Novel anaerobic fermentation paradigm of producing medium-chain fatty acids from food wastes with self-produced ethanol as electron donor

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ABSTRACT

As food waste (FW) is a largely available and carbon-rich feedstock, bulk production of medium-chain fatty acids (MCFAs) via chain elongation (CE) from FW is of great interest to biotechnology. However, the development of this emerging biotechnology is limited by the high cost from external electron donor (ED) input. To solve this bottleneck, replacing external ED input with self-produced ED in the system is a key. Therefore, this study provided a novel anaerobic fermentation paradigm of inoculating yeast to internally generate ethanol from FW as ED for subsequent MCFAs production. Batch experimental results demonstrated that cumulative 1540 mg COD/L of MCFAs was produced from FW with endogenous ethanol when inoculating 4.50×10^7 cells/ml-FW *Saccharomyces cerevisiae* in the fermenter over 20 days. In contrast, only a very small amount of MCFAs were detected in the control (58.98 mg COD/L). In continuous operation over 61 days, around 1300 mg COD/L of MCFAs was steadily obtained in the long-term fermenter with yeast assisted, which was almost 1.49 times higher than that from the control. The enriching *S. cerevisiae* responsible for on-site ethanol generation and chain-elongating bacteria including *Caproiciproducens* and *Oscillibacter* jointly promoted FW-derived MCFAs productions. Higher abundance of genes encoding substrate degradation, ethanol biosynthesis and CE further ensured the higher MCFAs production in the yeast-assisted system. This biotechnology does not require major changes to the design and operation of existing anaerobic fermentation infrastructure. Instead, the utilization of FW was enlarged through producing high-value MCFAs while waiving the cost of external ED by using inexpensive cheap yeast.

1. Introduction

Nowadays, the escalating depletion of non-renewable resources, including coal and oil, is driving the pursuit of renewable energy and chemical production [1]. Anaerobic fermentation is a widely used biotechnology to achieve resource recovery from biowastes by generating renewable biochemical or bioenergy [2]. Methane and short-chain fatty acids (SCFAs) were the main products derived from biowaste fermentation [3]. However, the low methane (CH_4) content in biogas (60 ~ 70 %) [4,5] and the difficulties of separating SCFAs from liquid phase [6] reduce their economic value and make them less attractive [7]. Compared to these two common metabolites, the easy-separated medium-chain fatty acids (MCFAs) have higher monetary value due to their high energy density and multifaceted application in the synthesis of biochemicals or biofuels [8]. MCFAs are straight chain mono-

carboxylates with 6–12 carbon atoms, mainly composed of caproate (C_6), heptylate (C_7), caprylate (C_8), etc. [9]. Chain elongation (CE) by microbiome is a cyclic bioprocess that yields these marketable products in the fermentative reactor, wherein two carbon atoms are added to the starter molecules and/or electron acceptors such as SCFAs [10].

Food waste (FW) is one of the most abundant organic wastes produced globally [11]. Nearly 1/3 food is wasted annually due to the perishability of food products, inefficiencies in supply and consumer behaviour [12]. Discharging FW to the landfill could lead to enormous environmental concerns including bad odour, pest transmission and groundwater contamination [13]. Additionally, the proper disposal of FW in landfills is greatly linked to greenhouse gas emissions, which are challenging to be captured or harness effectively [14]. Efforts have been made to reduce the negative impact of FW. Apart from avoiding undesirable food surplus or utilizing FW as animal feed, resource recovery

from FW via the means of anaerobic fermentation is a potential option and has attracted intensive attentions recently [15]. Compared to other organic wastes, FW attains less recalcitrant compounds regardless of its heterogeneous nature (Table S1). Given FW is rich in fermentable sugars with lower level of recalcitrant compounds in its structure [8], this carbon-rich biomass is a promising substrate for MCFAs production.

External provision of electron donors (ED) such as ethanol is generally required to supply electron and reducing equivalents (e.g., NADH) for CE reaction with acetyl-CoA working as intermediate [16,17]. The resulted acetyl-CoA would be integrated with short-chain fatty acids (e.g., acetate, etc.) to elongate the carbon chain of carboxylates each cycle [15]. One of the key factors hindering the sustainability of MCFA production is the high cost associated with the addition of external ED [18]. Therefore, the on-site production of ED in the fermentation system is encouraged to address this issue. Ethanol is an energy-rich compound and a preferable ED resource to start the CE process [19]. As most of the domestic/household FW contains high level of easily convertible carbohydrates such as starchy compounds, this sort of FW was employed as good substrate for bioethanol production for years [20,21]. A satisfactory ethanol yield exceeding 3000 mg/L was previously achieved when fermenting FW with distiller's grain [22]. Yeast was used to facilitate the breakdown of FW and achieve high ethanol formation simultaneously [23,24]. Most of soluble sugar derived from FW will be effectively utilized for ethanol fermentation by the inoculated yeast.

As such, a novel anaerobic fermentation paradigm was proposed herewith to produce MCFAs from FW with self-produced ethanol as ED by inoculating yeast. Both batch and long-term semi-continuous experiments were performed to evaluate the FW-derived MCFAs production in this novel anaerobic fermentation paradigm. To avoid the loss of yeast and the additional cost induced by the repeated yeast dosage, the yeast dosed into the long-term experiment was entrapped into the alginate beats before inoculation. To further disclose the metabolic pathways of MCFAs production from FW in the anaerobic fermentation system with yeast assisted, metagenomics and 16S rRNA gene amplicon sequencing were applied together to analyse the shifts in microbial community, associated genes, and functional enzymes. As a proof-of-concept study, this is expected to provide feasibility and mechanism support for the MCFAs production from FW in the large-scale pilot plant.

2. Materials and methods

2.1. Source of the food waste and yeast

The FW, mainly composed of rice, meat, and vegetables, was freshly collected from a university school canteen. 70 ± 2 % of the FW was composed of carbohydrate and 10 ± 3 % of the FW was composed of protein. The composite FW was firstly masticated using an electrical blender and screened with sieve to ensure that such organic matter was presented in the form of slurry. The volatile solids (VS) of the slurry form FW was 238.2 g/L. Before use, the slurry form FW was diluted 10 times with tap water, making the VS of FW was 23.82 g/L. The COD of the diluted FW was 33.11 g/L. Anaerobic digestion sludge (ADS) was harvested from an anaerobic digester, which has been used for treating sewage sludge and operated under steady status for nearly a year. The VS of ADS was 17.09 g/L. The harvested ADS was then added into the fermentation systems as the inoculum. The yeast used in the experiment was *Saccharomyces cerevisiae*, which is the most frequently used anaerobic yeast and an important model microorganism. The *S. cerevisiae* cells used in this study was purchased from Angel Yeast Co. Ltd. and were maintained in the form of dry cells powder. This is a common industrial strain which shows a good ethanol production and high biomass production [25]. The accurate weighing of the desired *S. cerevisiae* was achieved by considering the microbial concentration of dry yeast powder (3.57×10^7 cell/g) and the intended number of cells to be inoculated.

