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Chain Elongation Using Native Soil Inocula: Exceptional *n*-Caproate Biosynthesis Performance and Microbial Mechanisms

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ABSTRACT

This study demonstrates the feasibility and effectiveness of utilizing native soils as a resource for inocula to produce *n*-caproate through the chain elongation (CE) platform, offering new insights into anaerobic soil processes. The results reveal that all five of the tested soil types exhibit CE activity when supplied with high concentrations of ethanol and acetate, highlighting the suitability of soil as an ideal source for *n*-caproate production. Compared with anaerobic sludge and pit mud, the native soil CE system exhibited higher selectivity (60.53%), specificity (82.32%), carbon distribution (60.00%), electron transfer efficiency (165.00%), and conductivity (0.59 ms·cm^{-1}). Kinetic analysis further confirmed the superiority of soil in terms of a shorter lag time and higher yield. A microbial community analysis indicated a positive correlation between the relative abundances of *Pseudomonas*, *Azotobacter*, and *Clostridium* and *n*-caproate production. Moreover, metagenomics analysis revealed a higher abundance of functional genes in key microbial species, providing direct insights into the pathways involved in *n*-caproate formation, including *in situ* CO_2 utilization, ethanol oxidation, fatty acid biosynthesis (FAB), and reverse beta-oxidation (RBO). The numerous functions in FAB and RBO are primarily associated with *Pseudomonas*, *Clostridium*, *Rhodococcus*, *Stenotrophomonas*, and *Geobacter*, suggesting that these genera may play roles that are involved or associated with the CE process. Overall, this innovative inoculation strategy offers an efficient microbial source for *n*-caproate production, underscoring the importance of considering CE activity in anaerobic soil microbial ecology and holding potential for significant economic and environmental benefits through soil consortia exploration.

1. Introduction

Renewable bio-based chemicals have been used in recent years to replace a variety of fossil-fuel-based products, as fossil-fuel consumption and carbon dioxide (CO_2) emissions increase due to rising social demands in the manufacturing industry. Medium-chain fatty acids (MCFAs, which have 6–12 carbon atoms) are high-value platform compounds produced through the chain elongation (CE) bioprocess that feature high energy density and hydrophobicity; they have attracted unprecedented attention for their potential to reduce carbon emissions [1–4]. For example, *n*-caproate, one of the most attractive products from the CE platform, is an environmentally friendly compound that can be processed into manufactured products such as corrosion inhibitors, antimicrobials, and pharmaceutical precursors [5–7].

Thus far, attempts to enhance the efficiency of MCFAs production have predominantly focused on a mixed-culture-method approach considering three key elements: electron donors (EDs), electron acceptors (EAs), and microbial sources [7,8]. Various organic compounds such as ethanol, methanol, lactate, glycerol, and glucose have been successfully utilized as EDs for MCFAs production [2,9,10], interacting with short-chain fatty acids (SCFAs) as EAs and incrementally elongating the carbon chain length by two carbons each time during the CE bioprocess. Among these compounds, ethanol has emerged as the most favorable substrate for conducting CE via functional microbiomes [11–13]. Nonetheless, as a growth-dependent anaerobic metabolism, CE is intensely affected by microorganisms and their habitat conditions [6], and the inefficiency of inoculating bacteria in the CE system can hinder

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the performance of MCFAs production. While previous studies have employed natural and engineered microbial sources as CE biological reactor inocula, such as anaerobic sludge [4], municipal solid waste [12], and pit mud [14], a prolonged growth period is necessary for CE functional microorganisms to adapt to the culture environment conditions. In this sense, the exploration of superior microbial resources for inocula, such as natural soil [15], could significantly enhance *n*-caproate biosynthesis performance—a topic that remains almost entirely unexplored in the context of anaerobic soil environments.

In fact, the link between microbial CE and soils dates back more than a century; in 1868, Béchamp discovered a high concentration of caproate in an anaerobic bottle containing an ethanol solution with soil microbiomes from a dry riverbed [16]. In the 1940s, *Clostridium kluyveri* (*C. kluyveri*)—the model microorganism for CE—was isolated from a saturated soil sample and exhibited caproate production of 11.6 mmol·L^{-1} when supplied with ethanol and acetate derived from canal mud [17,18]. Moreover, as previously investigated, specific microorganisms belonging to the genera *Clostridium*, *Eubacterium*, *Pseudomonas*, *Azotobacter*, and *Rhodospirillum* are capable of producing MCFAs when supplied with ethanol [7,19,20], which may also be present in natural soil ecosystems. Considering the presence of specific microorganisms capable of conducting CE, natural soil holds promise as a potential inoculum source for MCFAs production. Recent research has demonstrated the occurrence and microbial ecology of CE in different soil types through microcosms and enrichments supplemented with CE substrates [15], shedding light on the possibility of utilizing natural soil as a superior microbial resource for inocula. Additional investigations have also revealed the predominant presence of acid-producing fermentative bacteria in anaerobic soils, providing further supportive evidence for the hypothesis that soil microbes can serve as favorable inocula for MCFAs production [21–24]. While previous studies have examined microbial CE as a carbon metabolism that involves anaerobic conditions in soils [14], the potential of CE as a soil process and as a resource for inoculum application remains unclear. A comprehensive understanding is warranted to elucidate the biosynthesis performance of MCFAs and the underlying microbial mechanisms in natural soil, as compared with other bacterial consortia sources.

In addition to the aforementioned functional microbial potential, anaerobic soils—similar to municipal or agricultural wastes—harbor a wealth of biodegradable organic compounds that can serve as valuable nutrients for functional bacteria engaged in soil fermentation events during CE processes [25]. Recent research has also highlighted the contribution of inorganic mineral components in enhancing electron transfer efficiency; for example, conductive materials and humus act as electron shuttles and promote microbial respiration in natural anaerobic soil models for the biodegradation of organic compounds, including hydrolytic, fermentative, and methanogenic reactions [21,26–28]. Akin to other inocula or additives, soil, with its inherent bio-stability, contains surface pores and crevices that provide a sheltered habitat for microorganisms and offer binding sites for toxic compounds [3,29]. Consequently, a comprehensive exploration of the potential role of natural soil—encompassing its microbiomes and associated environments—as a whole inoculum for MCFAs production is of significant value.

The contents of this study are as follows. First, we investigate the feasibility and potential of utilizing diverse soil types from different plots as inoculum sources for CE processes. Then, comparative assessments are conducted under identical initial conditions, employing anaerobic sludge and pit mud as reference inocula, and an examination of the remarkable biosynthesis performance of MCFAs is carried out via dynamic CE analysis and carbon flux measurements. Subsequently, high-throughput sequencing and metagenomic analysis are conducted to elucidate the taxonomic composition and functional genes within the different cultures, facilitating the identification of key metabolic pathways and discrepancies in the soil system compared with other inocula. Finally, we discuss the interesting results of the performance of natural soil, providing new insight into the concept of harnessing soil microbial communities for CE biotechnology on an industrial scale, specifically in the production of MCFAs using soil as an inoculum source.

