

# Enrichment Versus Bioaugmentation—Microbiological Production of Caproate from Mixed Carbon Sources by Mixed Bacterial Culture and *Clostridium kluyveri*

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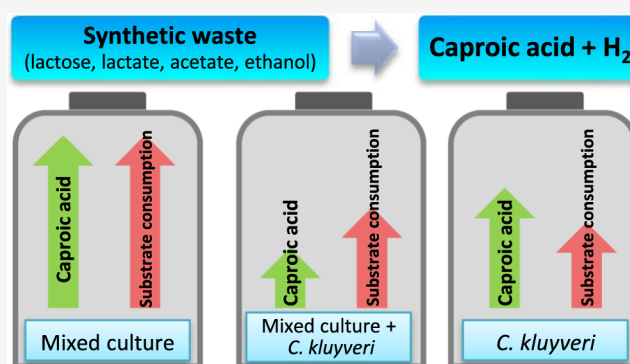
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**ABSTRACT:** Chain elongation is a process that produces medium chain fatty acids such as caproic acid, which is one of the promising products of the carboxylate platform. This study analyzed the impact of bioaugmentation of heat-treated anaerobic digester sludge with *Clostridium kluyveri* (AS + Ck) on caproic acid production from a mixed substrate (lactose, lactate, acetate, and ethanol). It was compared with processes initiated with non-augmented heat-treated anaerobic digester sludge (AS) and mono-culture of *C. kluyveri* (Ck). Moreover, stability of the chain elongation process was evaluated by performing repeated batch experiments. All bacterial cultures demonstrated efficient caproate production in the first batch cycle. After 18 days, caproate concentration reached  $9.06 \pm 0.43$ ,  $7.86 \pm 0.38$ , and  $7.67 \pm 0.37$  g/L for AS, Ck, and AS + Ck cultures, respectively. In the second cycle, AS microbiome was enriched toward caproate production and showed the highest caproate concentration of  $11.44 \pm 0.47$  g/L. On the other hand, bioaugmented culture showed the lowest caproate production in the second cycle ( $4.10 \pm 0.30$  g/L). Microbiome analysis in both AS and AS + Ck culture samples indicated strong enrichment toward the anaerobic order of Clostridia. Strains belonging to genera *Sporanaerobacter*, *Paraclostridium*, *Haloimipatiens*, *Clostridium*, and *Bacillus* were dominating in the bioreactors.



## 1. INTRODUCTION

Production of biochemicals and biofuels from biomass feedstock by fermentation could be facilitated through the carboxylate platform, which is a promising technology within a bio-based economy concept.<sup>1</sup> Chain elongation is an important part of this platform and an emerging biotechnological process which produces medium chain fatty acids (MCFAs) from volatile fatty acids (VFAs). MCFAs are straight-chain carboxylic acids with 6–12 carbon atoms and are promising precursors for the production of other added-value products.<sup>2</sup> During primary fermentation, the substrate is converted to a mixture of short-chain carboxylates and alcohols. *n*-Caproate is a product of secondary fermentation, in a biochemical process of carboxylic acid chain elongation. *n*-Caproate is one of the most attractive MCFAs because of its broad industrial and agricultural applications. It could be used as a feedstock in the chemical industry, feed additive, antimicrobial agent, or as a biofuel precursor.<sup>3</sup>

The most important chain elongation pathway is a cyclic process of reverse  $\beta$ -oxidation (RBO).<sup>4</sup> Chain elongation requires energy-rich, reduced compounds such as ethanol or lactic acid, which can be transformed to acetyl-CoA molecule. Then, acetyl-CoA is added to carboxylic acid, resulting in chain elongation by two carbon atoms per reaction cycle.

Consequently, acetate (C2) is elongated to butyrate (C4) and then to caproate (C6) and so forth.<sup>2,3</sup> It is proposed that other pathways can also be responsible for chain elongation, like fatty acid biosynthesis (FAB) pathway.<sup>5</sup> FAB is also a cyclic process but here malonyl-CoA plays the role of a 2-C donor. Moreover, it is less efficient and consumes more adenosine 5'-triphosphate than the RBO process.<sup>5</sup>

*Clostridium kluyveri* is a well-known and efficient caproic acid producer that simultaneously utilize acetate and ethanol for growth.<sup>6</sup> However, it is unable to utilize simple sugars such as glucose or lactose, which are easily fermentable by many other bacteria.<sup>7</sup> The fact that different pure bacterial cultures are unable to utilize substrates in the form of lipids, carbohydrates, and proteins for *n*-caproate production makes it necessary to use mixed cultures.<sup>3</sup> Microbiomes can convert complex organic wastes into valuable products due to their metabolic diversity. Anaerobic microbiomes are composed of different hydrolytic

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and fermentative bacteria which degrade complex substrates to compounds suitable for chain elongation. Thousands of microbial species are present within such microbial communities and their structure depends on the process conditions, which often makes it difficult to control the bioaugmented process. There is still limited understanding on the key metabolic interactions in this complex system, which could lead to bioconversion of the substrate to nonspecific products. However, one of the potential ways to improve the conversion of VFAs into MCFAs may be increasing the content of efficient chain-elongating microbes, such as *C. kluyveri*, in the microbial community.

