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RESEARCH ARTICLE

Co-substrate composition is critical for enrichment of functional key species and for process efficiency during biogas production from cattle manure

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Abstract

Cattle manure has a low energy content and high fibre and water content, limiting its value for biogas production. Co-digestion with a more energy-dense material can improve the output, but the co-substrate composition that gives the best results in terms of degree of degradation, gas production and digestate quality has not yet been identified. This study examined the effects of carbohydrate, protein and fat as co-substrates for biogas production from cattle manure. Laboratory-scale semi-continuous mesophilic reactors were operated with manure in mono-digestion or in co-digestion with egg albumin, rapeseed oil, potato starch or a mixture of these, and chemical and microbiological parameters were analysed. The results showed increased gas yield for all co-digestion reactors, but only the reactor supplemented with rapeseed oil showed synergistic effects on methane yield. The reactor receiving potato starch indicated improved fibre degradation, suggesting a priming effect by the easily accessible carbon. Both these reactors showed increased species richness and enrichment of key microbial species, such as fat-degrading *Syntrophomonadaceae* and families known to include cellulolytic bacteria. The addition of albumin promoted enrichment of known ammonia-tolerant syntrophic acetate- and potential propionate-degrading bacteria, but still caused slight process inhibition and less efficient overall degradation of organic matter in general, and of cellulose in particular.

INTRODUCTION

Treatment of cattle manure (CM) by anaerobic digestion (AD) provides many benefits, such as production of renewable energy (biogas), recirculation of nutrients and reduction of GHG emissions from agricultural production (Holm-Nielsen et al., 2009; Liebetrau et al., 2013; Petersen et al., 2013; Pucker et al., 2013; Zhang, Wang, Yin, & Dogot, 2021). The total amount of manure produced in Europe has been estimated to correspond to a biogas potential representing 4.5% of the consumption of nature gas, if collected entirely (Scarlat et al., 2018). Unfortunately, the development of manure-based AD processes is hampered by CM having low levels of degradable organic matter, resulting in low methane production and efficiency and difficulties to achieve economic feasibility (Møller et al., 2004; Ruile et al., 2015; Triolo et al., 2011; Tufaner & Avşar, 2016). To achieve reasonable levels of degradation, the retention time in the reactor needs to be sufficiently long (Linke

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et al., 2013; Ruile et al., 2015). Unfortunately, CM has a low concentration of organic matter and a high content of water, making it difficult to achieve long retention time at reasonable organic loads (Ruile et al., 2015). Different strategies can be used to improve microbial degradation of manure, such as application of different reactor technologies and pre-treatments or use of process additives and co-digestion (Nasir et al., 2012). Co-digestion is an interesting approach since, if applied for energy-dense materials, it can allow a higher load without a marked decrease in retention time, resulting in improved volumetric and specific methane production (Esposito et al., 2012; Labatut et al., 2011; Li et al., 2021; Tufaner & Avşar, 2016). Moreover, co-digestion can overcome any imbalances in nutrients and improve overall biodegradation (Ma et al., 2020; Mata-Alvarez et al., 2014; Zhou et al., 2021). It has also been suggested to give a greater reduction in global warning impact than mono-digestion of daily manure (Zhang, Wang, Yin, & Dogot, 2021).

Many substrates with different chemical composition have been evaluated and shown to work as co-substrates for AD of manure, such as straw, energy crops, food waste, slaughterhouse waste and residual fat (Ahlberg-Eliasson et al., 2018; Mata-Alvarez et al., 2014; Søndergaard et al., 2015). Most studies on co-digestion report improved methane production compared with digesting CM alone, but in many cases, the increase in gas yield is attributable solely to the co-substrate, and not to improved degradation of the CM per se (Li et al., 2021). However, some studies also suggest synergistic effects, with improved methane formation and/or degradation as a consequence of addition of co-substrate (summarised in Li et al., 2021). Synergistic effects have, for example, been proposed for co-digestion of CM with the organic fraction of municipal solid (Macias-Corral et al., 2008), switchgrass (Zheng et al., 2015) and sheep manure (Li, Achinas, et al., 2020).

Degradation of organic material in a biogas process is performed by an array of different microorganisms, working in a synchronised manner (Schnürer & Jarvis, 2017). The process involves four different microbial degradation steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis), requiring the combined activity of several different groups of microorganisms. To create a stable, efficient biogas process, it is important to meet the growth requirements of all microorganisms involved. To provide favourable conditions for microbial growth, the substrate needs to supply growth factors, macronutrients and micronutrients and contain low levels of microbial inhibitors (Westerholm & Schnürer, 2019). By itself, CM can supply sufficient nutrients to maintain microbial growth during mono-digestion, but the addition of a suitable co-substrate can give a more balanced nutrient composition and thus result in synergistic effects, with improved degradation, higher methane yields and promotion of a more diverse microbial community (Mata-Alvarez et al., 2014). However, the co-digestion substrate needs to be chosen carefully, since instead of giving positive effects, some may result in antagonistic interactions, resulting in lower biogas productivity and biodegradability. For example, high ammonia levels have been shown to inhibit degradation of cellulose during co-digestion of cow manure and protein-rich material* (Li, Zhao, et al., 2020). Therefore, co-digestion of animal manure and lignocellulosic feedstocks (crops) has been proposed as a solution to reduce the risk of ammonia inhibition and to bring the C/N ratio closer to the optimum value for microbial growth. The response of the microbial community to a co-digestion substrate will depend on the character and composition of the co-substrate and on operating conditions in the reactor (Westerholm & Schnürer, 2019). Many studies have investigated the response of microbial communities to co-digestion, including in reactors operating with manure (Ahlberg-Eliasson et al., 2018; Li, Achinas, et al., 2020; Song & Zhang, 2015; Wang et al., 2018; Wei et al., 2019; Xu et al., 2018; Zhang, Wang, Xing, et al., 2021), but only a few have assessed responses specifically related to enhanced biodegradability of the manure. One such study investigated co-digestion of CM with sheep manure in continuously stirred tank reactors (CSTR) and observed improved degradation of lignocellulose compared with in mono-digestion of CM (Li, Achinas, et al., 2020). Their analysis revealed enrichment of *Firmicutes*, genus *Romboutisia* and *Turicibacter*, and particularly *Candidatus* Cloacimonas and *Methanoculleus*, all showing a positive correlation with cellulose degradation (Li, Achinas, et al., 2020). Enrichment of *Firmicutes* has also been found to be linked to enhanced hydrolysis during co-digestion of cattle manure and apple waste fructose (Lin et al., 2022).

The general concept of co-digestion of co-substrate with cow manure is thus well known and investigated. However, most studies have focused mainly on methane productivity and only a few have included a deeper chemical and/or microbiological evaluation of potential synergistic effects on degradation of the cow manure. In addition, less attention has been devoted to examining effects of different categories of macromolecules, for example, fats, proteins or carbohydrates, in co-digestion with manure or the effect of co-digestion on residual methane potential (RMP) in the digestate. In theory, a well-designed co-digestion strategy would improve degradation efficiency and gas production, balance the nutrient content in the digestate and reduce RMP of the digestate. High RMP poses a risk of methane losses from storage and decreases the overall environmental benefits of biogas production from manure (Clemens et al., 2006; Liebetrau et al., 2013; Rodhe et al., 2012). The optimal co-substrate to achieve the most efficient process in co-digestion with manure is still not completely clear.

The aim of this study was to assess the suitability of different co-substrates for optimal biogas production from cattle manure. The specific objective was to identify links between co-substrate composition and overall process efficiency and stability, levels of nutrients and RMP of the digestate. The co-substrates selected for assessment were egg albumin (protein), rapeseed oil (fat) and potato starch (carbohydrates), alone and in combination, which were co-digested with CM. Process performance was evaluated using different chemical parameters, such as gas production, degradation efficiency of different macromolecules and RMP. To capture changes in microbial community development caused by the different co-substrates and assess the stability of the process, analyses of the microbial community were conducted. These analyses considered the overall microbial community, targeting the 16S rRNA gene, and also specifically the acetogenic/syntrophic community, targeting the FTHFS gene. Analysis of the FTHFS gene has recently been proposed as a useful method for detection of community changes before effects emerge in physico-chemical profiles in biogas processes (Singh, 2021; Singh, Moestedt, et al., 2021).

EXPERIMENTAL PROCEDURES

Substrates and inoculum

Manure substrate, liquid and solid manure and inoculum were collected from a biogas plant located on a farm in south-west Sweden. Manure from the same biogas plant was evaluated as a sole substrate for biogas production in a recent publication, where detailed information about the farm and operation of the biogas plant can be found (Ahlberg-Eliasson et al., 2017). The manure mix used for the present experiment was collected directly from the mixing tank and from the mixing wagon for the substrates, and had a high dry matter content. All substrates were frozen at −18°C and stored for further use. Materials evaluated as co-substrate were egg albumin (Källbergs Industri AB, Sweden), rapeseed oil (ICA, Sweden) and potato starch (Alfa Aesar, Thermo Fisher (Kandel) GmbH). The chemical composition of the substrate mix is presented in Table S1.

Batch and continuous biogas processes

The biochemical methane potential (BMP) of all substrates investigated (CM, egg albumin, rapeseed oil, potato starch and mixtures of these) was determined using the commercial system AMPTS II (Bioprocess Control, Sweden) as described elsewhere (Ahlberg-Eliasson et al., 2018). In brief, inoculum for the test was collected from a municipal wastewater treatment plant in Uppsala and degassed at 37°C for 4 days. The inoculum had a total solids (TS) content of 3.1% of wet weight and a VS content of 2.0%. All substrates, single or mixed, in the same proportions as used in the CSTR, were combined with inoculum in a ratio of 1:3 (VS basis) in 500-mL reactors (final liquid volume 400 ml). The organic load was set to 3 g VS L^{-1} day^{-1} and tap water was used to adjust to the final volume. Triplicate batch reactors were started for each substrate combination and for cellulose (medium fibre, Sigma-Aldrich), which was used as a control substrate to ensure an active inoculum and resulted in a BMP of 337 ± 10 ml CH_4 g VS^{-1}. Furthermore, inoculum alone was added to the last set of batch reactors (also triplicates), without any addition of substrate, to measure background levels of methane production. Incubation was performed at 37°C and was terminated when daily methane production fell below 1% of the accumulated methane production on a volume basis. The gas volumes produced were normalised to 1.01325 bar and temperature 273.2 K.

