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Thermal and alkaline pre-treatments of inoculum halt methanogenesis and enable cheese whey valorization by batch acidogenic fermentation

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Abstract

BACKGROUND: Carboxylates such as volatile fatty acids (VFA) can be produced by acidogenic fermentation (AF) of dairy wastes including cheese whey, a massive residue produced at 160.67 million m^3 of which 42% are not valorized and impact the environment. In mixed-culture fermentations, selection pressures can favor AF and halt methanogenesis. In this study, inoculum pre-treatment was evaluated as a selective pressure for AF demineralized cheese whey in batches. Alkaline (NaOH, pH 8.0, 6 h) and thermal (90 °C for 5 min, ice-bath until 23 °C) pre-treatments were tested with batch operations runs at initial pH 7.0 and 9.0, food-to-microorganism (F/M) ratios of 0.5 to 4.0 g COD g^{-1} VS, and under pressurized (P) and nonpressurized (NP) headspace, in experiments duplicated in two different research institutes.

RESULTS: Acetic acid was highly produced on both Unicamp and TU Delft samples (1.36 and 1.40 g COD_{AcOH} L^{-1}, respectively), at the expense of methanogenesis by combining a thermal pre-treatment of inoculum with a NP batch operation started at pH 9.0. Microbial communities comprising VFA and alcohol producers, such as *Clostridium*, *Fonticella* and *Intestinimonas*, and fermenters such as *Longilinea* and *Leptolinea*. The lipid-accumulating *Candidatus microthrix* was observed in both bulk material and foam. Despite the absence of methane production, *Methanosaeta* were detected within the microbial community. An F/M ratio of 0.5 g COD g^{-1} VS led to the best VFA production of 1769.4 mg L^{-1}.

CONCLUSION: Overall, inoculum thermal pre-treatment, initial pH 9.0 and NP headspace acted as a selective pressure for halting methanogenesis and producing VFAs, valorizing cheese whey via batch acidogenic fermentation.

Keywords: acidogenic fermentation; physicochemical pre-treatments; alkaline; thermal; volatile fatty acids; cheese whey

INTRODUCTION

Anaerobic digestion (AD) has been used from the 19th Century onwards to obtain biogas as an energy carrier.[1] Since then, AD has been constantly improved[2,3] to process various waste streams,[4] and to address energy production, environmental burdens and the circular economy. The primary goal remains to reduce organic matter and remove nutrients from agro-industrial, food and municipal solid wastes.[5-7]

Cheese whey (CW) is a by-product of the dairy industry with a high organic load [i.e. 50 to 80 g O_2 L^{-1}, in terms of chemical oxygen demand (COD)].[8] It has an annual production of 160.7 million m^3 where 58% of the production is absorbed by various industries (e.g. food, nutrition, cosmetics and pharmaceutical). However, a staggering amount of 66.5 million m^3 yr^{-1} of CW[9-11] is currently transformed into low-added-value products such as animal feed and fertilizer, or discharged into water bodies leading to eutrophication.[10,12] Hence, cheese whey can be an excellent substrate for acidogenic fermentation.

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Acidogenic fermentation (AF) focuses on the valorization of organic matter via the carboxylate platform, by combining the inhibition of methanogenesis and production of volatile fatty acids (VFA) at high yields, which are of great economic interest owing to their potential industrial applications (e.g. biofuels, biopolymers and chemicals).[13-16]

Selective pressures (e.g. pH, physicochemical pre-treatments, reactor headspace pressure and F/M ratio) can dictate microbial diversity and dynamics, interactions, energy requirements and preferred metabolisms.[17] Most studies in AD and AF focus on the pre-treatment of the substrate and waste-activated sludge.[18] The present study focused on various selective pressure mechanisms for enhancing VFA production and halting methanogenesis in AF. We also investigated the effects of alkaline and thermal inoculum pre-treatments, variations in the initial pH in headspace pressure, and different F/M ratios on the acidogenic fermentation of cheese whey.

Alkaline pre-treatments have been shown to improve AD by increasing sludge solubilization and enhancing methane (CH_4) production,[7,19-22] whereas thermal pre-treatments have been used to inhibit methanogenesis.[23-25]

However, thermal pre-treatments, which can be performed at temperatures ranging from 30 to 180 °C, are time-consuming (up to several hours), which would increase AF's overall costs.[26] Contradictory results also have been observed regarding the effects of such pre-treatments on methanogenesis, with an inhibition observed after alkaline pre-treatment[27] and an enhancement observed after thermal pre-treatment.[28] The lack of consensus on the best conditions for inoculum pre-treatment and their influence on AF still needs to be elucidated. To date, studies on thermal pre-treatments are mostly performed on waste-activated sludge. Their use as a selective pressure mechanism for VFA production remains scarce.

Variations in the initial pH can positively influence VFA production.[29] In addition, uncontrolled pH approaches can reduce AF's costs because there is no further need for chemical utilization for stabilizing pH.[18]

Headspace gas composition and pressure play a role in product formation and metabolic pathways preferentially used. However, most studies still focus on hydrogen (H_2) production.[30] According to Zhou[31] and Sarkar,[23] low H_2pressure favors VFA formation. Finally, the F/M ratio, which is inoculum and substrate-dependent, is a parameter that impacts acidogenesis, with lower F/M ratios being beneficial to VFA production.[32,33]

In this work, we aimed at: (i) identifying how abiotic factors (i.e. pH, inoculum pre-treatment, headspace pressure) and F/M ratio influence the product spectra of cheese whey via AF; (ii) validating thermal pre-treatment efficiency for halting methanogenesis; and (iii) identifying the parameters that increase acetate level of production for other biological processes (e.g. microalgal photoorganoheterotrophic biomass production).

MATERIALS AND METHODS

Figure 1 depicts the overall methodology, involving experiments performed at the University of Campinas (UNICAMP, Brazil) and reproduced at Delft University of Technology (TU Delft, The Netherlands). Substrate and inoculum preparation and inoculum pre-treatment are common to all experiments apart from a few modifications described hereafter. Although replicates, these experiments cannot be considered *sensu stricto* biological replicates, as they consist of different microbial communities. The location where each set of experiments was performed was used to facilitate the correlation between both replicates.

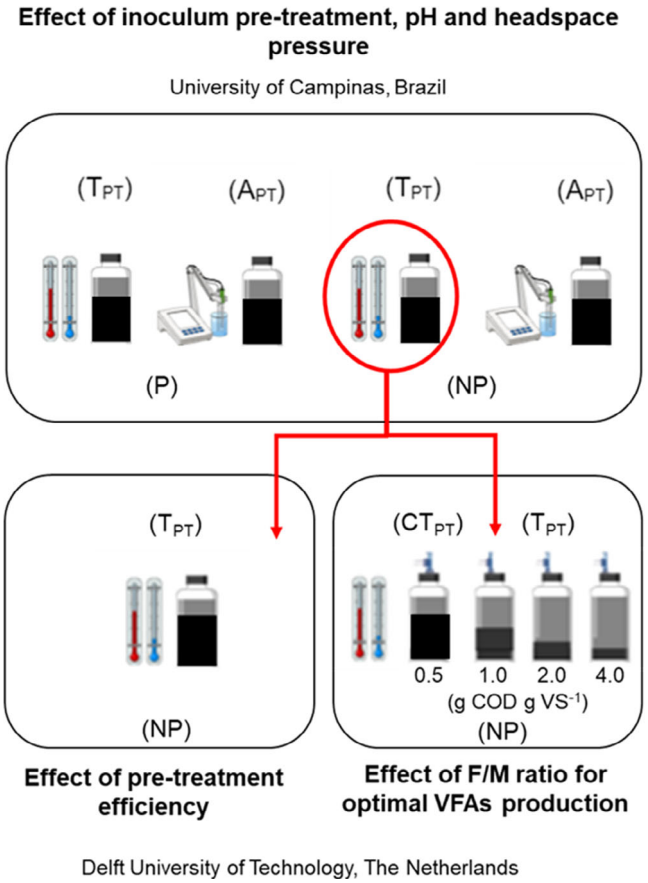

Figure 1. Overall experiment methodology. The inoculum went through alkaline (A_{PT}) and thermal (T_{PT}) pre-treatments. The initial pH at the start of the digestions was set at 7.0 and 9.0 and headspace assays were carried out in pressurized (P) and nonpressurized (NP) batches. Analyses encompassed gas and VFA profiles, total organic carbon (TOC), total nitrogen (TN), chemical oxygen demand (COD), solid series, carbohydrates and amplicon sequencing (16S rRNA gene).

