Adaptation of Methanogenic Communities to the Cofermentation of Cattle Excreta and Olive Mill Wastes at 37°C and 55°C[∇][†]

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The acclimatization of methanogens to two-phase olive mill wastes (TPOMW) was investigated in pilot fermenters started up with cattle excreta (37°C) and after changing their feed to excreta plus TPOMW (37°C or 55°C) or TPOMW alone (37°C) until a steady state was reached (28 days). Methanogenic diversity was screened using a phylogenetic microarray (AnaeroChip), and positive targets were quantified by real-time PCR. Results revealed high phylogenetic richness, with representatives of three out of the four taxonomic orders found in digesters. *Methanosarcina* dominated in the starting excreta (>96% of total 16S rRNA gene copies; over 45 times more abundant than any other methanogen) at high acetate (0.21 g liter^{-1}) and ammonia N concentrations (1.3 g liter^{-1}). Codigestion at 37°C induced a 6-fold increase of *Methanosarcina* numbers, correlated with CH_4 production ($r_{Pearson} = 0.94$; $P = 0.02$). At 55°C, the rise in temperature and H_2 partial pressure induced a burst of *Methanobacterium*, *Methanoculleus*, *Methanothermobacter*, and a group of uncultured archaea. The digestion of excreta alone resulted in low but constant biogas production despite certain oscillations in the methanogenic biomass. Unsuccessful digestion of TPOMW alone was attributed to high Cu levels inducing inhibition of methanogenic activity. In conclusion, the versatile *Methanosarcina* immediately adapted to the shift from excreta to excreta plus TPOMW and was responsible for the stimulated CH_4 production at 37°C. Higher temperatures (55°C) fostered methanogenic diversity by promoting some H_2 scavengers while yielding the highest CH_4 production. Further testing is needed to find out whether there is a link between increased methanogenic diversity and reactor productivity.

Turning residues into energy is a societal and scientific priority due to climate change, fossil fuel exhaustion, and waste accumulation. In 2006, in Europe (EU27), less than 3% of electricity production came from biomass and wastes (11). Biogas plants, which anaerobically treat organic wastes to produce energy, are increasingly promoted in Europe, but their distribution is highly biased (35). While thousands of full- and farm-scale biogas plants are spread over central and northern Europe, anaerobic digestion technology in Mediterranean countries—Portugal, Spain, Italy, Greece, and Turkey—is in its early stages (35). These nations and other circum-Mediterranean countries lead in the production of olive oil and thus in olive mill wastes and wastewaters, which have a huge biogas production potential due to their lipid composition (1). Spain alone generates one-third of the world's oil production and millions of tons of two-phase olive mill wastes (TPOMW) per year. TPOMW are mostly burned or composted (28), hence releasing methane into the atmosphere. This compels a change in strategy: methane production from TPOMW should be optimized in engineered environments and transformed into energy.

TPOMW is a humid residue containing the olive pulp and stone. Its anaerobic digestibility is hampered by its low pH, low ammonia N, and high content in antimicrobial substances (1). However, it has been successfully fermented under laboratory conditions by supplementing it with nutrients and increasing the reactor organic loading rate stepwise (2) or by codigesting it with residues with a high buffering capacity, e.g., cattle excreta (17). These approaches seem to facilitate the adaptation of the methane-producing anaerobic community to the environmental conditions that TPOMW impose.

Methanogenic archaea—microbes clustered within five orders of the *Euryarchaeota*—constitute the last step in the trophic chain of decomposers degrading organic matter in oxygen-free environments (36). Methanogenesis is often the rate-limiting step of anaerobic digestion of organic wastes (3) due to the fast duplication times of bacteria, which generate all substrates for the slow-growing methane-producing archaea. It is also the most sensitive step in processing imbalances (4), likely due to the lack of functional redundancy among methanogens (8). High concentrations of volatile fatty acids, salts, ammonia, and heavy metals can be inhibitory for methanogens (5, 22) and are the most common reasons for reactor failure (3). Our objective was to understand the adaptation of methanogenic communities to TPOMW. We investigated methanogenic diversity and abundance in pilot digesters fed with cattle excreta and after changing their feed to TPOMW or TPOMW plus excreta. We expected that mixing both residues would allow a faster adaptation and more efficient performance of the methanogenic communities in digesting TPOMW. The cofermentation was evaluated at 37°C and 55°C. During an acclimatization period of 28 days, we screened the methanogenic

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diversity using an in-house-devised phylogenetic microarray, the AnaeroChip (13), and quantified dominant genera by real-time quantitative PCR (qPCR). We have taken primers from the literature, and we present four new sets of genus-specific primers and SYBR green I-optimized assays for quantifying methanogens in anaerobic environments.