A considerable limitation in mixed-culture systems is activity of other metabolic pathways which compete with chain elongation—mainly acetoclastic and hydrogenotrophic methanogenesis. Methane formation can be mitigated by adding methanogen inhibitors<sup>8,9</sup> or by application of mildly acidic conditions (pH about 5.5) during the process.<sup>2,10</sup> However, these methods require constant controlling of the inhibitor concentration or pH. Another possibility is a physical pretreatment of inocula, which is performed only once at the beginning of the process. The principle behind the use of pretreatment is that many bacteria are able to form spores under severe conditions of temperature, pH, radiation, electric current, and so forth.<sup>11</sup> The formed endospores survive the extreme conditions, unlike non-sporulating bacteria which are suppressed. Pretreatment is especially helpful in suppressing not only methanogenic microorganisms<sup>12</sup> but also homoacetogens, sulfate reducing bacteria, propionate producers, or lactic acid bacteria.<sup>11</sup> However, other desirable microorganisms may also be inhibited, which reduces metabolic diversity of a microbiome. The heat treatment is widely used because of its simplicity, easy control, and effectiveness in inhibiting methanogens.<sup>13</sup>

Several studies have been carried out to characterize the process of MCFA formation from acetate and ethanol.<sup>9,14,15</sup> Hegner et al.<sup>15</sup> performed upscaling experiments with caproic acid production of 11.3 g/L, while *C. kluyveri* was the most abundant microorganism detected in the mixed culture. Steinbusch et al.<sup>9</sup> obtained 8.17 g/L of caproic acid in the batch experiments with ethanol and hydrogen as electron donors. However, real wastewaters are composed of a wide range of complex compounds. For example, disposing of acid whey from dairy industry is an important environmental problem because of its large production and difficulties with its disposal.<sup>16</sup> Acid whey contains high concentrations of lactose and lactic acid, and therefore it is an attractive substrate for anaerobic fermentation and MCFA production. Thus, in this study, a medium with mixed carbon source consisting of lactose, lactate, acetate, and ethanol was applied. The aim of this work was to evaluate the impact of bioaugmentation of heat-treated anaerobic digester sludge with *C. kluyveri* on the caproic acid production from a mixed substrate. Anaerobic digester sludge is a mixture of anaerobic bacteria with a capability to degrade more complex compounds to VFAs. Therefore, a combination of *C. kluyveri* and anaerobic digester sludge could increase the overall process efficiency. The production of liquid and gaseous metabolites was analyzed and comprehensively assessed. Another aim was to evaluate the stability of the bioaugmented culture in a repeated batch process. In addition, the bioaugmented microbial community structure was analyzed and compared with processes without the addition of *C. kluyveri*.

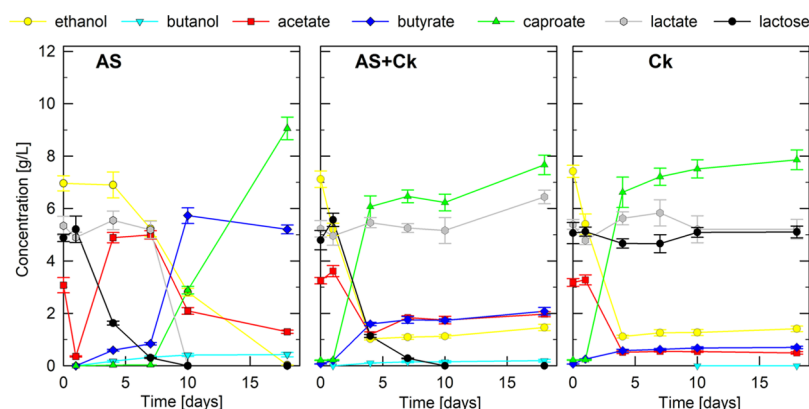
## 2. MATERIAL AND METHODS

**2.1. Inoculum.** *C. kluyveri* bacteria (DSM 555) were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ) and were cultivated on DSM-52 medium containing (per liter) 10 g of potassium acetate, 0.31 g of  $K_2HPO_4$ , 0.23 g of  $KH_2PO_4$ , 0.25 g of  $NH_4Cl$ , 0.20 g of  $MgSO_4 \cdot 7H_2O$ , 1 mL of trace element solution SL-10 (1.5 mg of  $FeCl_2 \cdot 4H_2O$ , 0.070 mg of  $ZnCl_2$ , 0.1 mg of  $MnCl_2 \cdot 4H_2O$ , 0.006 mg of  $H_3BO_3$ , 0.190 mg of  $CoCl_2 \cdot 6H_2O$ , 0.002 mg of  $CuCl_2 \cdot 2H_2O$ , 0.024 mg of  $NiCl_2 \cdot 6H_2O$ , and 0.036 mg of  $Na_2MoO_4 \cdot 2H_2O$ ), 1 mL of selenite-tungstate solution (0.003 mg of  $Na_2SeO_3$  and 0.004 mg of  $Na_2WO_4$ ), 1.00 g of yeast extract, 0.50 mL of resazurin solution (0.1% w/v), 20.00 mL of ethanol, 2.50 g of  $NaHCO_3$ , 1 mL of vitamin solution (0.1 mg of vitamin B<sub>12</sub>, 0.08 mg of *p*-aminobenzoic acid, 0.08 mg of D(+)-biotin, 0.2 mg of nicotinic acid, 0.1 mg of calcium pantothenate, 0.3 mg of pyridoxine hydrochloride, and 0.2 mg of thiamine-HCl), 0.25 g of L-cysteine-HCl-H<sub>2</sub>O, and 0.25 g of  $Na_2S \cdot 9H_2O$ . The initial pH was adjusted to 6.8. The medium was dispensed in 120 mL bottles and flushed with N<sub>2</sub> to create anaerobic conditions. The bottles were autoclaved, and bicarbonate, vitamins, cysteine, and Na<sub>2</sub>S were added from sterile stock solutions. Then, *C. kluyveri* were inoculated into the bottles, and they were incubated at 37 °C. Bacteria were transferred into a fresh medium every 4–5 days.

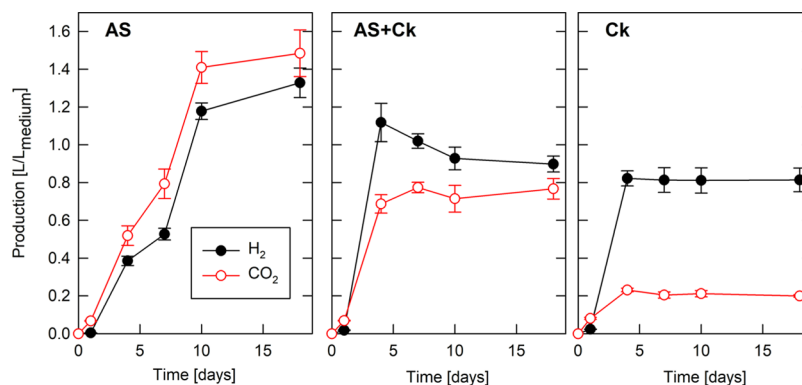
Anaerobic digester sludge (suspended) from a municipal wastewater treatment plant (WWTP) (city of Poznań, Poland) was applied as the source of mixed microorganisms. A full-scale WWTP anaerobic digestion reactor was treating primary and waste activated sludge in proportion 1:1 (by volume). The sludge was pretreated at 100 °C for 15 min with continuous mixing to inhibit the methanogenic activity and promote the spore-forming bacteria.<sup>10,17,18</sup>