Demineralized cheese whey media

The chosen carbon source for all experiments consisted of 40% demineralized whey powder (WPC40), which was the closest substrate to raw cheese whey. The substrate medium for experiments held at UNICAMP was composed of (per L): WPC40 (Pic-Nic, Brazil) (4 g)[34] and the following reagents: $NaHCO_3$ (1 g), NaCl (250 µg), $MgCl_2.6H_2O$ (7 µg) and $CaCl_2{\cdot}2H_2O$ (9.5 µg).[35] The medium was prepared fresh before experiments and its initial pH was ≈7.0. No pH corrections were made after medium preparation. The sludge used as inoculum was obtained from a UASB reactor in Brazil (25° 05′ 10.1″ S, 47° 58′ 49.7″ W). The National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) register number is AE43468.

For the experiments held at TU Delft, the medium was composed of (per L): WPC40 (Subo International, The Netherlands) (4 g) amended with 0.3 mL micronutrient solution per g substrate COD. The micronutrients solution was composed of $FeCl_3{\cdot}6H_2O$ (2 g L^{-1}), $CoCl_2{\cdot}6H_2O$ (2 g L^{-1}), $MnCl_2{\cdot}4H_2O$ (0.5 g L^{-1}), $CuCl_2{\cdot}2H_2O$ (32 mg L^{-1}), $ZnCl_2$ (50 mg L^{-1}), HBO3 (50 mg L^{-1}), $(NH_4)6Mo_7O_2{\cdot}4H_2O$ (90 mg L^{-1}), $Na_2SeO_3{\cdot}5H_20$ (100 mg L^{-1}),

$NiCl_2{\cdot}6H_2O$ (6 mg L^{-1}), EDTA (1 g L^{-1}), HCl 36% (1 mL L^{-1}), Resazurine (0.5 g L^{-1}) and yeast extract (2 g L^{-1}). Sludge used as inoculum was obtained from an anaerobic digester at WWTP Harnashpolder in The Netherlands (52° 00′ 48.8″ N, 4° 19′ 02.5″ E). Note that 1 g L^{-1} of WPC40 corresponds to 1 g O_2 L^{-1} in COD terms.

Mechanical homogenization, thermal and alkaline pre-treatments of inocula

The anaerobic granular sludge used as inoculum at UNICAMP was mechanically disaggregated, consequently increasing their superficial area. The anaerobic granular sludge used as inoculum in TU Delft was used as is.

Thermal pre-treatment, which was performed both at UNICAMP and TU Delft, was adapted from Mockaitis *et al.*[36] Briefly, the sludge was heated at 90 °C in a water bath for 20 min under constant stirring. Heating was then halted by decreasing the sludge temperature to 23 °C in an ice bath.

Alkaline pre-treatment consisted of increasing the sludge pH up to 8.0 using 1 mol L^{-1} NaOH under continuous mixing, and controlling it for the next 6 h, with a correction to 8.0 if needed. This pre-treatment was only performed at UNICAMP.

Effect of thermal and alkaline pre-treatments of inocula, initial batch pH, and headspace pressure on acidogenic fermentation

Batch experiments were conducted at UNICAMP. They assessed the effects of three factors evaluated at two levels in WPC40 acidogenic fermentation, namely: (i) inoculum pre-treatment [alkaline (A_{PT}) and thermal (T_{PT})] to enhance sludge biodegradability and select for alkaline-tolerant acetoclastic microorganisms, and to inactivate methanogenic microorganisms, respectively; (ii) variation in the initial pH of digestion (7.0 and 9.0) to further select microorganisms adaptable to neutral and alkaline pH environments; and (iii) gas headspace pressure [pressurized (P) and nonpressurized (NP)] to investigate the influence on AF products spectra. Table 1 shows the two-level factorial experimental design with three factors (2^3 designs) applied to this set-up.

Table 1. The 2^3 experimental designs for phase one

	Factors		
Experiment	Pre-treatment (binomial)	pH (continuous)	Headspace (continuous)
A7P (*ac*)	Alkaline (+1)	7.0 (−1)	P (+1)
A9P (*abc*)	Alkaline (+1)	9.0 (+1)	P (+1)
A7NP (*a*)	Alkaline (+1)	7.0 (−1)	NP (−1)
A9NP (*ab*)	Alkaline (+1)	9.0 (+1)	NP (−1)
T7P (*c*)	Thermal (−1)	7.0 (−1)	P (+1)
T9P (*bc*)	Thermal (−1)	9.0 (+1)	P (+1)
T7NP (*1*)	Thermal (−1)	7.0 (−1)	NP (−1)
T9NP (*b*)	Thermal (−1)	9.0 (+1)	NP (−1)
Control (P)*	–	8.25	P (+1)
Control (NP)*	–	8.25	NP (−1)

Note: The (±1) values indicate the upper and lower levels of the investigated factors. The design aimed at testing the main effects (referred to as a, b and c), the two-factor interaction effects (ab, ac and bc), and the three-factor interaction effect (abc). Two control experiments were included.

Experiments (Fig. 1) were performed in 1-L Duran flasks (total volume 1130 mL) with an initial working volume of 730 mL and an initial headspace volume of 398 mL. Bottles were inoculated at a concentration of 6.6 g total volatile solids (TVS) L^{-1}, as depicted in Mockaitis *et al.*[36] Batch reactors were continuously agitated at 50 rpm in an orbital incubator (MA420; Marconi, Sao Paolo, Brazil) at a mesophilic temperature of 35 °C for 30 days.

Batch headspace pressure assays

The influence of headspace pressure (P and NP batches) on VFA production was investigated according to Peixoto *et al.*[37] Manometric ambient pressure was measured, and then, 3 mL samples of gas were collected with a syringe containing a pressure lock (Thermo Fisher Scientific, Waltham, MA, USA). Each sample corresponded to a batch condition.

In P headspace assays, butyl rubber stoppers of each flask were covered with silicone sealant after manometric measurement and gas sample collection, allowing gas accumulation in the batch headspace.

In NP experiments, the headspace of each flask was punctured with a needle after pressure manometric reading and gas sampling, allowing the remaining gas to be released until reaching the ambient atmospheric pressure value. After this step, butyl rubber stoppers also were covered with silicone sealant avoiding any gas release into the atmosphere.

Once collected, gas samples were analyzed with a gas chromatograph (GC) equipped with a thermal conductivity detector GC-TCD (Construmac, São Carlos/SP, Brazil) with hydrogen as a carrier gas.

The volumetric production of biogas and its constituents were inferred through Eqn (1) as a discrete function from sampling timestep $t_0 = 0$ to t for NP assays.

$$\Gamma_n = \frac{T^{STP}}{T \cdot P_{STP}} \cdot \sum_{i=1}^{n} V_{i-1}^{H} \left(P_i \cdot {}^{A}\chi_i - P_{atm} \cdot {}^{A}\chi_{i-1} \right) \quad (1)$$

where Γ_n is the volumetric production of a gas of interest (N_2, H_2, CH_4 or CO_2) in standard temperature and pressure (STP) equivalents (L), at a given time n; P_i is the measured pressure at sampling time (atm); V_{i-1}^{H} is the headspace volume before sampling (L); T is system's temperature (K), T^{STP} is the standard temperature (273 K), P^{STP} is standard pressure (1 atm), ${}^{A}\chi_i$ is the molar fraction of the gas of interest at sampling time and ${}^{A}\chi_{i-1}$ is the molar fraction of the gas of interest before sampling.