MATERIALS AND METHODS

Reactor operation and performance. Four 75-liter (working volume) anaerobic continuously stirred tank reactors were completely loaded with cattle excreta (mixed excrements and urine), progressively heated up to 37°C, and fed daily with 3.5 liters day^{-1} of cattle excreta, corresponding to a hydraulic retention time (HRT) of 21.4 days. Similar steady conditions were achieved in all reactors, that is, stable biogas production of 241 ± 18 ml biogas liter^{-1} sludge day^{-1} with 68.7% ± 0.4% (vol/vol) CH$_4$ for three consecutive days and similar physical and chemical properties of the effluent sludge (average data are given in Table 1). This initial step was performed in order to set up common starting conditions for all reactors and prove reproducibility of reactor performance under equal operating conditions. On day 4, cattle excreta were replaced with the following specific feed solutions at a flow rate of 3.5 liter day^{-1}. Reactors EX_OL37 and EX_OL55 were fed with a mixture of 3:1 excreta/TPOMW and operated at 37°C or 55°C. Both showed an immediate and sharp increase in CH$_4$ production that stabilized as soon as 4 and 14 days, respectively, after changing of the feed. Biogas production averaged 1,096 and 1,289 ml biogas liter^{-1} sludge day^{-1} at 37°C and 55°C after the complete turnover of the reactor load (21.4 days). The other two reactors were loaded with either cattle excreta/water (EX37) or water/TPOMW (OL37) at a ratio of 3:1 and operated at 37°C to assess the contribution of each residue to the mesophilic codigestion. Reactor EX37 performed constantly, and after one HRT, it produced 251 ml biogas liter^{-1} sludge day^{-1}. Reactor OL37 acidified, its volatile fatty acid/alkalinity ratio exceeding 0.8 after 21 days, and CH$_4$ production ceased completely on day 30. A detailed analysis of all operational parameters can be found elsewhere (17).

Sampling of sludge, DNA extraction, and AnaeroChip analyses. Three sludge samples (ca. 500 ml) were collected every 3.5 days from each reactor. One subsample was used for determination of physical and chemical parameters. Table 1 summarizes the physical and chemical properties of the influent and effluent sludge after one HRT. A second subsample was kept at −20°C until DNA extraction.

Sludge samples (1 ml) were submitted to three freeze-thaw cycles consisting of 30 min at −80°C and 5 min at 65°C. After centrifugation (5 min, 14,000 rpm), DNA was extracted from the pellet using the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA). Sludge DNA from samples collected on days 4, 14, and 28 was subjected to PCR amplification of the 16S rRNA gene using the universal archaeal primers 109F and 934R (Table 2) to screen the methanogenic diversity in the start, middle, and end stages of the codigestion. PCR amplifications were performed in a Flexcycler instrument (Analytik Jena, Germany) in 50-μl volumes, with each reaction mixture containing a final concentration of 1× reaction buffer [16 mM (NH$_4$)$_2$SO$_4$, 67 mM Tris-HCl [pH 8.8], 1.5 mM MgCl$_2$, 0.01% Tween 20], 200 μM each deoxynucleoside triphosphate (dNTP), 0.8 μM primer 109F with a Cy5 label in the 5′ end, 0.2 μM primer 934R with a PO$_4$- group in the 5′ end, 0.1 mM MgCl$_2$, 0.4 mg ml^{-1} bovine serum albumin (BSA), 1.25 U BioTherm DNA polymerase (GeneCraft, Germany), 10 mM tetramethylammonium chloride, and sterile water. Two μl DNA was directly applied to the reaction mix. Thermal cycling, preparation of single-stranded PCR products, array hybridization, washing, and scanning were performed as described in reference 13.

Real-time quantitative PCR. Sludge DNA extracted from samples collected on days 4, 7, 14, 21, and 28 was subjected to real-time quantitative PCR (qPCR) amplification with the genus-specific primers in Table 2. Selection of target organisms was based on positive AnaeroChip results. Primers were either obtained from the literature or designed using the ARB software package (24) as described in reference 13. Standard curves were constructed with PCR-amplified 16S rRNA sequences from pure cultures or environmental clones using the group-specific primers in Table 2 and the source DNA in Table 3. Endpoint PCR amplifications were performed in 25-μl volumes as above with slight modifications (0.2 μM each primer, 0.63 U BioTherm DNA polymerase [GeneCraft, Germany], 1× enhancer [Peqlab, Germany], and no extra MgCl$_2$ added). One μl DNA was applied directly to the PCR mix. Thermal cycling was initiated with 5 min at 95°C, followed by 30 cycles consisting of 45 s at 95°C, 45 s at 51 to 60°C (see specific annealing temperature for endpoint PCR in Table 3), and 45 s at 72°C and terminated with 10 min at 72°C. PCR products were purified (Nucleo-

TABLE 1. Physical and chemical sludge properties in influent and effluent solutions and gas quality parameters in all reactors under initial steady-state conditions and after 1 HRT[a]

