall biogas production, including specific biomethane production, and overall process stability and rate. Additionally, increased rate and gas production was observed during the days when starch was not added to the PD, indicating a "priming memory" in the system. A shift in the microbial community potentially boosted the activity of cellulolytic bacteria as an increased degradation of cellulose, hemicellulose, and total sugars were observed after priming. The potential mechanistic role of starch priming consists of enhancement in both hydrolytic, fermentative, and methanogenic activities. The application of starch for achieving such priming effects would be relatively easy in a full-scale context if a PD and a starch-rich substrate are present. The application could improve the degradability of residual organic structures and provide additional economic benefits to post-digestion processes.

CRediT authorship contribution statement

Mette Axelsson Bjerg: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fredrik Heino:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Sepehr Shakeri Yekta:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Luka Šafarič:** Writing – review & editing, Supervision, Resources, Investigation. **Alex Enrich-Prast:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Jan Moestedt:** Writing – review & editing, Supervision, Methodology. **Ebba Perman:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Anna Schnürer:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Annika Björn:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, 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.biteb.2025.102168.

Data availability

16S rRNA-gene sequence data can be accessed at BioProject accessing numbers PRJNA1190856 and at Sequence Read Archive (SRA), National Center for Biotechnology Information (NCBI)

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