**2.2. Procedures.** The batch experiments were carried out in triplicate in 120 mL serum bottles capped with butyl rubber stopper and aluminum cap (working volume of 55 mL). The growth medium (DSM-52) with modified carbon sources was used in fermentation experiments. The composition of carbon sources were lactose 5 g/L, acetate 2.5 g/L, ethanol 7 g/L, and lactate 5 g/L. In bioaugmentation experiments actively growing *C. kluyveri* (3.5% v/v) and pretreated anaerobic digester sludge (6.5% v/v) were inoculated into the medium (AS + Ck). In addition, two controls were carried out in which pretreated anaerobic digester sludge (AS) or *C. kluyveri* (Ck) were used (10% v/v). In order to evaluate the stability of the caproic acid production and to enrich caproic acid producers, batch experiments were performed in consecutive cycles—culture from previous experiment was used as an inoculum for the new batch process (10% v/v). During batch fermentation process anaerobic cultures were not mixed, and the pH of the medium was not controlled. Initial pH was set to  $7.5 \pm 0.1$ . Bioreactors were kept in an incubator at 30 °C. Gas and liquid samples were taken for analysis of gas composition, substrate degradation, and VFA formation.

**2.3. Analytical Methods.** The composition of the collected gases was measured by the gas chromatographic method and gas-tight syringe (Shimadzu GC2014 with Porapak N column, TCD detector). Nitrogen was used as the carrier gas at a flow rate of 15 mL/min, and the temperature of the injector, column, and detector were 110, 50, and 80 °C, respectively. All gas volumes are reported at 1 atm and 273.15 K. Details of the gas amount calculations are



**Figure 1.** Concentration of substrates and liquid products during the 1st cycle of batch experiments. Propanol, propionate, valerate, and caprylate were detected in concentrations below 50 mg/L and are not shown.



**Figure 2.** Hydrogen and carbon dioxide production during the 1st cycle of batch experiments.

described in the [Supporting Information](#). Organic acid and alcohol concentrations were monitored with gas chromatography (GC) using Shimadzu 2014 GC System based on the method described by Vasquez et al.<sup>19</sup> Samples were analyzed for methanol, ethanol, propanol, *i*-propanol, butanol, *i*-butanol, acetate, propionate, *i*-butyrate, butyrate, *i*-valerate, valerate, caproate, heptate, and caprylate. Prior to the analysis, samples were acidified with H<sub>3</sub>PO<sub>4</sub> and filtered with 0.45  $\mu$ m syringe filters. A flame ionization detector (FID) and a high-performance capillary column with a free fatty acid phase (Zebron ZB-FFAP, Phenomenex) were used. Helium was supplied at a flow rate of 7.38 mL/min. The initial oven temperature was 70 °C, maintained for 1 min, raised to 240 °C at 10 °C/min, and finally held at 240 °C for 3 min. The temperature of the FID and the injection port was 250 and 200 °C, respectively. Concentrations of lactate and lactose were determined with high-performance liquid chromatography (HPLC 20AT, Shimadzu equipped with Rezex<sup>TM</sup> ROA-Organic Acid H+ (8%) column and refractive index detector). 5 mM aqueous sulfuric acid was used for elution at the flow rate of 0.6 mL/min at 63 °C.<sup>20</sup> Each bioreactor was sampled for gas and liquid metabolites. Therefore, the results present the mean and standard deviations of three biological replicates. Specificity was calculated as product-to-all carboxylates concentration ratio in % mol C (eq S2). Conversion efficiency was calculated by dividing caproate concentration by concentration of the substrates (% mol C) (eq S3).

**2.4. DNA Extraction, Sequencing, and Microbial Population Analysis.** To evaluate composition of microbial consortia enriched during fermentations and identify com-

munity changes along the subsequent batch cycle, total DNA was isolated from post-fermentation and inoculum (prior to heat-treatment) samples and subjected to 16S rRNA gene fragment amplification and sequencing. Total genomic DNA was isolated from 150 mg of collected cell biomass as previously described in Duber et al.<sup>21</sup> and submitted to Genomed (Poland) for library construction and sequencing using Illumina Miseq instrument (250 bp paired-end sequencing, Miseq v2). Libraries were prepared according to 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, Part #15044223, Rev. B), with Herculease II Fusion DNA Polymerase Nextera XT Index Kit V2n. Amplification of V3 and V4 region of 16S rRNA gene was carried out with Q5 Hot Start High-Fidelity DNA Polymerase, primers 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC-TACGGGNGGCWGCAG) and 785R (5'-GTCTCGTGG GCTCGGAGATGTGTATAAGAGACAGGAC-TACHVGGGTATCTAATCC) according to manufacturer's recommendations. This primer set is universal for both bacteria and archaea. Sequences containing primers were trimmed with cutadapt, and all other reads were filtered out.<sup>22</sup> Subsequently, filtering, generation of operational taxonomic units (OTUs), and mapping of reads to OTUs were performed using the UPARSE/unoise3 pipeline.<sup>23</sup> Taxonomy was assigned to OTUs using SINTAX and Silva LTPs v132 database containing 13899 curated 16S ribosomal RNA sequences (taxonomic ranks below 80% USINTAX bootstrap confidence threshold measure<sup>23</sup> were considered unclassified). Each sample was normalized to the depth of the sample with the least read count. Downstream analyses were performed