| Parameter[b] | Initial value | | | Value after 1 HRT for reactor: | | | | | | | | | | | |
| | | | | EX_OL37 | | | EX_OL55 | | | EX37 | | | OL37 | | |
	IN	EF	Gas quality	IN	EF	Gas quality	IN	EF	Gas quality	IN	EF	Gas quality	IN	EF	Gas quality
TS (% [wt/vol])	4.7	4.0 (0.1)		11.0	5.6 (0.4)		11.0	5.3 (0.4)		3.6	3.2 (0.0)		7.5	3.7 (0.1)	
VS (% [wt/wt] of TS)	71.1	66.5 (0.8)		75.1	74.7 (1.0)		75.1	72.3 (0.8)		71.1	63.8 (0.4)		21.8	71.4 (2.1)	
Particulate COD (g liter^{-1})	25.5	20.0 (0.4)		74.9	28.8 (0.9)		74.9	26.1 (0.2)		19.2	16.5 (0.05)		52.7	23.0 (0.3)	
Soluble COD (g liter^{-1})	14.7	7.0 (0.7)		43.6	12.3 (0.8)		43.6	19.0 (1.7)		11.0	5.7 (0.4)		28.4	13.7 (0.1)	
pH	7.4	7.80 (0.02)		6.7	7.67 (0.04)		6.7	7.85 (0.06)		7.4	7.60 (0.06)		4.9	7.20 (0.06)	
EC (mS cm^{-1})	17.2	16.4 (0.8)		14.4	16.0 (0.1)		14.4	16.6 (0.1)		17.2	15.1 (0.2)		4.1	12.2 (0.4)	
Acetate (g liter^{-1})	3.34	0.21 (0.01)			0.21 (0.09)			0.20 (0.03)			0.03 (0.01)			2.70 (0.4)	
Propionate (g liter^{-1})	0.64	BDL[c]			0.55 (0.03)			0.60 (0.14)			BDL			1.21 (0.12)	
NH$_4$-N (g liter^{-1})	1.20	1.32 (0.04)		0.83	0.88 (0.01)		0.83	1.02 (0.01)		0.90	1.07 (0.03)		BDL	0.58 (0.02)	
Alkalinity (mmol H$^+$ eq liter^{-1})	166	188.7 (6.4)		124	188.6 (2.4)		124	191.6 (1.4)		125	172.8 (2.4)		10	110.3 (10.3)	
CH$_4$ (liters day^{-1})			12.9 (0.7)			54.3 (4.1)			66.5 (7.5)			12.9 (1.5)			2.4 (2.6)
H$_2$ (ml day^{-1})			0.34 (0.07)			4.26 (0.63)			38.38 (7.67)			0.27 (0.07)			0.34 (0.55)

[a] IN, influent; EF, effluent; "Initial," initial steady-state conditions (excreta at 37°C); one HRT = 21.4 days. Reactor conditions: for EX_OL37, 3:1 excreta/TPOMW at 37°C; for EX_OL55, 3:1 excreta/TPOMW at 55°C; for EX37, 3:1 excreta/water at 37°C; for OL37, 3:1 water/TPOMW at 37°C. "Initial" data are averages for all four reactors. Effluent sludge data after 1 HRT are averages for the last two sampling dates. CH$_4$ and H$_2$ data are averages of seven consecutive daily measurements. Standard deviations are given in parentheses.

[b] TS, total solids; VS, volatile solids; COD, chemical oxygen demand; EC, electrical conductivity; NH$_4$-N, ammonia nitrogen.

[c] BDL, below detection limit.

TABLE 2. Primers used in this study

Target	Primers	Sequences (5′–3′)	E. coli positions	Reference
Archaea	109F	ACKGCTCAGTAACACGT	109–125	18
	934R	GTGCTCCCCCGCCAATTCCT	915–934	
Methanosarcina	240F	CCTATCAGGTAGTAGTGGGTGTAAT	240–264	14
	589R	CCCGGAGGACTGACCAAA	589–606	
Methanosaeta	MS1b	CCGGCCGGATAAGTCTCTTGA	585–606	30
	SAE835R	GACAACGGTCGCACCGTGGCC	835–851	
Methanoculleus	298F	GGAGCAAGAGCCCGGAGT	298–315	14
	586R	CCAAGAGACTTAACAACCCA	586–606	
Methanothermobacter	410F	CTCTTAACGGGGTGGCTTTT	410–440	14
	667R	CCCTGGGAGTACCTCCAGC	667–686	
Uncultured group	195F	AAAACTCCGGTGCCTTAGGATT	195–230	14
	330R	CCCGTAGGGCCTGGACTCA	330–348	
Methanobrevibacter	210F	TTTCGCCTAAGGATGGGTCT	210–365	This study
	367R	CGATTTCTCACATTGCGGAG	367–386	After 32[a]
Methanobacterium	fMbium	CGTTCGTAGCCGGCYTGA	576–593	32
	748R	TTCGTTACTCACCGTCAGGT	748–767	This study
Methanosphaera	594F	TAAGTCTTTGGTGAAAGCTT	594–613	This study
	747R	GTTACTCACCGTCAAGAT	747–764	
Methanocorpusculum	193F	TCCTCGAAAGATCCGTC	193–219	This study
	488R	CTGCCCTTTCTTCACATA	488–504	

[a] Modified from reference 32.

Spin kit extract II; Macherey-Nagel, Germany), eluted with prewarmed 1× DNase-free Tris-EDTA (TE) buffer in low-DNA binding tubes (Biozym, Austria), quantified in triplicate with the Quant-iT PicoGreen double-stranded DNA kit (Invitrogen, Carlsbad) using low-autofluorescent-microtiter plates (Nunc, VWR International, Austria), and stored at −20°C. The total number of copies of the 16S rRNA gene in the stock DNA was calculated from the length of the amplified fragment and the DNA concentration, assuming that the average weight of a base pair is 650 Da.