2. Material and methods

2.1. Source of inoculum and medium

The Chinese city of Ziyang has a wine-making history of 6000 years, including 300 years of distilling history, and its wine-making industries still generate a great deal of ethanol- and acetate-containing wastewater that should be treated with resource recovery. Therefore, five different soils in Ziyang were selected for this study; their descriptions can be found in Table S1 in Appendix A. Each soil sample was named based on its respective sampling location: ① in a distillery sewage facility; ② near a river; ③ near a medical wastewater treatment workshop; ④ near a leachate treatment plant; and ⑤ in a fruit forest. To ensure representative sampling, three soil cores were randomly collected from each plot using a hand trowel, reaching a depth of 15–20 cm below the ground surface. Subsequently, the soil cores from each plot were meticulously mixed to create a composite sample for each site, which was carefully packed in paper sampling bags and promptly transported to the laboratory. As anaerobic sludge and pit mud are often used as microbial resources in ethanol-fed CE experiments [1,3], these were selected as reference inocula in this study for comparative purposes. The anaerobic sludge was obtained from the secondary sedimentation tank of the Wenchang wastewater treatment plant (Harbin, China), and the pit mud was sourced from the brewing fermentation pit of a strong-aroma type of liquor distillery (Ziyang, China). Detailed information regarding the anaerobic sludge, pit mud, and growth medium can be found in Tables S1 and S2 in Appendix A, respectively.

2.2. Comparison of MCFA-production performance for different inocula

To demonstrate the viability of soil as inocula for CE, duplicate soil experiments were first conducted using 500-mL glass serum bottles containing a predetermined amount of growth medium (Table S2). Each bottle was filled with a mixture of 150 mmol·L^{-1} ethanol (the ED) and 50 mmol·L^{-1} acetate (the EA), maintaining an optimal ED/EA molar ratio of 3/1 as recommended by Wu et al. [30]. The pH was adjusted to 6.0 ± 0.1, using 6 mol·L^{-1} HCl and 6 mol·L^{-1} NaOH solutions. Prior to the experiments, all bottles were flushed with high-purity nitrogen for 1 min. Subsequently, the bottles were placed in an air-bath shaker operating at 150 r·min^{-1} and 37 °C. Blank reactors, devoid of ethanol and acetate, were included to account for any contribution from the inoculum itself in MCFA production. Specific soil types, as described above, were selected for investigation, along with two control reactors inoculated with anaerobic sludge and pit mud, aiming to examine the effects of the inocula and identify potential discrepancies in subsequent batch experiments. The culture conditions for the second set of comparative batch experiments mirrored those of the initial soil experiments. Table 1 provides a comprehensive overview of the experimental design. Samples of the fermentation broth were collected daily for compositional analysis. Additional operational details are provided in Section S1 in the Appendix A.

Table 1
Experiment design of different inocula for MCFA production.

Tests and experiments	Groups	Reactors	Inoculum[a]
Feasibility tests	Experimental groups	s1[c]	Soil1 (Table S1)
		s2[c]	Soil2 (Table S1)
		s3	Soil3 (Table S1)
		s4	Soil4 (Table S1)
		s5	Soil5 (Table S1)
Comparative experiments	Experimental groups[b]	ES	Fresh soil from distillery sewage treatment
		EP	Raw pit mud from a Chinese strong-aroma type of liquor distillery
		SBS	Anaerobic sludge from a secondary sedimentation tank
	Blank group	BS	Fresh soil (the same as ES without an ED and EA)

[a] For feasibility tests, each reactor consisted of 25 g of fresh soil and 450 mL of reduced anaerobic mineral medium; for comparable tests, each reactor was inoculated with the same solid mass (=1 – moisture content) by tested solid content, and ES consisted of 25 g of fresh soil and 450 mL of reduced anaerobic mineral medium.
[b] Samples of the second experimental reactors were selected for high-throughput DNA sequencing and metagenomics analysis for comparison.
[c] Selected soil for enrichment in the later enrichment experiments. One of the duplicates for each soil type was made by removing half the liquid and replacing it with fresh medium with ED (150 mmol·L^{-1}) and EA (50 mmol·L^{-1}); the other one was made by removing the solid components and utilizing the fermentation broth to continue enrichments with the addition of fresh medium with ED (150 mmol·L^{-1}) and EA (50 mmol·L^{-1}) in terms of equal volume. The total broth volume was 450 mL.

All experiments, from the substrate allocation of each sample to the end of the reactor operation, were conducted on a sterile workbench.

2.3. High-throughput sequencing for microbial community analysis

High-throughput sequencing analysis was conducted to assess the microbial community structures of the CE inocula in the ES, EP, and SBS reactors (referred to Table 1 in Section 2.2), as well as the original inoculum for comparative analysis. A Soil DNA Kit (Omega Biotek, USA) was employed to extract DNA from the aforementioned samples following standard protocol. Polymerase chain reaction (PCR) amplification of the V4–V5 region of the microbial 16S rRNA gene was performed using the primers 515F (5′-GTGCC AGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTC TAAT-3′) [3,30], which were specifically chosen to obtain bacterial and archaeal information simultaneously. Subsequently, sequencing libraries were prepared, and paired-end sequencing was performed using an Illumina Miseq platform at Shanghai Majorbio Bio-pharm Technology Co., Ltd. (China).

2.4. Metagenomic analysis to identify the CE pathway of different inocula

In order to comprehensively investigate the impact of different inocula on the microbiomes involved in CE, a metagenomic analysis was conducted. The results of the enrichment experiments indicated that the *n*-caproate production performance was predominantly influenced by the microbiomes associated with the solids rather than the liquid fractions in the experimental groups. Hence, the samples were collected from the solid components of the CE reactors designated as SBS, ES, and EP, following centrifugation at 10 000 r·min^{-1} at the conclusion of the experiment. The metagenomic analysis focused on the reactors exhibiting the highest *n*-caproate productivity within the three groups, aiming to identify the key microbial species involved in the CE process and elucidate their variation with different inocula. Gene annotation, taxonomy assignment, and functional enzyme analysis related to critical pathways were carried out following the methods described by Wu et al. [30]. Detailed information regarding DNA extraction, sequencing, and bioinformatic analysis is provided in Sections S2–S3 in Appendix A.

2.5. Chemical analysis

Daily samples of 5 mL were collected from each reactor and subjected to component analysis. The samples were filtered using a 0.45-μm filter prior to analysis. Ethanol, SCFAs, and MCFAs were quantified using a gas chromatograph (7890; Agilent Technologies, USA) equipped with a flame ionization detector and a 30 m × 0.25 mm DB WAX polyethylene glycol capillary column [31]. The inlet temperature was set at 220 °C, while the detector temperature was set at 240 °C. The temperature program for the column involved an initial increase from 70 to 170 °C at a rate of 20 °C·min^{-1}, followed by a 5-min hold at 170 °C and a subsequent temperature ramp of 20 °C·min^{-1} to 240 °C. Prior to analysis, liquid samples were acidified with 3% (v/v) formic acid [9]. Moreover, the electron-transporting system (ETS) activity was measured as an indicator of the microorganisms' respiratory activity and sludge bioactivity [32], as detailed in Section S4 in Appendix A. Conductivity was tested using an electrical conductivity pocket meter (CTS1 Oakton35462-11, Cole-Parmer, China) to evaluate the electron transfer efficiency.