2.2. Batch experimental procedure

A series of identical 150 ml serum bottles were employed as batch reactors to explore the MCFAs production from FW via anaerobic fermentation with yeast assisted. Six of the bottles were added with 35.14 ml FW and 64.86 ml ADS, individually. Three FW-contained bottles were used as the control without yeast. The other three FW-contained bottles were worked as the experimental group with 4.50×10^7 cells/ml-FW *S. cerevisiae* inoculation. Blank group was also set up to exclude the interference of MCFAs production from ADS. In the blank group, the FW was substituted with an equivalent volume of tap water, while maintaining the same volume of ADS feed. pH condition of the batch test was adjusted to and maintained at 5.0 ± 0.1 with 3 M HCl and 3 M NaOH solutions. This is because mildly acidic pH was proved to be the optimal condition for the ethanol type fermentation [26–28] and CE process [29]. Sodium 2-Bromoethanesulfonate (2-BES) was added into the systems to suppress methanogen activity. All batch reactors were then purged with nitrogen gas for 5 min to ensure the anaerobic environment in the systems. Afterward, each bioreactor was sealed with a gas-tight robber stopper and wrapped with parafilm to prevent the release of the produced gas as well as oxygen contamination. The batch reactors were kept in suspension mode by placing them in a shaker-incubator at 180 rpm under 37 ± 1℃.

The 1 ml gas sample was taken out of the bottle to analyse the H_2 and CH_4 content contained in each bottle. Afterwards, 1 ml liquid sample was extracted from the system for the detection of alcohols, SCFAs, and MCFAs every 2–3 days. The method used for abovementioned metabolites analysis was described in detail in Section 2.6. The batch tests were lasted for 20 days until the MCFAs concentration were no longer increased evidently.

2.3. Long-term semi-continuous experimental procedure

Two 500 ml anaerobic fermenters in a semi-continuous operational mode were employed to test the feasibility of this studied anaerobic fermentation paradigm with yeast assisted to continuously produce MCFAs from FW. The total volume of FW and ADS was 300 ml with VS ratio of 1:1. One fermenter was control, and the other was experimental with 4.50×10^7 cells/ml-FW *S. cerevisiae* inoculation, which was same with batch test. To avoid the loss of yeast in the semi-continuous operational mode, the *S. cerevisiae* was immobilized in alginate beads. This is a widely used entrapment approach to avoid the washout of cells in the continuously operated fermentative system [30]. Calcium alginate was recommended as the best yeast cell carrier due to its low cost, ease of availability and good biocompatibility [31]. Therefore, calcium alginate was employed for the entrapment of yeast. Details of performing calcium alginate entrapment were referred to the previous study and described specifically in SI [31]. In short, the pre-activated *S. cerevisiae* suspension was added to 3 % (w/v) sodium alginate and mixed thoroughly. The cell-alginate mixture was then dropped to $CaCl_2$ solution to form beads using a hypodermic syringe. After the gelling and washing process, the yeast-contained calcium alginate beads was then well prepared. The yeast entrapped in the alginate would exhibit high activity and attain similar ability to yield ethanol [32,33]. Pure nitrogen was purged for 5 min to ensure the anaerobic conditions of the systems. Next, the two anaerobic fermenters were placed in a shaker-incubator at 180 rpm under 37 ± 1℃ for mixing (22 h 40 min). The shaker-incubator will firstly stop shaking for around 1 h to settle the sludge contained in the bioreactors before sampling. As the solid retention time (SRT) of the reactors was maintained at 9 days, 33.34 ml of liquid supernate was discharging, followed by the feed of 33.34 ml FW to each reactor on daily basis. The whole sampling and feeding process consumed 10 min in total. The gas content in the fermenter was analysed to evaluate the accumulated biogas levels in the system. The SCFAs, MCFAs, and alcohols contained in the discharged liquid were then analysed everyday using the gas chromatography following the methods described in

Section 2.6. The fermentation pH was adjusted at 5.0 and maintained manually every day at this value throughout the operation of the reactor.

2.4. Analytical methods

The biogas consisted of H_2, CO_2 and CH_4 was determined at mL using gas chromatography (HP5890, GC) equipped with a thermal
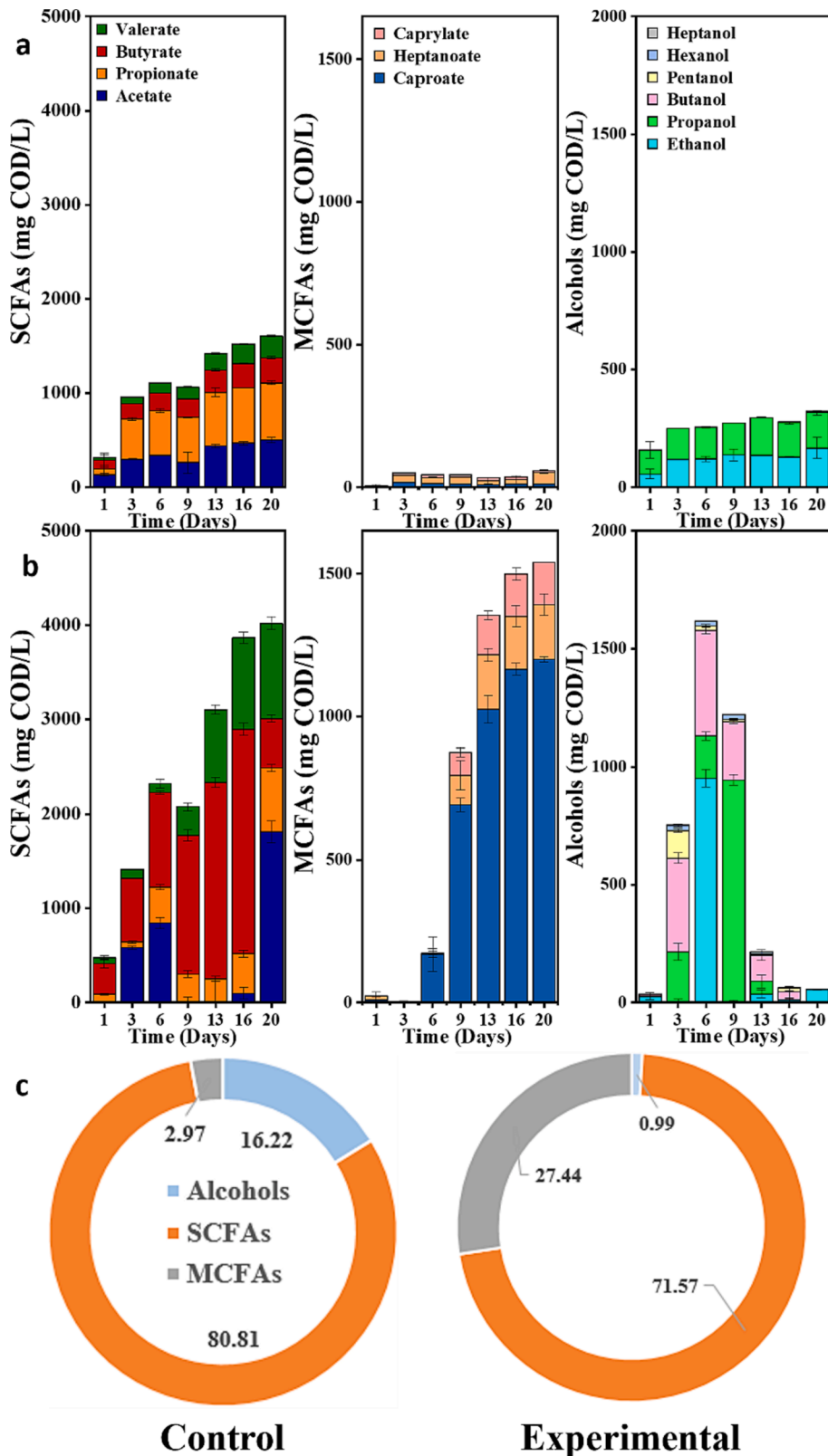