Real-time PCR amplifications were conducted using a Rotor-Gene 6000 system (Corbett Life Sciences, Australia) in 20-μl volumes, with each standard reaction mix containing 1× Quantimix Easy SYG kit (Biotools, Spain; 0.8 × for Methanosphaera), 100 nM each primer (150 nM for Methanoculleus and Methanobacterium and 200 nM for Methanosphaera), 0.4 mg ml⁻¹ BSA, and distilled water. Two μl sludge DNA was applied to the PCR mix. Ther-

mocycling was as follows: 95°C for 5 min, 40 cycles of 20 s at 95°C, 20 s at 58 to 65°C (see specific annealing temperature for real-time PCR in Table 3), and 20 s at 72°C. Runs were completed with a melting analysis (65°C to 95°C; ramp, 0.5°C/min) to check for product specificity and primer dimer formation. Each assay included five standard solutions that were freshly prepared from the stock (Table 3). All standards and samples were run in duplicate.

Shannon's diversity index (H') and evenness (E) were calculated using the equations $H' = -\Sigma\ (p_i \times \ln p_i)$ and $E = H'/\log S$ (25), where p_i denotes the ratio of the corrected 16S rRNA gene copy number of each methanogenic genus to the sum of gene copies of all quantified methanogens, and S denotes the number of methanogenic genera detected at a percentage greater than 0.01. Univariate analysis of variance (ANOVA) was used to test for differences in diversity (Shannon's index) among reactors at the start (day 4) and end (day 28) of the experiment with the SPSS software program, v. 17.0.

TABLE 3. Source DNA, annealing temperature, and standard curve parameters used for real-time PCR quantification of several groups of methanogens

Target	Source DNA[a]	AT, endpoint PCR (°C)[b]	AT, real-time PCR (°C)[b]	Standards (gene copies μl⁻¹)[c]	Slope	Intercept	r^2 value
Methanosarcina	M. barkeri (DSM 800)	51	64	10^8–10^4	−4.12	36.50	0.998
Methanosaeta	M. concilii (DSM 2139)	60	60	10^4–10	−3.79	31.87	0.996
Methanoculleus	Clone F_2FA36 (AM947508)	58	65	10^5–10	−3.85	34.58	0.994
Methanothermobacter	M. wolfeii (DSM 2970)	51	61	10^4–1	−3.58	31.86	0.996
Uncultured group	Clone F_1FA28 (AM947509)	58	64	10^4–1	−4.13	38.43	0.994
Methanobrevibacter	M. smithii (DSM 861)	54	59	10^5–10^2	−3.77	33.39	0.999
Methanobacterium	M. formicicum (DSM 1535)	54	58	10^4–10	−3.66	37.32	0.993
Methanosphaera	M. stadtmanae (DSM 3091)	56	61	10^5–10	−3.63	31.58	0.994
Methanocorpusculum	M. bavaricum (DSM 4179)	56	58	10^6–10^2	−4.02	37.72	0.999

[a] All reference strains from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Environmental clones are described in reference 16.
[b] AT, annealing temperature.
[c] Range of standard solutions used for quantification by real-time PCR.

RESULTS AND DISCUSSION

Screening of methanogenic diversity. Sludge DNA extracted from samples taken from all four reactors on days 4, 14, and 28 was used for hybridization with the AnaeroChip microarray, an oligonucleotide microarray targeting methanogenic genera clustered within *Methanomicrobiales*, *Methanosarcinales*, *Methanobacteriales*, and *Methanococcales*, which thrive in digesters (13, 20).

Based on the fluorescence intensity data, the signal-to-noise ratio (SNR) was calculated for all probes, and those with an SNR of ≥2 in one or more samples were used for subsequent analyses (see Table S1 in the supplemental material). Microarray data are semiquantitative due to the use of PCR-amplified templates that overrepresent the dominant microbes and the inherent differences in hybridization efficiencies among oligonucleotide probes (29, 37). Still, the SNR values provide an estimate of a target's abundance (7). The facultative acetoclastic *Methanosarcina* genus dominated all reactors. All six probes targeting this genus fluoresced in most samples, with SNRs up to 271. No clear differences in fluorescence intensity were detected among either samples or sampling dates. Other genera detected with the AnaeroChip microarray in the samples were as follows: the strict acetotrophic genera *Methanosaeta*, the hydrogenotrophic genera *Methanobrevibacter*, *Methanobacterium*, *Methanoculleus*, *Methanothermobacter*, *Methanocorpusculum*, *Methanolobus*, *Methanomicrobium*, *Methanocalculus*, and *Methanospirillum*, the methanol-consuming genus *Methanosphaera*, and a group of uncultured archaea. These results indicate a metabolically diverse methanogenic community comprised of organisms capable of methane formation through all known biochemical pathways. Our results also point out an extremely uneven methanogenic community completely dominated by a single genus but highly rich in phylogenetic terms, with representatives of three out of the four taxonomic orders which are commonly found in digesters (20).

Dominance of *Methanosarcina* has been reported before for many anaerobic environments (23), including manure-digesting reactors (9). This is thought to be due to their tolerance for substances which can be inhibitory to other methanogens at high concentrations, such as total ammonia N, which averaged 1.32 ± 0.04 g liter^{-1} at the start of our experiment (while all reactors were operated with excreta). Ammonia is presumed to inhibit methanogenesis through diffusion into the cells and subsequent induction of an intracellular pH rise or enzyme inhibition (3). The higher volume-to-surface ratio of the coccoid *Methanosarcina* and its growth in clusters might result in comparatively lower ammonia diffusion per unit mass and thus increased tolerance to high ammonia levels (5). On the other hand, the relatively high acetate concentration, averaging 212 ± 10 mg liter^{-1} in the reactors at the start of the experiment, would explain *Methanosarcina*'s ability to outcompete *Methanosaeta* (39). The former is known to grow at faster specific rates (38) and to prevail at high acetate concentrations (33).