**Table 1. Process Parameters and Carbon Balance for Repeated Fed-Batch Processes with Anaerobic Sludge (AS), *C. kluyveri* (Ck) and Anaerobic Sludge + *C. kluyveri* (AS + Ck) with Mixed Substrate<sup>a</sup> (Days with the Highest Concentration of the Caproic Acid Were Selected for the Calculations)**

inoculum	cycle	carbon provided [mM C]	caproate production [mM C]	substrate carbon provided [mM C]	substrate carbon consumed [mM C]	substrate carbon remaining [mM C]	caproate-to-carboxylates specificity [% mol C]	substrate-into-caproate conversion efficiency [% mol C]	pH final
AS	1	755	468	754	709	44	62.2	62.1	6.33
	2	788	591	749	669	80	67.9	78.8	6.08
Ck	1	800	406	785	357	430	88.6	51.7	6.70
	2	796	416	762	321	441	89.3	54.6	6.80
AS + Ck	1	774	396	759	456	343	70.9	52.2	5.48
	2	800	212	766	375	441	47.1	27.7	5.15

<sup>a</sup>Substrate—lactose, lactate, ethanol, acetate.

with qiime<sup>24</sup> and Phyloseq R package.<sup>25</sup> Raw sequences obtained in this study were submitted to NCBI Sequence Read Archive (SRA) database and are available under BioProject ID PRJNA587706 (BioSample accessions SAMN13198906–SAMN13198910).

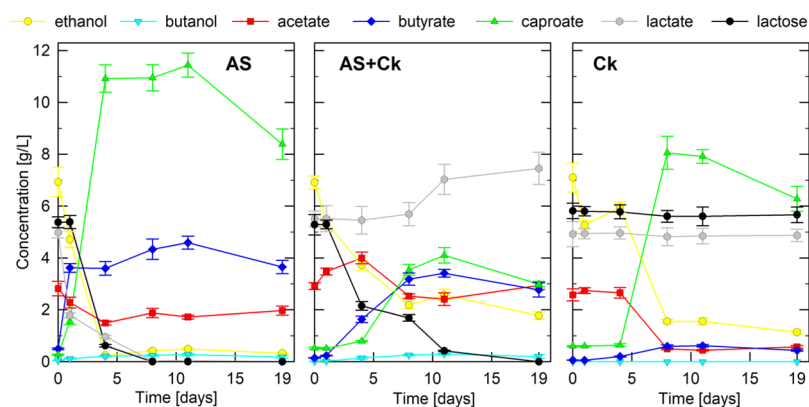
### 3. RESULTS AND DISCUSSION

**3.1. First Cycle of the Batch Processes.** Bioaugmentation of anaerobic digester sludge with *C. kluyveri* (AS + Ck) was tested in batch fermentation experiments. Fermentation with anaerobic digester sludge (AS) or *C. kluyveri* (Ck) as inoculum was used as a reference. Production of liquid metabolites in the 1st cycle is presented in Figure 1, while gaseous product formation is shown in Figure 2. AS culture quickly regained its activity after heat pretreatment. Surprisingly, acetate was utilized after 1 day, while no significant changes in the concentration of lactose, lactate, and ethanol were observed (Figure 1 AS). This indicated the presence of acetate-consuming microorganisms (e.g., sulfate reducers which oxidize acetate to CO<sub>2</sub>).<sup>26</sup> In the following days, the utilization of lactose was observed with the simultaneous production of acetate and hydrogen, which reached  $4.89 \pm 0.20$  g/L and  $0.39 \pm 0.03$  L/L<sub>medium</sub> ( $16.0 \pm 1.0$  mmol/L<sub>medium</sub>) after 4 days, respectively. The rapid production of hydrogen is characteristic for the fermentation of sugars in the dark fermentation process.<sup>27</sup> Lactose was consumed nearly completely after 7 days, and then bacterial culture began to utilize the remaining substrates. Acetate, ethanol, and lactate were utilized with simultaneous production of butyrate ( $5.73 \pm 0.30$  g/L) and caproate ( $2.89 \pm 0.14$  g/L). Lactate was completely consumed within 2 days. Production of butyrate and caproate during the chain elongation of acetate with lactate as a substrate was also reported by other researchers.<sup>3,28</sup> After the 10th day most of caproate came from the transformation of acetate, butyrate, and ethanol (Figure 1 AS). After 18 days of the process, the only metabolites measured in the bioreactor were acetate ( $1.29 \pm 0.06$  g/L), butyrate ( $5.20 \pm 0.16$  g/L), caproate ( $9.06 \pm 0.43$  g/L), and butanol ( $0.43 \pm 0.08$  g/L). Therefore, the heat pretreated mixed bacterial culture quickly specialized in the production of caproate. Similarly, Hegner et al.<sup>15</sup> observed MCFA production from acetate and ethanol after 10 days with inoculum derived from a leach-bed reactor. Most of the input carbon substrate was consumed (Table 1), while the pH in the bioreactor dropped to 6.33. As shown by Kenealy et al.,<sup>29</sup> caproate production can be significantly increased when acidification of the medium is prevented by base addition, and pH is kept above 6.0.