VFA measurements

Quantification of VFAs, alcohols and carbohydrates was by high-performance liquid chromatography (HPLC) based on Penteado *et al.*[38] The VFA and alcohol profile consisted of the following: lactic acid, formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, iso-valeric acid and ethanol.

The chromatograph was equipped with two LC-20 AD pumps, one DGU-20A3R degasser, a SIL-20AHT autosampler, a CTO-20A column oven, an SPD-20 UV detector with readings at 210 nm (Shimadzu, Tokyo, Japan), an Aminex HPX-87H 300 × 7.8 mm column (BioRad, Hercules, CA, USA), a RID-10A index refraction detector and a CBM-20A controller (Shimadzu).

Two millilitres of mixed liquors were centrifuged at 16 025×*g* for 5 min. Forty microlitres of sulfuric acid (H_2SO_4; 2 mol L^{-1}) were added to 1 mL supernatant to acidify the sample for ideal analyte separation. The samples were then filtered through regenerated

cellulose (RC) syringe filters (RC 0.20 μm) (GVS, Bologna, Italy), and transferred to 1.5-mL pre-washed vials (H_2SO_4 at 2 mol L^{-1} to avoid any contamination) before HPLC analyses. The HPLC run time was 60 min per sample with a constant column oven temperature of 43 °C and 0.005 mol L^{-1} H_2SO_4 mobile phase at a flux rate of 0.5 mL min^{-1}.

Inoculum thermal pre-treatment and nonpressurized headspace efficiency

Inoculum thermal pre-treatment and NP headspace assays were reproduced in TU Delft to confirm the efficiency of such imposed conditions in halting methanogenesis while enhancing VFA production. Each factor was tested at one level, based on the effects identified at UNICAMP (see the 'Effect of thermal and alkaline pretreatments of inocula, initial batch pH, and headspace pressure on acidogenic fermentation' section): inoculum thermal pretreatment, initial pH 9.0 and NP headspace. A batch with no inoculum pre-treatment, initial pH 9.0 and NP headspace acted as a control. The experimental design with three factors applied to this set-up is shown in Table 2.

Similar to the first experiments conducted, the initial working volume of the 1-L Duran flasks was 750 mL with an initial headspace volume of 398 mL, for a total working volume of 1.15 mL. The inoculum concentration was identical at 6.6 g TVS L^{-1} as depicted in Mockaitis *et al.*[36] Batch reactors were incubated for 10 days at a mesophilic temperature of 35 °C in a Certomat® BS1 incubator (Sartorius AG, Goettingen, Germany) under continuous agitation (145 rpm). The headspace was released with the aid of Cole-Palmer stopcocks with Luer connection, a one-way male lock (Cole-Parmer, Vernon Hills, IL, USA). Experiments were performed in triplicate and lasted 12 days.

VFA measurements

The VFAs and carbohydrates were quantified by HPLC. One millilitre of mixed liquors was centrifuged at 5.07×*g* for 5 min and supernatants were filtered using 0.45-μm syringe filters. Filtered samples were then transferred to 300-μL Waters total recovery vials, from which 10 μL were injected with a 2707 Waters HPLC autosampler at 15 °C. The chromatograph was equipped with a Waters M515 HPLC (Waters, Milford, MA, USA) pump, a Waters 2414 refractive index (RI) detector, with 1024 of sensitivity and a Waters 2489 UV-visible detector at 210 nm. The column was an HPX-87H (300 × 7.8 mm) with a Cation-H refill cartridge (30 × 4.6 mm) guard column (both BioRad) and the column oven was built in-house. The flow rate of the pump was 0.6 mL min^{-1} and the temperature of the column was set at 59 °C. The RI detector was operated at 30 °C. The mobile phase was 1.5 mmol L^{-1} phosphoric acid diluted in ultrapure water (MilliQ, Merck Millipore, Darmstadt, Germany). The VFAs measured were acetic acid, butyric acid, formic acid, caproic acid, propionic acid, valeric acid, iso-butyric acid, iso-caproic acid and iso-valeric acid.

Table 2. Experimental design for the F/M ratio experiments with inoculum thermal pretreatment and nonpressurized headspace assays. All experiments were done in duplicate

	Factor
Experiment	F/M ratio (continuous)
FM0.5	0.5
FM0.5	0.5
FM1.0	1.0
FM1.0	1.0
FM2.0	2.0
FM2.0	2.0
FM4.0	4.0
FM4.0	4.0

Ideal F/M ratio for optimal VFA production with thermally pre-treated inocula

The F/M ratio is a measurement used to determine the amount of substrate needed for the quantity of microorganisms present in a system. It is an important parameter to evaluate in an approach aiming at maximizing VFA production. To determine the best FM ratio for VFA production when working with a thermal pretreated sludge at a NP headspace, four different F/M ratios were tested: 0.5, 1.0, 2.0 and 4.0 g_{COD} g_{VS}^{-1}. These ratios were obtained by dividing the COD of the substrate by the VS of the sludge. The initial pH of all experiments was 9.0. In the 'Inoculum thermal pretreatment and nonpressurized headspace efficiency' section, both control and thermal batches presented an F/M ratio of 0.5 g $_{COD}$ g $_{VS}$ $^{-1}$. g $_{COD}$ g $_{VS}$ $^{-1}$. Assays were conducted in duplicates for 14 days. The experimental design is depicted in Table 2.

Biogas measurements

The presence of CH_4 in the gas was detected by injecting 10 mL samples of gas from the headspace of the bottles in a GC (Agilenttech 7890 A; Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an HP-PLOT Molesieve GC column (Agilent 19095P-MS6, Agilent Technologies Inc.) of 60 m × 0.53 mm × 200 μm and a thermal conductivity detector (TCD). The carrier gas was helium (14.8 psi, 23 mL min^{-1}) and the operating temperature was 200 °C.

Physicochemical analyses

Mixed liquors and gas phases of the experimental flasks were sampled for physicochemical analyses in both original and replicated experiments. For the experiments held at UNICAMP, every 15 days, 150 mL mixed liquors were collected and used in the following analyses and respective code methods: solid series (2540 B-F; 50 mL), COD (5220 B; 1 mL), sulfate (4500-SO_4^{2-} E; 1 mL), sulfide (4500-S^2 D, 1 mL), total organic carbon (5310-TOC; 15 mL)[39] and total nitrogen (TN, ASTM D8083; 15 mL).[40] Additional analyses were performed consisting of pH measurements (4500 H) (Federation 2005), headspace assays (CH_4, N_2 and CO_2, 3 mL gas phase), HPLC (2 mL) for VFAs, sugar and alcohol characterization, total carbohydrate measurements[41] (1 mL), total alkalinity and VFA measurements[42,43] (60 mL). The remaining sludge was discharged. On alternate days, these analyses also were performed.

For the replicated experiments performed at TU Delft, 100 mL mixed liquors were collected on the first and last days of the process, and were used in the following analyses in the same volume as mentioned above: solid series, COD and TOC/TN. Other analyses also were performed, consisting of pH measurements, biogas composition determination, HPLC for VFA and sugar characterization, and total carbohydrate measurements. In addition, on alternate days 12 mL mixed liquors were sampled for pH, HPLC, COD, TOC and TN measurements.

16S rRNA gene amplicon sequencing

Sample collection

Microbial community analyses were performed through 16S rRNA gene amplicon sequencing on: (i) the initial and final samples from inoculum thermal pre-treatment efficiency assays and (ii) the final samples of each F/M ratio experiment.