A previous report of methanogenic diversity in TPOMW-fed mesophilic reactors indicated the dominance of *Methanosaeta*

(27). This discrepancy might be due to differences in the following: (i) the start-up strategy, since we initially loaded all reactors with excreta whereas Rincón and coworkers (27) used inocula from an olive mill wastewater-digesting reactor plus a nutrient-trace element solution; (ii) organic loading rates, which were rather low in the work described in reference 27 (0.75 to 3 g chemical oxygen demand [COD] liter^{-1} day^{-1}), allowing total volatile fatty acid (VFA) levels to be kept at ≤440 mg liter^{-1}. This contrasts with the 3.8- to 5.5-g COD liter^{-1} day^{-1} fed in our digesters, which determined total VFA concentrations between 535 and 3760 mg liter^{-1} in the reactors containing TPOMW (either alone or mixed with excreta) after the period corresponding to one HRT.

Finally, the codigestion of excreta and TPOMW at 55°C (reactor EX_OL55) induced an increase in *Methanoculleus* abundance, as indicated by the consistently higher SNR values of the probes Mcul574 and Mcul393 on days 14 and 28 (see Table S1 in the supplemental material).

Quantification of methanogenic genera detected with the AnaeroChip microarray. All methanogenic genera found to be more abundant and ubiquitous in the samples according to the AnaeroChip data were targeted with real-time quantitative PCR. Primers designed in this study (Table 2) were specific for their targets after amplification with endpoint and real-time PCR. Prior to the quantification assays, PCR efficiency reduction due to the presence of inhibitors in the sludge matrix was tested by serially diluting the DNA extracts and spiking the standard curves with 2 μl sludge DNA or purified sludge DNA. No differences in amplification efficiencies compared to those for unspiked standards were found (data not shown).

Table 3 summarizes the standard curve parameters (slope, intercept, and r^2) developed for all quantification assays. Figures 1 to 4 show the number of 16S rRNA gene copies per gram of sludge, after correction of amplification efficiency, detected in the four reactors on days 4, 7, 14, 21, and 28 for all the methanogenic genera quantified. As expected from the microarray data, *Methanosarcina* was the most abundant methanogen in all reactors (Fig. 1), with its gene copy numbers representing between 96.2 and 97.5% of the total in the initial methanogenic community (day 4). This was followed by *Methanobrevibacter* (1.4 to 2.1%), *Methanocorpusculum* (0.4 to 1.4%), and *Methanosaeta* (0.3 to 0.6%). All other genera were found to represent <0.2% of all methanogens quantified. However, these figures must be interpreted with caution due to uncertainties in the number of *rrn* operons in methanogens and differences in amplification efficiency resulting both from the inherent differences among primer pairs and the various abundances of the target organisms, although this is partly solved by data correction.

The change from excreta to excreta plus TPOMW in the mesophilic reactor (EX_OL37) invoked an immediate increase in the 16S rRNA gene copy numbers of *Methanosarcina* (Fig. 1), which was strongly correlated with the rise in CH$_4$ production (Pearson's coefficient = 0.94; $P = 0.02$). The abundance of *Methanosarcina* in reactor EX_OL37 was correlated with that of total and volatile solids, COD, and with the decrease in ammonia N (for all variables, Pearson's coefficient was ≥|0.92|; $P \leq 0.03$). No correlation was found between *Methanosarcina* 16S rRNA gene copy numbers and acetate or H$_2$, which are two major substrates for most of the species of the genus (23),
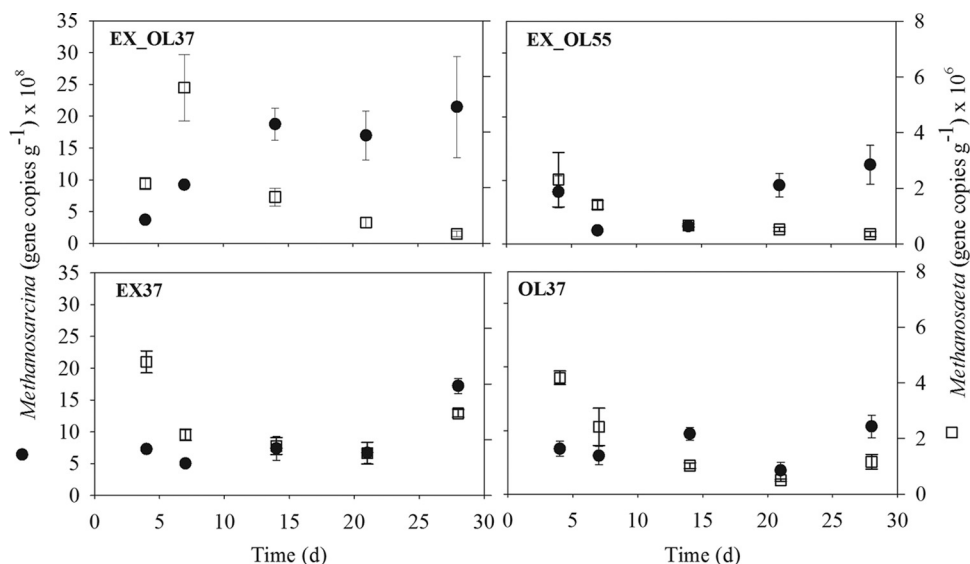
FIG. 1. 16S rRNA gene copy numbers of *Methanosarcina* and *Methanosaeta* in reactors EX_OL37 (3:1 excreta/TPOMW at 37°C), EX_OL55 (3:1 excreta/TPOMW at 55°C), EX37 (3:1 excreta/water at 37°C), and OL37 (3:1 water/TPOMW at 37°C). Bars represent standard errors ($n = 3$).