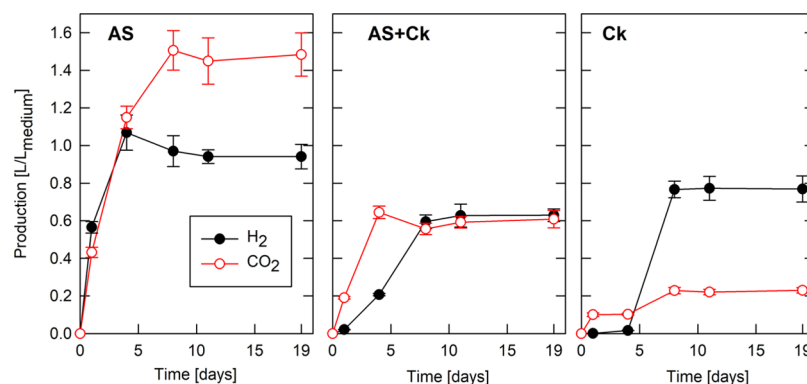
Bioreactors with pure culture of *C. kluyveri* proved to be a system with less complex profile of metabolites than bioreactors with AS (Figure 1 Ck). No changes in metabolites were observed after 1 day, as the bacteria were adapting to the conditions in the new bioreactor. Then, acetate and ethanol were quickly consumed with simultaneous caproate accumulation to  $6.62 \pm 0.58$  g/L after 4 days (Figure 1 Ck). Caproate production started earlier when compared to the AS process. Maximum butyrate concentration reached only  $0.68 \pm 0.05$  g/L, which indicated that it was quickly converted to caproate. Hydrogen and CO<sub>2</sub> amount reached  $0.81 \pm 0.06$  L/L<sub>medium</sub> ( $33.8 \pm 2.6$  mmol/L<sub>medium</sub>) and  $0.20 \pm 0.1$  L/L<sub>medium</sub> ( $8.3 \pm 0.3$  mmol/L<sub>medium</sub>), respectively (Figure 2 Ck). Ding et al.<sup>30</sup> showed that caproate production was inherently coupled with hydrogen formation. Lactose and lactate remained at the initial concentrations. This confirms that *C. kluyveri* have rather limited metabolic abilities and are unable to utilize these substrates.<sup>31</sup> Yin et al.<sup>14</sup> also studied caproate production by *C. kluyveri* DSM 555 from ethanol (3 g/L) and acetate (2.3 g/L) as carbon sources. However, in contrast to our research, they observed accumulation of butyrate in the bioreactor (1.6 g/L). As a result, they obtained lower concentration of caproate (2.9 g/L), while in our study, it reached  $7.86 \pm 0.38$  g/L after 18 days (Figure 1). Their results were not a consequence of lower substrate concentration because ethanol and acetate were still present at the end of the batch run (0.75 and 0.5 g/L, respectively). Yin et al.<sup>14</sup> observed pH drop from 7.5 to 5.7 due to accumulation of VFAs, while in our study, pH decreased only to 6.7. This pH drop to 5.7 was probably the cause of low caproate concentration because *C. kluyveri* prefers neutral pH for chain elongation.<sup>32,33</sup> Actually, Yin et al.<sup>14</sup> observed lower pH drop (to 6.3) at high initial ethanol concentration (23 g/L), which resulted in higher caproate concentration (8.42 g/L).

Ethanol ( $1.41 \pm 0.10$  g/L) and acetate ( $0.49 \pm 0.05$  g/L) were still remaining in the fermentation broth of Ck culture after 19 days (Figure 1). Yin et al.<sup>14</sup> also observed incomplete substrate utilization and suggested that it was probably due to product inhibition or pH decrease to around 6.0. However, in our study, final pH was around 6.7 (Table 1). The upper toxicity limit of an undissociated form of caproic acid reported in the literature is 0.87 g/L.<sup>2</sup> At pH 6.7, only about 1.5% of caproic acid exists in this form (0.12 g/L). Therefore, other effects were likely responsible for the incomplete utilization of substrates in our study.

Analysis of the combined process showed similarity to both AS and Ck cultures (Figure 1). Consumption of acetate and ethanol started after 1 day with concurrent production of



**Figure 3.** Concentration of substrates and liquid products during the 2nd cycle of batch experiments. Propanol, propionate, valerate, and caprylate were detected in concentrations below 50 mg/L and are not shown.



**Figure 4.** Hydrogen and carbon dioxide production during the 2nd cycle of batch experiments.

caproate to  $6.07 \pm 0.41$  g/L at the 4th day. Similar relationships were observed in the process with pure culture of *C. kluyveri*. On the other hand, production of caproate started after 10 days during the AS process. Therefore, in the AS + Ck system, caproate production could be attributed mainly to *C. kluyveri* bacteria. After the initial drop in acetate concentration, its concentration started to increase after the 4th day due to lactose fermentation by bacteria present in the anaerobic digester sludge. However, in contrast to the AS process, lactate was not utilized. This shows that bioaugmentation with *C. kluyveri* repressed somehow bacterial activity responsible for lactate utilization. However, the mechanism of this repression is not yet known. Addition of *C. kluyveri* could have inhibited the growth of some microorganisms present in the anaerobic digester sludge because *C. kluyveri* bacteria utilized a significant part of the substrate (acetate and ethanol). Additionally, the rapid increase in caproic acid concentration could result in the product inhibition for some microorganisms. As a result, concentration of butyrate in the AS + Ck system was lower than for AS culture because its production in the AS culture was associated with lactate utilization. The change of microbiological activity of bioaugmented culture was also represented by a different profile of hydrogen and  $\text{CO}_2$  production when compared to the AS culture (Figure 2). Caproate concentration reached  $7.67 \pm 0.37$  g/L after 18 days of the AS + Ck process.

In all tested systems, high hydrogen production was observed (Figure 2). The hydrogen partial pressure above 0.03 atm is crucial because it thermodynamically inhibits competing processes such as anaerobic oxidation of MCFAs

and excessive ethanol oxidation.<sup>2,31</sup> The highest hydrogen amount was observed in the AS experiments— $1.33 \pm 0.08$  L  $\text{H}_2/\text{L}_{\text{medium}}$  ( $55.2 \pm 3.3$  mol/ $\text{L}_{\text{medium}}$ ). This corresponds to the hydrogen partial pressure of 0.65 atm. This elevated hydrogen partial pressure likely resulted in the biological reduction of butyrate to butanol<sup>34</sup> (Figure 1 AS)—its concentration reached  $0.43 \pm 0.08$  g/L. Agler et al.<sup>1</sup> described the process of carboxylate reduction to corresponding alcohols by hydrogen at higher partial pressures. In the AS + Ck culture, lower  $\text{H}_2$  production resulted in a lower butanol concentration ( $0.19 \pm 0.05$  g/L).

It is worth noting that no methane was detected in any of the bioreactors. Hydrogen and  $\text{CO}_2$  were the only gaseous metabolites. Methane production is unfavorable because it contributes to the loss of carbon and energy, which negatively affects the efficiency of the chain elongation. Methane formation was reported in other chain elongation studies with anaerobic sludge as an inoculum,<sup>35</sup> but it was not detected in this study. Hence, heat pretreatment was sufficient to inhibit methanogenic activity. An analogous effect was observed by Ding et al.<sup>30</sup> during hydrogen and caproate formation by heat-treated seed sludge.