Samples (0.5 mL) of mixed liquors were collected from each condition in 1.5-mL Eppendorf tubes completed with demineralized water for initial washing. Samples were centrifuged at 10000×*g* at 4 °C for 3 min, and the supernatant was discarded. DNA was extracted using the DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and concentrations of the DNA extracts were assessed using a Qubit fluorometer and the Qubit dsDNA HS assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

For each sample, a minimum of 20 μL DNA extracts with concentrations ranging from 13.9 and 95 ng μL^{-1} were sent to Novogene (Beijing, China) for 16S rRNA gene amplicon sequencing. All sequencing experiments (i.e. 16S rRNA sequence amplifications, library preparations and sequencing runs) were performed at Novogene, according to their protocols. The targeted hypervariable regions V3–V4 of the 16S rRNA gene were amplified by PCR with the pair of barcoded primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT).[44] This set of primers is known to cover both bacteria and archaea with high specificity, except for *Planctomycetes*.[45]

Sequences analyses

All sequencing data received from Novogene were analyzed using AmpliconTagger.[46] Briefly, raw reads were scanned for sequencing adapters and PhiX spikes in sequences. The remaining paired-end reads were processed to remove primer sequences (pTrimmer v1.3.3 –[47] and discard reads having average quality Phred score <20. The remaining sequences were processed for generating amplicon sequence variants (ASVs) (DADA2 v1.12.1) (Callahan 2016) using the following key parameters: filterAndTrim (maxEE =2; truncQ = 0; maxN = 0; minQ = 0) and learnErrors (nbases = 1e8) functions for both forward and reverse filtered reads. Reads were then merged using the mergePairs (minOverlap = 10; maxMismatch = 0) function. Chimeras were removed with DADA2's internal removeBimeraDeNovo (method = 'consensus') method followed by UCHIME reference (48). ASVs were assigned a taxonomic lineage with the RDP classifier[49] using training sets containing the complete Silva release 138 databases[50] supplemented with a customized set of mitochondria and plastid sequences. The RDP classifier gave a score of (0 to 1) to each taxonomic depth of each ASV. Any taxonomic depth having a score of ≥0.5 was kept reconstructing the final lineage. Taxonomic lineages were combined with the cluster abundance matrix obtained above to generate a raw ASV table from which the bacterial or fungi ASV tables were generated. Five hundred 1000-read rarefactions were then performed on these ASV tables and the average number of reads of each ASV of each sample was computed to obtain consensus rarefied ASV tables. Alpha diversity metrics (RTK v0.93.2[51] and taxonomic summaries microbiomeutils v0.9.3[52]) were then computed using the consensus rarefied ASV tables. Figures were generated in R using the ggplot2 package.

RESULTS AND DISCUSSION

Effects of inoculum thermal and alkaline pre-treatments and initial pH on the anaerobic digestion

Inoculum thermal pre-treatment efficiently halted methanogenesis

Figure 2 shows the CH_4 and carbon dioxide (CO_2) production in alkaline and thermal batches with both P and NP headspaces. Inoculum thermal pre-treatment successfully inhibited methanogenesis in all tested conditions (i.e. P and NP headspace assays with initial pH of 7.0 and 9.0).

Inoculum alkaline pre-treatment halted methanogenesis in both P and NP assays but only with an initial pH of 7.0 (i.e. A_{PT}7P 0.0 mL and A_{PT}7NP, 0.54 mL), whereas initial pH 9.0 presented a total CH_4 production of ≈52.29 mL for P assays and 0.67 mL for NP ones.

As a strategy to maximize VFA production, inoculum thermal pre-treatment appears to be the best selective pressure for impairing methanogens in batches. It is of utmost importance to confirm whether this selective pressure also is successful in halting methanogenesis considering different types of inocula (e.g. waste-activated sludge and secondary sludge), reactors (e.g. sequence-batch reactors and continuous-stirred tank reactors) and configurations (e.g. organic loading rate, hydraulic retention time, coupled reactors). If so, then inoculum thermal pre-treatment can be implemented in full-scale AF, decreasing their overall costs.[26]

Some studies have used alkaline pre-treatment of inoculum to increase the biodegradability of waste-activated sludge[7] and to stabilize the sludge originating from aerobic processes, while treating the mineralization of its remaining organic compounds.[22] Sludge alkaline pre-treatment increased the COD: COD_{Total} ratio[22] while improving VS reduction.[53,54] Hence, their CH_4 production also would increase.

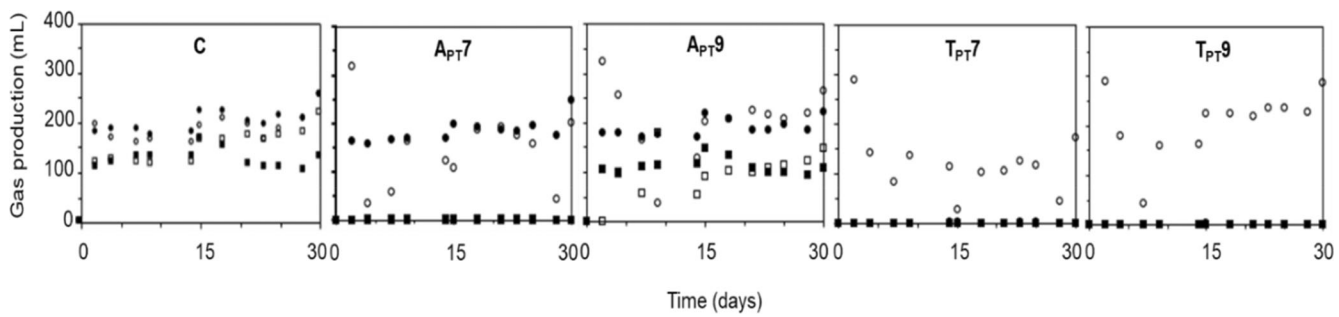

Figure 2. Methane (CH_4) and carbon dioxide (CO_2) gas production profiles for control (C), inoculum alkaline (A_{PT}) and thermal (T_{PT}) pre-treatment with initial pH of 7.0 and 9.0 during 30 days. Nonpressurized (NP) and pressurized (P) headspace assays also are represented. In the figure: ■ CH_4NP, □ CH_4P, ● CO_2NP and ○ CO_2P.

Table 3. Volatile fatty acid (VFA) and alcohol product spectra of pressurized (P) and nonpressurized (NP) headspace batches with alkaline (A_{PT}) and thermal (T_{PT}) pre-treatments and initial pH of 7.0 and 9.0. Experiments lasted for 30 days

Batch operational conditions (mg COD compound L^{-1})											
		Control		$A_{PT}7$		$A_{PT}9$		$T_{PT}7$		$T_{PT}9$	
	Time	P	NP	P	NP	P	NP	P	NP	P	NP
Acetic acid	t_0	53.9	154.3	97.5	0	0	180.1	183.9	314.6	154.6	308.3
	t_f	267.6	1109.1	369.8	1056	280.2	0	377.4	1479.1	417.9	1558.2
Butyric acid	t_0	0	0	0	0	0	0	0	0	0	0
	t_f	929.8	317.6	662.5	177.7	773.3	0	881.1	1207.5	821.6	1425.4
Iso-butyric acid	t_0	0	0	0	0	0	0	536	10.8	537.8	0
	t_f	108	0	7.5	113.9	78.	7677.3	57.7	29.8	182.4	85.1
Ethanol	t_0	0	0	0	0	0	0	0	0	0.	0
	t_f	494.9	0	489.7	0	598.6	13 683.2	621.3	303.0	479.0	303.5
Formic acid	t_0	0	0	0	0	0	0	0	0	0	0
	t_f	0.3	0	0.5	0	0.5	0	0.52	0	0.44	0
Lactic acid	t_0	0	0	0	0	94.1	0	37.6	1524.3	38.7	1137.6
	t_f	0.5	0	0.4	0	0	0	0.22	0	0	0
Propionic acid	t_0	0.	0	0	0	0	0	0	0	0	0
	t_f	69.4	189.4	34.8	130.5	65.8	0	15.53	91.2	20.0	111.2
Valeric acid	t_0	0	0	0	0	0	0	0	0	0	0
	t_f	0	0	0	0	0	0	44.1	0	48.0	0
Iso-valeric acid	t_0	0	0	0	0	0	0	270.1	0	260.8	0
	t_f	45.4	194.4	45.4	179.1	143.4	0	142.8	303.6	182.7	625.4