Operation in semi-continuous CSTR processes

The manure, alone or in co-digestion, was evaluated during semi-continuous operation in five 10-L laboratory-scale CSTRs (Dolly, Belach Bioteknik AB). At start-up, the reactors were filled with 5 L of fresh inoculum. The reactors were then fed once a day, for practical reasons 6 days a week, and stirred continuously at 90 rpm during the whole experiment. Process parameters were set to simulate the conditions in a corresponding large-scale plant, that is, 42°C, organic loading rate (OLR) of 2.5 g VS L^{-1} day^{-1} (average calculated for weekly substrate load) with manure and using a hydraulic retention time (HRT) of 32 days. The reactors were designated R0 (control), R1, R2, R3 and R4. After 60 days of operation with only CM, protein (egg albumin), fat (rapeseed oil) and carbohydrates (potato starch) were added as co-substrate to R1, R2 and R3 respectively. Reactor R4 received a mix of the co-substrates in equal amounts of VS. The co-substrate corresponded to an additional total load of 0.5 g VS L^{-1} day^{-1}, resulting in a final organic load in reactors R1-R4 of 3 g VS L^{-1} day^{-1}. R0 was operated throughout as a control reactor with only CM, with OLR 2.5 g VS L^{-1} day^{-1}. The reactors were operated for a total of 224 days. During operation, the process was evaluated by daily measurement of total gas production, weekly analysis of pH, gas composition and levels of volatile fatty acids (VFA) and digestate compositional analysis after each HRT (for details, see analytical methods below). Gas volumes were calibrated on each reactor by collecting the gas produced in bags and determining the volume using a drum meter (TG 0.5, Ritter, Germany). For microbial analysis, addition of co-substrate was considered the start of the experiment

and samples (15 ml) were taken at day 3, 29, 66, 143 and 164 and stored frozen (−20°C) for further use.

Residual methane production

Residual methane production (RMP) was measured in digestate taken from all five reactors on the last day (224 days) of operation. Aliquots of 300 ml were added to the 500-ml reactors in the AMPTS II system (Bioprocess Control) and production of methane was monitored over time. The incubation was performed at 42°C for a total of 90 days and, as in the batch experiment, gas volumes produced were normalised to 1.01325 bar and temperature 273.2 K. Loss of VS in VFA was calculated according to Vahlberg et al. (2013).

DNA extraction, amplicon sequencing and data analysis

Total genomic DNA was isolated in triplicate from the frozen samples using the FastDNA™ Spin Kit for Soil (MP Biomedicals, 2019a) and FastPrep®-24 instrument (MP Biomedicals, 2019b) according to the method described by Sun et al. (2016). The total genomic DNA isolated was quantified by Qubit® 3.0 Fluorometer (Invitrogen 2014). Qualitative PCR analysis of syntrophic acetate-oxidising bacteria (SAOB) was conducted using primers and a method described by Westerholm et al. (2011). A 16S rRNA gene library was constructed for Illumina sequencing of the V4 region (515F-805R) (Hugerth et al., 2014), according to the method described by Müller et al. (2016). Raw sequencing data were quality-controlled (Q-score > 20) and adapters/primers were trimmed with Cutadapt (v3.5) (Martin, 2011). The paired end reads were merged and amplicon sequence variants were analysed and generated by removing the chimera sequences using R (v4.1.3) (R Core Team, 2021) in the RStudio (v 2021.09.0 + 351) (RStudio Team, 2020) using package *dada2* (v1.22.0). The taxonomic profile of the microbial community was visualised with the packages *phyloseq* (v1.38.0) (McMurdie & Holmes, 2013) and *ggplot2* (v3.3.6) (Wickham, 2016). Differential abundance testing (using normalised mean of control vs treatment in pairwise analysis) was performed with the package *DESeq2* (v1.34.0) (Love et al., 2014) and linear discriminate analysis (LEfSe method) with the package *microbial* (v0.0.20) (Guo & Guo, 2021). Multivariate analyses (non-metric multidimensional scaling [NMDS] and principal coordinate analysis [PCoA]) were performed using the packages *phyloseq* and *vegan* (v2.5.7) (Oksanen et al., 2019). Formyltetrahydrofolate synthetase (FTHFS) gene-amplicon sequencing and data analysis (using the AcetoScan pipeline) were performed as described by Singh et al. (2020). For the data analysis, raw paired-end reads for forward and reverse sequence were concatenated in a single file and used as single-end reads (with parameters -m 300, -n 150, -q 20, -t 1.0, -c 2, -e 1e-30) as described elsewhere (Singh, Moestedt, et al., 2021). For taxonomic annotations, the AcetoBase database (v2.0) was used (Singh & Schnürer, 2022). The raw sequencing data have been submitted to the Sequence Read Archive (SRA) database at the National Center for Biotechnology Information (NCBI), under the study numbers PRJNA507984 (16S rRNA gene-amplicon sequencing) and PRJNA873909 (FTHFS gene-amplicon sequencing). The FTHFS amplicon OTUs have been submitted to AcetoBase with accession numbers UN_0000029650 – UN_0000030098.

Analytical methods

Gas analysis

Concentration of carbon dioxide (CO_2) in the raw biogas was measured by liquid displacement in a saccharometer filled with 7 M sodium hydroxide. Concentration of hydrogen sulphide (H_2S) and methane percentage (% CH_4) in the raw biogas was determined using a Biogas 5000 gas analyser (Geotechnical Instruments). Methane was also analysed by gas chromatography according to a previously described method (Westerholm et al., 2012).

Digestate analysis

The pH in the reactors was monitored by direct measurements of outlet digestate using a bench pH meter (3510 pH Meter, Jenway). Short-chain VFAs were identified and quantified by HPLC according to the method described by Westerholm et al. (2016). An external standard (0.25–8.00 g L^{-1}) consisting of acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid and isovaleric acid was used for identification and quantification. Moreover, 500-ml digestate samples were taken from each of the five reactors on three occasions (days 145, 175 and 222) for chemical analysis of fibre, fat and protein content. These analyses were performed at the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden. Concentration of fat was analysed according to European Commission Directive 98/64/EC (1998) and concentration of protein according to Nordic Committee on Food Analysis (1976). Fibre fractions, that is, cellulose and hemicellulose, were calculated using analysed levels of neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL). Concentration of NDF was analysed according to Chai and Udén (1998), while ADF and ADL

were determined according to Van Soest et al. (1991). Analysis of TS and VS in substrates and digestates was performed according to APHA (1998).

Calculations

Operating parameters for OLR and HRT were calculated according to Schnürer et al. (2016). Reduction in VS (VSred) was determined as a measure of degree of degradation (DD) according to Ahlberg-Eliasson et al. (2017). Process efficiency in the biogas plant was calculated according to Rico et al. (2015). This calculation includes the parameters methane production, hydraulic retention time and residual methane production.

Statistical analysis

Daily biogas production (GP), specific methane production (SMP), VFA content, DD, quality of the raw biogas (i.e. H_2S, CH_4 and CO_2 content) and nutrient concentrations were statistically evaluated pairwise for the reactors, using the t-test procedure in R (v4.1.3). Values with $p < 0.05$ were taken as significant.

RESULTS

Biomethane potential of single substrates and substrate mixes

Cattle manure reached final BMP of 195 ± 4 ml CH_4 g VS^{-1} after 74 days of incubation, with 80% of this potential reached after 25 days (Table 1). For the rapeseed oil, albumin and potato starch co-substrates, final BMP was 676 ± 56, 333 ± 10 ml and 321 ± 7 ml CH_4 g VS^{-1}, respectively, with 80% of final BMP in these cases reached after 27, 4 and 3 days respectively (Table 1, Figure S1). For the batches running with the substrates in co-digestion, the highest BMP was found for manure combined with rapeseed oil (274 ± 0 ml CH_4 g VS^{-1}), followed by manure combined with egg albumin and manure with the blended mix of co-substrates (Table 1). The lowest BMP (190 ± 22 ml CH_4 g VS^{-1}) was found for the mixture with manure and potato starch (Table 1). Comparing these BMP values with calculated additive values based on analysis of the individual co-substrates showed similar values for manure in combination with rapeseed oil and egg albumin (Table 1). However, for starch and the mixture of co-substrates combined with manure, higher values were obtained for the calculated co-digestion (216 ± 11 and 245 ± 7 ml CH4 g VS^{-1} respectively) than in the actual experiment.

Co-digestion in continuous stirred-tank reactor

In the initial phase of the experiment and during operation with manure alone, volumetric yield was 3284 ml day^{-1} and specific methane yield was 172 ml CH_4 g VS^{-1}. After 60 days of operation the co-substrates were added, whereupon specific methane production (SMP) and volumetric methane gas production (MP) both increased, to differing levels in the different reactors. The control reactor R0, with manure only, showed MP of 3340 ± 145 ml day^{-1} and SMP of 172 ± 6 ml CH_4 g VS^{-1} (Table 2, Figure S2). Among the co-digestion reactors, R2, receiving rapeseed oil, showed significantly higher values of MP (6202 ± 203 ml day^{-1}) and SMP (302 ± 9 ml CH_4

TABLE 1 Biochemical methane potential (BMP) of the test co-subtrates in mono-digestion (MD) and in co-digestion (CD) with cattle manure (CM), including time in days to reach 50, 80 and 100% of final BMP.

Substrate	Type of digestion	Days to reach a certain share of final BMP			Final BMP [Nml CH_4 gVS^{-1}]	Sum of individual BMP[a] [Nml CH_4 gVS^{-1}]
		50%	80%	100%		
Potato starch	MD	2	3	75	321 ± 7	
Egg albumin	MD	2	4	8	333 ± 10	
Rapeseed oil	MD	16	27	77	676 ± 56[b]	
Cattle manure	MD	6	25	74	195 ± 4	
CM + potato starch	CD	3	11	77	190 ± 22	216 ± 11
CM + egg albumin	CD	4	20	77	217 ± 13[b]	219 ± 6
CM + rapeseed oil	CD	4	11	76	274 ± 0.04[b]	277 ± 14
CM + mixture	CD	3	8	76	216 ± 24	245 ± 7
Mixture (1:1:1)	CD	3	8	16	489 ± 20	

[a]Sum of BMP for the individual substrates in the same ratio as in the reactor.
[b]Mean value of two replicates.

TABLE 2 Mean value and standard deviation (SD) of process data collected from reactor operation during day 69–224 and nutrient content in the digestate collected from the same reactors based on the mean of samples collected at 2, 3 and 5 hydraulic retention times.