**3.2. Second Cycle of Batch Processes.** After 18 days of the first cycle, 10% v/v of all bacteria cultures were transferred to a fresh medium for the second cycle. The AS system after 1 day was utilizing mainly lactate with the simultaneous production of butyrate (Figure 3). Caproate concentration reached  $11.4 \pm 0.5$  g/L after only 4 days. Lactate, lactose, and ethanol were consumed nearly completely at this point. The obtained concentration of caproate exceeded most of those

reported in the literature during batch experiments—Weimar et al.<sup>35</sup> reported 6.1 g/L and Steinbusch et al.<sup>9</sup> reported 8.27 g/L; however, San-Valero et al.<sup>33</sup> demonstrated 21.2 g/L. In another study, with acetate (2.5 g/L), lactate (5.7 g/L) and ethanol (2.9 g/L) as substrates, caproate concentration reached 3.0 g/L.<sup>36</sup> Such high activity in our study resulted from an earlier adaptation to the substrates in the first cycle as well as proper composition of substrates. Lactate utilization was observed only in the AS system, and its utilization was associated with high CO<sub>2</sub> production in both cycles with the AS. Caproate concentration was higher than in the first cycle, and it was associated with lower H<sub>2</sub> amount (Figure 4). Moreover, electron balance (Table S2) revealed that less electron equivalents were distributed to hydrogen, butyrate, and butanol when compared to the first cycle. Therefore, high caproate concentration in the second cycle resulted from redirecting the reducing power from butyrate, butanol, and hydrogen to the chain elongation.

Pure culture of *C. kluyveri* showed similar metabolic activity as in the first cycle. Acetate and ethanol were nearly completely consumed, while a caproate concentration reached  $8.05 \pm 0.64$  g/L. However, the lag phase of caproate production was about 5 days because the inoculum did not come from the actively growing culture and bacteria required more time for adaptation.

The bioaugmented culture in the second cycle (AS + Ck) showed much lower caproate production than expected. Initially, lactose was fermented into acetate and butyrate. Then, these intermediates were elongated to caproate with ethanol as a reducing agent (Figure 3). Similar to the first cycle, lactate was not consumed. However, caproate concentration reached only  $4.10 \pm 0.30$  g/L after 11 days, and it was much lower than those in AS and Ck systems. Although, the initial pH in all systems was identical (7.5), the final pH for AS, Ck, and AS + Ck were 6.08, 6.80, and 5.15, respectively (Table 1). Other studies showed that caproate production in the fermentation process was sensitive to the pH conditions. Vasudevan et al.<sup>37</sup> and Agler et al.<sup>38</sup> demonstrated that caproate could have an adverse effect on microorganisms when pH was lower than 5.5. A recent study on caproate production from acetate, butyrate, and ethanol by *C. kluyveri* showed that pH controlled at 6.8 resulted in high caproate concentrations.<sup>33</sup> Therefore, one of the reasons for a low concentration of caproate in the AS + Ck system was a decrease of pH. As a result, different metabolic pathways were active in the microbiome. Caproate production stopped even though the required substrates (acetate, butyrate, and ethanol) were available in the bioreactor at high concentrations (Figure 3).

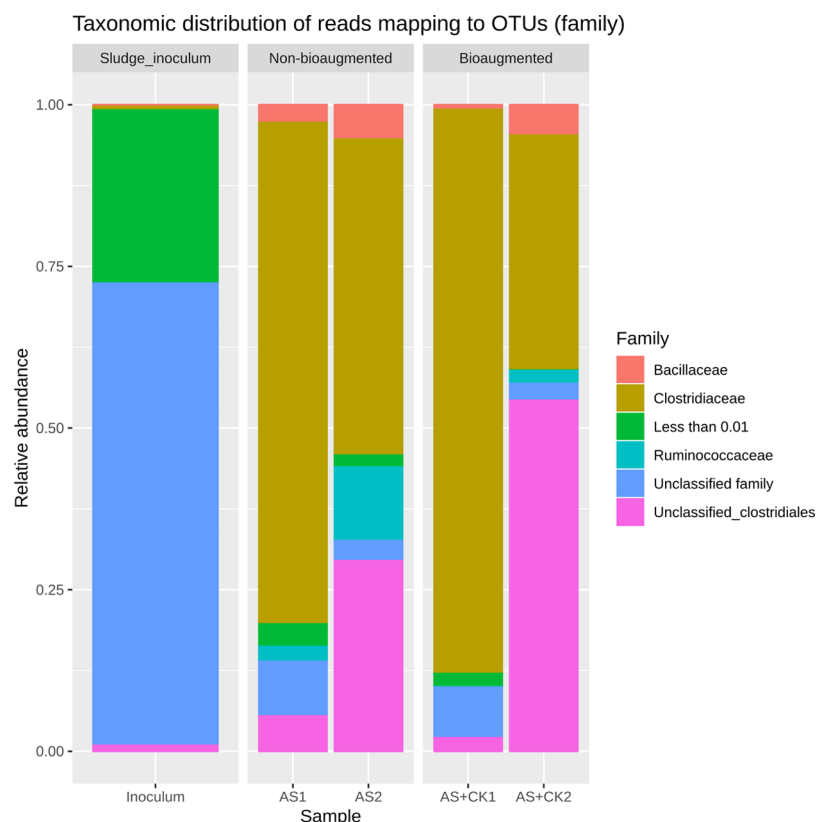
**3.3. Carbon Balance and Conversion Efficiency Analysis.** Carbon balance analysis (Tables 1 and S1) revealed that caproate concentrations during the first cycle in all experiments were comparable and reached 468, 406, and 396 mM C for AS, Ck, and AS + Ck, respectively. The highest concentration of caproate for the AS coincided with the highest substrate carbon consumed (709 mM C) and the highest substrate-into-caproate conversion efficiency (62.1% mol C). It was an effect of lactate utilization by the AS culture. Also fractional distribution of substrate electron equivalents to caproic acid was the highest and reached 68.9% (Table S3). Lactate was not utilized by Ck, while it was produced in the AS + Ck co-culture (Figure 1). On the other hand, the highest

specificity was obtained for Ck—88.6% mol C because these bacteria are specialized in the production of caproate.