2.6. Data analysis

Carbon (C) and electron balances were meticulously conducted to elucidate the distribution of carbon and electrons from the utilized CE substrates to the identified metabolites under the selected culture conditions. The C/electron balances were determined by dividing the millimolar carbon (mmol C) or millielectron equivalents (me$^-$ equiv.) of each metabolite at the conclusion of an experiment cycle by the total mmol C or me− equiv. consumed from the added substrate(s) [15,33]. The chemical oxygen demand (COD) was used to calculate the *n*-caproate yield, with a unit of 'g COD/g COD', which represents the generation of *n*-caproate product per unit of g COD for each substrate consumed. For the second set of comparable experiments, the cumulative concentration of MCFAs was further assessed using a modified Gompertz Model [34]. Selectivity, which characterizes the energy flow of the bacterial metabolism and estimates the conversion rate of substrates into MCFAs [35]. A detailed analysis can be found in Sections S5−S8 in Appendix A.

3. Results and discussion

3.1. The performance of n-caproate production for each tested inoculum

Based on the sample collection locations, it was anticipated that the unacclimated and untreated soils would exhibit varying degrees of microbial CE feasibility, influenced by their specific characteristics. However, it was observed that *n*-butyrate and *n*-caproate were the primary metabolic products ($p < 0.05$) when high concentrations of ethanol and acetate were supplied (Table S3 in Appendix A). During the initial batch tests, 10.58%–

55.40% of the maximal consumed millimolar carbon per litre (mmol C·L^{-1}) and 13.46%–53.37% of the maximal consumed me^{-} equiv. from the substrates were recovered as C4–C6 fatty acids (Table S4 in Appendix A). These results indicate that, while soil from different origins did exhibit different CE performance with varied *n*-butyrate and *n*-caproate concentration, the significant CE activity occurred when the soils were employed as inocula—a finding that aligns with previous studies on soil anaerobic incubations [15]. In subsequent enrichment fermentations (Fig. 1), *n*-butyrate and *n*-caproate remained the dominant metabolites produced, with solids (178–226 mmol C·L^{-1}) exhibiting better performance in *n*-caproate production compared with broth (144–161 mmol C·L^{-1}). Although the *n*-butyrate and *n*-caproate concentration sometimes declined, the phenomenon of their increasing concentration in general demonstrates the sustained capacity of soils for MCFA production and suggests that the enhanced microbial CE activity encompassed the entire soil matrix, including its biotic and abiotic components. To some extent, it can also be preliminarily inferred that some soils used for CE fermentation have a certain stability of *n*-caproate production, which was the focus of our follow-up research to further explore the complexity and stability of soil inocula.

The profiles of the main products (*n*-butyrate and *n*-caproate) and substrates (ethanol and acetate) were examined to further investigate the MCFA production performance using different inocula types (i.e., soil, sludge, and pit mud) in the CE experiments (Figs. 2(a–c)). Over the 15-day experimental period, the ethanol was completely consumed in all reactors, while the ES group exhibited the highest acetate consumption rate (96.0%) compared with the other groups (81.8%–86.6%). Comparing the ES group with the SBS and EP groups, the concentration variation in Fig. 2(b) shows that the soil inoculum in the ES group exhibited significant *n*-caproate productivity (249.69 mmol C·L^{-1}). Moreover, the synthesis of *n*-butyrate, serving as an intermediate product, occurred from the conversion of ethanol and acetate, which was subsequently elongated into *n*-caproate in the ES reactor [36]. The accumulation of *n*-butyrate is known to have a negative impact on the efficiency of MCFA production in the CE process. Thus, it is crucial to prevent the buildup of *n*-butyrate so that it can enter the cell through phosphorylation and subsequently convert to butyryl-CoA, which is the active state involved in the CE reaction [35]. In this study, the ES reactor exhibited a minimized concentration of *n*-butyrate (54.96 mmol C·L^{-1})), indicating superior transformation compared with the other groups. Conversely, the significant

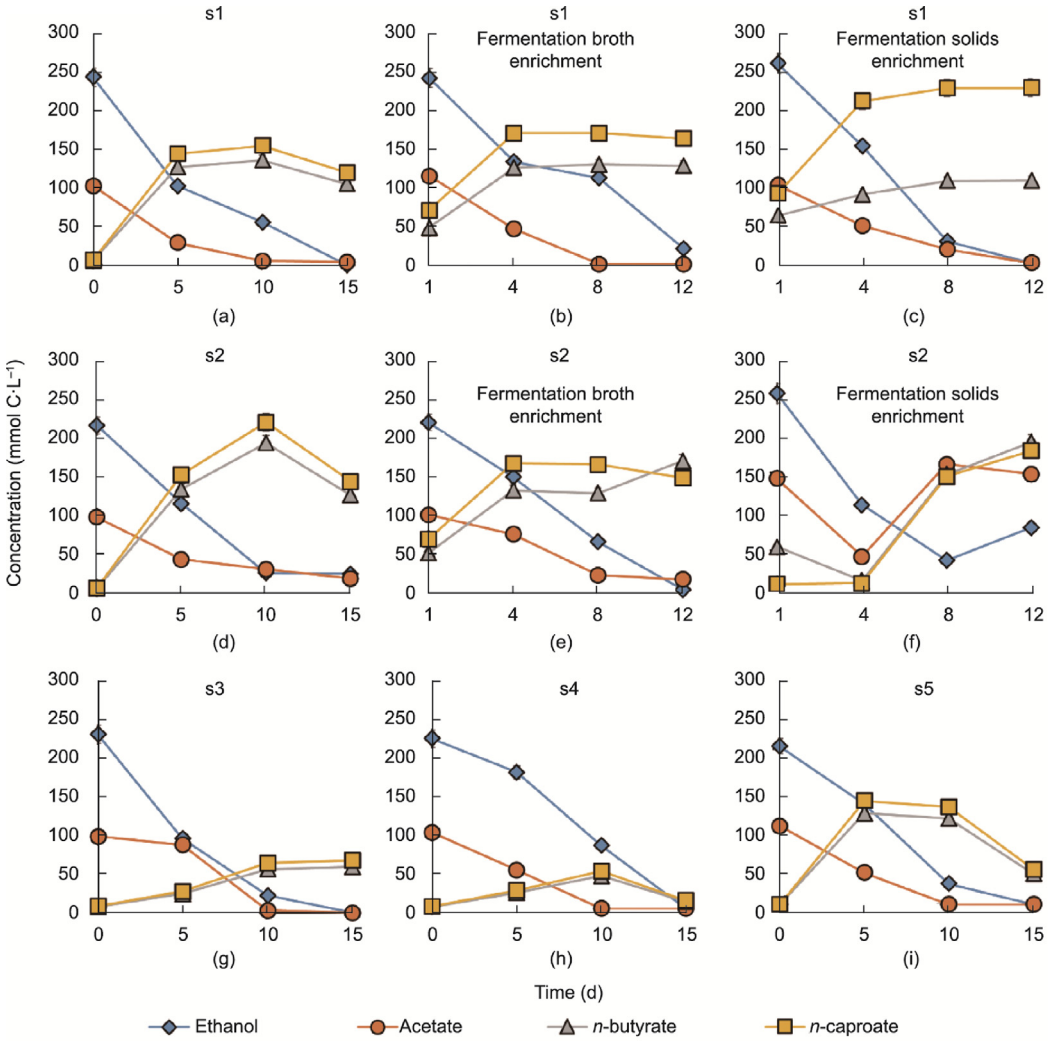

Fig. 1. Results of microbial chain elongation (CE) feasibility tests and enrichment batches with different soil inocula amended with 150 mmol·L^{-1} ethanol and 50 mmol·L^{-1} acetate. s1–s5 represent experimental groups with different types of soil inoculum from different origins: (a–c) a distillery sewage facility; (d–f) near a river; (g) in a medical wastewater treatment workshop; (h) in a leachate treatment plant; and (i) in a fruit forest.