Fig. 1. Metabolite profiles derived from the control (a) and the experimental with yeast-assist (b) anaerobic fermentation system and product distributions (c) in batch mode.

conductivity detector throughout the period of batch experiment. The SCFAs, MCFAs and alcohols were evaluated by a gas chromatography (SHIMADZU GC, 2010 Plus, Japan) equipped with a hydrogen flame ionization detector. The 250 °C and 300 °C were the temperature for the inlet and detector, respectively. The temperature program applied for column was designed based on the description by Wu et al. (2020) [34]. Specifically, the temperature was firstly maintained at 70 °C for 3 min, then raised to 180 °C at the rate of 10 °C/min and hold for 10 min. Finally, the temperature was increased to 250 °C at the rate of 35 °C/min. The temperature for the inlet and detector was set at 300 and 250 °C. The TS and VS of the ADS and FW were determined based on the standard methods [35].

2.5. Microbial community analysis

The microbial community analysis was characterised to identify the variation of the key microbial community involved in the chain elongation in the two long-term operational systems with or without yeast inoculation. DNA was extracted from the composting mixture obtained on day 61 of the long-term experiment. E.Z.N.A. soil DNA kit (Omega Biotek, Norcross, GA, U.S.) was adopted to extract the microbial geonomic DNA of the fermentation mixture based on the manufacturers' protocols. More details for microbial community analysis can be found in **SI**.

2.6. Metagenomic analysis

For metagenomics analysis, samples collected from the long-term operational systems were obtained to check the variations in the microbial structure as well as the biological pathway at genetic level. Specifically, the sludge samples were same to **Section 2.5** and were withdrawn on the last day of the reactor operation. No yeast-contained beads were collected during sampling process. The samples were then transferred to 10 ml centrifuge tubes and centrifuge at 6000 rpm for 10 min. The supernatant of the samples was discarded. The remaining pellets were then preserved at −80 °C and sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for analysis. More details for microbial community analysis can be found in **SI**.

3. Results and discussion

3.1. MCFAs production from FW with self-produced ethanol in batch experiments

Fig. 1 exhibited the SCFAs, MCFAs and alcohols productions from FW in the batch control and experimental anaerobic fermenters over 20 days. In general, SCFAs were difficult to be converted into MCFAs via CE with little ethanol in the control (yeast-free) system (Fig. 1**a**). However, the inoculation of yeast (*S. cerevisiae*) in the experimental fermenter induced higher carbon recovery from FW by forming greater amounts of metabolites, i.e., SCFAs, MCFAs and alcohols (Fig. 1**b**). For the control reactor, the concentration of FW-derived ethanol or acetate remained basically unaffected from Day 3 to Day 20. Only 58.98 mg COD/L of total cumulative MCFAs yield were obtained throughout 20 days' fermentation in the control, far less than that attained in the experimental system. The low MCFAs recovery efficiency in the control was further revealed by the product distribution (Fig. 1**c**). Only 2.97 % of the final metabolite was composed of MCFAs in the control, which was much lower than that in the experimental system (27.44 %). Simultaneously, both the percent conversion of the fed FW (0.51 %) and productivity of MCFAs (29.49 mg COD/L·day) exhibited low levels within the control group. The low MCFAs yield could be ascribed to the ethanol shortage [36]. Similar finding was also reported in other study wherein ethanol to acetate ratio was used as a steering parameter to control the efficiency of CE, and lower ethanol to acetate ratio was found to be linked with lower CE efficiency [6]. The low ethanol to acetate ratio was

mainly resulted from the lower in-situ ethanol biosynthesis in this study, as the substrate was not converted to ethanol efficiently in the control.

In contrast, a marked increase in the MCFAs production from FW (1540 mg COD/L) was observed over 20 days in the experimental system, which is largely higher than that in the control. Such MCFAs yield aligned with previous studies when using in-situ ethanol to perform CE [37–39]. The much higher percentage of conversion of the fed FW (13.24 %) and the productivity of MCFAs (770 mg COD/L·day) further validated the greater MCFAs production in the yeast-assisted system. Statistically, the accumulated MCFAs production in the experimental group was significantly higher than that in the control (p < 0.05). Such increase was likely attributed to the higher in-situ ethanol production from FW, facilitated by the yeast assisted (Fig. 1). This is in line with previous studies, where high and quick ethanol production was also reported in the FW-contained fermentation systems with yeast assisted [40,41]. Higher ethanol to acetate ratio was therefore attained in the experimental system than the control. Revealing the real-time ethanol production throughout the duration of the experiment proves challenging. This was due to the dynamic nature of MCFAs production in the experimental system, where intracellular ED and EA were simultaneously formed and consumed. Also, the complex microbial interactions in the one-stage fermentative system further complicated the determination of actual ethanol formations. The follow-up content focuses on the changes of the metabolites in the experiment system by deliberating the trend of MCFAs productions and the impact of SCFAs production on MCFAs synthesis.

Specifically, despite no obvious ethanol accumulation was observed over the whole time, a large amount of MCFAs was detected, indicating the successful achievement of CE process in the yeast-inoculated system. This was because ethanol was rapidly consumed for CE process. Ethanol was accumulated transiently on Day 6 but was consumed rapidly to yield MCFAs with the further proceeding of FW fermentation. Caproate was the main MCFAs in the experimental system, followed by heptanoate and caprylate. The final MCFAs concentration remained still, and no obvious increase in MCFAs production was observed in the later stage of the experiment. Similar finding was also reported by other researchers where a stagnant MCFAs composition and level were found when external dosed ethanol was almost exhausted [42]. This suggested that the limited availability of internal ethanol in the experimental system could potentially contribute to the sustained MCFAs levels observed in the later stage of the experiment.

In addition, SCFAs biosynthesis in the experimental system was also greater than that in the control. Previous studies found that yeast was able to enhance the degradation of the carbohydrates and protein [43,44], therefore more complex organic matters could be hydrolysed and fermented for SCFAs generation. Considering MCFAs was formed using SCFAs as electron acceptor, greater SCFAs availability then led to more abundant MCFAs production once ethanol was sufficient. Notably, hydrogen (H_2) is formed as the co-product of CE process, which was also observed in many other CE-concerning studies [45,46]. Correspondingly, more H_2 was accumulated in the experimental reactor (**Fig. S1**).

3.2. MCFAs production from FW with self-produced ethanol in semi-continuous tests

Semi-continuously operated reactors were conducted to investigate the FW-derived MCFAs yield with endogenous electron donor, i.e., ethanol. The metabolites profiles showed in Fig. 2 indicated that the experimental reactor with *S. cerevisiae* assisted drove stable and increased MCFAs production from FW with self-produced ethanol compared to that in the control.