During the second cycle, the AS culture was specialized in the formation of caproate because even higher caproate specificity (67.9% mol C) and substrate conversion efficiency (78.8% mol C) were obtained. On the other hand, in the bioaugmented culture, the values of these parameters were only 47.1 and 27.7% mol C, respectively. The *C. kluyveri* system showed the highest stability because caproate concentration, specificity, and substrate conversion efficiency were nearly identical to those in the first cycle. AS culture maintained the highest substrate consumption in the second cycle, which resulted in the highest caproic acid concentration (669 mM C). Bioaugmented culture showed 17% higher substrate consumption than the Ck in the second cycle, but the caproate concentration was nearly two times lower. As revealed by carbon balance, the AS + Ck culture completely consumed lactose, but in addition to caproate, carbon was to a large extent distributed to butyrate and lactic acid (Table S1). Fractional distribution of electron equivalents to hydrogen was generally low in all the experiments and did not exceed 4% (Table S3). Carbon recovery in various metabolites was in the range 109.1–112.1 and 92.1–99.2% for the first and second cycles, respectively (Table S1). Higher carbon recoveries in the first cycle could have resulted from a release of carbon present in the initial sludge used as an inoculum.

Our study showed the difficulty in obtaining stable bioaugmented co-culture. Introducing *C. kluyveri* to the anaerobic digester sludge was not beneficial for an increasing caproate concentration. Other studies also indicated doubts, whether the bioaugmented organisms and the introduced property would be established in the bioaugmented system.<sup>39</sup> On the other hand, Kenealy et al.<sup>29</sup> demonstrated improved caproate production (4.4 g/L) with the co-culture of *Fibrobacter succinogenes* and *C. kluyveri* grown on cellulose and ethanol. However, this process was performed in only one short cycle (120 h). To the best of our knowledge, only two studies reported bioaugmentation of mixed bacteria culture with *C. kluyveri*.<sup>35,40</sup> Mixed ruminal microflora bioaugmented with *C. kluyveri* and cellulosic biomass and ethanol as substrates showed caproate concentration of 6.1 g/L, while without bioaugmentation, it was only 0.9 g/L.<sup>35</sup> Reddy et al.<sup>40</sup> used food waste leachate supplemented with ethanol for MCFA production by mixed anaerobic culture and *C. kluyveri*. The bioaugmented culture produced mainly caproate (8.11 g/L), while the mixed anaerobic culture itself produced only 2.68 g/L caproate. Caproate concentration in the bioaugmented culture in our study was comparable with these reported in the literature and reached  $7.67 \pm 0.37$  g/L (first cycle). On the other hand, much higher concentration was obtained for the non-augmented system (AS)— $9.06 \pm 0.43$  g/L. Batch tests of Reddy et al.<sup>40</sup> and Weimer et al.<sup>35</sup> were performed with only one batch cycle; so the stability of these bacterial cultures is unknown. Our experiment contradicted with findings that a bioaugmentation with caproate producers is always beneficial for MCFA production. Our study indicated that stability of bioaugmented batch cultures was limited, while high caproate concentration could be obtained with heat-treated anaerobic digester sludge even without bioaugmentation with *C. kluyveri*.

**3.4. Microbiome Composition.** After adapter trimming and joining paired reads, a total of 363,704 sequences were obtained for the five investigated samples (19,346–92,705 reads per sample). Quality-filtering, dereplication, and employ-



**Figure 5.** Relative abundance of reads mapped to OTUs at family taxonomic level in the enriched microbial communities and starting inoculum. “Less than 0.01” corresponds to the percentage of reads mapped to OTUs with read abundance lower than 0.01% in the sample, and undefined family refers to the percentage of reads mapped to OTUs with bootstrap confidence lower than 80%. The number after the process abbreviation on the *x*-axis indicates the cycle number.

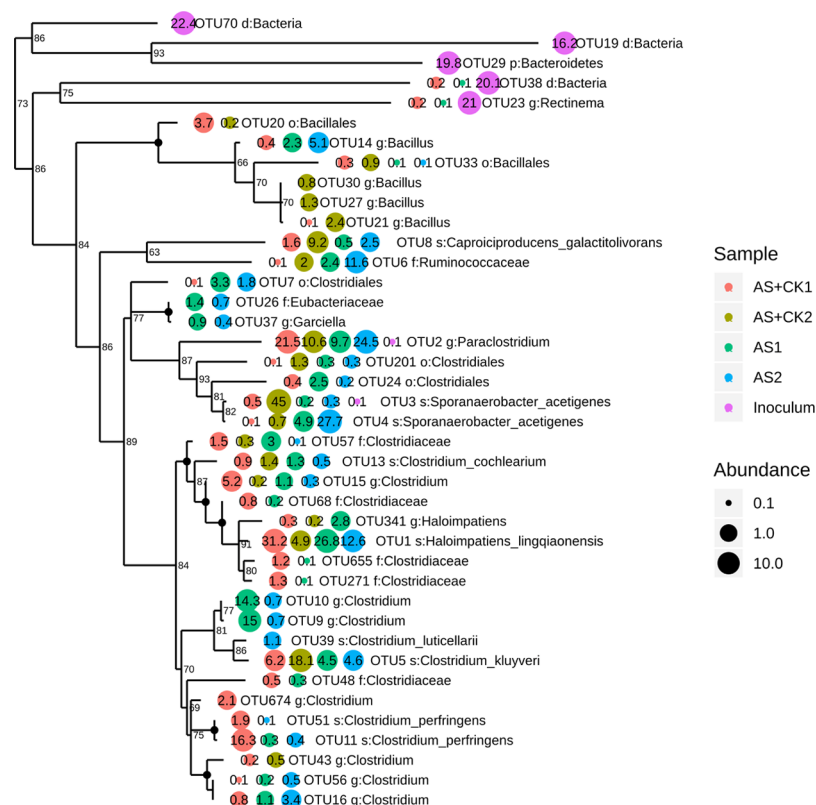
ment of UNOISE denoising resulted in 731 OTUs. The analysis of the microbial composition of the enrichment samples at all taxonomic levels (Supporting Information) revealed significant differences between the initial anaerobic digester sludge inoculum and the enriched fermentation cultures. While anaerobic sludge community is represented by a number of bacterial phyla, including Bacteroidetes, Proteobacteria, and Actinobacteria (18.6, 14.1, and 9.9% of reads mapped to corresponding OTUs, respectively), Firmicutes accounted for the largest fraction (97.5–99.8%) in all fermentation samples—represented mainly by families Clostridiaceae, Bacillaceae, Ruminococcaceae, and unclassified family from Clostridiales order (Figure 5). The first two are commonly associated with the capability to produce heat-resistant endospores,<sup>41</sup> and this was in line with the pretreatment of the anaerobic digester sludge used in this study.