Table 4. Initial and final results of the main physicochemical analyses performed in the AF experiment for 30 days

Batch operational conditions											
		Control		$A_{PT}7$		$A_{PT}9$		$T_{PT}7$		$T_{PT}9$	
Analyses (mg L^{-1}) * × •	Time	P	NP	P	NP	P	NP	P	NP	P	NP
CH_4•	t_0	0	0.	0	0	0.0	0	0	0	0	0
	t_f	11 086.4	5.7	0	0.5	52.3	0.7	0	0	0	0
COD*	t_0	8368.6	8634.3	7336.9	11 174.5	8583	15 294.5	9325.3	8758	10 000	9736
	t_f	7391.1	11 054	7694.8	10 658.8	7610	10 584.7	8859.7	14 289.6	8779.3	13 845
TOC	t_0	2005.7	1695.3	3280	1326	2890	1636	3467	2230	3691.	2237
	t_f	2284	1181	2229	1213	2385	1176	3133	1842	3266	1784
TN	t_0	333.4	618.0	309.8	69.2	306.5	100.3	468.3	219.2	463.5	216.1
	t_f	279.3	175.0	268.2	157.4	403.6	136.8	544.9	317.6	484.1	284.2
C:N ratio	t_0	6.02:1	2.7:1	10.59:1	19.15:1	9.43:1	16.31:1	7.40:1	10.17:1	7.96:1	10.35:1
	t_f	8.18:1	6.75:1	8.31:1	7.71:1	5.91:1	8.60:1	5.75:1	5.80:1	6.75:1	6.28:1
TS	t_0	0.0052	5.7	0.005	6.7	0.005	6.7	0.007	7.7	0.007	8.5
	t_f	0.003	5.5	0.003	5.1	0.003	4.7	0.004	6.1	0.004	5.6
TVS	t_0	0.004	4.98	0.004	5.9	0.005	5.8	0.006	6.8	0.006	7.4
	t_f	0.003	4.66	0.003	4.23	0.003	4.0	0.003	5.1	0.003	4.6
pH	t_0	8.25	8.25	7.0	7.0	9.0	9.0	7.0	7.0	9.0	9.0
	t_f	4.8	5.1	4.6	5.0	5.0	5.0	4.9	5.1	5.1	5.2

Note: Batches consisted of control, alkaline and thermal pre-treatments with initial pH 7.0 and 9.0 ($A_{PT}7$, $A_{PT}9$, $T_{PT}7$ and $T_{PT}9$) with pressurized (P) and nonpressurized (NP) headspaces. Analyses considered are chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen (TN), total solids (TS), total volatile solids (TVS) and pH.

The CH_4 production levels observed in our experiments over 30 days were negligible in most setups except alkaline pre-treatment initial pH 9.0 and P headspace (i.e. $A_{PT}7$NP, 0.54 mL; $A_{PT}7$P, 0.00 mL; $A_{PT}9$NP 0.67 mL and $A_{PT}9$P, 52.29 mL).

This could be a consequence of the adaptation of the microbial community in this setup. The main products were acetic acid, propionic acid and ethanol. Because the acetic acid production was low (i.e. 280.2 mg COD_{AcOH} L^{-1}) and there was CH_4 production,

it can be conjectured that acetic acid with the dissolved H_2 was partially converted to CH_4 and CO_2.[7,54]

Another correlation between acetate and CH_4 production was the fact that although there was no CH_4 production in inoculum alkaline treatment with initial pH 7.0 in both NP and P headspace batches, acetate production increased 185.6% from NP headspace assay to P headspace (i.e. A_{PT}7NP 1056 mg COD_{AcOH} L^{-1} and A_{PT}7P 369.8 mg COD_{AcOH} L^{-1}). So, headspace pressure can favor acetate production in acetoclastic methanogens.

The detected levels of CO_2 in both A_{PT}7NP and A_{PT}7P (i.e. 1.84 and 60.8 mL, respectively) were low as was the average of the final pH for both conditions (i.e. 4.8). Hence, instead of methanogenesis being inhibited owing to disturbance in the buffer system,[55] we can infer that most CO_2 was dissolved in the liquid phase, decreasing the pH.[56]

Initial pH, headspace pressure and inoculum pre-treatments influenced VFA product spectra

Cheese whey has a natural tendency to acidify and because pH was not controlled during the experiments (P and NP batches displayed an average pH of 4.96 and 5.26, respectively), it is likely that hydrolysis was not a limiting step in AF.

An additional hypothesis to justify the different production of VFAs and alcohol in all batches was that together with uncontrolled pH, the headspace internal pressure variation from P and NP batches played a key role in the spectra and quantity of VFAs produced.

In P batches, the pressure would contribute to the increase of the concentration of dissolved H_2 within the liquid phase, which influenced both gas and VFA spectra. In anaerobic processes, $H_2$is formed in the liquid phase.[57] Because of its low solubility, which is influenced by its partial pressure and temperature,[58] a high amount of dissolved $H_2$is usually found in the liquid phase.[57]

Synthrophic microorganisms are thermodynamically constrained by the H_2partial pressure. A H_2partial pressure $>10^{-4}$ atm leads to VFA and alcohol accumulation, and methanogenesis inhibition.[23,59,60]

However, H_2utilization by bacteria varies according to H_2partial pressure, mass transfer rates, temperature, product spectra and microbial communities' composition.[59,61] In NP batches, due to headspace release, gas and VFA production restarted daily, after each sampling. Overall, NP batches yielded higher VFAs compared to P batches as evidenced by experiments. We can deduce that NP batches displayed a higher H_2 partial pressure. Special highlights can be given to A_{PT}9 NP (21 360.54 mg $COD_{compound}$ L^{-1}), T_{PT}9 NP (4108.7 mg $COD_{compound}$ L^{-1}) and T_{PT}7 NP (3414.2 mg $COD_{compound}$ L^{-1}) when compared to their counterparts (A_{PT}9 P 1940.1 mg $COD_{compound}$ L^{-1}, T_{PT}9 P 2152 mg $COD_{compound}$ L^{-1} and T_{PT}7 P 2140.6 mg $COD_{compound}$ L^{-1}). Both P and NP controls and A_{PT}7 did not present a substantial difference with an increase of ≈2.9% in VFAs produced in NP batches compared to P batches as seen in Table 3.