Consistent with more MCFAs yields with more in-situ produced ethanol observed herewith, other researchers also reported the close link between the endogenous ethanol concentration and the simultaneous formation of MCFAs in the biowaste fermenting system [47]. Specifically, 2198 mg COD/L ethanol was generated in the experimental
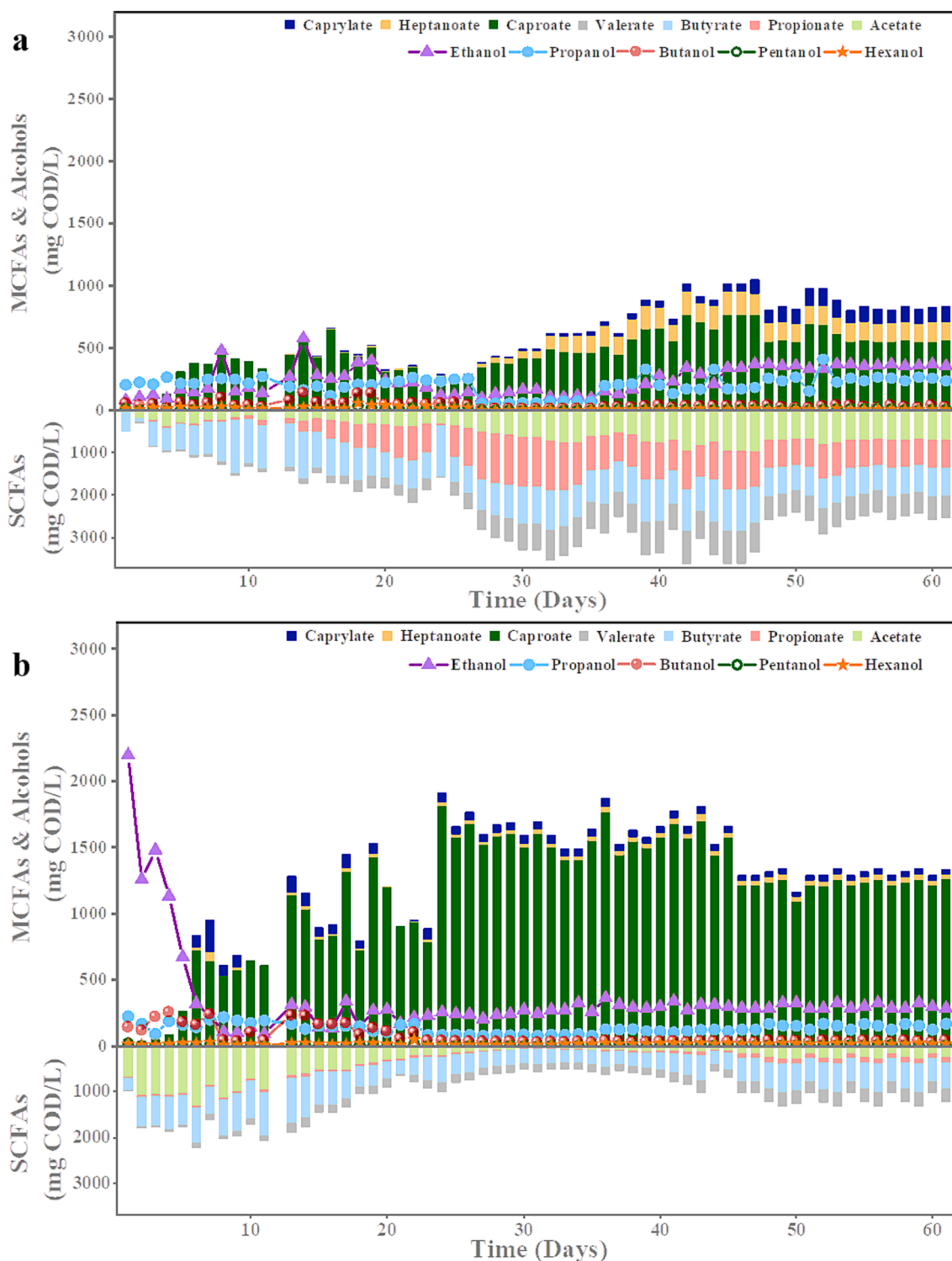
Fig. 2. MCFAs, alcohols, and SCFAs concentration during the fermentation in two reactors under continuous mode: a) the control system without yeast inoculation; b) the experimental reactor with yeast-assist.

system on Day 1 (Fig. 2), with around 15 % of the FW transformed into ethanol, much higher than that in the control (0.57 %). The high ethanol production in the experimental system was similar to that of previous researchers who also reported high and quick ethanol yield in the FW-contained fermentation systems with yeast assisted [40,41]. A dramatic decline in the ethanol level was subsequently observed in the experimental system in the first week of the operation, followed by the escalation of MCFAs yield from Day 5 to Day 24. After 24 days'

fermentation, the ethanol was yielded and consumed steadily. Around 250 mg COD/L ethanol level was stably accumulated in the experimental system on daily basis. As ethanol reduced quickly from Day 1 to Day 24, MCFAs production rate gradually increased and reached to a relatively stable state in the experimental system. FW-derived MCFAs production in the experimental system reached to 1659 mg COD/L. Caproate was the main MCFAs product with its concentration barely exceeded 1559 mg COD/L from Day 24 onwards. Notably, a slight

decreased but stable total MCFAs production from FW was observed from Day 45 to Day 61. Such slightly decreased phenomenon was also observed in other mixed-culture fermentation systems [48,49]. The biotoxicity of overmuch un-dissociated MCFAs probably resulted in the inhibition of CE process [50]. Correspondingly, the increment in SCFAs level was also observed in the experimental system [51]. During this period, the MCFAs production in the control also reached a stable level. The total MCFAs production from FW in the experimental system with yeast assisted was 1323 mg COD/L, 1.49 times higher than that in the control (883 mg COD/L). The percentage of COD conversion of MCFAs and associated productivity at this point were around 9.41 % and 22.27 mg COD/L·day, which were 1.6 and 1.22 times higher than that in the control. Statistically, yeast inoculation caused a highly significant MCFAs yield improvement compared to the control during long-term experiment ($p < 0.01$). Caproate was the primary MCFAs product in both the control and experimental reactors, which was agree with the results from many studies concerning biowaste-derived MCFAs fermentation [52].

Higher odd-chain carboxylates including valerate and heptanoate were attained from the control than the experimental group. Three reasons were proposed to explain this interesting fact. Firstly, the formations of these odd-chain products were probably ascribed to low ethanol availability in the control. Similar result was also attained in previous study where higher odd-chain products were likely to be attained at low ethanol: propionate ratios [53]. Secondly, the propanol transformed from propionate may be another trigger for higher odd-chain MCFAs production. Odd-chain MCFAs was likely to form when propanol and acetate co-existed in the fermentative systems [51]. Due to the lower in-situ ethanol production, the propanol transformed from propionate may work as additional ED and be utilized for later MCFAs production in the control [46,51]. However, no settled statement has

been published to strongly back up the MCFAs production with propanol as ED. Thirdly, the higher affinity of MCFAs producers to ethanol and acetate is the additional reason for the lower odd-chain MCFAs productions in the experimental systems [42].