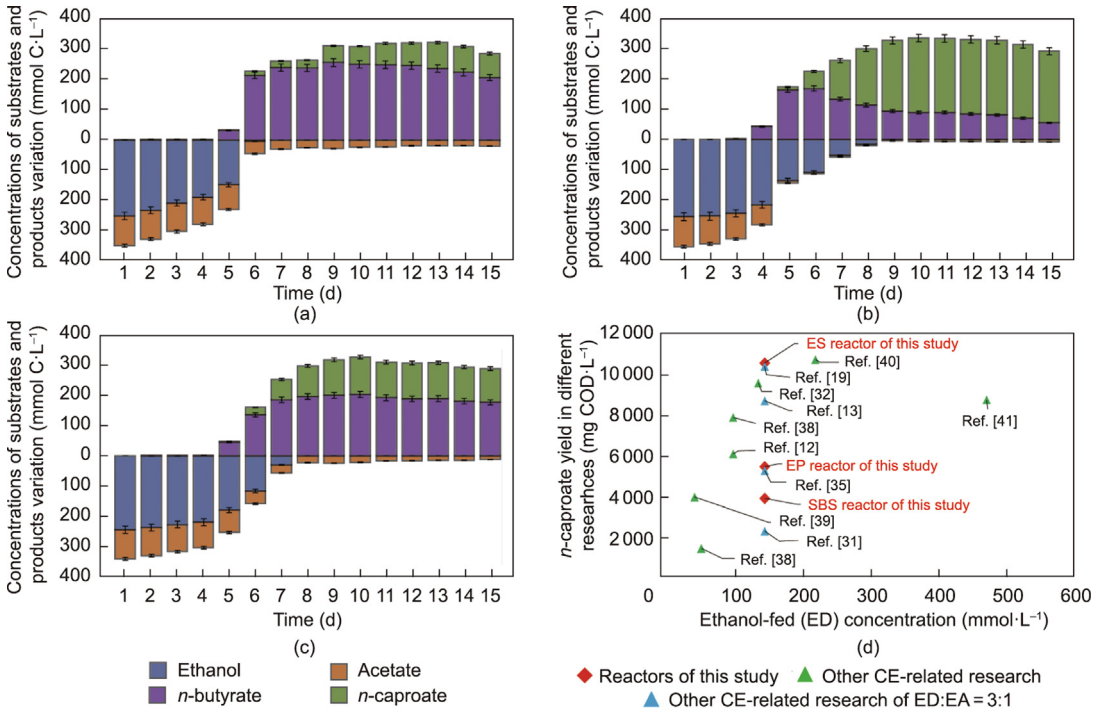

Fig. 2. Concentrations of substrates and products in (a) the SBS reactor (inoculating anaerobic sludge), (b) the ES reactor (inoculating soil), and (c) the EP reactor (inoculating pit mud). (d) Comparison of *n*-caproate production in different studies [12,13,19,31,32,35,38–41] (detailed in Table S6 in the Appendix).

amounts of *n*-butyrate observed in the SBS (209.67 mmol C·L^{-1})) and EP (178.85 mmol C·L^{-1})) reactors suggested the detachment of this compound from the carbon CE cycle, resulting in its accumulation as an intermediate product. Therefore, the utilization of soil-inoculated consortia can alleviate the adverse effects of inadequate carbon chain extension caused by *n*-butyrate accumulation, providing a feasible solution for optimizing MCFA production [37].

Previous studies on CE have predominantly focused on *n*-caproate production using ethanol as the ED and anaerobic sludge as microbial source [13,36], while this research utilize soil as inoculum. The *n*-caproate yields obtained in this study using different inocula were evaluated against the published literatures (Fig. 2(d) [12,13,19,31,32,35,38–41], Table S5 in Appendix A). The optimal *n*-caproate yield achieved by the ES reactor in this study surpassed some reported experiments when ethanol was fed as the ED under similar culture conditions. Taking only the maximal yield into consideration, the soil inoculum still exhibited a relatively significant superiority in *n*-caproate production over other inocula, despite the adoption of enhancement measures such as conductor materials or specific agents in some studies [19,32,35,42–45]. Compared with pure cultivation fed with ethanol, the maximum concentration and conversion rate achieved in this study were satisfactory, approaching the reported values of 74.2 mmol·L^{-1} and 49.49%. The *n*-caproate yield of 60.53% achieved by the ES reactor without an acclimatization process was notably high. These results demonstrate the potential of native soil as a superior inoculum source for CE, given its outstanding performance in *n*-caproate production.

A kinetic fitted curve demonstrated the superior *n*-caproate production performance of the ES reactor, which was characterized by a shorter lag-phase, higher maximal yield, and higher synthesis rate (Fig. 3(a); Table S6 in Appendix A). Over time, the ES reactor achieved the highest maximal yield of *n*-caproate (22.50 g COD/g COD), surpassing the EP and SBS reactors by 2.10- and 2.90-fold, respectively (Fig. 3(b)). Notably, ES exhibited the highest selectivity (60.53%) and specificity (82.32%) compared with SBS and EP, indicating that a greater proportion of electrons consumed from the substrates was directed toward *n*-caproate production rather than *n*-butyrate production when utilizing soil as the inoculum [19]. Carbon flux analysis further revealed the efficient conversion of substrates to products in the ES reactor, with 60.00% carbon distribution at the end of the experiment (Fig. S1 in Appendix A). These findings highlight the high efficiency of *n*-caproate biosynthesis achieved by soil incubation, which represents the primary target product of CE in the ES reactor. Furthermore, the daily pH variation (Fig. 3(c)) indicated greater stability in the soil CE system, demonstrating the soil's buffering capacity for pH retention. The ES group also exhibited a 65.00% and 25.49% increase in electron transfer efficiency and an increase of 0.35 and 0.15 ms·cm^{-1} in conductivity compared with SBS and EP, respectively, as depicted in (Fig. 3(d)).