			R0		R1		R2		R3		R4	
			Control		Egg albumin		Rapeseed oil		Potato starch		Mixture	
	Parameter	Units	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*
Reactor	MP	$ml\,day^{-1}$	3340	145	4027	266	6202	203	5027	128	5193	149
	SMP	$ml\,gVS^{-1}\,day^{-1}$	172	6	178	12	302	9	203	5	231	7
	CH_4	% of v/v	56.0	0.4	59.2	1.0	62.4	0.9	52.9	0.7	58.8	0.7
	CO_2	% of v/v	40.9	0.4	37.9	0.6	35.4	0.8	43.9	0.4	38.5	0.6
	H_2S	ppm of v/v	40.8	4.8	479	262	33	5	41	5	67	15
	VFA	gL^{-1}	0.04	0.01	0.88	0.61	0.14	0.12	0.032	0.01	0.049	0.02
	pH	AU	7.66	0.06	7.82	0.08	7.70	0.07	7.60	0.06	7.74	0.09
	DD	%	27.9	0.7	31.2	2.3	38.0	0.8	38.8	0.3	35.4	1.0
Digestate	TS	% of w/w	7.6	0.1	8.4	0.0	7.7	0.2	7.5	0.1	7.9	0.1
	VS	% of w/w	5.9	0.0	6.7	0.1	6.0	0.2	5.9	0.0	6.2	0.1
	NH_4^+-N	gL^{-1}	1.9	0.1	3.8	0.2	1.8	0.0	1.8	0.0	2.5	0.1
	Protein	% of TS	29.5	1.1	41.1	1.7	30.1	1.2	30.2	0.4	34.2	0.8
	Fat	% of TS	2.4	0.1	2.3	0.1	3.1	0.1	2.6	0.1	2.5	0.1
	Cellulose	% of TS	18.5	0.2	22.2	0.3	18.7	0.5	18.3	0.5	20.1	0.1
	Hemicellulose	% of TS	14.8	0.3	15.1	0.5	15.8	1.0	14.5	0.8	15.6	0.5
	Lignin	% of TS	13.0	0.9	13.2	0.6	12.7	0.9	13.2	0.3	12.1	1.0

Note: Reactors operated with manure in mono-digestion (R0) or in co-digestion with egg albumin (R1), rapeseed oil (R2), potato starch (R3) or a mix of these co-substrates (R4).

Abbreviations: DD, degree of degradation; MP, methane production; SMP, specific methane production; TS, total solids; VFA, volatile fatty acids; VS, volatile solids.

g VS^{-1}) than the control reactor (R0) and the other co-digestion reactors (Table 2, Figure S2). Reactors R3 and R4 also showed significantly higher values of MP and SMP compared with the control reactor. However, reactor R1, receiving egg albumin, had significantly higher MP, but not SMP, than R0 (Table 2, Figure S2). The total concentration of VFA was low in reactors R0, R3 and R4 (~0.1 g L^{-1}), while R1 had a significantly higher mean VFA concentration (0.88 ± 0.61 g L^{-1}) than all other reactors (Table 2). Reactor R1 showed an increasing trend in VFA level, starting approximately after day 75 (15 days after introduction of co-substrate) and increasing to a peak around 1.6 g L^{-1} at day 154 (Figure S3). In reactor R2, the mean VFA level was 0.14 ± 0.12 g L^{-1}, including two periods of moderate increases (Figure S3). The pH also showed some differences between the reactors, with average values ranging from pH 7.60 to 7.82 (Table 2). The methane concentration in the raw biogas in reactor R0 was 56.0% ± 0.4%, while R2, R1 and R4 all showed significantly higher values, with that in R2 reaching 62.4% ± 0.9% (Table 2). However, R3 had a significantly lower methane content (52.9% ± 0.7%) than R0. The concentration of carbon dioxide followed the same pattern as methane, but in the opposite direction (from high to low) for the reactors (Table 2, Figure S2). The concentration of H_2S was 41 ± 5 ppm for the reference R0 reactor and was significantly higher in R1 and R4 (479 ± 262 ppm and 67 ± 15.2 ppm respectively) (Table 2, Figure S2). The other reactors showed more moderate concentrations of H_2S, ranging from 33 to 67 ppm (Table 2).

In terms of degradation of organic matter, all reactors supplemented with a co-substrate (R1-R4) showed higher degradability of VS compared with R0 (Table 2, Figures S4 and S5). The nutrients added to the reactors with the co-substrates affected the digestate composition to different extents (Table 2). Digestate from R1 and R4 showed significantly higher concentrations of protein and ammonium-nitrogen than digestate from the other reactors (Table 2, Figures S4 and S5). Based on proportion of TS, the level of fat was highest overall in R2, which received additional fat in the substrate mix, while no significant difference was seen between the other reactors. The content of cellulose was higher in R1 and R4 than in R0, R2 and R3. Reactor R3, receiving potato starch, showed the lowest content of cellulose in the digestate, although the level was not significantly different from that in the reference reactor (Table 2, Figures S4 and S5). Moreover, the levels of hemicellulose and lignin did not differ significantly between the reactors receiving co-substrates compared with the reference reactor. However, R3, receiving starch, showed the lowest level of hemicellulose of all reactors (Table 2, Figures S4 and S5).

Residual methane production

Residual methane potential after 90 days of incubation at 42°C ranged from 67 to 78 ml CH_4 g VS^{-1}, with lowest value for the R1 digestate and the highest for the digestate from R2 and R3 (Table S2). These values, combined with the volumetric methane production values and the HRT, were used to calculate efficiency values according to Rico et al. (2015). The highest efficiency values were obtained for R2 and R4 (85% and 82% respectively), while for R3, R1 and R0 the values were 79%, 77% and 76% respectively (Table S2).

Microbial community structure

The 16S rRNA gene-amplicon sequencing reads were processed by the *dada2* algorithm. A total of 1173 amplicon sequence variants (ASVs) were recovered after quality control, filtering and chimera removal. The universal primer pair 515F-805R covered both the bacteria and archaeal community. Of the total of 1173 ASVs identified, 1152 (98.2%) belonged to the kingdom Bacteria while 21 (1.8%) belonged to the kingdom Archaea. Sequences representing phyla and present at abundance <1% were merged in a category called 'minor phylum', while sequences with relative abundance >1% (major phyla) were used for further analysis (Figure 1). At the phylum level, 20 major phyla were detected, among which *Firmicutes* (12%–67%) and *Bacteroidetes* (9%–48%) were the most abundant in all reactors, with an increasing trend for *Firmicutes* found in reactor R3. For *Bacteroidota*, the level varied over time in the different reactors and no clear trend could be seen. Besides, all reactors contained additional phyla, but often at low relative abundance. Phylum *Synergistetes* was present in R0, R1, R2 and R4 at levels fluctuating over time, with no presence after day 29 in R2, and was not observed at all in R3. The relative abundance of phylum *Cloacimonadota* also fluctuated somewhat over time in all reactors, with R3 (receiving potato starch) showing the lowest relative abundance and R4 (substrate mix) a slightly higher level compared with the control reactor R0 (Figure 1). The kingdom Archaea was dominated by phylum *Methanobacteriota*, with initially higher relative abundance in R0, R2 and R4 (12%–32%) than in R1 (4%) and R3 (<1%), but its presence gradually decreased over time in these reactors.

At the class level, 21 classes with relative abundance >1% were detected. In all reactors, *Clostridia* was the dominant class in phylum *Fimicutes* and *Bacteroidia* in phylum *Bacteroidota*, followed by class *Synergistia* and *Cloacimonadia*, in phylum *Synergistetes* and *Cloacimonadota* respectively. *Methanobacteria* and *Methanosarcinia* were the only dominant archaeal classes (phylum *Methanobacteriota* and

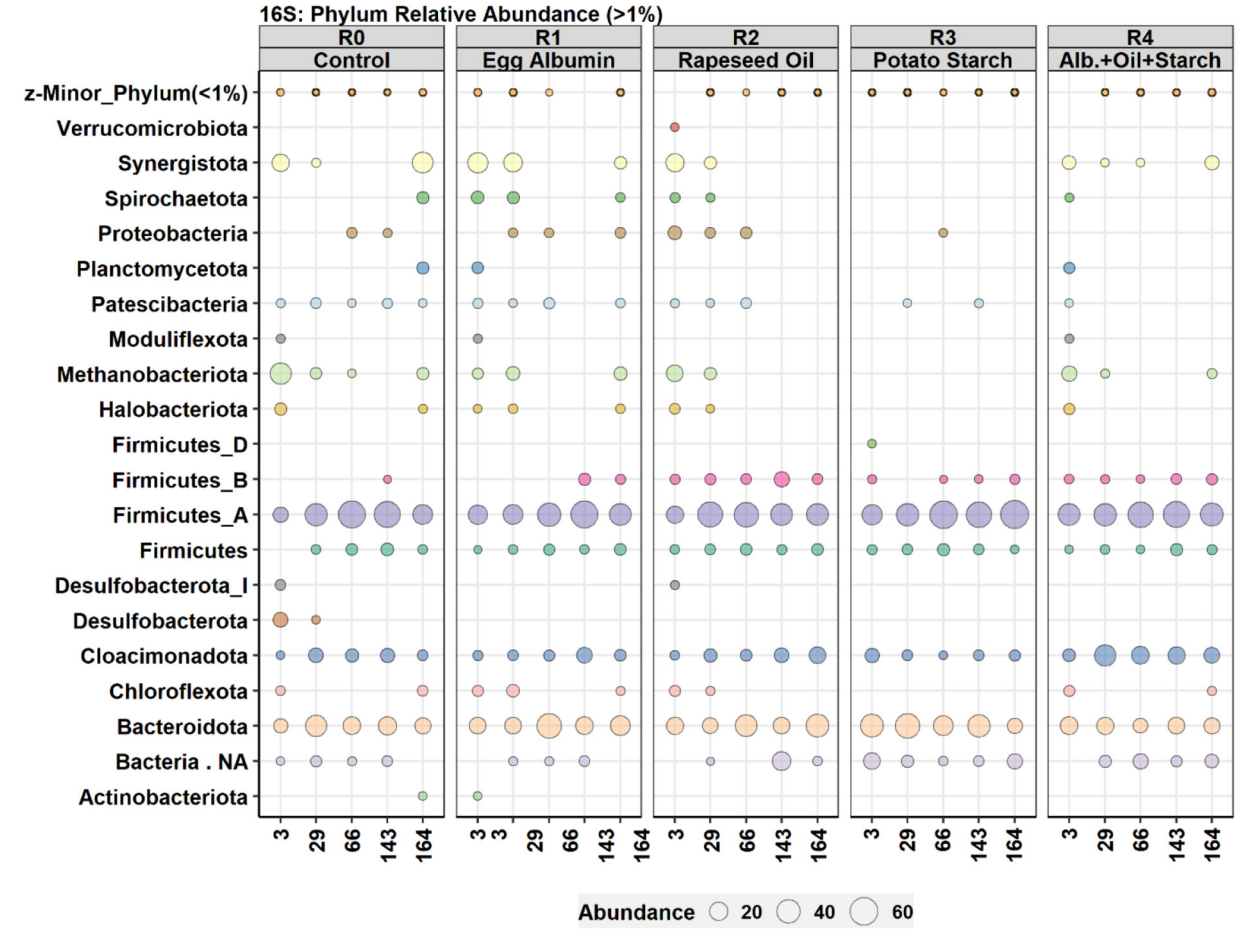

FIGURE 1 Relative abundance of the microbial community (identified using 16S rRNA gene amplicon sequencing) at phylum level (relative abundance >1%) in different reactors. Phyla with relative abundance <1% are merged in the category 'minor phylum'. Samples taken from reactors operating with manure in mono-digestion (R0) or in co-digestion with egg albumin (R1), rapeseed oil (R2), potato starch (R3) or a mix of these co-substrates (R4).