Both batch and continuously operated experiments suggested that this novel anaerobic fermentation paradigm of inoculating yeast enabled to generate ethanol on-site from FW, allowing the enough supply of ED to achieve continuous MCFAs biosynthesis from FW. External ED was no longer required, largely reducing the cost for MCFAs production. Notably, we would like to highlight that this is only a proof-of-concept study and therefore the continuous separation of MCFAs from fermentation system was not conducted in this study. This is worth studying in the future to avoid the biotoxicity of accumulated un-dissociated MCFAs towards microorganisms, resulting in the decreased MCFAs production.

3.3. Microbial community composition and interaction network analysis

Microorganisms are the key participants in the MCFAs production process [54]. The abundance and the composition of the microbes would dictate the fermentation performance including CE process. Microbial communities of control and experimental systems were therefore analysed using 16S rRNA sequencing technology. The rarefaction curve of each sample was constructed and approached a plateau at sequencing depth of 2000 as presented in Fig. 3**b**, reflecting the captured microbial OTUs were representative and reliable. A heatmap was plotted to show the differences in microbial community composition in the control and experimental system at phylum and genera level (Fig. 3**a**). *Bacteroidota*, *Firmicutes*, and *Proteobacteria* were the major phyla, with their total abundance over 99 % obtained in both samples (Fig. 3**a**). Some of the organisms belonging to these phyla were documented with the
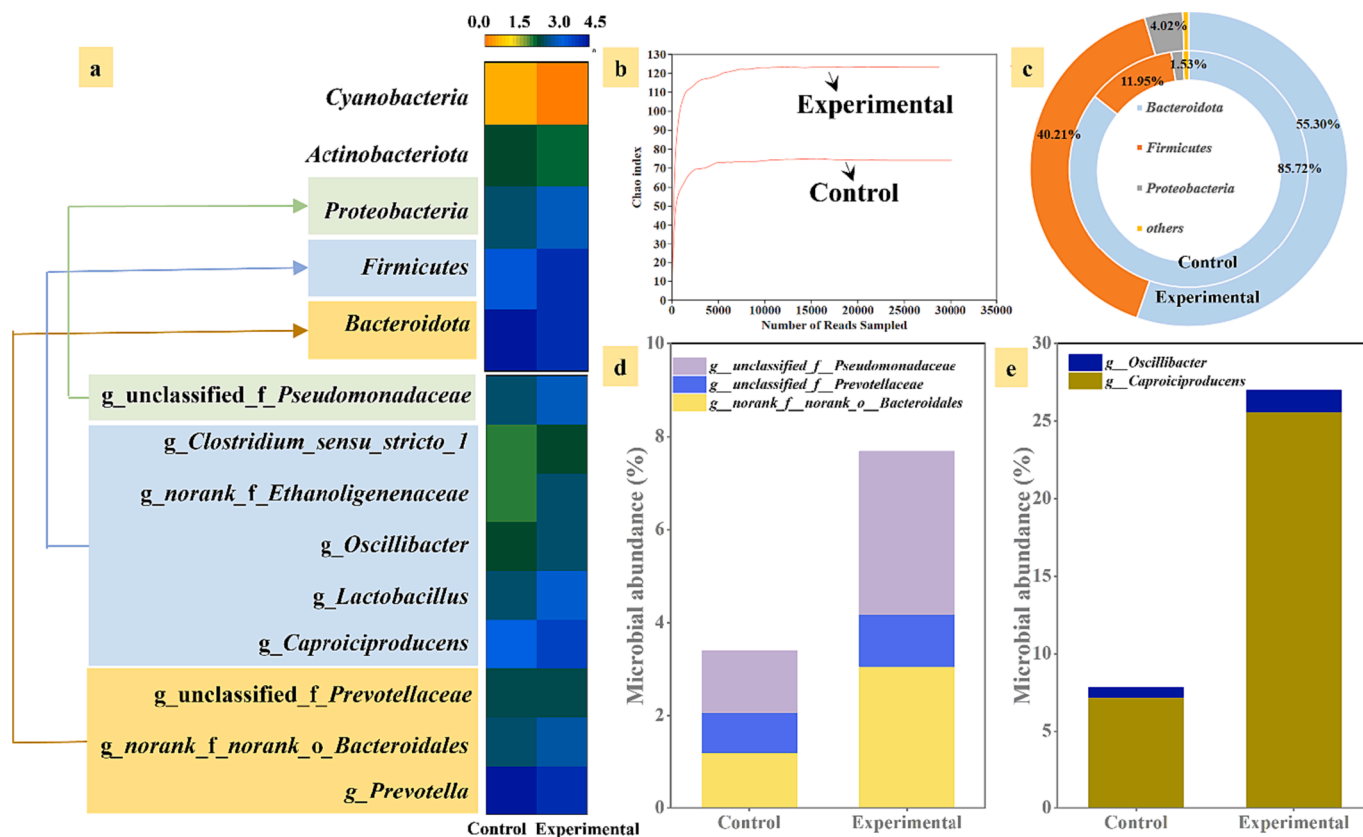

Fig. 3. 16 s rRNA-based microbial community analysis in the samples obtained from the long-term experimental system and the control: (a) heat map of the top microbial abundance on phylum and genus level; (b) rarefaction curve; (c) percent of microbial abundance on a phylum level; (d) relative abundance of key microorganisms involved in substrate degradation and SCFAs production; (e) relative abundance of chain elongator.

potentiality to act as the facultative organisms involved in hydrolysis, acidification, or CE process.

The top microbial genera identified hereby were affiliated to these three phyla as marked in Fig. 3a. Given the FW-derived MCFAs production was associated with the cooperation among substrate-degrading microbiota, SCFAs producers and chain elongators, the identified microbial genera were then clustered into different groups based on their functions as shown in Fig. 3d & e. Families *Pseudomonadaceae* [55] and *Prevotellaceae* [56] were documented with the capability to perform substrate degradation. Two unclassified microbial genera affiliating to *Pseudomonadaceae* or *Prevotellaceae* family were the predominant genera