3.2. Microbial community analysis for the n-caproate production of different inocula

To further understand the mechanisms of the CE process using different inocula, the microbial community compositions with ES, SBS, and EP were compared. Principal co-ordinates analysis (PCoA) showed that the microbial community structures were dramatically affected by the different inoculum genres (Fig. 4(a)). Different original environmental conditions may have shaped the different microbial compositions. Thus, the initial microbial community composition at the phylum level of the inocula before the experiments was also analyzed (Fig. S2 in Appendix A). At time = 0, the overwhelming majority of sequences in the soil sampled belonged to Proteobacteria (33.1%), Acidobacteria (13.4%), Actinobacteria (10.0%), and Bacteroidetes (8.1%), while the initial microbial communities in the SBS and EP groups were dominated by Actinobacteria (20.9% and 20.9%), Chloroflexi (11.1% and 4.1%), Bacteroidetes (7.90% and 7.92%), and Firmicutes (5.7% and 3.7%) (Fig. S3 in Appendix A). After the CE experiments, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* were found to be the dominant

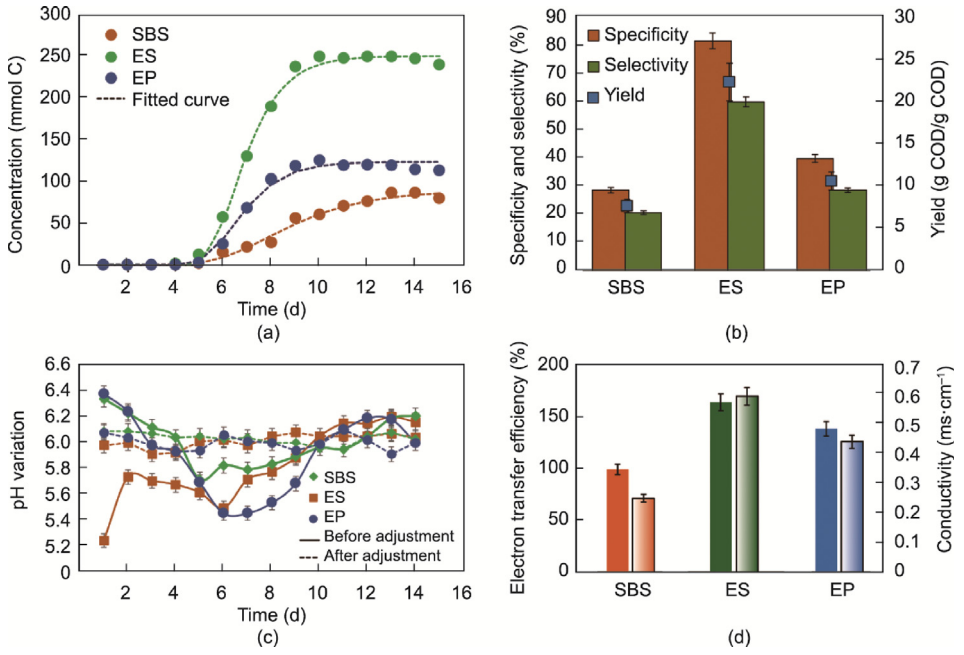

Fig. 3. (a) Kinetic fitted results; (b) specificity, selectivity, and yield calculation; (c) pH variation before and after adjustment; (d) electron transfer efficiency and conductivity among the three groups of ES, SBS, and EP.

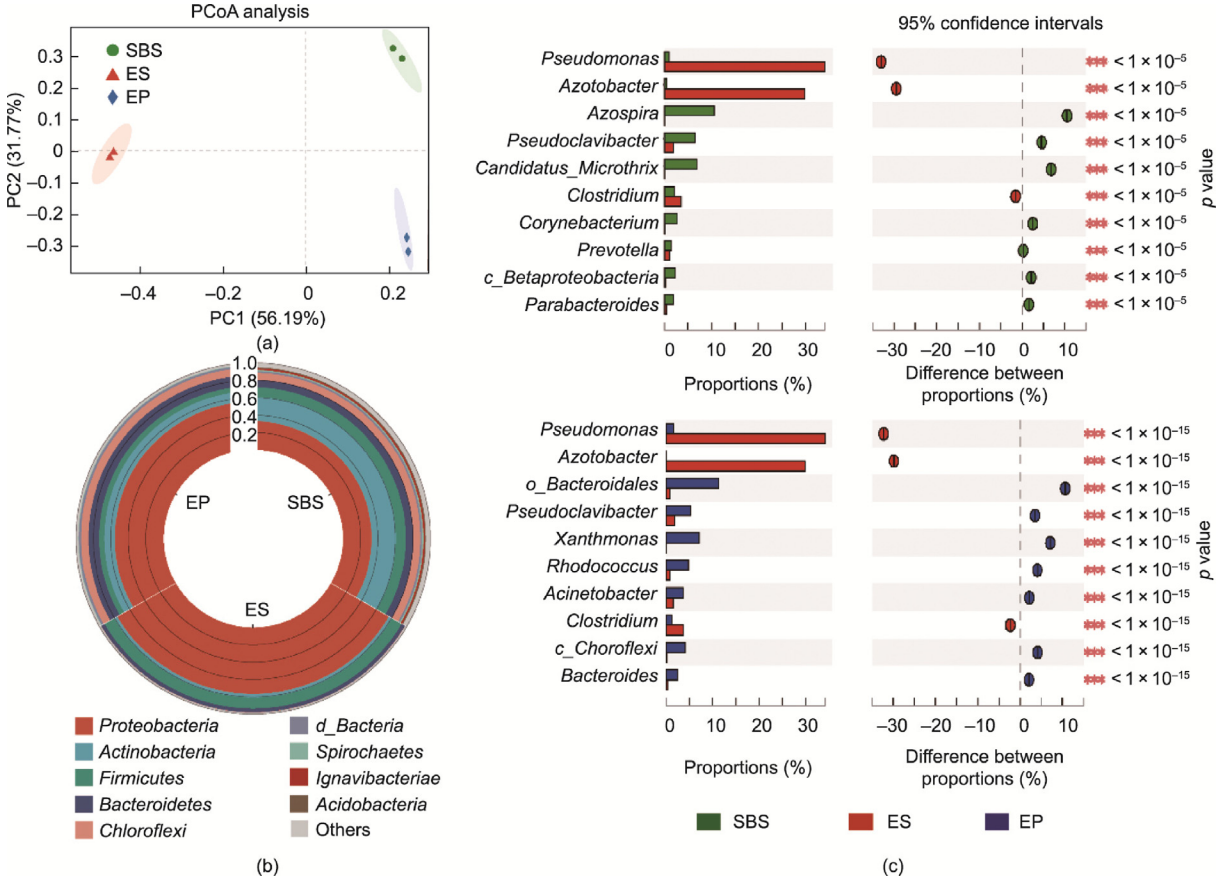

Fig. 4. Difference analysis of microbial communities among groups. (a) PCoA of the microbiomes with three different inocula (PC1: the principal coordinate component of the data change as much as possible; PC2: the principal coordinate component that accounts for the largest proportion of the remaining variability); (b) microbial communities at the phylum level; (c) differential comparison of the microbial community at the genus level.

phyla in all three groups, accounting for 97.9%, 80.6%, and 85.7% of the microbial population, respectively (Fig. 4(b)). *Firmicutes* has frequently been associated with MCFA production in anaerobic fermentation [46], it also showed a relatively higher abundance after the CE process, as expected, accounting for 12.7% of the final population (Fig. 4(b)). Notably, Proteobacteria was still found to be the major microbial community (76.7%) for soil CE fermentation in this research, compared with the initial composition. The ES group showed a great increase of 43.54% and 9.64% in Proteobacteria and Firmicutes, respectively, which have been recognized as key phyla involved in the production of *n*-caproate [11,13,47]. Therefore, although only a few strains could be isolated, as documented in CE laboratory batch cultures and bioreactors [48,49], the predominance of Proteobacteria in the soil that promoted CE biosynthesis could not be neglected as supporting evidence, regardless of the initial phylum distribution. Consequently, the soil was speculated to possess a strong ability to promote the growth of CE-related functional flora belonging to Proteobacteria and Firmicutes in particular, which may contribute to *n*-caproate production.

