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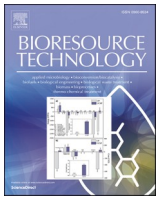

Lactobacillus inoculation mediated carboxylates and alcohols production from waste activated sludge fermentation system: Insight into process outcomes and metabolic network

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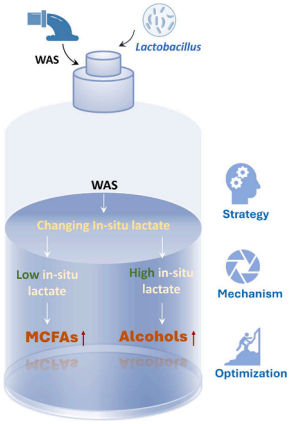
HIGHLIGHTS

- A new biotechnology was conceived to produce carboxylates and alcohols from WAS.
- Highest MCFAs yield from WAS was achieved by the lowest *Lactobacillus* inoculation.
- High alcohols production was achieved under higher *Lactobacillus* inoculation.
- A metabolic route for carboxylates and alcohols production was established.

GRAPHICAL ABSTRACT



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ABSTRACT

Producing medium chain fatty acids (MCFAs) from waste activated sludge (WAS) is crucial for sustainable chemical industries. This study addressed the electron donor requirement for MCFAs production by inoculating *Lactobacillus* at varying concentrations (7.94×10^{10}, 3.18×10^{11}, and 6.35×10^{11} cell/L) to supply lactate internally. Interestingly, the highest MCFAs yield (~2000 mg COD/L) occurred at the lowest *Lactobacillus* inoculation. Higher inoculation concentrations redirected more carbon from WAS towards alcohols production rather than MCFAs generation, with up to 2852 mg COD/L alcohols obtained under 6.35×10^{11} cell/L inoculation. *Clostridium* dominance and increased genes abundance for substrate hydrolysis, lactate conversion, and MCFAs/alcohol production collectively enhanced WAS-derived MCFAs and alcohols synthesis after *Lactobacillus* inoculation. Overall, the strategy of *Lactobacillus* inoculation regulated fermentation outcomes and subsequent

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carbon recovery in WAS, presenting a sustainable technology to achieve liquid bio-energy production from underutilized wet wastes.

1. Introduction

One aspect of the circular bio-economy paradigm aims to recover the carbon resource contained in the biowastes as profitable chemicals, thereby reducing the dependency on the finite fossil fuels. Waste activated sludge (WAS) was generated abundantly during wastewater treatment, which has been treated and disposed as an important source of pollution (Appels et al., 2008). The treatment of WAS is very costly. Around 50 to 60 % of the operational expense was used for WAS treatment during wastewater treatment (Huyard et al., 1994). Nevertheless, WAS represents a valuable resource rich in multiple organic matter including carbohydrates and protein (Xiao et al., 2017). Treating WAS as a potential resource rather than an unwanted waste should assist the development of bio-based economy (Wainaina et al., 2020). Anaerobic fermentation is a classic method for recovering carbon from WAS. Despite intensive efforts have been devoted to the production of short chain fatty acids (SCFAs) and biogas, the economic value of these fermentative products is limited (Kleerebezem et al., 2015). End products from anaerobic fermentation, including medium chain fatty acids (MCFAs) and long chain alcohols (LCAs), have received increasing attention due to their broad-spectrum application. MCFAs can be converted into longer-chain liquid fuels, or used directly as antimicrobial agents, corrosion inhibitors, livestock feed additive, and plant growth promoters, or building blocks for producing fragrance, lubricants, and dyes (Shrestha et al., 2023). LCAs refer to alcohols with 4 or more carbon atoms, such as butanol, pentanol, hexanol, which can be applied as solvents, fuels, flavoring agents, cosmetics and pharmaceuticals (de Leeuw et al., 2019).

MCFAs are the carboxylates resulting from chain elongation (CE). In this process, two carbon atoms are added to short-chain fatty acids (SCFAs) during each cycle, forming MCFAs. The carbon atoms contained in MCFAs usually range from six to twelve, with caproate formed as the main carboxylate (Xie et al., 2021). Reverse β oxidation (RBO) and fatty acid biosynthesis (FAB) are the CE processes for the synthesis of these valuable carboxylates. Generally, external supply of electron donors (EDs) is usually required to provide enough energy and reduce power to the chain elongating microbes (Liu et al., 2022; Zhu et al., 2021). However, the external EDs addition diminishes the economic revenue of the WAS-derived MCFAs production. This reliance on external EDs hampers the development of CE process (Wu et al., 2023d). Hence, promoting the self-production of EDs is a viable solution to address this technical challenge. Lactate is an easily metabolized molecule and compatible with most of the organisms in the fermentation system (Vuyst, 2000). Lactate is a common ED for assisting MCFAs production (Tang et al., 2022), which can be produced from carbon-rich substrates including WAS. Therefore, this study proposed a novel strategy to encourage in-situ lactate formation via *Lactobacillus* inoculation to boost economical MCFAs biosynthesis.