It is important to stress that although A_{PT}9 NP, T_{PT}9 NP and T_{PT}7 NP produced greater quantities of metabolites, they did not produce a great variety of VFAs and alcohol. A_{PT}9 NP produced 7677.3 mg COD_{IBA} L^{-1} iso-butyric acid and 13 683.2 mg COD_{EtOH} L^{-1} ethanol. The production of these metabolites together with the absence of acetic acid production is a clear result of a metabolic shift that was favored as a consequence of

Table 5. Volatile fatty acids (VFA) spectra of replicated control and thermal pre-treatment (pH 9.0) acidogenic fermentation experiment compared to the original experiment for 10 days

VFA production (mg COD compound L^{-1})

		UNICAMP		TU Delft	
Products	Time	C	T	C	T
Acetic acid	t_0	154.3	308.3	0.	19.3
	t_f	1037.3	1361.1	319.1	1407.9
Butyric acid	t_0	0	0	0	0
	t_f	187.3	1121.5	5.4	106.9
Iso-butyric acid	t_0	0.8	0	16.0	149.7
	t_f	0	0	16.0	58.8
Caproic acid	t_0	–	–	290.9	179.7
	t_f	–	–	479.2	2587.2
Iso-caproic acid	t_0	–	–	0	0
	t_f	–	–	0	42.8
Ethanol	t_0	0	0	–	–
	t_f	0	155.1	–	–
Formic acid	t_0	0	0	4.3	4.3
	t_f	0	0	0	0
Lactic acid	t_0	0	1137.6	–	–
	t_f	0	0	–	–
Malic acid	t_0	0	24.7	–	–
	t_f	0	0	–	–
Propionic acid	t_0	0	0	11.2	11.2
	t_f	161.	55.2	85.8	55.9
Valeric acid	t_0	0	0	0	6.9
	t_f	0	0	20.8	27.8
Iso-valeric acid	t_0	0	0	0	20.8
	t_f	46.5	235.9	20.8	83.3

Note: Replicate results are expressed as a means average of the triplicate assays. (–) The product was not evaluated.

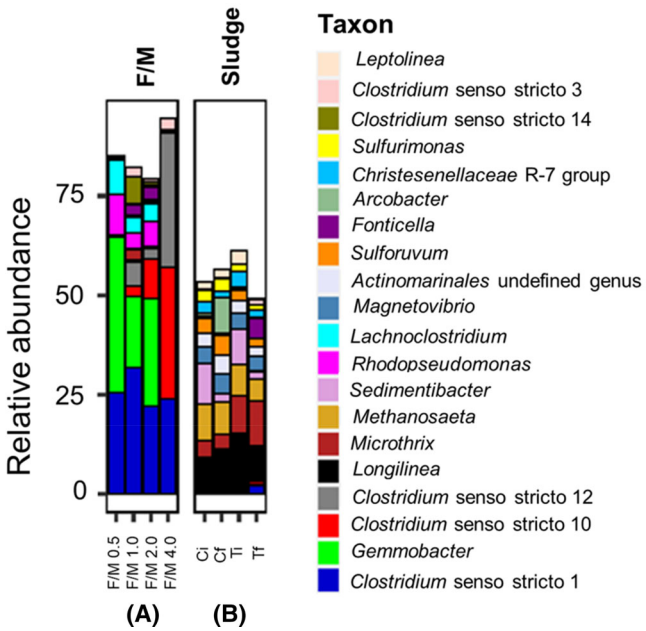

Figure 3. Microbial communities' compositions and evolution. The 20 most abundant archaeal and bacterial genera were identified through 16S rRNA gene amplicon sequencing. (A) microbial population from the F/M ratios experiments (FM). (B) microbial populations from the control and thermal pre-treated inoculum batches (T_{PT}9 NP).

the imposed selective pressures in this batch (i.e. alkaline inoculum pre-treatment, initial pH of 9.0 and NP headspace).

This was in opposition to all other conditions tested. It is likely that in such operational conditions, acetate served as the substrate for fermentative bacteria to form iso-butyric acid and ethanol, as already described by Thatikayala *et al.*[62] and Liu *et al.*[63] Both chemicals are of great industrial interest (i.e. pharmaceutical, feed, chemical, biofuels). Optimization of AF towards the production of either ethanol or iso-butyric acid or both can be an additional approach for cheese whey valorization.

T_{PT}9 NP and T_{PT}7 NP displayed production of acetic acid of 1479.1 and 1558.2 mg COD_{AcOH} L^{-1}, respectively. Acetic acid was consumed in A_{PT}9NP, whereas in A_{PT}7NP its production was slightly lower than both T_{PT}9 NP and T_{PT}7 NP with 1056 mg COD_{AcOH} L^{-1}.

Interestingly, CNP also presented a higher acetic acid production of 1109.1 mg COD_{AcOH} L^{-1}, which shows that headspace pressure is a more suitable selective pressure parameter than inoculum pre-treatment for acetic acid production. The produced acetic acid can thus be utilized as an organic carbon source for higher added-value biological processes (i.e. microalgal photoorganoheterotrophic biomass production).

The imposed parameters (i.e. inoculum pre-treatments, initial pH and headspace pressure) also acted as selective pressures in the batch experiments modifying the microbial fermentation end-products. In general, thermal pre-treatment, NP headspace and initial pH 9.0 displayed a trend for higher VFA production. Nevertheless, it is not possible to affirm what the best combination of parameters would be because there was no pattern of production observed for all VFAs in this given setup.

However, the combination of parameters can be suitable for the production of specific volatile acids or ethanol, as seen in T_{PT}7NP and T_{PT}9NP for the production of acetic (1479.1 and 1558.2 mg COD_{AcOH} L^{-1}, respectively) and butyric acids (1207.5 and 1425.4 mg COD_{BTA} L^{-1}), and A_{PT}9NP for iso-butyric acid and ethanol (7677.3 mg COD_{IBA} L^{-1} and 13 683.2 mg COD_{EtOH} L^{-1}).

Initial WPC40 carbohydrate concentration was ≈260 mg glucose L^{-1}, stabilizing at 65 mg glucose L^{-1} on Day 4. The