Halobacteriota respectively) observed overall in all reactors (Figure S6).

Comparisons of relatively and differentially abundant families illustrated that, compared with R0, the differential abundance pattern in all other reactors was specific to the substrate added (Figure S7, Figure 2). Family *Cloacimonadaceae* was one of the most abundant families in all reactors, but decreased in differential abundance in R1. *Syntrophomonadaceae* was differentially more abundant in R2 than in the control reactor. Families belonging to class *Clostridia* (viz. Acetivibrionales_NA, *Acetalibacteraceae*, *Anaerovoracaceae*, DTU013, DTU023, Firmicutes_A_NA), order *Bacteroidia* (UBA932) and order *Kiritimatiellae* (Lenti-01), were differentially abundant in reactor R3, fed with potato starch. For reactors R1 and R3, overall lower microbial community dynamics and higher differential abundance, respectively, compared with R0 were observed (Figure S7, Figure 2). To evaluate the diversity of the bacterial community in all reactors, alpha diversity indices, for example, observed richness, were used (Figure S8). The results for the Shannon and Simpson diversity indices showed no general trend in observed richness for reactors R0, R1 and R4. In R2 and R3, however, the diversity/richness was found to increase over time and by at the end of the experiment was higher than in R0. Reactors R0, R2, R3 and R4 showed similar Shannon index, while a lower value was observed for R1, except at the last two sampling points (days 143 and 164), when a sudden decrease and then increase in the diversity index was observed. Alpha diversity, calculated as Simpson index, revealed no specific trends for R0, R1 and R2, but an increase was observed in R3 except at the last sampling point. NMDS analysis using Bray–Curtis distance indicated dispersion in the multidimensional space, but with no clear correlation of individual samples to different operating parameters (Figure S9). However, the mean values calculated and represented as a centroid in NMDS analysis indicated overall influence of the process parameters on the samples from individual reactors. Similar results were obtained in the weighted PCoA analysis, where

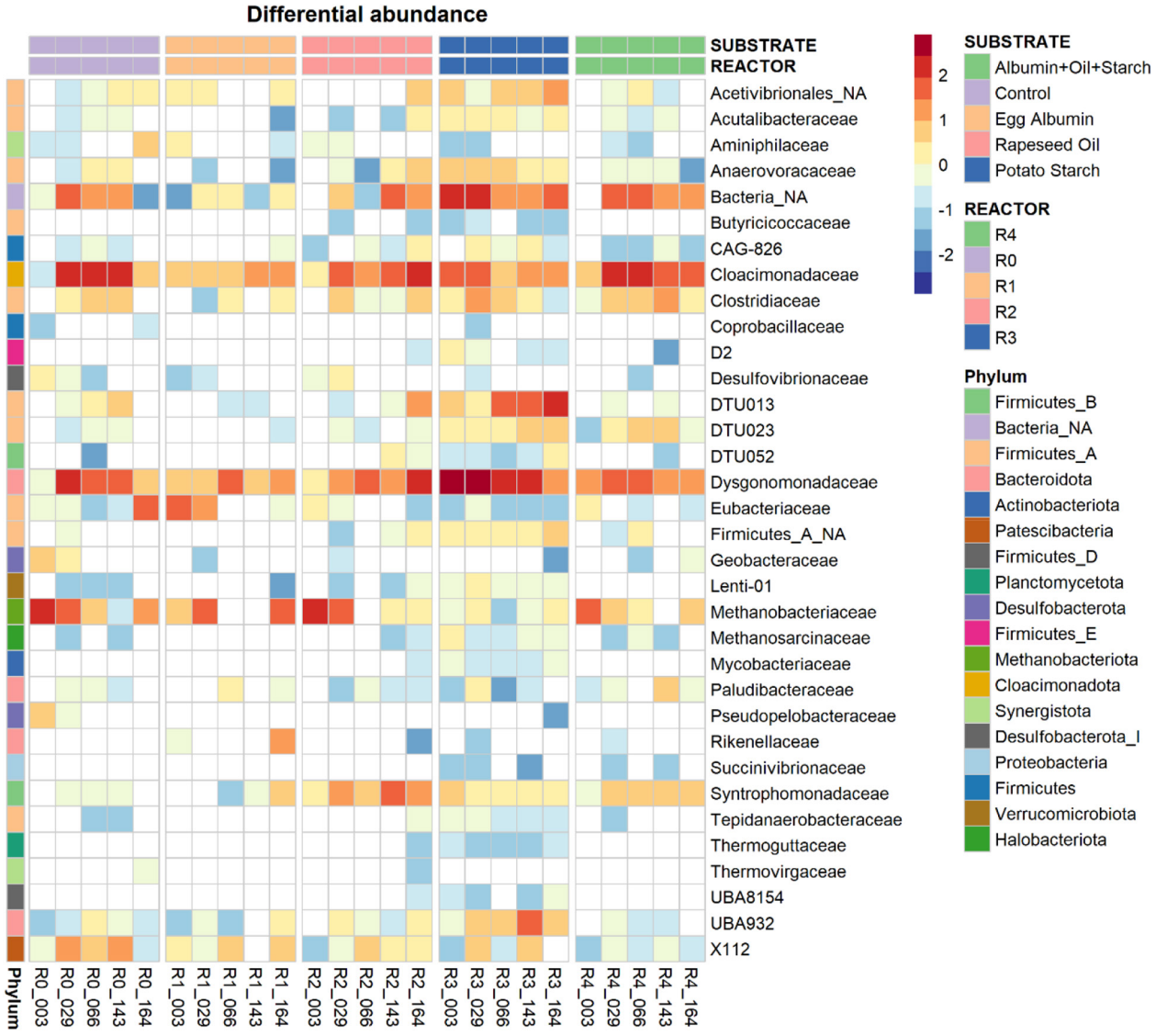

FIGURE 2 Heatmap of differentially abundant families (identified using 16S rRNA gene amplicon sequencing) in different reactors. Samples taken from reactors operating with cattle manure in mono-digestion (R0) or in co-digestion with egg albumin (R1), rapeseed oil (R2), potato starch (R3) or a mix of these co-substrates (R4). White colour represent not detected/not differentially abundant.

the samples from individual reactors were not clustered closely but the centroid of the samples moved along under the influence of the process parameters (Figure S10).

To further evaluate the specificity of the microbial taxa in relation to the co-substrate, differential abundance Log2 Fold Change analysis (LFCa) and Linear Discriminant analysis (LDa) in pairwise analysis of control versus individual reactors was used as a criterion for defining co-substrate specificity. In addition to the community profile changes described above, the LFCa and LDa results revealed co-substrate-specific positive and negative impacts on the microbial families. Egg albumin largely had a negative impact on the microbial community according to both LFCa (except minor positive effect on family *Porphyromonadaceae*) and LDa (Figures 2–4). The addition of rapeseed oil in R2 had a high (LFCa ~ 5 and LDa > 5) and significant ($p < 0.05$) positive impact on family *Syntrophomonadaceae*. In reactor R3, higher diversity of microbial community indicated a positive influence of addition of potato starch and, among other families, DTU013 and *Dysgonomonadaceae* were highly significant ($p < 0.05$) in both LFCa (>2) and LDa (>4) (Figures 3–4). Interestingly, according to both LFCa and LDa addition of potato starch had a significant positive effect on the methanogen family *Methanosarcinaceae*, but a negative effect on family *Methanobacteriaceae* (Figures 3–4). In reactor R4, families showing a significant positive effect ($p < 0.05$) in both LFCa (>2) and LDa (>4) values to addition of co-substrate mix were *Clostridiaceae*, DTU023 and *Syntrophomonadaceae* (Figures 3–4).

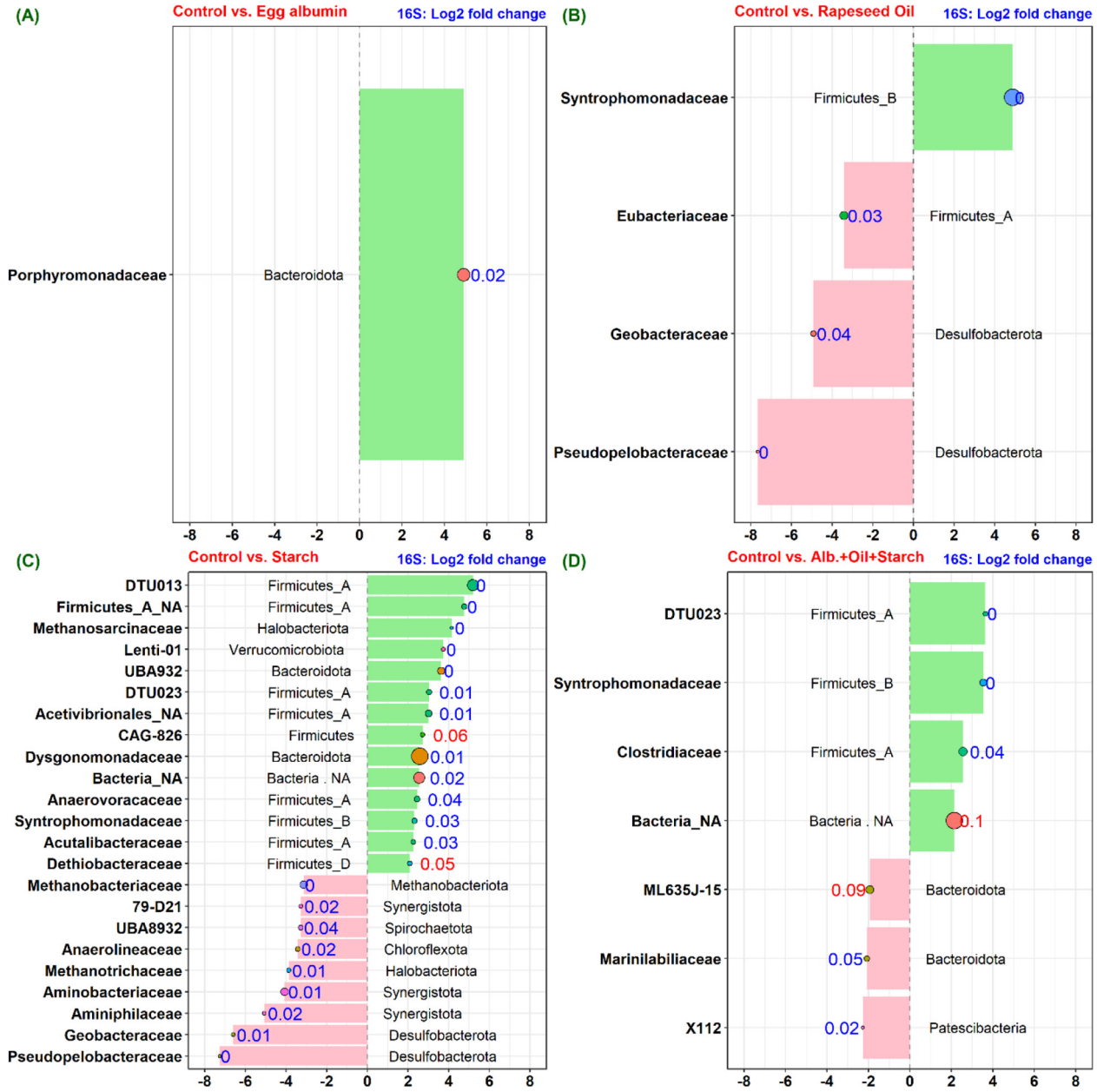

FIGURE 3 Differential abundance Log2 fold change analysis (LFCa) plot (16 S rRNA gene amplicon sequencing), showing Log2 fold change in microbial families. Positive and negative Log2 fold change represents a synergistic and inhibitory effect, respectively, on the microbial community at family level due to addition of co-substrate. (A) Control vs. egg albumin, (B) control vs. rapeseed oil, (C) control vs. potato starch and (D) control vs. albumin + rapeseed oil + potato starch. Bubble colour indicates phylum and bubble size baseMean in differential abundance analysis. The numerical value beside each bubble indicates the *p*-value of differential abundance analysis (blue $p < 0.05$, red $p > 0.05$).