The strong enrichment and decrease in the complexity of the microbial consortia was observed after each fermentation cycle for both non- and *C. kluyveri*-bioaugmented fermentations. This is reflected both in the number of OTUs observed in each sample but also in species diversity calculated as Shannon diversity index (Figure S2). While all cultures were enriched with similar microorganisms—the AS + Ck in the second batch cycle was the most distinct in terms of the microbial composition (Figures 6 and S3). Microbial communities from the AS and AS + Ck cultures were enriched with microorganisms belonging to the same genera. Significant numbers of reads were mapped to OTUs corresponding to *Clostridium*, *Bacillus*, *Paraclostridium*, *Sporoanaerobacter*, and

*Haloimpatiens*. The presence of Bacilli in caproate production reactors was already reported in the previous studies.<sup>36,42</sup> Bacteria belonging to this class were beneficial for caproate and caprylate production when ethanol or lactate were used as electron donors.<sup>36</sup> *C. kluyveri* was identified in both cycles of the AS culture, with relative abundance of reads mapped 4.3 and 4.4%, respectively. In bioaugmented culture samples, relative abundances of amplicon reads corresponding to this species were 5.7 and 17.8%, for the first and the second cycle, respectively. This indicated that *C. kluyveri* was enriched in the bioaugmented culture. However, increased caproate concentrations were not observed. In a mixed culture fed on yeast-fermentation beer (containing mainly ethanol as electron donor), *C. kluyveri* accounted for only 4% of the consortium, while other members of *Clostridium* had a relative abundance of 50%.<sup>38</sup> Interestingly, reads mapping to *Caproiciproducens galactitolivorans* were identified in samples from the second cycle in both AS and AS + Ck cultures. This strain belonging to Ruminococcaceae family was recently isolated from activated sludge and was capable of L-galactose utilization and production of caproic acid. Moreover, it was reported that its growth and caproic acid production were enhanced when co-cultured with other anaerobic strains producing ethanol, acetic acid, or butyric acid.<sup>43</sup>

The relative abundance of reads mapping to OTUs corresponding to *Sporanaerobacter* sp. increased in the second cycle for both AS (from 4.8 to 27.3%) and AS + Ck cultures (from 0.6 to 45%). The relative abundance of reads mapping to *Paraclostridium* spp. increased from 9.1% in the first cycle to 23.9% in the second cycle for AS experiments. However, the





**Figure 6.** Phylogenetic tree of 40 most abundant OTUs in analyzed samples and relative abundance of reads mapped to OTUs. Circle size represent the relative abundance (%) of reads mapped to OTUs (logarithmic scale). Taxonomy was assigned to each OTU at the highest possible level of detail based on a USINTAX bootstrap confidence threshold of 0.8.

reverse trend was noticed in the AS + Ck cultures. The relative abundance of *Paraclostridium* spp. decreased from 19.9 to 10.4% in the second cycle. *Sporanaerobacter* and *Paraclostridium* showed apparent correlations with caproate production in the recent study with ethanol and acetate as the substrates and heat-treated anaerobic sludge as the inoculum.<sup>42</sup> *Sporanaerobacter acetigenes* was a dominant microorganism during chain elongation with ethanol as an electron donor and was positively correlated with caproate production.<sup>44</sup> *S. acetigenes* can ferment sugars to acetate and H<sub>2</sub> as well as facultatively utilize sulfur as an electron acceptor to produce sulfide.<sup>44</sup> It can also play a role in an electron transfer between different species.<sup>42</sup> Therefore, even though *Sporanaerobacter* bacterium is not considered to be a caproate producer, it indirectly affected caproate production in this study. Phylotype related to Clostridiaceae family and annotated as *Haloimpatiens* spp. corresponded to a high relative abundance of mapped reads in the AS and AS + Ck samples, especially in the first cycle (25.2 and 29.0%, respectively). *Haloimpatiens* spp. is a recently discovered genus isolated from a wastewater sample; however, its role in chain elongation is not known.<sup>45</sup> The relative abundance of members of Ruminococcaceae was higher in the second cycle for both the AS and AS + Ck cultures. Ruminococcaceae were found in the reactor microbiomes associated with *n*-caproate production from ethanol<sup>29</sup> and lactate<sup>28,46</sup> in several previous studies.

Heat treatment of anaerobic digester sludge enabled an efficient production of two desirable products—hydrogen and caproic acid. All bacteria cultures—AS, CK, and AS + Ck demonstrated high caproate concentrations (7.7–9.1 g/L) from a mixture of lactate, lactose, ethanol, and acetate in the

first batch cycle. The AS culture was further enriched toward caproate production in the second cycle and showed the highest caproate concentration (11.4 ± 0.5 g/L). On the other hand, bioaugmented culture exhibited a lower caproate concentration in the second cycle (4.10 ± 0.30 g/L). This adverse effect may be caused by changes in the composition of the mixed bacterial culture. *C. kluyveri* mono-culture showed the most stable caproate production. Results indicated that the specific microorganisms responsible for caproate production would eventually establish by themselves. This study showed that bioaugmentation of mixed culture with *C. kluyveri* could have an adverse effect on caproic acid production.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.9b07651>.

Calculations of gas amount; other equations; carbon material balance; electron equivalents balance; and additional information on bacterial community analysis (PDF)

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## Notes

The authors declare no competing financial interest.

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