suggesting that substrates did not limit growth at any stage. Therefore, *Methanosarcina* easily adapted to the new environmental conditions that resulted from changing the feed from excreta to excreta-TPOMW at 37°C and was responsible for the enhanced methane production because of the codigestion. *Methanosarcina*'s competitive advantage under conditions of system instability has been reported before (26).

The codigestion of excreta and TPOMW at 55°C (EX_OL55) provoked an initial increase of volatile fatty acids, mainly acetate, which averaged 0.21 g liter^{-1} on day 4 and peaked at 4 g liter^{-1} on day 7, indicating an imbalance of the microbial guilds involved in anaerobic digestion. This was co-

incident with a decrease in the gene copy numbers of *Methanosarcina* (Fig. 1). From day 14 on, concurrent with acetate stabilization to a final concentration of 0.18 g liter^{-1} on day 28, *Methanosarcina* grew again in the reactor. Presumably, *Methanosarcina thermophila*, which is the single thermophilic species known in the genus (15), adapted to the new conditions. This organism has been found to be present in low numbers in mesophilic reactors (39) and to codominate a thermophilic cattle manure-fed reactor together with *Methanoculleus thermophilicus* (6).

In the same reactor (EX_OL55), setting the temperature at 55°C increased the H$_2$ production 34 times in 3 days, from 0.36
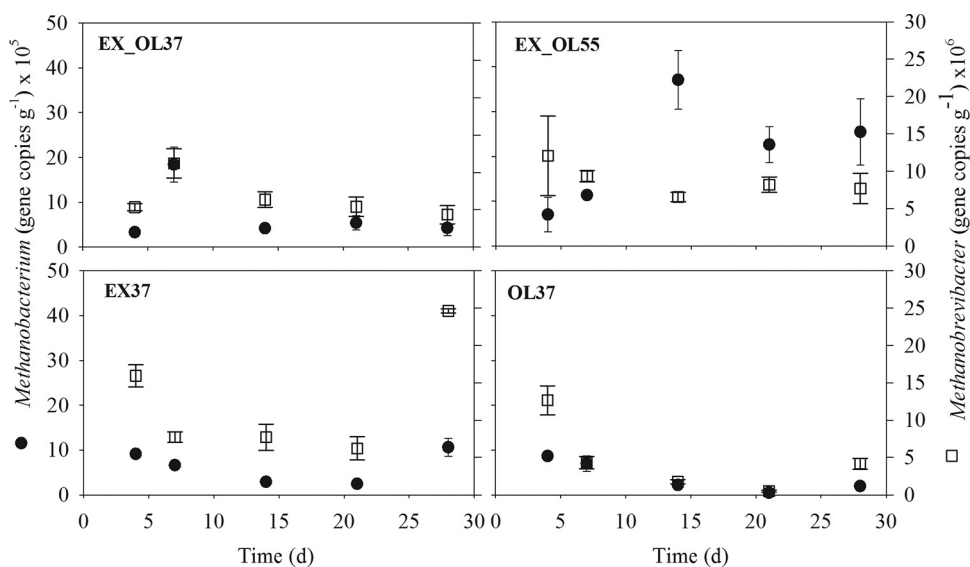

FIG. 2. 16S rRNA gene copy numbers of *Methanobacterium* and *Methanobrevibacter* in reactors EX_OL37 (3:1 excreta/TPOMW at 37°C), EX_OL55 (3:1 excreta/TPOMW at 55°C), EX37 (3:1 excreta/water at 37°C), and OL37 (3:1 water/TPOMW at 37°C). Bars represent standard errors ($n = 3$).
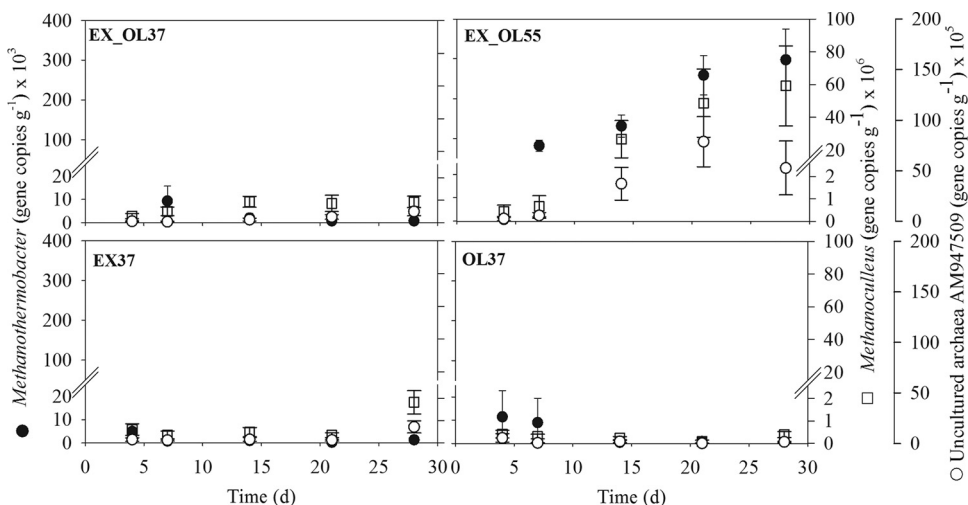
FIG. 3. 16S rRNA gene copy numbers of *Methanoculleus*, *Methanothermobacter*, and a group of uncultured archaea (AM947509) in reactors EX_OL37 (3:1 excreta/TPOMW at 37°C), EX_OL55 (3:1 excreta/TPOMW at 55°C), EX37 (3:1 excreta/water at 37°C), and OL37 (3:1 water/TPOMW at 37°C). Bars represent standard errors ($n = 3$).