FTHFS-harbouring microbial community

Formyltetrahydrofolate synthetase, which is a marker gene for the acetyl-CoA pathway, has recently been proposed and demonstrated as a marker gene candidate for microbiological surveillance of biogas plants (Singh, 2021; Singh et al., 2020; Singh, Moestedt, et al., 2021; Singh, Müller, & Schnürer, 2021). As a surveillance tool, FTHFS analysis provides the possibility to zoom into the microbial community based on physiological function. We used the FTHFS amplicon sequencing to analyse the bacterial community and its dynamics in co-digestion of CM with co-substrates. Compared with the reference reactor, a very distinctive community profile was observed for the reactors fed with protein, fat and carbohydrates. A reduction in family unclassified Cloacimonetes (LFCa ~ 0.5, LDa > 6), with a corresponding increase in family *Peptococcaceae* (LFCa > 2, LDa > 6), was observed in reactor R1 (Figures 5–7, Figure S11). Enrichment of family *Syntrophomonadaceae* (LFCa > 3, LDa > 5) was seen in reactor R2 and an increase in relative abundance of family *Eggerthellaceae* (LFCa ~ 1) and *Lachnospiraceae*

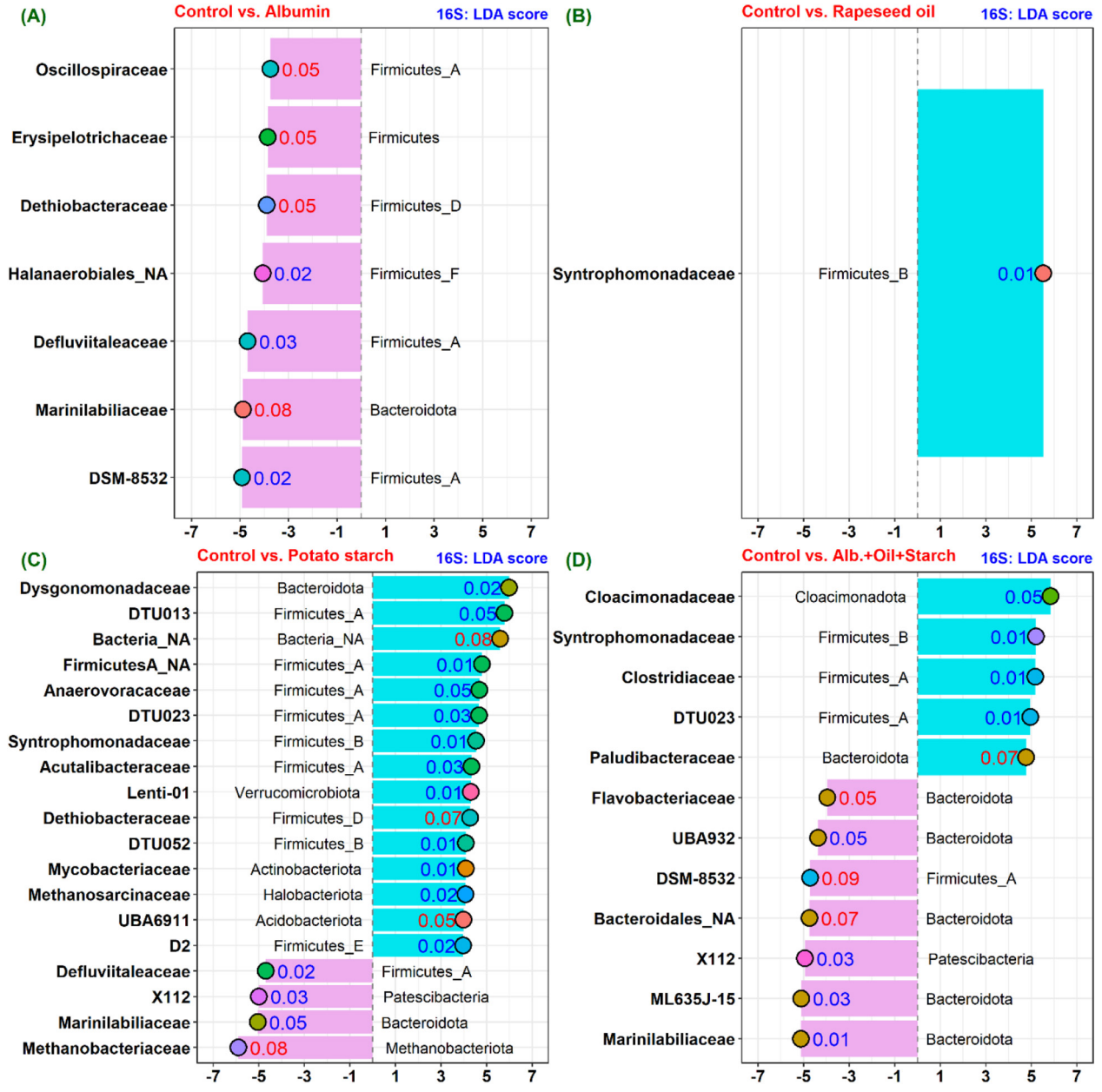

FIGURE 4 Linear discriminant analysis (LDa) plots (16S rRNA gene amplicon sequencing) showing microbial families positively or negatively associated with treatment in pairwise analysis with the control. (A) Control vs. egg albumin, (B) control vs. rapeseed oil, (C) control vs. potato starch and (D) control vs. albumin + rapeseed oil + potato starch. The numerical value beside each bubble indicates the *p*-value of LDa (blue $p < 0.05$, red $p > 0.05$).

(LFCa > 1, LDa > 5) in reactor R3. Family *Oscillospiraceae* (LFCa ~ 1, LDa ~ 5) was only observed in reactor R3, in all except the first sample, but relative abundance of this family was very low (1%–2%). Control reactor R0 and the reactor receiving a mixture of protein, fat and carbohydrates (R4) showed similar community profile, with relatively slightly higher abundance of family *Eggerthellaceae* (LFCa > 1, LDa > 5), *Lachnospiraceae* (LFCa > 1, LDa > 5), *Peptococcaceae* (LFCa ~ 0.5, LDa > 5) and family *Peptoniphilaceae* (>1%, LDa ~ 5) in all samples, although *Peptoniphilaceae* was only observed once (>1%) in R0 (Figures 5–7, Figure S11).

DISCUSSION

Importance of co-substrate for methane production in batch and continuous reactors

When the substrates were evaluated individually in batch reactor tests, fat (rapeseed oil) showed the highest BMP, followed by protein (egg albumin) and carbohydrates (potato starch), which is in line with the theoretical values for these macromolecules (Angelidaki & Sanders, 2004). For CM, previously