A previous study has reported the presence of both butanol and hexanol, the common LCAs, in fermentation systems containing undefined mixed cultures, particularly in reactors with sufficient external electron donor sources (Wu et al., 2020). The microbial consortium within the open-culture fermentation system can be significantly influenced by external organism inoculation, including microbial structure and functionality subsequently (Liu et al., 2019; Wu et al., 2023c). For instance, some *Clostridium* species, which attain the ability to convert carboxylates to alcohols, was previously found to be enriched in an open-culture system with yeast inoculation (Wu et al., 2023c). Therefore, the change of in-situ lactate production resulting from *Lactobacillus* inoculation alternation may regulate or even expand the product portfolio in the WAS anaerobic fermentation system.

This study developed an innovative *Lactobacillus* inoculation technology to encourage sustainable carboxylates and alcohols production from WAS fermentation system without the need for external ED dosage. Then, the dynamics of profiles and the mechanisms of carboxylates as well as alcohols synthesis driven by *Lactobacillus* inoculation were further elaborated through unveiling the changes in microbial community and fermentative pathways. The implication of this novel fermentation paradigm was finally evaluated.

2. Materials and methods

2.1. Source of the waste activated sludge and Lactobacillus

WAS applied in this study was freshly collected from a secondary tank of a local municipal wastewater treatment plant and maintained under 4 °C before commencing the experiment. The main characteristics of WAS were as follows: total solids (TS) at 38.31 g/L, volatile solids (VS) at 21.87 g/L, and TCOD at 37.04 g/L. The *Lactobacillus* adopted in this study was contained in the commercial yoghurt starter powder, which was purchased from a local store. Seven *Lactobacillus* species were contained in this yogurt stater powder, including *L. mucosae*, *L.panis*, *L. delbruekii*, *L. amylovorus*, *L. amylolyticus*, *L* sp., and *L harbinensis*. The desired *Lactobacillus* cell number inoculated in various reactors can be attained by weighing it, as the weight of *Lactobacillus* cell is fixed. The *Lactobacillus* contained in the yogurt starter powder was produced for direct industrial use, exhibiting high microbial activity, and not requiring acclimation before inoculation.

2.2. Batch experimental procedure

A series of 150 ml serum bottles were used as anaerobic reactors to study the change of metabolite structure in the WAS anaerobic fermentation systems operating in batch mode. The batch test was lasted for 57 days, as the concentrations of fermentative products under all tested conditions remained almost unaltered. 100 ml WAS was firstly added into the serum bottles before the commencement of the experiment. Except for the control group, all experimental groups were inoculated with yogurt stater powder containing *Lactobacillus* at 7.94×10^{10}, 3.18×10^{11}, and 6.35×10^{11} cell/L, respectively. As the weight of the *Lactobacillus* inoculation was increased by 0.5, 2, and 4 times, 0.5L, 2.0L and 4.0L were then used to indicate various experimental 150 ml bottles. 10.5 g/L 2-bromoethanesulfonate (2-BES) was then added to the fermentative systems to prevent the occurrence of methanogenesis. The pH was adjusted to 4.5 and maintained at this value by manually adding 3 M HCl or 3 M NaOH throughout the experiment. All serum bottles were then purged with nitrogen gas for 5 min and were subsequently sealed up with rubber stopers along with parafilm to ensure the anaerobic conditions. All prepared bottles were placed in an incubator at 120 rpm and 37 ± 0.5 °C to perform anaerobic fermentation. Unless otherwise stated, all the tests were performed in triplicate.

2.3. Analytical methods

1 Ml liquid sample was collected regularly to test the change in the lactate, SCFAs, MCFAs, and alcohols contained in the fermentation systems. These fermentative products were then measured using a gas chromatograph (GC system 7890 A, agilent Technologies, USA) equipped with a flame ionization detector. The specific method for detecting these fermentative products via GC were following Wu et al., 2020a. Lactate concentration was determined through liquid chromatograph (LC, waters 2695 + 2489, USA) equipped with a UV/Visible detector.