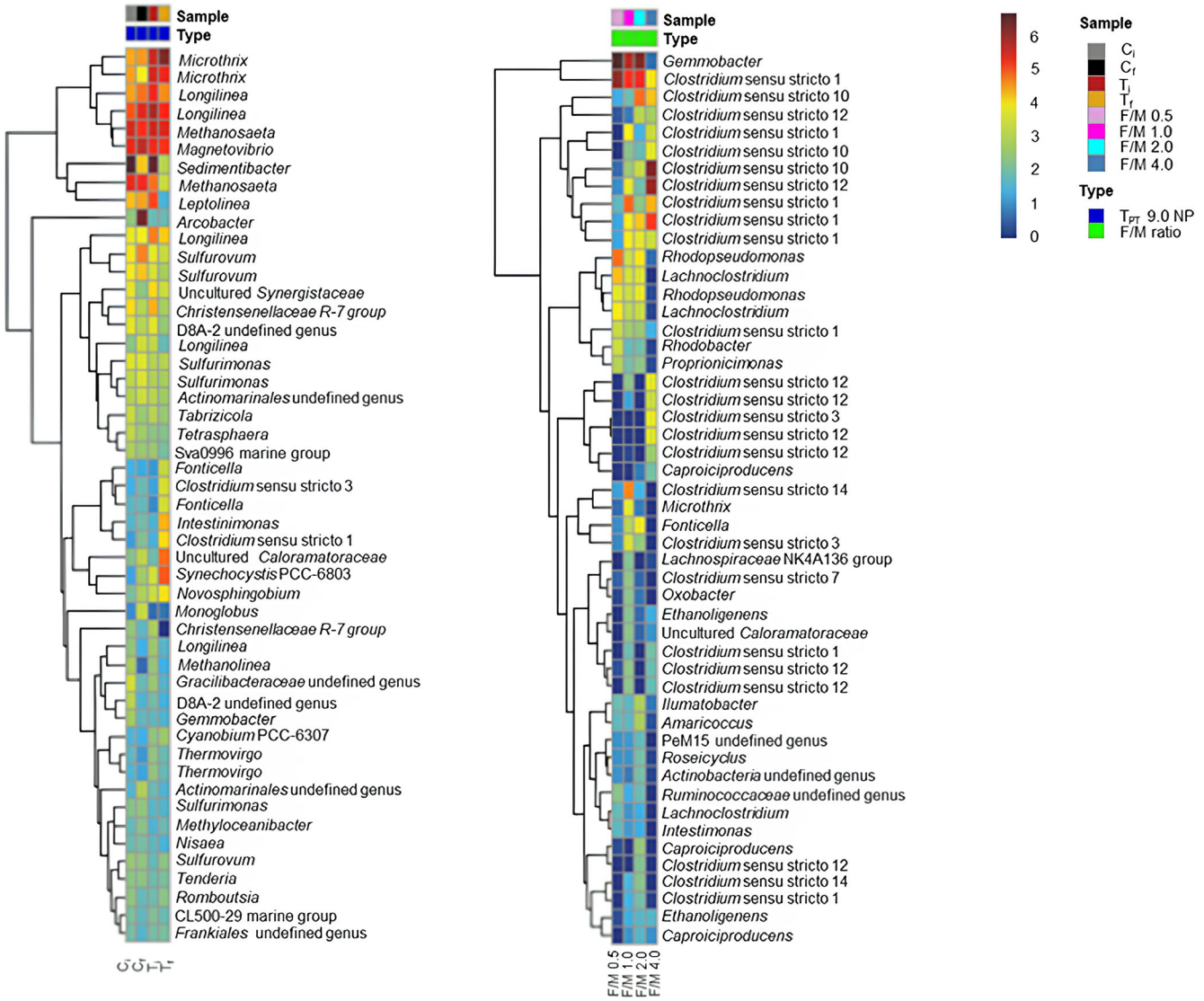

Figure 4. Amplicon sequence variant (ASV) abundance. The 50 most abundant archaeal and bacterial ASVs detected by the AmpliconTagger pipeline. FM, F/M ratios experiments; T_{PT}NP9, thermal pre-treated inoculum batches.

acidogenesis rate of 1.9 day^{-1}, was close to the typical rate of 2 day^{-1} observed in similar studies.[64] COD removal and VS showed similar patterns, with sCOD decreasing from 4.2 to 2.4 g L^{-1} and VS decreasing from 4.9 to 4.7 g L^{-1}. This islikely to have been the consequence of biomass production and organic matter oxidation. Control batches showed a decrease from 4.9 to 4.7 g L^{-1}, corroborating the COD removal results. Cheese whey alkalinity varied by 6% in all batches (1.53 to 1.45 $CaCO_3$ L^{-1}). Acidification of whey might thus have led to the production of VFAs, as seen in both control and thermal pre-treatment pH 9.0. Table 4 shows the results of the main physicochemical analyses. Table 5 depicts the VFA spectra of the control and thermal pre-treatment (pH 9.0) acidogenic fermentation experiment at both Unicamp and TU Delft during the first 10 days of the experiment. Inoculum thermal pre-treatment, initial pH and NP headspace acted as selective pressure mechanisms on microbial communities during cheese whey acidogenic fermentation.

In the replicate experiment performed in TU Delft, both control and inoculum thermal pre-treated microbial communities' composition and evolution were determined through 16S rRNA gene amplicon sequencing (Figs 3 and 4). Initial microbial communities were highly similar, with few quantitative and only minor qualitative differences being observed. They both shared seven of their eight main detected phyla, 10 of their 11 main detected classes, and 18 of their 19 main detected orders, as well as their 10 and five most important detected families and genera, respectively.

As shown in Figs 3 and 4, the common genera included fermenters and VFA producers (such as *Sedimentibacter*,[65] *Longilinea*, *Leptolinea*[66] and *Christensenellaceae* R-7 group),[67] the lipid-accumulating *Candidatus microthrix* (found in both bulk and foam),[68] and microorganisms involved in sulfur (S) and nitrogen (N) metabolisms (*Magnetovibrio*,[69] *Sulfurovum* and *Sulfurimonas*,[70] as well as the acetoclastic methanogen *Methanosaeta*).[71]

The only noticeable difference consisted of *Cyanobacteria* being detected in the inoculum thermal pre-treated population (Fig. 4). Cyanobacteria are aerobic photosynthetic microorganisms.[72] However, they display mechanisms that enable them to survive in unfavorable conditions such as in hypoxic or anaerobic[70] environments. Although chlorophyll (Chl) deficiency can be caused by low levels of O_2, these microorganisms can regulate Chl production by activating O_2-independent oxidases or by inducing the transcription of genes that encode enzymes that work in microoxic conditions.[73] In the given scenario, facultative anoxygenic photosynthesis uses sulfide as the electron donor with photosystem I-driven photo assimilation.[74]

Control and inoculum thermal pre-treated microbial communities evolved differently along the process. The final populations presented highly similar compositions at the phylum, class, order and family levels, sharing all or almost all the main detected taxons with only quantitative differences being perceived. Noticeable qualitative and quantitative differences were, however, observed at the genus level (Figs 3 and 4). Figure 5 depicts the beta diversity analysis of the samples.

In the control final microbial community, an increase was observed in fermenters, such as *Longilinea* and *Leptolinea*,[66] and microorganisms involved in S and N metabolisms, such as *Magnetovibrio*,[69] *Sulfurovum* and *Sulfurimonas*.[70] *Arcobacter*,[75] a sulfide oxidizer and denitrifier, also appeared and became the second genus of importance, whereas the two VFA producers *Sedimentibacter*[65] and *Christensenelaceae* R-7 group[67] strongly decreased.

In opposition, however, in the inoculum thermal pre-treated final microbial community, an increase was observed in VFA and alcohol producers, such as *Clostridium*,[76] *Fonticella*[77] and *Intestinimonas*,[78]

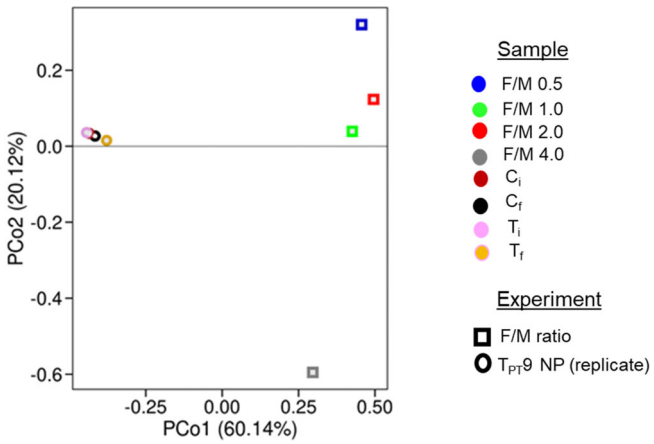

Figure 5. Beta diversity analysis of the microbial populations. Principal coordinates analysis computed from bray–curtis dissimilarity distance matrix. FM, F/M ratios experiments; T_{PT}NP9, thermal pre-treated inoculum batches.

whereas fermenters such as *Longilinea* and *Leptolinea* strongly decreased. The lipid-accumulating and bulk and foaming *Candidatus microthrix*[68] and the cyanobacteria *Synechocystis*[73] increased, and *Magnetovibrio*,[69] *Sulfurovum* and *Sulfurimonas*,[70] involved in sulfur and nitrogen metabolisms, slightly decreased.

Those results could explain the differences in performance observed in both processes. Methanogenesis was likely halted in the inoculum thermal pre-treated assay by the strong decrease observed in microorganisms such as *Longilinea* and *Leptolinea*, which are fermenters known to provide substrates to methanogens,[79,80] concomitantly with the strong increase in VFA and alcohol producers.

The decrease, even if limited, observed in *Methanosaeta* tends to confirm that methanogens were inhibited. The presence of *Methanosaeta* could be explained by several hypotheses: (i) samples detected were dead biomass or spores; (ii) inoculum pre-treatments were sufficient to inhibit or diverge the metabolic pathway for CH_4 formation; or (iii) they could have been outcompeted by acetate fermenters.[81-84] Further -omics studies can clarify this finding. The presence of denitrifiers could be a response to batches' O_2 limitation because they are aerobic facultative.[85] They also can compete for nitrate content in cheese whey.[64,86]

Impact of F/M ratio on VFA production during acidogenic fermentation of cheese whey

Lower F/M ratio is more efficient for VFA production

Four different F/M ratios of WPC40 were evaluated in an experiment performed with a thermal pre-treated inoculum at an initial pH of 9.0 and under NP conditions. Ratios were F/M 0.5, F/M 1.0, F/M 2.0 and F/M 4.0, corresponding to 0.5, 1.0, 2.0 and 4.0 g COD g VSS^{-1}, respectively.