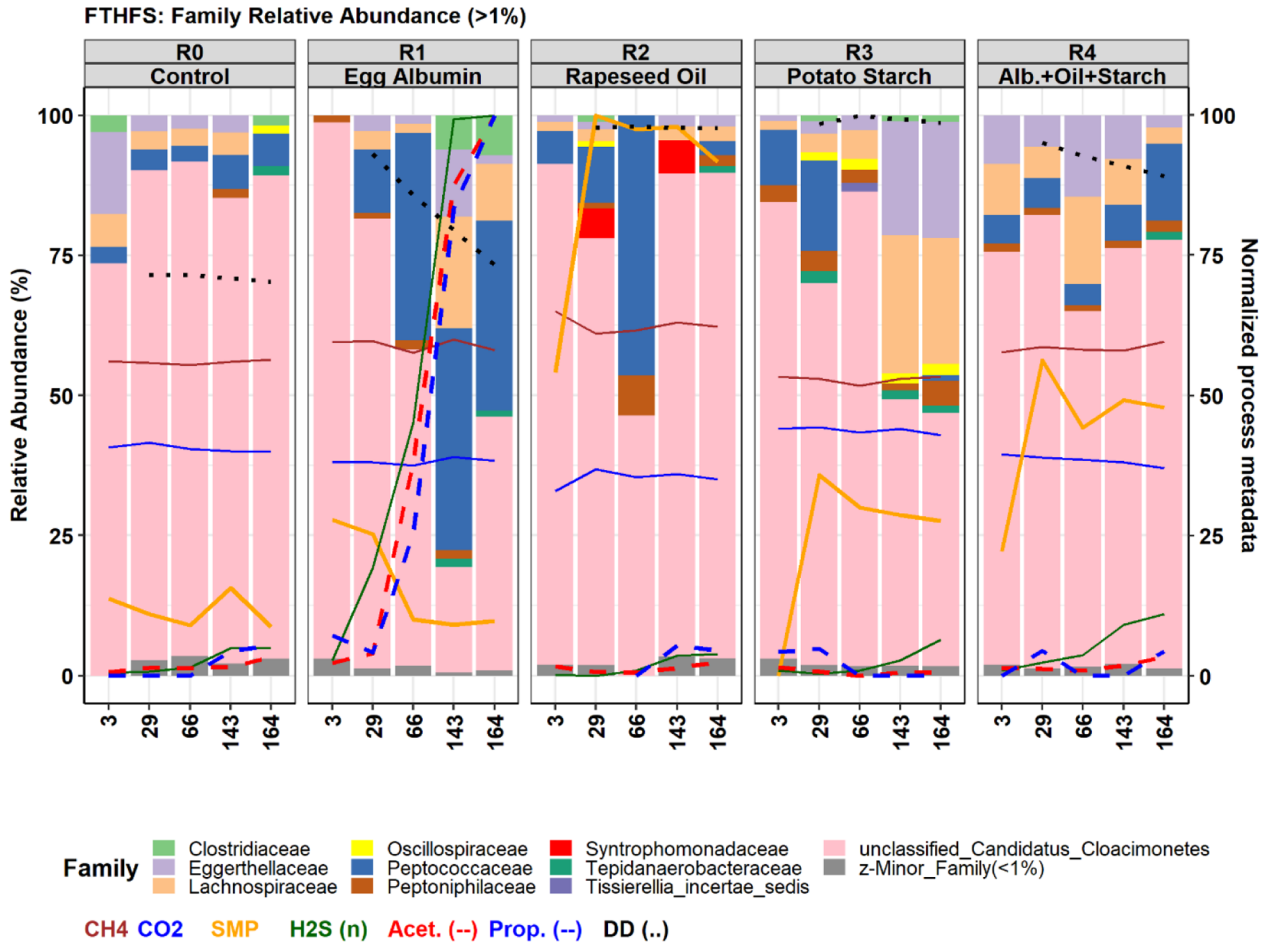

FIGURE 5 Relative abundance of the microbial community (identified using FTHFS gene amplicon sequencing) at family level (relative abundance >1%) in different reactors. Families with relative abundance <1% are merged in the category 'minor family'. Samples taken from reactors operating with manure in mono-digestion (R0) or in co-digestion with egg albumin (R1), rapeseed oil (R2), potato starch (R3) or a mix of these co-substrates (R4). Secondary y-axis shows normalised values (0–100) of individual process parameters, for which the unit of measurement is dependent on the parameter. The overlay lines represent normalised process parameters corresponding to the legends at the bottom of the diagram, where: CH_4 = methane (%), CO_2 = carbon dioxide (%), SMP = specific methane production (ml g VS^{-1} day^{-1}), H_2S = hydrogen sulphide (ppm), Acet. = acetate concentration (g L^{-1}), prop. = propionate concentration (g L^{-1}) and DD = degree of degradation (%).

reported values typically range between 150 and 265 ml CH_4 g VS^{-1} (Møller et al., 2004; Ruile et al., 2015; Triolo et al., 2011). The CM used in this study had BMP of 195 ± 4 ml CH_4 g VS^{-1}, that is, within the reported range. On digesting manure with the co-substrates no synergistic effects were seen, with the mixtures giving similar results as for additive values based on analysis of the single substrates. Previous studies investigating co-digestion of CM in batch reactors have used more complex co-substrates than in the present study, and thus direct comparison is difficult. However, a recent study identified synergistic effects during batch wise co-digestion of CM with food waste (rich in protein and lipids), and maize straw (rich in carbohydrates) (Zhang, Wang, Xing, et al., 2021). In the study by Zhang, Wang, Xing, et al. (2021), the effect varied depending on the proportion of co-substrate in the mix, which in all cases included a higher load of co-substrate than in the present study. The synergistic effects observed in that study were attributed to high relative abundance of both hydrogenotrophic and acetotrophic methanogens.

The CSTRs in the present study reached SMP values of 170–302 ml CH_4 g VS^{-1} (Table 2). These were in line with values reported in a meta-analysis of different studies on methane yield during anaerobic co-digestion of animal manure with other feedstocks, with mean methane yield in continuous reactors of 175.3 and 298.8 ml CH_4 g VS^{-1} for mono- and co-digestion of cattle manure respectively (Ma et al., 2020). Methane production in our CSTRs R0-R4 was in proportion to expected values based on batch trials, but with some indications of both synergistic and antagonistic effects during co-digestion. Reactors R1, receiving protein, and R2, receiving fat, showed significantly ($p < 0.05$) lower and higher methane production, respectively, than expected from the batch results. A recent meta-analysis identified a recommended synergy interval of carbon-nitrogen ratio of 20–27 for anaerobic co-digestion of

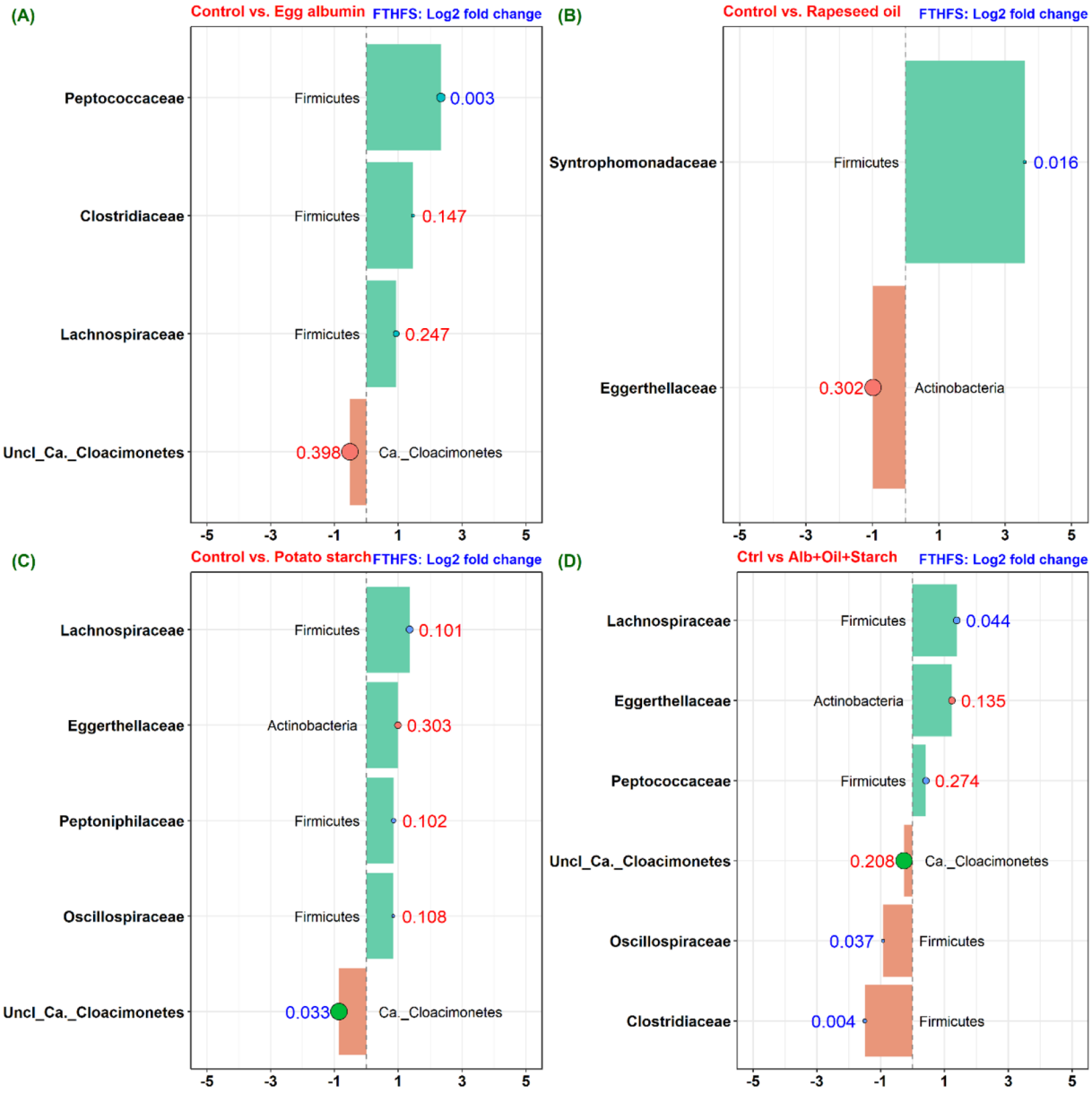

FIGURE 6 Differential abundance Log2 fold change analysis (LFCa) plot (FTHFS gene amplicon sequencing) showing Log2 fold change in microbial families. Positive and negative Log2 fold change represents a synergistic and inhibitory effect, respectively, on the microbial community at family level due to the addition of co-substrate. (A) Control vs. egg albumin, (B) control vs. rapeseed oil, (C) control vs. potato starch and (D) control vs. albumin + rapeseed oil + potato starch. Bubble colour indicates phylum and bubble size baseMean in differential abundance analysis. The numerical value beside each bubble indicates the *p*-value of differential abundance analysis (blue $p < 0.05$, red $p > 0.05$).

livestock manure, with a higher probability of synergy during co-digestion when the fat/carbohydrate ratio exceeds 0.13 and the protein/carbohydrate ratio exceeds 0.26 (Zhou et al., 2021). In line with this suggestion, the reactor showing synergistic effects on methane production, R2, was the only reactor receiving substrate with ratio values in the recommended range (0.11 and 0.67 respectively) (Figure S5). In contrast, reactor R1 showed significantly lower methane production, as expected from theoretical values and from results in the batch trial. This was most likely a consequence of ammonia inhibition. In R1, degradation of protein originating from the added egg albumin resulted in high total ammonium-nitrogen (TAN) concentration (up to 3.8 g L^{-1}), resulting in a free ammonia concentration of 0.4 g NH_3^+ L^{-1} (Table 2). In contrast, reactor R4, receiving a lower inclusion rate of albumin, reached more moderate levels (around 0.2 g NH_3^+ L^{-1}), with no apparent negative effect on process performance. Inhibition of biogas processes has been reported at various levels of free ammonia, depending on operating conditions, but is typically observed at around 0.15–0.5 g NH_3^+ L^{-1}