The detailed method for lactate detection was based on the wei et al., (2021)

2.4. Metagenomic analysis

Samples from the control and 0.5L system were collected to perform metagenomic analysis, hypothesizing the changes in the microbial structure, metabolite pathways, and associated enzymology. The supernatant of the sludge samples was discarded, and the remaining pellet was preserved under −80 °C for further metagenomic analysis. The DNA extraction, sequencing, and bioinformatics analysis were all performed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (SMBBC, Shanghai, China). The raw reads, each 150 bp in length, underwent quality control using the FASTp Toolkit (version 0.20.0) and retained over 97 % of the reads. This process yielded 49,238,932 clean reads for the control group and 45,770,166 clean reads for the 0.5L group. Subsequently, the filtered data was assembled using the MEGAHIT program (Version 1.1.2). ORF prediction was carried out on the contigs from the assembly using Prodigal software (Version 2.6.3). The CD-HIT program was utilized to cluster biological sequences and create a non-redundant gene set. High-quality reads from each sample were aligned against this non-redundant gene set using SOAPaligner software, enabling the quantification of gene abundance in each sample. Identified genes were annotated and classified based on their species and functions, utilizing the Non-Redundant (NR) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases for function and annotation assignments. The sequence data is publicly available at NCBI under the project ID: PRJNA1127703.

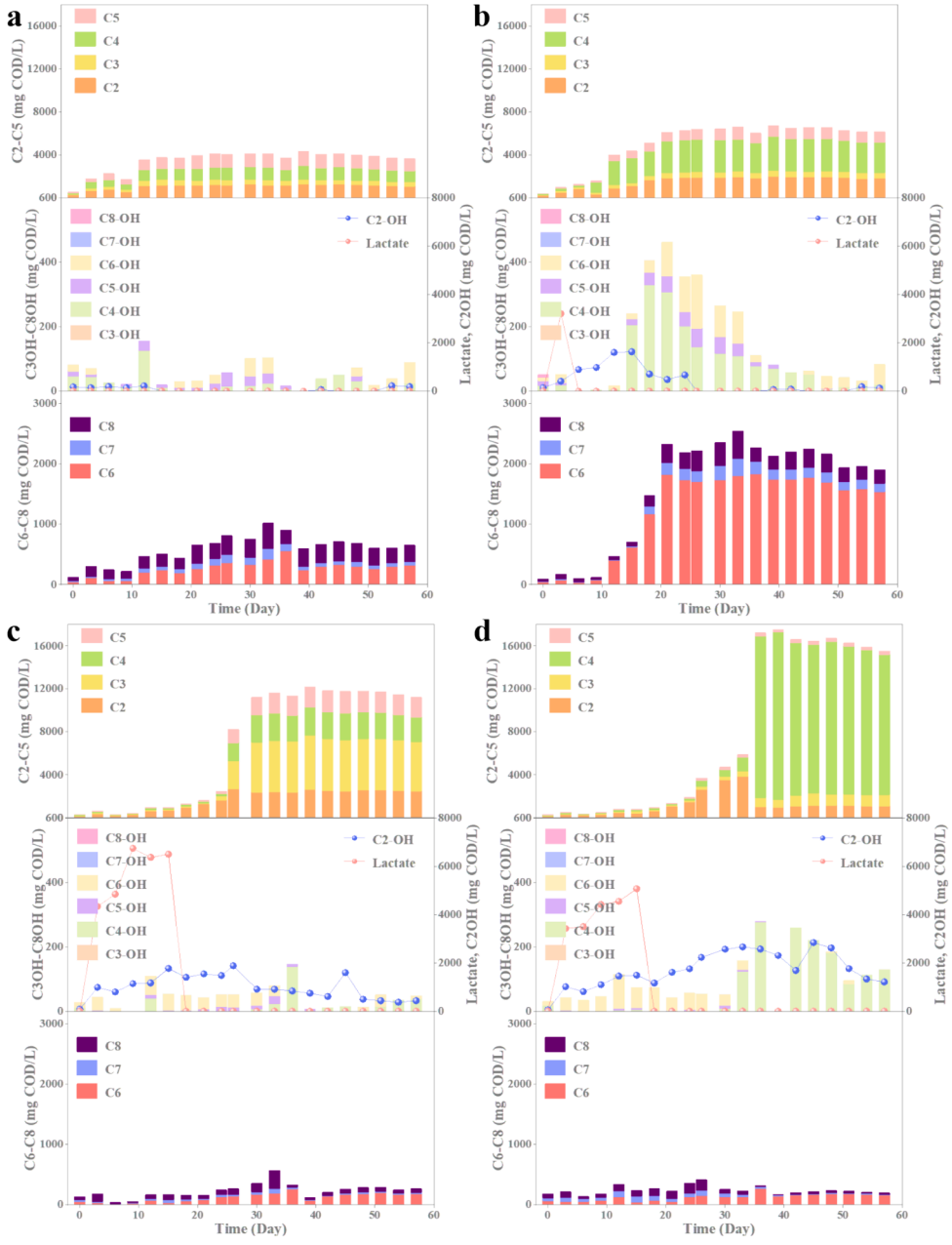

Fig. 1. Fermentative products derived from the control (a), 0.5L (b), 2.0L (c) and 4.0L (d) experimental groups.