ml day^{-1} (day 3) to 12.3 ml day^{-1} (day 6). This increase in the H$_2$ concentration with temperature is consistent with previous observations of methanogenic environments and has been suggested to respond to thermodynamic control (19). From day 6 on, H$_2$ production in EX_OL55 continued to rise progressively, peaking at 42.7 ml day^{-1} on day 15, which is a 3.5-fold increase during the eight subsequent days. The H$_2$ production in the thermophilic reactor after one HRT (21.4 days) averaged 38.4 ml day^{-1}, multiplying by a factor ranging from 9 to 143 the data corresponding to the three mesophilic reactors (Table 1). The evolution of H$_2$ partial pressure with the fermentation was mirrored in that of the gene copy numbers of several genera of hydrogenotrophic methanogens, which include thermophilic species (15), mostly *Methanobacterium*,

Methanoculleus, and *Methanothermobacter*, which rose by 1 to 2 orders of magnitude (Fig. 2 and 3). From day 14 on, *Methanoculleus* became the second most abundant methanogen in the sludge, confirming the AnaeroChip results (see Table S1 in the supplemental material). The immediate growth of these H$_2$ scavengers was probably crucial for equilibrium reestablishment, since an uncontrolled increase in H$_2$ partial pressure might have led otherwise to reactor acidification and instability. Furthermore, a group of uncultured archaea (AM947509), which was found to be abundant in a biowaste-digesting thermophilic full-scale reactor (16), was present and grew rapidly when the temperature had risen to 55°C (Fig. 3). Therefore, this group of uncultured organisms most likely includes hydrogenotrophic thermophilic methanogens. Strong correla-
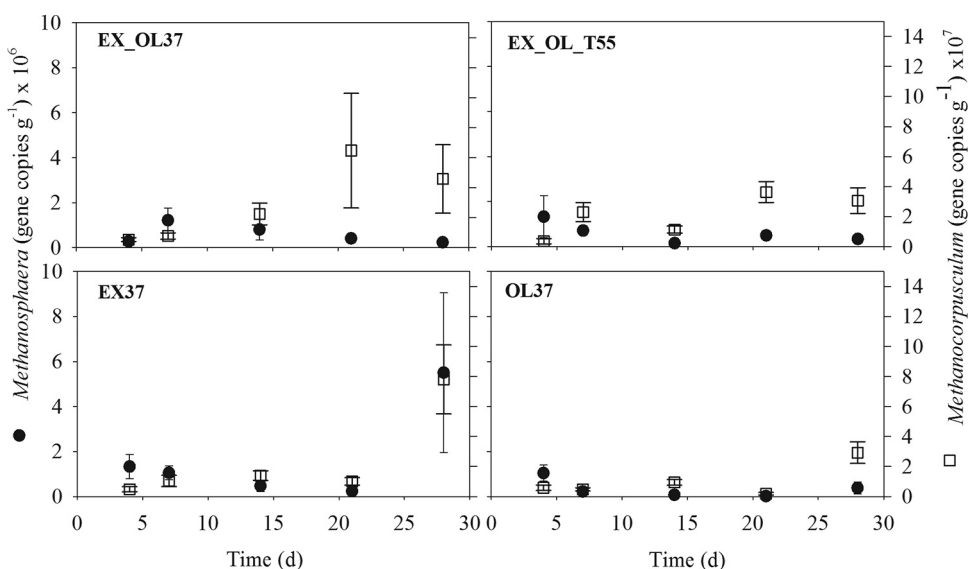

FIG. 4. 16S rRNA gene copy numbers of *Methanosphaera* and *Methanocorpusculum* in reactors EX_OL37 (3:1 excreta/TPOMW at 37°C), EX_OL55 (3:1 excreta/TPOMW at 55°C), EX37 (3:1 excreta/water at 37°C), and OL37 (3:1 water/TPOMW at 37°C). Bars represent standard errors ($n = 3$).