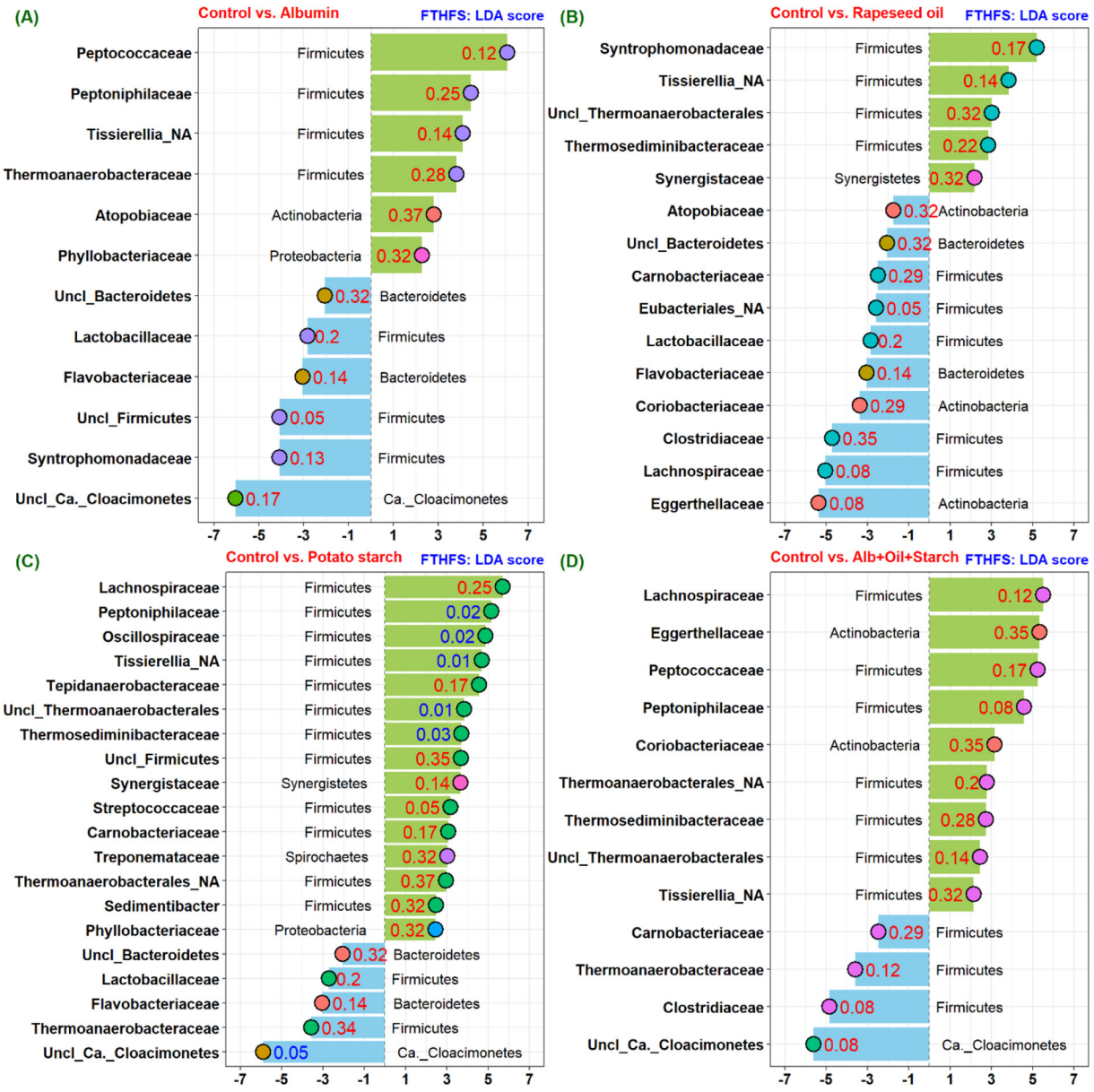

FIGURE 7 Linear discriminant analysis (LDa) plot (FTHFS gene amplicon sequencing) showing microbial families positively or negatively associated with treatment in pairwise analysis with the control. (A) Control vs. egg albumin, (B) control vs. rapeseed oil, (C) control vs. potato starch, and (D) control vs. albumin + rapeseed oil + potato starch. Bubble colour indicates phylum and the numerical value beside each bubble indicates the *p*-value of LDa (blue $p < 0.05$, red $p > 0.05$).

(Calli et al., 2005; Rajagopal et al., 2013; Westerholm et al., 2016). The inhibitory effect in R1 was illustrated by greater build-up of fatty acids than in the other reactors (Figures S2 and S3).

The reactors operating with co-digestion showed a smaller increase (Δ 0.5 g VS L^{-1} day^{-1}) in load than the reference reactor R0 (3.0 compared with 2.5 g VS L^{-1} day^{-1}). However, this increase still resulted in much more efficient use of the reactor volume for all reactors, with an increase in volumetric methane production of between 27% and 100% in the co-digestion reactors. The overall increase in yield brought about by co-digestion depends on both composition and VS contribution of the co-substrate. As all reactors in the present study were supplemented with the same amount of VS from the co-substrate, the composition of the co-substrate was the main influencing factor. Reactor R2, supplemented with fat, showed the highest gas production and highest methane content in the gas, resulting in the highest efficiency value (Table 2, Table S2). This is a reasonable observation considering the high energy content and high gas potential of fat (Angelidaki et al., 2011; Schnürer, 2016; Schnürer et al., 2016). In this study, the added fat was well degraded (~85%, Figures S4 and S5) and the reactors showed no sign of VFA accumulation. However,

co-digestion with fat-rich material can be challenging and, if fat is included at high rates, it can result in accumulation of fatty acids and inhibition of methanogenesis (Holohan et al., 2022). This was clearly illustrated in a study by Wang et al. (2021) where CM was co-digested with glycerol trioleate (fat, oil, grease (FOG)) or glucose, applied in an increasing load from 3.2 to 5 g VS L^{-1} day^{-1}. The load was increased in two or four successive steps but, regardless of loading strategy, the reactors receiving lipids suffered from inhibition caused by accumulation of long-chain fatty acids (LCFA). In contrast, no significant inhibition was seen on addition of glucose. The optimal load of FOG for co-digestion with sludge has been found to be 0.5%–1.5% (v/v), giving 80%–90% degradation of the lipid, while above this inclusion level FOG causes VFA accumulation and low LCFA degradation (Usman et al., 2020). The final load of rapeseed oil in reactors R2 and R4 corresponded to 1.6% and 0.5% (v/v), respectively, that is, it was within the suggested optimal range. In a meta-analysis/regression analysis by Ma et al. (2020), VS concentration and C/N ratio in the mixed substrate were identified as significant factors in improved methane yield compared with mono-digestion of CM, with optimal VS content of 18.2 g L^{-1} and C/N ratio of 35. In the present study, inclusion of co-substrate gave VS content close to this value (17.2 g L^{-1}, calculated based on the organic load and VS values from Table S1).

Degradability and nutrient content of digestate

In all cases, co-digestion gave a greater VS reduction than mono-digestion, with the highest values obtained for reactors R2 and R3, supplemented with fat and carbohydrates respectively (Table 2, Figure S5). Reactor R3 also showed the greatest degradation of cellulose and hemicellulose, suggesting a small synergistic effect of co-digestion. Insam and Markt (2016) suggested that co-digestion with small amounts of easily accessible substrates can result in a *priming effect*, that is, a non-additive interaction between decomposition of organic matter and the added substrate. In line with this suggestion, labile carbon (fructose) has been suggested to trigger a positive priming effect during co-digestion of swine manure (Lin et al., 2022). The results in the present study indicate a priming effect, but this cannot be completely proven as the differences seen were not statistically significant. In contrast to R2 and R3, reactor R1 showed the lowest degree of degradation, likely caused by ammonia inhibition as discussed. However, even with residual protein left in the digestate, this reactor showed higher degradation of proteins compared with the other reactors. Instead, among the different macromolecules present cellulose showed the lowest degradability in this reactor. The effect of ammonia on methanogens has been widely investigated, but less is known about ammonia inhibition of cellulolytic bacteria. One study on solid-state anaerobic digestion of maize stover revealed significant inhibition of the cellulose hydrolysis rate at a TAN concentration above 2.5 g L^{-1} (Wang et al., 2013) compared with 3.8 g L^{-1} in the present study. Inhibition of cellulose degradation by ammonia during digestion of cow manure and cellulose in batch reactors initiated with different inoculums has also been suggested (Li, Zhao, et al., 2020; Sun et al., 2016). In comparison, reactor R4, receiving a mixture of co-substrates and with TAN of 2.5 g L^{-1}, did not show lower cellulose degradability than the other reactors. In addition to ammonia inhibition, the low degradability in R2 could have been partly caused by general inhibition of microbial activity through trace element limitation, since degradation of albumin in this reactor resulted in production of a significantly higher level of H_2S in the raw biogas (Table 2, Figure S2). Elevated levels of H_2S decrease biogas quality and can also trap trace metals that are essential for microbial activity (Choong et al., 2016; Wang et al., 2016). As mentioned, the reactor receiving rapeseed oil (R2) had the highest efficiency value (Table 2 and Table S2).

Residual methane production

During optimisation of agricultural biogas processes, it is important to consider not only gas production and nutrient content in the digestate, but also residual methane production. An optimisation approach giving increased gas production can sometimes increase the risk of methane emissions during storage of digestate, decreasing the environmental benefits of biogas production from manure (Ahlberg-Eliasson et al., 2021). In the present study, the RMP values were in the lower range (68–78 L CH_4 kg^{-1}) as compared to previously reported values (range 20–240 L CH_4 kg^{-1}) (Ahlberg-Eliasson et al., 2017, 2021; Ruile et al., 2015). Comparing all reactors, R1 had a lower degradation rate and less accumulation of VFA, representing potential for residual methane production. However, this digestate had a rather low RMP value, suggesting that high ammonia levels hamper methane production not only in the biogas process but also during storage of digestate. This is supported by previous findings that RMP is lowered at ammonium-nitrogen levels above >2.7 g L^{-1} NH_4^+-N (as reviewed in Monlau et al., 2015). However, as illustrated in a recent study on biogas production from manure, RMP lowering at high ammonium-nitrogen levels is not a general rule (Ahlberg-Eliasson et al., 2021). Inhibition of methane production is thus most likely influenced also by other factors, such as temperature and pH, which affect the actual level of ammonia. The digestates from reactors R2 and R3 showed the highest RMP values, most likely represented by degradation of

residual co-substrate, for example, fat in R2. However, reactor R3 showed similar values despite high degradation of the added co-substrate. Thus, the indicated synergistic effect in degradation of cellulose and hemicellulose probably persisted during RMP measurement (Table S2).

Microbial community response

Sampling for microbial analysis began on the third day after addition of the co-substrate and analysis of these samples indicated an immediate effect that was most pronounced in the reactor (R3) receiving carbohydrates as co-substrate (Figures 1, 2, 5). However, the reference reactor R0 also showed changes in community structure and dynamics over time, in line with previous findings in studies of manure digestion that stable, non-altered anaerobic digesters can have a highly dynamic community structure (Fernández et al., 1999; St-Pierre & Wright, 2014). The addition of different co-substrates resulted in enrichment of candidate microbes specific to the co-substrate added to the reactors, supporting previous findings that several sets of bacterial species are associated with specific substrate categories used in anaerobic reactors (Amani et al., 2010; Zhang, Wang, Xing, et al., 2021). We also identified key candidate taxa that were specific to the different macromolecules, which has not been done previously at this level of resolution in CM-based reactors. In addition to the 16S rRNA gene amplicon sequencing results, FTHFS gene amplicon sequencing further helped in visualisation of microbial community profile. Acetogens and FTHFS-harbouring bacterial communities are phylogenetically very diverse and metabolically dextrous components of the overall microbial community (Singh et al., 2019) and are associated with many degradation steps in biogas reactors. The FTHFS analyses revealed some specialist bacterial taxa with potential for metabolic tasks such as degradation of complex plant material (family *Lachnospiraceae*) (Beaumont et al., 2021; Koeck et al., 2015; Lebuhn et al., 2014; Suksong et al., 2019), known syntrophic acetate/propionate/butyrate-oxidising bacteria (*Tepidanaerobacter*, *Syntrophomonas*, etc.) (Singh & Schnürer, 2022) and proposed acetate/propionate-oxidising bacteria (phylum *Cloacimonadota* and family *Clostridiaceae*, *Peptococcaceae*) (Ahlert et al., 2016; Singh & Schnürer, 2022; Singh, Schnürer, & Westerholm, 2021; Westerholm et al., 2022). Specific changes in community profile in the control reactor and reactors with co-substrates are further discussed below.