Given the featured role of phyla in microbial CE, we further examined the identified genera at the genus level using Fisher's exact tests in order to investigate the differential microbial taxa (Fig. 4(c), Table S7 in Appendix A). The results indicated that *Pseudomonas* and *Azotobacter*, both of which belong to the *Proteobacteria* phylum, exhibited the highest significant differences in abundance among the ES and other groups. *Pseudomonas* has been previously studied for its role in anaerobic processes such as biohydrogen production and acidogenic fermentation [4,50,51], but it had a low abundance in our research with the anaerobic sludge and pit mud inocula. In our research, *Pseudomonas* was identified as the first predominant population in the CE reactor when using soil as the inoculum, accounting for 34.1%; it was found to contain CE-related genes and functions, and contributed to *n*-caproate biosynthesis. Previous studies have also shown that *Azotobacter* can generate low-potential electrons that facilitate the reduction of the ferredoxin and flavodoxin electron-bifurcating complexes involved in acetogenesis and nitrogen fixation [52,53]. Thus, it may may also contribute to the CE process in soil fermentation. Moreover, the genus *Clostridium*, which has been reported to be a strictly chain-elongating bacterium and is a common model organism across many reports [19,46,48], was found to substantially increase in relative abundance under the soil CE conditions in our research. Other fermentative bacteria such as *Acinetobacter*, *Bacteroides*, and methanogens may also be extremely significant in their proliferation from the inoculum, as shown in some studies with similar inocula [35,54], even though these populations did not make up the main discrepancies among the three groups in this study. It should be noted that the abundance of other microorganisms alone does not directly determine their relevance to CE, as they may have other roles or dependencies in the system. Moreover, the entire fermentation period in this study was relatively short (15 days), so longer experiments in future studies may provide more information on microbial structures related to *n*-caproate production.

3.3. Variation analysis of n-caproate production pathways based on metagenomics

The functional genes were annotated against the KEGG database to identify key metabolic pathways relevant to *n*-caproate production. The abundances of genes related to fatty acids were enriched differently with different inoculum types. The abundances of fatty acid biosynthesis (FAB) were higher in ES than in the other groups. Genes involved in carbohydrates, amino acids, and cellular activity metabolism (e.g., bacterial secretion, pyruvate metabolism, and amino sugar and nucleotide sugar metabolism) were more abundant in the ES group (Table S8 in Appendix A). This finding might suggest a more active metabolism or a plentiful associated microbial increment in the soil group, which is possible with more energy and active synthesis of DNA and RNA in the soil CE reactor [45,46].

To further investigate the discrepancies in CE among the three inocula, the typical metabolic pathways and functional enzymes were researched (Fig. 5, Tables S9–S10 in Appendix A). Acetyl-CoA, the key intermediate in CE, was primarily generated from ethanol oxidation in this study. When ethanol was used to produce acetyl-CoA, the abundances of genes encoding *yiaY*, *adhP*, and *yahk* in ES were significantly higher compared with those in the SBS and EP groups, indicating a greater energy supply and the provision of reducing equivalents for acetaldehyde synthesis [36,55]. The abundance of acetaldehyde dehydrogenase encoding *mhpF* was also higher in ES, which indicated that the ethanol oxidation process performed by soil microbes was more efficient. Interestingly, we observed an expansion phenomenon of the collection gas bag in the entire experiment, although no CO_2 accumulation was detected at the end of the CE reaction. However, significant differences were detected in the gene abundance of CO_2 reduction to carbon monoxide (CO) and in CO reduction to acetyl-CoA in the ES reactor compared with the SBS and EP reactors. The genes encoding anaerobic CO dehydrogenase and acetyl-CoA decarboxylase/synthase (CODH/ACS) were also found to be more abundant in the ES reactor. This finding may indicate that acetyl-CoA can be primarily generated from CO_2 via an *in situ* reduction to CO as the ED, which requires further verification in future studies. Although the total abundance of this process was not as high as that of ethanol oxidation, the result of no CO_2 accumulation may substantiate the better catalytic activity of the relevant enzymes in the ES reactor. This phenomenon indirectly indicates that the *in situ* production and utilization of CO_2 was better conducted in the ES reactor—a finding that holds positive significance for reducing carbon emissions via the use of CE bioprocesses. It should be pointed out that the phenomenon of CO_2 reduction to acetyl-CoA is founded in previous studies in which anaerobic sludge was utilized as the inoculum and substrate [9,31,46]. However, this study has elaborated a detailed potential mechanism by means of metagenomics, as well as demonstrating the superiority of soil microbes in acetyl-CoA synthesis.

In the ES group, the relative abundances of some key enzymes involved in the RBO pathway were increased. More specifically, the genes encoding acetyl-CoA C-acetyltransferase (*atoB* and *ACAT*), which initiates the first cycle of RBO by coupling acetyl-CoA and CoA derivative to form acetoacetyl-CoA, exhibited significant abundance in the ES group compared with the other groups [56]. In addition, the abundances of the genes encoding acetyl-CoA acyltransferase (*fadA* and *fadI*) were observably up-regulated in the ES group. These enzymes facilitated the transformation of butyryl-CoA and effectively prevented butyrate accumulation in ES reactor, which was consistent with the observed *n*-caproate production performance among the three inocula. The genes related to the FAB pathway, another route for *n*-caproate production, which starts with malonyl-CoA, produced by the carboxylation of acetyl-CoA and the conversion of malonate, in the role of a 3-C donor, also exhibited a higher abundance in the ES group, as shown in Fig. 5(c) [54]. Moreover, the genes (*accA*, *accC*, and *accD*) encoding acetyl-CoA carboxylase were significantly higher in the ES group. The abundances of the genes encoding 3-oxohexanoyl-acp (*fabB*) and trans-hex-2-enoy-acp (*fabZ*) were also higher in the ES group in comparison with the other groups, which may suggest a highly efficient conversion of butyryl-ACP as a key intermediate, regardless of enzyme activity. Although other genes involved in the process did not show high abundance, their importance in the production of *n*-caproate cannot be neglected. In gen-