The F/M 0.5 ratio acted as a positive control because the thermal batch in the replicate experiment presented the same F/M ratio of 0.5 g COD g VSS^{-1}. About 95% of carbohydrates were consumed by Day 3, whereas substrate acidification started in the first couple of days F/M 0.5 (1056 mg $COD_{compound}$ L^{-1} day^{-1}), F/M 1.0 (789 mg $COD_{compound}$ L^{-1} day^{-1}), F/M 0.2 (486 mg $COD_{compound}$ L^{-1} day^{-}1nd F/M 4.0 (390 mg $COD_{compound}$ L^{-1} day^{-1}).

As shown in Table 6, the F/M 0.5 ratio showed the best VFA production (3317.9 mg $COD_{compound}$ L^{-1}), whereas the F/M ratio 2.0 (345.9 mg L^{-1}) had the lowest. The NP thermal pre-treatment

Table 6. Volatile fatty acids (VFA) spectra of F/M ratio experiments

VFA production (mg CODcompound L^{-1})					
Products	Time	F/M 0.5	F/M 1.0	F/M 2.0	F/M 4.0
Acetic acid	t_0	34.0	9.2	0	0
	t_f	900.5	956.5	493.4	865.8
Butyric acid	t_0	0	0	0	0
	t_f	349.8	25.3	34.6	36.5
Iso-butyric acid	t_0	1707.0	79.0	60.7	25.6
	t_f	43.2	38.9	19.3	13.0
Caproic acid	t_0	154.3	30.4	0	0
	t_f	1823.4	1042.2	3620.9	194.2
Iso-caproic acid	t_0	0	0	0	0
	t_f	0	0	0	0
Formic acid	t_0	1.8	2.2	2.9	3.3
	t_f	0	0	0.3	0
Propionic acid	t_0	5.3	0	0	0
	t_f	66.3	34.4	7.3	23.3
Valeric acid	t_0	9.8	0	0	0
	t_f	45.2	0	27.2	4.9
Iso-valeric acid	t_0	32.6	17.6	0	0
	t_f	89.5	58.2	40.3	27.4
Total production		3317.9	2155.5	4211.5	1165.1

Note: F/M 0.5, F/M 1.0, F/M 2.0 and F/M 4.0: 0.5, 1.0, 2.0 and 4.0 g COD g VSS^{-1}, respectively. F/M 0.5 acted as a positive control as it has the same 0.5 g COD g VSS^{-1} ratio as the replicated thermal pre-treatment batch in the 'Impact of F/M ratio on VFA production during acidogenic fermentation of cheese whey' section. The experiment was performed in triplicate and lasted 10 days.

batch in the replicate experiment had a VFA production of 2568.3 mg L^{-1}. There was no production of CH_4 in all samples which corroborates the results observed in previous experiments.

F/M ratios affected microbial communities' evolution

Microbial communities evolved differently during the F/M experiments. As shown in Fig. 5, increasing F/M ratios significantly impacted the microbial populations' evolution, with the strongest impact observed at the highest ratio of 4.0. Under such conditions, the microbial population was ultra-dominated by a single genus, *Clostridium* (Figs 3 and 4). At lower F/M ratios, the community was mainly composed of the same four genera, which altogether represented 84.3%, 77.6% and 74.7% of the F/M-0.5, F/M-1 and F/M-2 populations, respectively (Fig 3 and 4).

Those four genera were: *Gemmobacter*, a poly-hydroxybutyrate-accumulating and denitrifying bacteria;[87] *Clostridium*, a known fermenter and VFA producer;[76] *Lachnoclostridium*, a known butyrate producer;[88] and *Rhodopseudomonas*, a highly metabolically versatile bacteria capable of H_2 production through N_2 fixation or poly-hydroxybutyrate production.[89] The operational conditions applied in the F/M-0.5 experiment were thus apparently those that favor the best microbial combination to produce the highest level of VFAs. Further characterizations (i.e. metagenomics and/or metatranscriptomics analyses) should be performed to better understand why such close microbial populations could generate different amounts of VFAs.

CONCLUSIONS

Acidogenic fermentation is a successful alternative for producing VFAs of economic interest out of high-strength residues such as cheese whey. Inoculum pre-treatments can play selective pressures on microbial communities, selecting microorganisms that can thrive in imposed conditions and producing diverse end products. The main conclusions from this work are the following:

- Thermal pre-treatment of inocula in halting methanogenesis regardless of headspace pressure, pH and F/M ratios.
- Contrary to the literature, alkaline pre-treatment did not improve methanogenesis. However, A_{PT}9NP produced significant quantities of iso-butyric acid (7677.3 mg COD_{IBA} L^{-1}) and ethanol (13 693.2 mg COD_{EtOH} L^{-1}). Further studies on the mechanisms of this pre-treatment are necessary to optimize the process.
- Although *Methanosaeta* was present in both control (pH 8.25) and thermal (pH 9.0) batches, no CH_4 production was detected in thermal pre-treatment conditions. The detection of *Methanosaeta* in the 16S rRNA gene amplicon sequencing analysis could be a result of the inactivation or destruction of this microorganism during pre-treatment.
- A low F/M ratio of 0.5 g COD g^{-1} VS selected a microbial community that produced a high level of VFAs during AF.
- Headspace pressure shifts the metabolic pathways towards VFAs and alcohol production in AF, with P headspace batches having more diverse VFA spectra, and P ones with higher amounts of VFAs.
- Initial pH of 9.0 produced the greatest number of products, regardless of headspace pressure and inoculum pre-treatment. The drastic pH drop influences the redox potential of the medium facilitating the uptake of compounds while setting the grounds for ecologic relationships within the microbial community

This work aimed to investigate the impact of imposed parameters (i.e. headspace pressure, inoculum thermal and alkaline pre-treatment, and initial pH) on the microbial community during cheese whey AF. The objectives were successfully achieved by halting methanogenesis and increasing acetic acid, iso-butyric acid and ethanol production. Acetate, a product of interest, can be used for different biological conversions (e.g. microalgal photoorganoheterotrophic processes), whereas iso-butyric and ethanol have applications in pharmaceutical, feed, chemical and biofuel industries.

However, further understanding is needed, especially regarding the influence of these parameters on metabolic pathways and microbial interaction. Although methanogenesis was halted with thermal pre-treatment, there is a need to comprehend the competition for the substrate.

Thermal inoculum pre-treatment can be used in short fermentation, but its efficiency with different substrates, types of inocula and reactor configurations requires further studies for process implementation and optimization.

In order to drive fermentation product spectra towards desired compounds it is important to understand VFA and H_2 production and consumption mechanisms. Future 16S rRNA metagenomics analyses will be fundamental to bridge these knowledge gaps.

AUTHOR CONTRIBUTIONS

M.P.G.dA conceptualized the experiment and wrote the manuscript with direct core inputs by D.G.W and G.M. M.P.G.dA performed the analysis for the acidogenic fermentation in Brazil and in The Netherlands, the latter with the assistance of C.M. The analysis and conceptualisation the F/M experiments were performed by C.M. The experiment was designed by MPGdA, DGW and GM by confronting ideas, concepts and solutions to technological, outcomes on microbial communities' genomics were provided by G.B and J.T. All authors read, edited, and provided critical feedback to the manuscript.

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CONFLICT OF INTEREST STATEMENT

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

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon request.

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