in all samples (Fig. 3d). Considering the upward shift in their abundance, these two unclassified organisms may also possess putative ability to degrade macromolecular substances like proteins and carbohydrates. Specifically, family *Pseudomonadaceae* could oxidise carbohydrates and breakdown aromatic rings [57]. The abundance of the unclassified genus affiliated to *Pseudomonadaceae* was higher in the experimental system (3.50 %) than that in the control (1.32 %). This indicated that more fermentable carbohydrate contained in FW could be transformed into glucose by this unclassified genus, ensuring sufficient substrate supply for later SCFAs or ethanol productions subsequently. Family *Prevotellaceae*, which belongs to phylum *Bacteroidota*, also attains
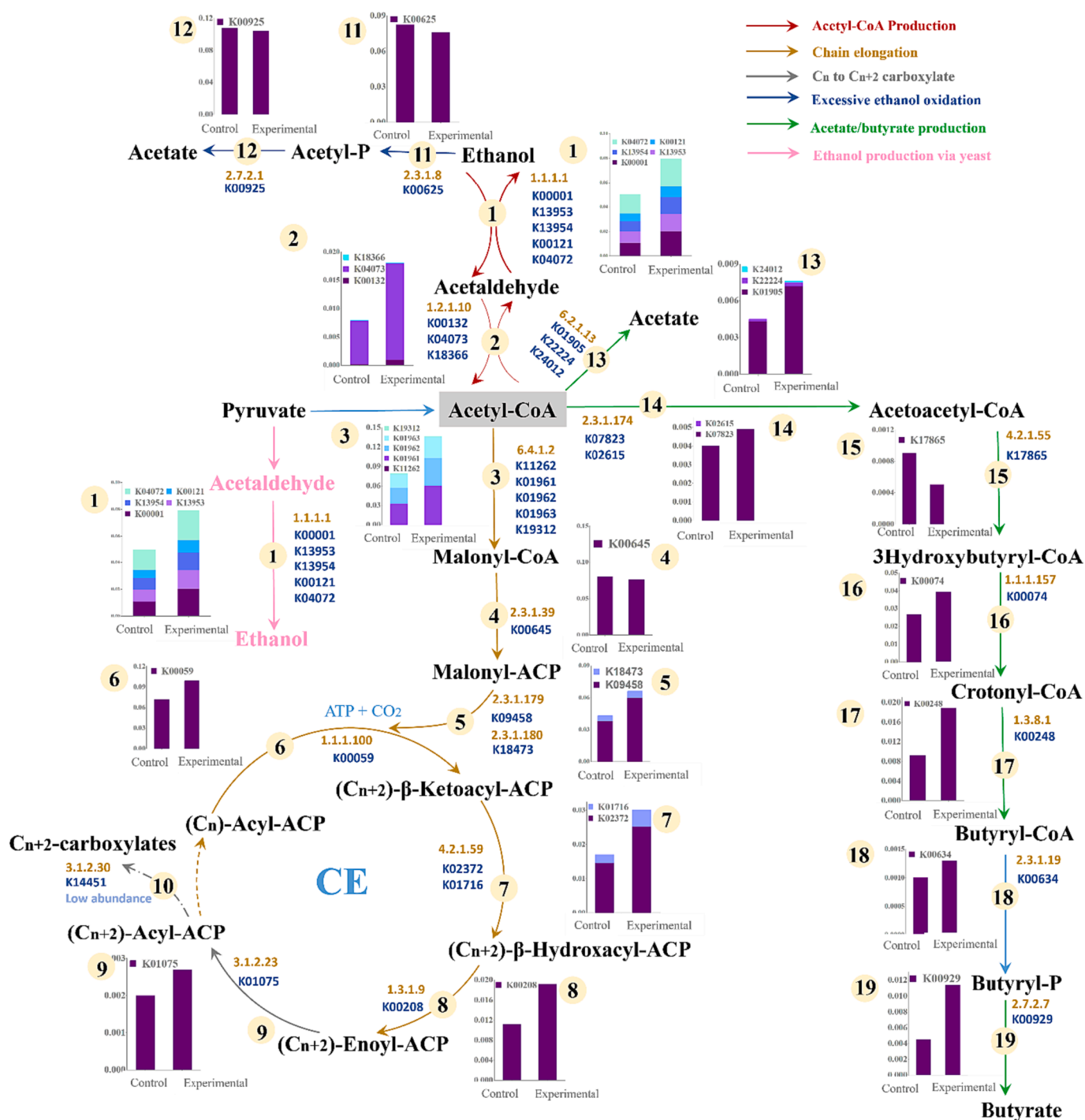

Fig. 4. MCFAs production pathways constructed according to KEGG database together with the relative abundance of functional genes responsible for MCFAs production from FW with self-produced ethanol.

potentiality to degrade the carbohydrate-rich substances contained in the FW with acetate generated subsequently [58]. The higher bacterial abundance of a unclassified genus affiliating to *Prevotellaceae* in the experimental system (1.12 %) implied that the degradation of FW was likely enhanced a bit compared to the control (0.87 %). More efforts are required to test the potentiality of these yet-to-be characterized genera.

Notably, *Bacteroidale*, which abundantly enriched in the experimental system, could be a multi-functional organism in substrate degradation and SCFAs productions (Fig. 3**d**). Specifically, *Bacteroidale* may appear to be an efficient degrader of protein or carbohydrate [59,60]. The higher abundances of *Bacteroidates*, together with the abovementioned the unclassified genera classified within *Pseudomonadaceae* & *Prevotellaceae*, may therefore jointly facilitate the efficient substrate degradation in the experimental system. *Bacteroidates* may also play a vital role in SCFAs production during fermentation based on the statement of previous studies [61,62]. Since higher proportion of this genus was attained in the experimental system than that in the control, more SCFAs would be formed for later CE process.

The detected genera related to MCFAs production herewith were identified within the *Firmicutes* phylum, which was suggested as the positive contributor for MCFAs production in previous studies [29,63,64]. Higher abundance of phylum *Firmicutes* was attained in the experimental system (Fig. 3**c**). *Caproiciproducens* and *Oscillibacter* were the dominant bacteria genera in *Firmicutes*, which were proposed to be responsible for CE process in some studies [29,65,66]. Compared to the control, the abundances of *Caproiciproducens* & *Oscillibacter* increased by 255 % and 132 % (Fig. 3**e**). The change of these microbial abundance may therefore lead to stronger MCFAs biosynthesis capacity in the experimental system. In sum, the changes in the microbial community were consistent with the trend of metabolites in the fermentative systems. The established enriched microbial community and enhanced cooperation network in the experimental system with yeast assisted could ensure more carbon contained in FW were utilized for ethanol and SCFAs generations, allowing stable and more MCFAs formation via CE platform.

3.4. Potential pathways for MCFAs synthesis

The functional genes in each sample were annotated against KEGG database to in-depth analyse the potential metabolic pathways of FW-derived MCFAs production. Fig. 4 mapped the route for MCFA production with in-situ ethanol provision. Table S2 showed the abundance of the associated genes and enzymes encoding by these genes. As the proceedings of CE was influenced by other essential biological reactions such as substrate degradation, it is necessary to reveal the changes in these metabolism categories and their associated interactions through analysing the molecular ecological networks. Therefore, metagenomic analysis at KEGG level 3 was conducted to predict a general picture of the alternations in these reactions.

The top 20 metabolisms identified in this study were associated with substrate degradation or key intermediate biosynthesis with their abundance shown in Fig. S3. Generally, protein and carbohydrate were suggested to be degraded more easily after inoculating yeast based on the metagenomic analysis at level 3 (Fig. S3**a**), as the associated predicted metabolisms were slightly more abundant in the experimental system than the control. Given protein and carbohydrate were the two main components of FW, the improvement of their metabolic pathways appeared to be beneficial to the follow-up MCFAs production by forming essential intermediate products. Cysteine metabolism (ko00270) and threonine metabolism (ko00260) were the two representative amino acid transformation processes with pyruvate formed as the shared intermediate. The improvement in the abundance of the genes controlling pyruvate formation could further confirm that more cysteine or threonine were transformed to pyruvate, facilitating the acetyl-CoA synthesis and following metabolites production (e.g., SCFAs, MCFAs, ethanol, etc.) (Fig. S3**b**). Similarly, the key genes encoding glucose synthesis via

carbohydrates transformation, the conversion of glucose to pyruvate, and the following acetyl-CoA synthesis via pyruvate transformation were all enriched in the experimental system compared to the control (Fig. S3**b**). The alternations in this genetic abundance can further validate the possible enhanced substrate degradation in the fermentative system with yeast inoculation.