In all experimental reactors, the microbial community was dominated by two phyla, *Firmicutes* and *Bacteroidota*, which in turn were dominated by class *Clostridia* and *Bacteroidia* respectively. This is in line with previous findings for biogas processes operating with manure (Ahlberg-Eliasson et al., 2021; Chen et al., 2016; Güllert et al., 2016; St-Pierre & Wright, 2014; Sun et al., 2015). *Firmicutes* and *Bacteroidota* are primarily involved in initial decomposition of organic matter via hydrolysis and acidogenesis of complex polysaccharides, but can also be engaged in protein degradation (Vanwonterghem et al., 2016; Westerholm et al., 2018).

Families belonging to phylum *Firmicutes*, class *Clostridia*, were observed to be present in higher and differential abundance in R3. Additionally, families (DTU013, DTU023, *Dysgonomonadaceae*) that harbour known and potential cellulolytic bacteria (e.g. *Clostridiaceae* spp., *Fermentimonas*, *Hungateiclostridiaceae* spp., *Ruminiclostridium* spp. etc.), together with unknown family candidates (Acetivibrionales_NA, Bacteria_NA, Firmicutes_NA), showed higher differential abundance (high LFCa and LDa values) in R3 compared with the control or other reactors (Figures 2–4). FTHFS analyses of reactor R3 showed significant differential abundance also of family *Oscillospiraceae*, *Lachnospiraceae*, *Eggerthellaceae* and *Peptoniphilaceae*. Family *Oscillospiraceae* and *Lachnospiraceae* include members with saccharolytic capacity and with glycoside hydrolase enzymes responsible for the degradation of cellulose and hemicellulose (Beaumont et al., 2021; Laptev, 2021). Representatives of these families are common in rumen/gut environments, but have also been isolated from biogas environments (Flaiz et al., 2020; Rettenmaier et al., 2021). The availability of readily accessible carbohydrates in the form of soluble starch in R3 likely increased the relative abundance of the saccharolytic families, and potentially also increased their metabolic activity. Combined with the process data, this confirms that addition of starch has the potential to give a priming effect by boosting microbial abundance and their physiological activity and improving degradation of lignocellulose. Priming is a well-known process in nature and has also been suggested to occur during co-digestion of sewage sludge and whey (Aichinger et al., 2015). The overall increase in microbial community richness seen in reactor R3 could explain the greater degree of lignocellulose degradation than in the reference reactor (Figure S5). The enrichment of family *Eggerthellaceae* and family *Peptoniphilaceae* suggests that the addition of starch was also positive for protein degradation. Both families contain known protein- (peptone, polypeptide) and amino acid-degrading species (Ezaki & Kawamura, 2015; Gupta, 2021; Johnson et al., 2014). Enhanced protein degradation at the end of operation of reactor R3 was also indicated by an increase in H_2S levels (Figure 5).

In an opposing trend to R3, reactor R1 showed decreased richness and evenness over time, likely explained by the increasing levels of ammonia (Lv et al., 2019). High ammonia levels result in inhibition of methanogens, giving overall less efficient degradation

and VFA accumulation (Capson-Tojo et al., 2020), as also seen in present study for reactor R1 (Figures S2–S4). High ammonia levels typically result in a shift from acetoclastic methanogenesis to syntrophic acetate oxidation (SAO), enabling methane production at high ammonia levels (Liu et al., 2017; Sun et al., 2016; Westerholm et al., 2016). Such a shift was not detectable in the sequencing data, but qPCR analysis revealed the presence of two known syntrophic acetate oxidisers, *Syntrophaceticus schinkii* and *Schnuerera ultunensis* (Figure S12). *Syntrophaceticus schinkii* was present in all reactors and showed higher abundance at day 164 than at day 3 in both R0 and R2, while *S. ultunensis* was present in significantly higher abundance at day 164 compared with day 3 only in R2, suggesting higher SAO activity in this reactor.

As discussed above, previous studies have reported an inhibitory effect of ammonia not only on methanogens, but also on cellulose degradation consortia, which would explain the higher levels of cellulose seen in the digestate from reactor R1. In line with this, lower differential abundance was indicated for several known cellulolytic families, such as *Oscillospiraceae* and *Defluvitaleaceae*, based on LFCa and LDa values for R1 compared with the control (Figures 3 and 4). In FTHFS-based analysis, reduced relative and differential abundance of phylum *Ca*. Cloacimonetes was shown in R1. Members within this phylum are proposed to have the capacity to use both amino acids and carbohydrates and to perform propionate oxidation (Johnson & Hug, 2022; Westerholm et al., 2022). In previous studies on biogas processes, this phylum has been suggested as a biomarker for process disturbance (Klang et al., 2019, 2020; Singh, 2021; Singh, Moestedt, et al., 2021; Singh, Müller, & Schnürer, 2021). In the present study, the reduced abundance of families belonging to phylum *Ca*. Cloacimonetes, under the influence of increased VFA, ammonia and reduced pH, can likely be seen as indicating an approaching disturbance. This confirms that functional FTHFS gene-based analysis is a strong method for detecting process disturbance. The FTHFS-based analysis also revealed that family *Peptococcaceae* is a more tolerant (to ammonia and VFA levels) and efficient propionate degrader than *Ca*. Cloacimonetes, and is thus a more sensitive indicator of process disturbance. A recent study on phylum *Cloacimonadota* showed that propionate oxidation is not a characteristic feature of this phylum (Johnson & Hug, 2022).

Irrespective of the target gene used for microbial community analysis (FTHFS or 16S rRNA gene), family *Syntrophomonadaceae* increased in relative and differential abundance over time in reactor R2, as confirmed by the LCFa and LDa values (Figures 1–7). In family *Syntrophomonadaceae*, >13 species have been characterised as capable of LCFA degradation (Alves et al., 2009; McInerney et al., 2008; Sousa et al., 2009). This could explain the high degradability of fat in R2 (Figure S5). Degradation of fat results in glycerol and LCFA, with the latter being a known (microbial) inhibitor often seen accumulating and resulting in foaming (He et al., 2017; Rodríguez-Méndez et al., 2017). A possible strategy to overcome this problem is to use pulse feeding instead of continuous feeding, which improves the conversion rate of the LCFA-degrading community dominated by *Syntrophomonadaceae* (Ziels et al., 2018). In the present study, all fat was added at once, combined with the manure, and apparently this feeding approach was sufficient to allow the enriched population of *Syntrophomonadaceae* (genus JAAYJK01, also *Syntrophomonas* sp. according to older taxonomy) to efficiently degrade the fat, resulting in enhanced overall efficiency of the process (Figure S1, Figures 2–7).

The community pattern in R4 was similar to that in R0, which is likely explained by the balanced co-substrate mixture (1:1:1 egg albumin: rapeseed oil: potato starch) and lower load of each substrate than in reactors R1-R3. Thus, the microbial community was probably not exposed to high selection pressure from increased amount of any specific nutrient-rich co-substrate. Although the overall community structure in reactor R4 was similar to that in the control reactor, an interesting and very sensitive insight was obtained by the FTHFS analysis of the microbial community response to addition of a mix of co-substrates. Initially, the easily digestible carbohydrates were likely degraded mainly by *Lachnospiraceae* and *Eggerthellaceae* (day 3) (Figure 5, Figure S11), which probably caused the slight increase in VFA levels (day 29). With increasing VFA level, a decrease in the relative abundance of phylum *Ca*. Cloacimonetes (day 66) was observed and the increase in VFA, especially propionate, instead probably stimulated the propionate-degrading *Peptococcaceae*, causing the reduction in propionate levels (day 66). In parallel, the higher microbial abundance and probable activity of *Lachnospiraceae* and *Eggerthellaceae* together with *Peptoniphilaceae* (Figure 5, Figure S11) and continuous addition of proteins in the co-substrate mix were associated with a gradual increase in H_2S levels over time (Figure 5). As mentioned, *Ca*. Cloacimonetes has been proposed as a process biomarker, but the results for reactors R2 and R4 suggest that *Ca*. Cloacimonetes, together with *Peptococcaceae*, increases resilience of the process to disturbance (increased levels of VFA) and allows recovery to relative stability.

CONCLUSIONS

This study clearly showed that manure-based biogas production can be improved by addition of co-substrates with different chemical composition, but with some differences in microbial community development/

dynamics, gas production, digestate nutrient values and residual methane production. All investigated co-substrates resulted in higher total gas production compared with manure alone and in the case of albumin also a higher ammonium-nitrogen level in the digestate. The addition of rapeseed oil gave the overall highest gas yield, with values indicating synergistic effects, possibly due to the highly enriched population of *Syntrophomonadaceae*. The addition of albumin as co-substrate caused some instability, with increasing VFA levels and less efficient degradation of cellulose, probably due to ammonia inhibition of cellulolytic activity and methanogenesis. In contrast, co-digestion with potato starch suggested a priming effect, with slightly more efficient degradation of the fibre fraction than in mono-digestion of manure, and enrichment of the known cellulolytic *Acetivibrionales*, *Lachnospiraceae* and *Oscillospiraceae*. However, the digestate from the reactor with potato starch showed higher RMP, suggesting continued degradation of fibre in the digestate. Interestingly the protein-supplemented reactor showed the lowest RMP values, suggesting that ammonia inhibition could be a measure to reduce the risk of methane emissions during storage of digestate. Comparison between the FTHFS and 16S rRNA gene-based microbial analysis illustrated that the former more clearly could identify a link between the applied co-substrate and the related microbial community, such as dynamics of propionate-degrading bacteria in the albumin-supplemented reactors (*Ca*. Cloacimonetes and *Peptococcaceae*),

In conclusion, several parameters should be considered when selecting a suitable co-substrate for biogas production from manure. To optimise process outputs, it is important to target the desired outcome, that is, gas yield or digestate nutrient content, while also considering overall efficiency, risk of instability and methane emissions during storage. A mixture of proteins, fats and carbohydrates can be used as co-substrate to increase resilience of the microbial community to process disturbance in a manure-based biogas process. This will also increase digestion rates and the fertiliser quality of the digestate (higher nitrogen content), without any harmful effects on microbial community or on biogas quality and quantity. It is thus a more balanced approach than mono-digestion of animal manure.

AUTHOR CONTRIBUTIONS

Karin Ahlberg-Eliasson: Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (supporting); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Abhijeet Singh:** Conceptualization (supporting); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Simon Isaksson:** Formal analysis (supporting); methodology (supporting); visualization (supporting). **Anna Schnürer:** Conceptualization (equal); data curation (supporting); formal analysis (supporting); funding acquisition (equal); investigation (equal); methodology (equal); project administration (lead); resources (lead); supervision (lead); validation (equal); visualization (supporting); writing – original draft (supporting); writing – review and editing (lead).

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CONFLICT OF INTEREST

The authors delare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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