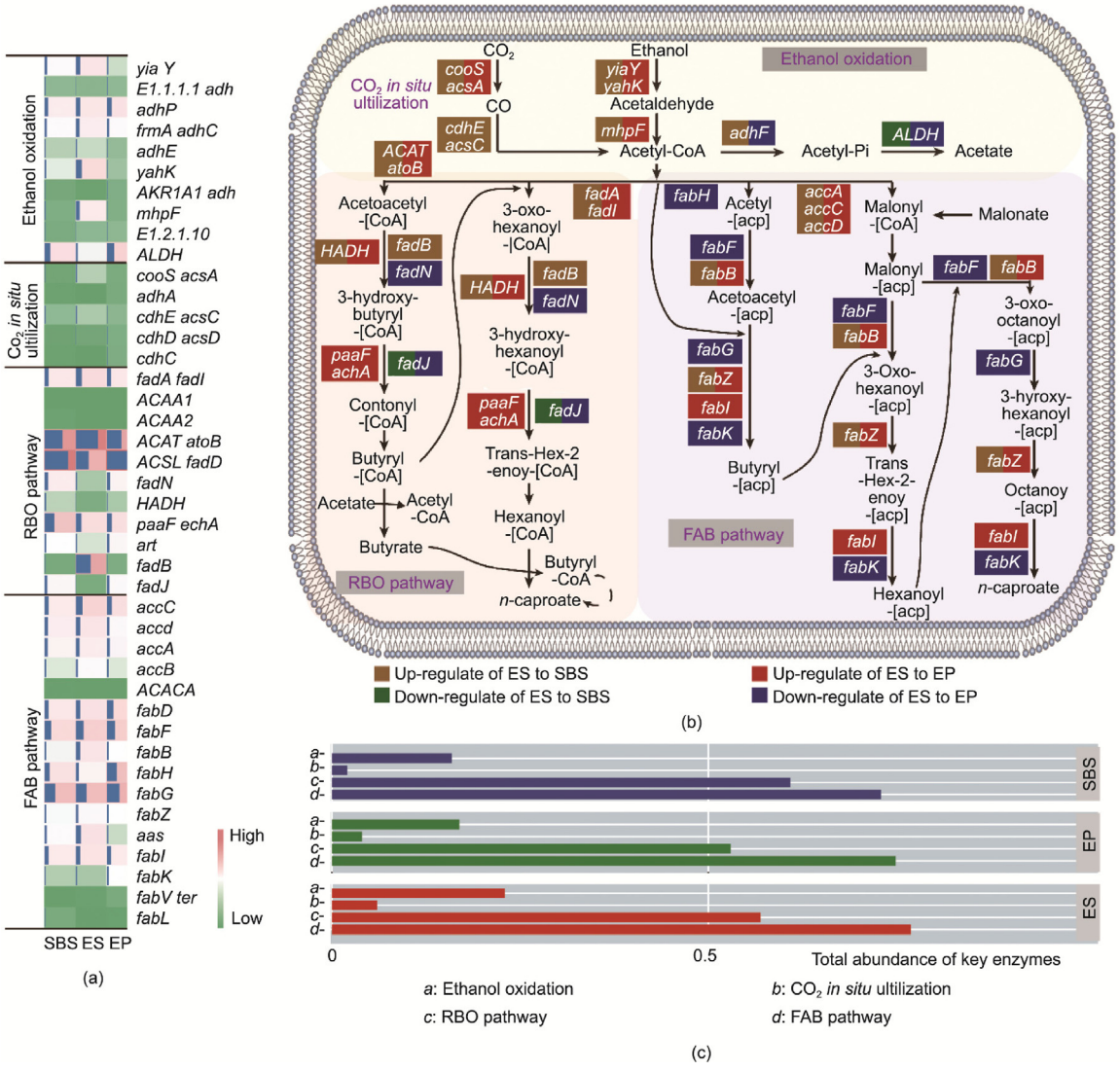

Fig. 5. Microbial synthesis process of *n*-caproate and its metabolism-related functional genes and enzymes. (a) Heatmap for the relative abundances of functional genes; (b) comparison of gene abundance values; (c) total abundance of enzymes in different pathways among the three groups. The corresponding definitions of genes abbreviations are attached to the Table S10 in the Appendix.

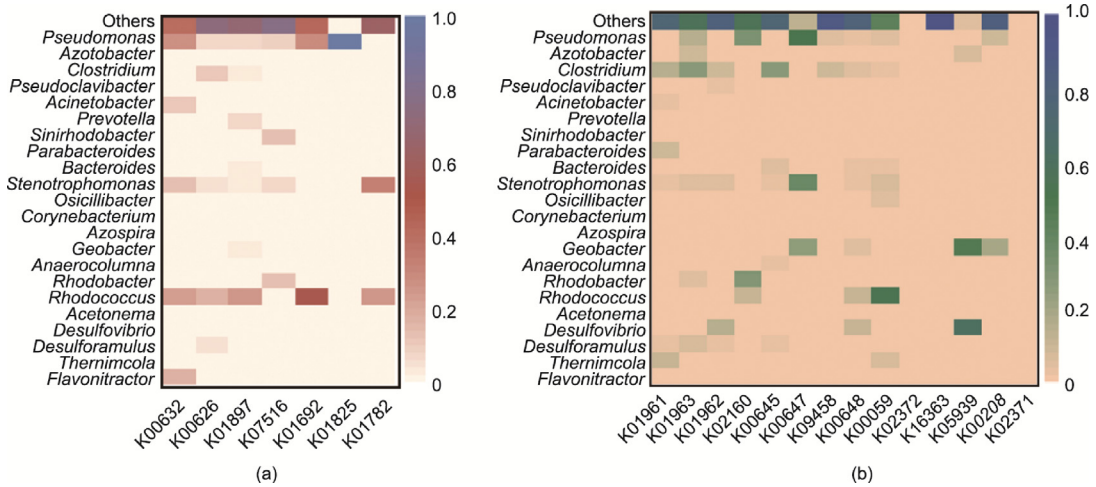

Fig. 6. Microorganisms connected with the key functions detected in (a) the RBO pathway and (b) the FAB pathway. The abundance was calculated based on RPKM (hit reads per kilo-base pair per million reads) and graphed logarithmically (the codes standing for gene functions and their involvement in metabolic pathways are provided in Tables S9–S10 in the Appendix A).

eral, the abundances of the enzymes involved in the RBO and FAB pathways in the ES group were higher than those in the SBS and EP groups, and the increased abundances of the genes associated with the RBO and FAB pathways may have affected the CE efficiency. The changed genes are likely to indicate a better performance of the inoculated soil microbes in producing *n*-caproate.