The shift in the genes encoding SCFAs productions might be the additional explanation for the higher MCFAs productions via FW fermentation. To generate deeper insight into the pathway associated with SCFAs production, the associated genes were also mapped to the KEGG database of pathways (Fig. 4). Specifically, as the relative abundance of the genes encoding acetate thiokinase in the experimental system (0.0075 %) was 1.67 times higher than the control (Fig. 4), more acetate should be theoretically and potentially produced via this enzyme with acetyl-CoA acting as intermediate [67]. Similarly, the production of butyrate via acetyl-CoA was also likely promoted in the experimental system based on the metagenomics analysis. Most of the genes controlling the butyrate production were more abundant in the experimental system than the control. Overall, as most of the genes encoding acetate and butyrate productions were more abundant in the experimental system, higher SCFAs was predicted to be formed for the subsequent MCFAs productions. The exact number of each genetic abundance were listed in Table S2.

The possible higher pyruvate production resulted from the enhanced protein and carbohydrate breakdown could also facilitate the in-situ ethanol production through yeast based on the changes in gene abundance. The higher ethanol biosynthesis resulted from yeast inoculation would, in turn, assist higher MCFAs production. Specifically, yeast can utilize pyruvate to generate ethanol as shown in Fig. 4. Higher abundance of genes encoding the enzyme of this pathway was attained in the experimental system, indicating that more ethanol might be formed onsite and provide electron and energy for the MCFAs production in the experimental system, with acetyl-CoA serving as the intermediate. The obvious increase in the abundance of genes encoding alcohol dehydrogenase (EC. 1.1.1.1) and acetaldehyde dehydrogenase (EC. 1.2.1.10) for acetyl-CoA generation in the experimental system may further back up this statement (Fig. 4). The relative abundance of these genes is provided in Table S2 for reference.

Interestingly, only FAB pathway was observed in chain elongation process in both systems, while RBO was not detected. This was also be found in the previous study [68], in which FAB was annotated as the only pathway of CE. A set of genes and associated enzymes involved in this cyclic process were annotated individually and mapped in detail as shown in Fig. 4. Specifically, acetyl-CoA derived from ethanol was utilized via FAB pathway to generate malonyl-CoA, which was used as the cyclic molecule interacting with SCFAs, extending their carbon chain by adding 2-carbon atoms each time. Acetyl-CoA C-acyltransferase (EC: 6.4.1.2) was the stater enzyme to initiate FAB by converting acetyl-CoA into malonyl-CoA. The abundance of the genes coding this enzyme in the experiment system was 1.73 times higher than that in the control. Enoyl-[acyl-carrier-protein] reductase (EC: 1.3.1.9) was the key enzyme acting on CH_2 and CH-CH group when $NADP^+$ or NAD^+ were electron acceptors [69]. Considering the gene encoding enoyl-[acyl-carrier-protein] reductase was more abundantly found in experimental system (0.019 %), the energy was assumed to be flowed to MCFAs biosynthesis more easily. Genes coding the other enzymes (i.e., EC: 2.3.1.39, EC: 2.3.1.179, EC: 2.3.1.180, EC: 1.1.1.100 & EC: 4.2.1.59) participated in CE process were also identified in this study. These genes were generally enriched in the experimental system with higher abundance attained than the control. The overall improvement in the genetic abundance could then probably contribute to higher MCFAs production. Note that, excessive ethanol oxidation (EEO) was another pathway existed in this study, which was an unwanted reaction for MCFAs production. Based on the decreasing abundance of the genes encoding the enzymes involved in EEO, less internally formed ethanol was likely to be wasted for forming acetate in the experimental system. Specifically, genes encoding

phosphate acetyltransferase (E.C. 2.3.1.8) and acetate kinase (E.C. 2.7.2.1) exhibited lower abundance in the experimental system. The yeast-inoculated system demonstrated a reduction of 7.75 % and 3.40 % in the genetic abundance of these specific genes than the control (**Table S2**).

3.5. Metabolic pathways of the key microbial communities

Caproiciproducens and *Oscillibacter* were identified as the main MCFA producers in both the control and experimental reactors (Fig. 3). Therefore, the genes and associated enzymes within these two genera were studied in detail to find out the potential specific chain elongating species by annotating to KEGG database. Previous researchers also evaluated the correlation between bacterial species and their potential functions in CE or other mechanism based on the detection of the genes [34,70,71].

Caproiciproducens sp. *NJN*-50 and *Caproiciproducens galactitolivorans* were the only two species belonging to *Caproiciproducens* identified in this study (Fig. 5**a**). *C. sp. NJN*-50 is the unclassified *Caproiciproducens* species encoding the genes associated with CE process [72]. *C. galactitolivorans* is a well-studied chain elongating bacteria which can produce MCFAs at satisfactory amount under mildly acidic condition (pH = 5 ~ 6) [72]. The pH 5 applied herewith therefore encouraged the MCFAs production controlled by *C. galactitolivorans*. Apart from the genes encoding enoyl-[acyl-carrier-protein] reductase (K00208), most of the enzymes required for CE were identified in *Caproiciproducens*. The higher abundance of *Caproiciproducen* along with its abundant functional genes and enzymes may jointly contribute to higher MCFAs formation in the experimental system.

Oscillibacter Valericigenes and *Oscillibacter ruminantium* were the key species within *Oscillibacter* (Fig. 5**b**). These two bacterial species can grow fermentatively on various carbohydrate resources including D-glucose, L-arabinose, D-ribose, and D-xylose, with butyrate and valerate as the main metabolites [73,74]. Caproate was also found in the system enriched with these two bacterial species [74]. Taxonomic assignments showed that more functional genes required for CE pathway (i.e., FAB pathway) were identified in *O. Valericigenes* compared to *O. ruminantium* (Fig. 5). Only the genes responsible for the transformation of malonyl-CoA to (C_{n+2})-β-Ketoacyl-ACP with Malonyl-ACP as intermediate were identified in *O. ruminantium*. *O. Valericigenes* was therefore more likely to be the main chain elongator for MCFAs production via FW

fermentation. Other species within the genera *Oscillibacter* only contained some but not all genes required for CE pathway. The missing genes of these species were specifically listed and presented in Fig. 5 and **Table S3**, respectively. Previous researchers also reported the similar findings that not all functional genes can be found in the associated functional organisms [34,70,71]. No confirmative experimental results have been published to substantiate the possible collaboration among these organisms for the MCFAs production. Further investigation is needed to be done to reveal this knowledge gap from a metagenomics perspective. The overall abundance of the functional genes contained in *O. Valericigenes* and *O. ruminantium* was slightly higher in the experimental system than that from the control, which has been reflected clearly in Table S3. Such differences may result in more MCFAs production from FW, as higher genetic abundance could be related to higher MCFAs biosynthesis. Metatranscriptomics analysis is wanted to better reveal the actual role of the species affiliating to *Oscillibacter* in CE process.