TABLE 4. Richness, evenness, and diversity of methanogens in all reactors at the start (day 4) and end (day 28) of the experiment[a]

Reactor	Time (days)	S	E	H'
EX_OL37	4	8.0 (0.0)	0.23 (0.04)	0.20 (0.04)
	28	7.3 (0.6)	0.14 (0.01)	0.12 (0.01)
EX_OL55	4	8.0 (0.0)	0.15 (0.03)	0.14 (0.03)
	28	9.0 (0.0)	0.38 (0.04)	0.36 (0.04)
EX37	4	8.0 (0.0)	0.23 (0.04)	0.21 (0.03)
	28	8.0 (0.0)	0.33 (0.03)	0.30 (0.03)
OL37	4	8.0 (0.0)	0.22 (0.06)	0.20 (0.05)
	28	8.0 (0.0)	0.19 (0.05)	0.17 (0.04)

[a] Data are averages ($n = 3$) (standard deviations are given in parentheses). S, richness; E, evenness; H', diversity.

tions were detected between the mentioned hydrogenotrophic microbes and both reactor H_2 partial pressure and temperature (Pearson's coefficient ≥ 0.7; $P \leq 0.001$). Previous surveys have also suggested methanogenesis from H_2/CO_2 under thermophilic conditions in reactors (9, 16) and soils (12). However, a higher H_2 partial pressure does not necessarily imply that more energy is available for the hydrogenotrophic methanogens (10). Finally, the evolution of the abundance of *Methanoculleus*, *Methanothermobacter*, and the uncultured group in reactor EX_OL55 was highly and positively correlated to the concentration of propionate (Pearson's coefficient ≥ 0.89; $P < 0.05$). This suggests the involvement of the mentioned H_2 scavengers as syntrophic partners of propionate oxidizers, an interaction that is widely known to energetically facilitate propionate degradation (34). Obviously, evidences in this respect would require researching the bacterial community and carrying out experiments to establish the pathways for methanogenesis.

All reactors had a similar diversity of quantified methanogens at the start of the experiment (F = 2.32; $P = 0.162$), but H' was significantly different after 28 days (F = 30.46; $P < 0.001$). The reduced abundance of the dominant *Methanosarcina* genus in the reactor run at 55°C (EX_OL55) and the increased levels of several groups of hydrogenotrophs account for the doubling of evenness and hence diversity of the methanogenic community (Table 4). Karakashev et al. observed that methanogenic communities examined by fluorescence *in situ* hybridization with family-specific probes were generally richer in digesters operated at 37 to 38°C than in those operated at 51 to 55°C (21). This led them to conclude that mesophilic reactors bear more-diverse methanogenic communities. Our data demonstrate that the shift from 37°C to 55°C can increase the diversity of a highly uneven methanogenic community by imposing growth conditions that are both suboptimal for the dominant population and beneficial for other, less-frequent populations. This might theoretically result in a more efficient use of the resources and thus enhanced system productivity.

The reactor fed exclusively with excreta had a stable performance in terms of biogas production. It showed small oscillations in the numbers of 16S rRNA gene copies for the methanogenic genera quantified, with no clear temporal trends (EX37 in Fig. 1 to 4). This could simply be a response to various numbers of methanogens being inoculated with the

excreta day by day. The magnitude of these differences was not enough to affect the performance of the reactor, which had a constant biogas production throughout the experiment (17). Also, Silvey et al. reported fluctuations in archaeal biomass, estimated from the levels of phospholipid fatty acids, but constant methane production for 14 days of mesophilic digestion of municipal solid waste (31).

More overwhelming was the lack of noticeable variability in methanogenic gene copy numbers in the reactor fed with TPOMW (OL37 in Fig. 1 to 4) despite the complete cessation of biogas production after 30 days. We initially hypothesized that methanogenic biomass could have been washed out from the reactor due to the shift from the initial load (75 liters of cattle excreta) to its content after one HRT (18.75 liters of TPOMW plus 56.26 liters of tap water). However, real-time PCR results demonstrated that the amount of methanogenic biomass in reactor OL37 was comparable to that of the other reactors. Therefore, we concluded that methanogens were present in sufficient numbers but their activity stopped. All necessary nutrients were present in the reactor, and 3 times higher copper availability (192 ppm; C. Mondini, personal communication) under the more acidic conditions imposed by TPOMW was hypothesized to be the cause of methanogenesis inhibition (22).

Stepwise forward regression was used to search for variables able to predict methane production in the reactors. The linear combination of four variables (log-transformed 16S rRNA gene copy numbers of *Methanoculleus*, *Methanosarcina*, *Methanosphaera*, and *Methanobacterium*) significantly (F = 19.91; $P < 0.001$) predicted 84.2% of CH_4 production. All other methanogenic genera quantified in the reactors were excluded from the model, since they did not increase its ability to explain CH_4 production.

Conclusions. A phylogenetically and metabolically rich methanogenic community that stably digested cattle excreta rapidly adapted to the change in feed to excreta plus TPOMW both at 37°C and 55°C but not to the digestion of TPOMW alone. The thermophilic codigestion of the residues was the most productive and held the most diverse methanogenic communities. This is consistent with the theoretical expectation of a more efficient exploitation of resources due to a better occupation of available niches. Linking of the system's diversity and functioning would require further investigating other microbial guilds involved in the complex food web of anaerobic decomposers and experimentally defining the pathways used for methane formation.

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