The taxonomic origins of the functions involved in the CE processes were investigated and are presented in Fig. 6. Regarding both the RBO and FAB pathways, the *Pseudomonas* and *Clostridium* genera were found to be primarily associated with these functions, indicating their crucial role in conducting the CE process within the fermented soil system, in line with the microbial community discussion above. However, many other microorganisms, such as *Rhodococcus* and *Stenotrophomonas*, also contain parts of genes associated with these functions. This finding suggests the possibility that the CE process is fulfilled through the interaction of different microorganisms, which warranted further investigation. Therefore, the presence of functional enzymes in the predominant species in *Pseudomonas* and *Clostridium* was further investigated to gain more information about the microbes responsible for the CE process using soil as an inoculum. As shown in Table S11 in Appendix A, the CE process cannot be accomplished by individual strains; rather, it depends on the interaction among different microorganisms. For example, as expected, most of the enzymes required for acetyl-CoA production, the RBO pathway, and the FAB pathway were assigned to *C. Kluyveri*. Moreover, *C. magnum*, *C. homopropionicum*, and *C. botulinum* were also observed to be associated with all the functional enzymes required for the FAB pathway and for acetyl-CoA production. Thus, we considered it worthwhile to conduct an in-depth investigation on the syntrophic cooperation of microbes for *n*-caproate synthesis in such an effective mixed-bacteria system.

Surprisingly, we discovered that some of the abovementioned predominant microbiomes belonged to conductive bacteria. Previous studies have reported that direct interspecies electron transfer (DIET) is an effective mode of energy transfer between microorganism species and can conserve energy better than electron transfer between substances; DIET is widely used in anaerobic systems [57,58]. Research has also shown that direct DIET can occur through various mechanisms, such as electrically conductive pili (e-pili), cytochromes, and biotic and abiotic electron transfer components (e.g., conductive materials) [59,60]. Therefore, this study suggests that the functional bacteria involved in improved CE biosynthesis may be engaged in DIET syntrophic relations. For example, *Pseudomonas*, which is known as a typical electroactive bacteria, exhibited high abundance in the functions and key genes of RBO and FAB in the ES reactor in this study, which may be attributed to its participation in the electron transport process mentioned above. These bacteria are recognized for their ability to conduct extracellular electron transfer via e-pili and to form syntrophic microbe-pili-microbe networks [61,62]. *Clostridium*, *Rhodococcus*, *Stenotrophomonas*, and *Geobacter* also showed relatively high abundances in specific functions within the RBO and FAB pathways. It is likely that these specific microbes in the ES system possess common features (Fig. S3): ① the presence of e-pili as conductive structures [59]; ② the secretion of cytochromes as electrical connection components [59]; and ③ the utilization of membrane vesicles and flagella [63,64], which might facilitate DIET. Aside from microbial components, abiotic factors may also contribute to accelerating *n*-caproate production via DIET in the CE process [65]. Therefore, multispecies aggregates may have formed; these have been verified as an effective structure for interspecies electron transfer (IET) or DIET for syntrophic partners in this kind of anaerobic system, as their formation is accompanied by a higher metabolic rate. In particular, such aggregates are very important for ethanol oxidization [62,66,67]. The higher *n*-caproate concentrations observed in our study could be partially attributed to the formation of aggregates in the soil inoculated system. Although there is no direct evidence to show whether DIET was established in the CE process or whether its presence had a promoting effect on the CE, DIET might become a common phenomenon in this unique system. Further in-depth investigations are warranted to understand the establishment of DIET in the soil CE process.

3.4. Implications of this work

This study investigated the utilization of soil as an inoculum source for producing *n*-caproate and has provided a comprehensive understanding of the exceptional performance of *n*-caproate production and the mechanisms of soil microbes in comparison with other inocula. The utilization of soil as an inoculum significantly promoted *n*-caproate generation, improved substrate consumption, and increased both product selectivity and carbon distribution. The soil CE system had a distinct microbial community structure that differed from traditional anaerobic fermentation consortia. In the soil-inoculated reactor, *Clostridium*, *Pseudomonas*, and *Azotobacter* were the most predominant microbiomes and were capable of involving CE functions. Metagenomics analysis revealed that the soil CE system had higher abundances of functional genes and enzymes in the RBO and FAB pathways compared with the other groups, likely indicating more abundant microbiomes or enhanced functions. Notably, CO_2 *in situ* utilization might have also boost the CE bioprocess for the production of *n*-caproate in a soil-inoculated reactor in our study. The produced *n*-caproate was positively related to the remarkable ETS activity and conductivity, which likely suggests that potential DIET-based electron syntrophy was established in the CE system performed by soil microbes.

To further advance the process of the microbial CE synthesis of MCFAs, future challenges may primarily arise in the following aspects: constructing efficient acid-producing fermentation systems, promoting directed carbon conversion, researching controllable synthesis strategies for target products, and developing product-separation and -extraction techniques and equipment. The development of microbial resources from soil for the construction of efficient functional microbial communities in CE provides a valuable reference toward these aims.

This study provides new insights into the utilization of soil as an inoculum source for MCFA production that possesses repeatability and applicability. The results from this study indicate that elongation as an energy-conserving pathway should not be overlooked in soil microbial studies. The soil inoculant experiments showed a superior performance in terms of *n*-caproate yield, selectivity, and carbon distribution. Moreover, the use of CO_2—which might act as an indirect ED—has the potential to enhance the carbon-fixation capability of the soil CE system. This could subsequently be determined by using a labeled-primer tracing technique.

Furthermore, this study reports for the first time that functional microbes (e.g., *Pseudomonas*, *Azotobacter*, *Clostridium*, *Rhodococcus*, *Stenotrophomonas*, and *Geobacter*) may achieve syntrophic cooperation in the CE process via DIET, which may have been established in the soil CE system in our research, considering the unique characteristics of soil and microbes. Further molecular analyses such as meta-transcriptomics and *in situ* spectroelectrochemical analysis are likely to better support the documentation of the extent of DIET in future research [68,69]. Henceforth, the technical feasibility of exploring soil inoculant microbiomes on the CE platform may be considered a beneficial idea for MCFA production at the industrial level as well, and the extent and role of microbial CE, as well as its dynamics under natural soil conditions, will be a pertinent research focus in our future study.

4. Conclusions

Current efforts to utilize complex microbial communities have popularized CE as a promising biotechnological platform for the production of MCFAs and other biochemicals. This study not only demonstrated the feasibility of utilizing different natural soils as inocula for *n*-caproate production using ethanol as an ED through the CE process but also investigated the microbial mechanisms through metagenomics analysis. The following conclusions can be drawn from this study:

(1) The soil inocula exhibited outstanding performance in *n*-caproate biosynthesis, achieving higher selectivity, specificity, and yield compared with other inocula. Kinetic analysis further confirmed the excellent CE activity, with a shorter lag-phase and maximal *n*-caproate production.

(2) The relative abundances of *Pseudomonas*, *Clostridium*, and *Azotobacter* increased with *n*-caproate production, suggesting their potential benefits in the soil CE system.

(3) Both the RBO and FAB pathways contributed to the soil CE system. Functions and genes were primarily associated with *Pseudomonas* and *Clostridium*, while other conductive bacteria also showed potential in enhancing the CE process in anaerobic soil.

The results of this study emphasize the importance of CE as an energy-conserving pathway in soil microbial studies. Investigating the extent and role of microbial CE under natural soil conditions remains a pertinent research focus in our group.

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Compliance with ethics guidelines

Lin Deng, Yang Lv, Tian Lan, Qing-Lian Wu, Wei-Tong Ren, Hua-Zhe Wang, Bing-Jie Ni, and Wan-Qian Guo declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2023.10.017.

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