3.6. Implication, from technique and economics perspectives

This study proposed a novel technology of producing MCFAs from FW with self-supplied ethanol instead of external ED provision, improving the economic benefits and practicality of waste-to-energy technology accordingly. To augment the ethanol productivity and avoid the labour intensity, the yeast cells immobilized in the alginate were dosed in the fermentative system before commencing the semi-continuous experiment. Specifically, 1540 mg COD/L MCFAs was attained in the batch reactors over 20 days of operation. A consistent yield of MCFAs at approximately 1323 mg COD/L was achieved in the semi-continuous system with yeast assisted, which was nearly 1.49 times greater than the yield in the control. The immobilized yeast in the alginate beads were reused for 60 days while ensuring a constant ethanol supplement and the succeeding MCFAs productions based on the product profiles in this study. Rupture of the alginate beads was observed at the later stage of the experiment. However, despite it is inevitable to observe the burst of CO_2 during the ethanol production process, the alginate-based yeast beads were not impaired extensively. Given that the around 84 % of yeast cells immobilized in the alginate beads were estimated to survive with 95 % of them remaining active during the ethanol-based fermentation [75], it is reasonable to assume that the inoculated yeast functioned normally throughout the long-term
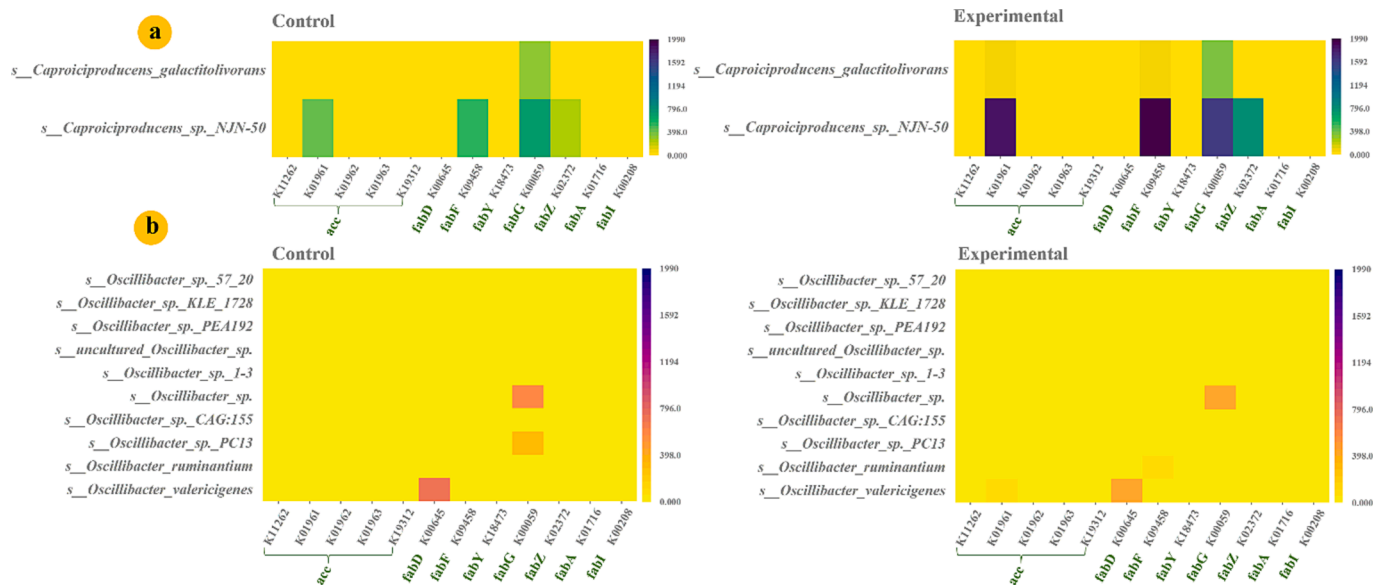

Fig. 5. Taxonomic origins of the key enzymes from long-term operated system detected in CE platform. a) Genes and the encoded enzymes contained in the species affiliated to *Caproiciproducens*. b) Genes and the encoded enzymes contained in the species affiliated to *Oscillibacter*. The abundance was calculated as reads number.

experiment. The yeast (*S. cerevisiae*) adopted in this study was a prevalent and persistent strain, which has been widely applied to the industrial context. Given to 1) the low price of the inoculated yeast strain, 2) the easiness to immobilize yeast into calcium alginate, and 3) the higher market value of MCFAs (4.0 $/kg) than ethanol (0.8 $/kg) [76], it seems promising to apply this technology beyond lab scale with profitable economic income.

To apply the novel fermentation paradigm with yeast inoculation on a larger scale, several research directions were proposed hereby. First is the optimization of yeast immobilization to achieve persist MCFAs production with longer and more robust in-situ ethanol provision. The application range of this study could then be wider when the immobilization material attains better mechanical properties and permeability to organic compounds. Some efforts have been devoted to improving the stability of the beads by optimizing the gelling or curing process. For instance, sodium alginate was previously blended with some special polymers such as polyvinyl alcohol and polyethylene oxide [77,78]. The chemical and physical properties of beads can then be improved, facilitating the following ethanol production via yeast. More efforts are required to further optimize the stability of yeast-contained beads and their mass transfer efficiency. Second is the adoption of multiple-stage systems to perform this novel fermentation paradigm, as the optimal conditions for ethanol producers and chain elongators are not the same. Specifically, a two-stage fermentation system, where ethanol synthesis and CE are separately controlled, might be the good idea to enhance in-situ ethanol production and the following MCFAs production. This is because the optimal pH conditions for ethanol production (4.0 ~ 4.5) and MCFAs synthesis (5.0–7.0) are different [79], and pH plays an important role in governing the microbial reactions [37]. The two-stage fermentation system has been widely adopted to recover the carbon resource from biowastes [8]. The reconfiguration of anaerobic fermentation system could therefore be a useful strategy to extend the application of the yeast-inoculation strategy. Third is combining the metagenomic and metatranscriptomic analysis together to better understand how yeast inoculation strategy alternate the mechanisms of genes responsible for MCFAs production in FW fermentation systems. A deeper and better understanding of mechanism would lay the foundation for the wider applications of this novel fermentation paradigm.

4. Conclusion

A novel anaerobic fermentation paradigm with yeast inoculation was proposed to achieve abundant MCFAs productions from FW using in-situ produced ethanol. The inoculation of yeast drove in-situ ethanol generation, which, in turn, improved the biosynthesis of MCFAs with 6 to 8 carbon atoms. 1540 mg COD/L MCFAs was attained in the batch reactors over 20 days of operation. A consistent yield of MCFAs at approximately 1323 mg COD/L was achieved in the semi-continuous system with yeast assisted, which was nearly 1.49 times greater than the yield in the control. The coordinated metabolic interactions and metabolic complementarity are important driving forces for metabolite exchange in anaerobic microbial communities. The promoted MCFAs production from FW with yeast assisted was mainly attributed to the enhanced SCFAs production via carbohydrate and protein metabolism, the higher in-situ ethanol productions with yeast-assisted, reinforced CE process and less ethanol waste. This pioneer study is of great importance in recovering the economic value of FW as MCFAs by offsetting the external ethanol addition costs. The strategy concerning yeast inoculation was a viable and economically promising means to produce MCFAs sustainably from FW, as no big change was required to the existing anaerobic fermentation infrastructure. Future study may need to test the feasibility of applying yeast-inoculation strategy on a larger scale by optimizing beads material and adopting multiple-stage systems.

CRediT authorship contribution statement

Lan Wu: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Wei Wei:** Writing – review & editing, Supervision, Project administration, Data curation, Conceptualization. **Zhijie Chen:** Writing – review & editing. **Xingdong Shi:** Investigation. **Jin Qian:** Writing – review & editing. **Bing-Jie Ni:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

The sequence data is publicly available at the European Nucleotide Archive under the project ID: PRJNA1026155.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2024.149236.

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