3. Results and discussion

3.1. Profiles of the fermentative products from WAS with self-supplied lactate in batch experiment

Batch tests were conducted to validate the feasibility of producing higher value-added products abundantly and economically via *Lactobacillus* inoculations. The profiles of fermentative products in four sets of anaerobic fermentation systems were shown in Fig. 1. Overall, more substrate was converted to the target end products (i.e., SCFAs, MCFAs, & alcohols) with the improvement in *Lactobacillus* inoculation. Lactate was produced abundantly after *Lactobacillus* inoculation. The MCFAs production was dose-dependently affected by the amount of *Lactobacillus* inoculation. The maximum MCFAs production (2000 mg COD/L) was attained under the lowest *Lactobacillus* inoculation (i.e., 0.5L system), much higher than that from the control (653 mg COD/L). Further improvement in *Lactobacillus* inoculation did not improve the MCFAs yield. But, on the contrary, the final cumulative MCFAs productions under those conditions were a bit lower than the control, where nearly 250 mg COD/L of MCFAs attained in the 2.0L and 4.0L systems.

Regardless of the decline of MCFAs in the 2.0L and 4.0L systems, ethanol, butanol and hexanol were surprisingly detected as the additional monetary fermentative products under these conditions, with the butanol acting as the main component of LCAs. Specially, 2663 mg COD/L ethanol, 277 mg COD/L butanol, and 106 mg COD/L hexanol were achieved in the 4.0L system. This is probably because the adopted pH (4.5) was favorable to ethanol (Zhao et al., 2018) and butanol production from carbon-rich substrates (Romans-Casas et al., 2024). Some ethanol may be derived from externally dosed *Lactobacillus*, as some *Lactobacillus* species were obligate heterofermentative organisms with the ability to produce both lactate and ethanol simultaneously by fermenting WAS (See more in Section 3.3) (Rich et al., 2015). The production of butanol and hexanol may result from MCFAs reduction through reacting with the internally formed ethanol in the systems (Richter et al., 2013; Wu et al., 2023a; Wu et al., 2018). Butanol dehydrogenase or alcohol dehydroagenase were reported to be the enzymes catalyzing the conversion of butyrate (Dai et al., 2016). However, the high ethanol synthesis attained under high *Lactobacillus* inoculation might be toxic for MCFAs producer, inducing the lower MCFAs production. The higher levels of in-situ formed ethanol in the 4.0L system would, in turn, stimulate the conversion of carboxylates to the corresponding alcohols.

3.2. Product distribution in WAS fermentation

To better reflect the desired products under varying *Lactobacillus* inoculations, the product distribution based on the division between metabolites and their sum detected on the final day of the experiment was analyzed (Fig. 2). In general, most of the carbon contained in WAS was diverted to SCFAs, with butyrate being the main component. The inoculation of *Lactobacillus* facilitated an increased accumulation of SCFAs, with its portion improved from 80 % to over 90 % in response to the increasing *Lactobacillus* inoculation. This result was in line with the product structure as discussed in Section 3.1. Previous studies also observed similar trends that butyrate concentrations exceeded other carboxylates in the MCFAs-producing fermentative systems (Cavalcante et al., 2020; Spirito et al., 2018). The acidic pH was probably one of the reasons affecting substrate conversion to MCFAs and the high SCFA production, as it hindered MCFA producers from using the ED pathway effectively to perform the CE process (Cavalcante et al., 2020). Except for 4.0L system, around 20 % of SCFAs were acetate under other testing conditions. As lactate was used up, the product distribution for this metabolite was zero in both *Lactobacillus*-free and *Lactobacillus*-inoculated reactors.

The 0.5L system with 7.94×10^{10} *cell/L Lactobacillus* inoculation was the best reactor for MCFAs production. More than 20 % of the final metabolites under such condition was composed of MCFAs, with caproate being the main component (18.61 %). The effective utilization of lactate for performing CE were the main drivers for such results (Zhu et al., 2015). Moreover, 0.5L system was the only reactor where heptanoate and caprylate accounted for proportions larger than 1.6 %, further supporting the successful proceeding of CE under low

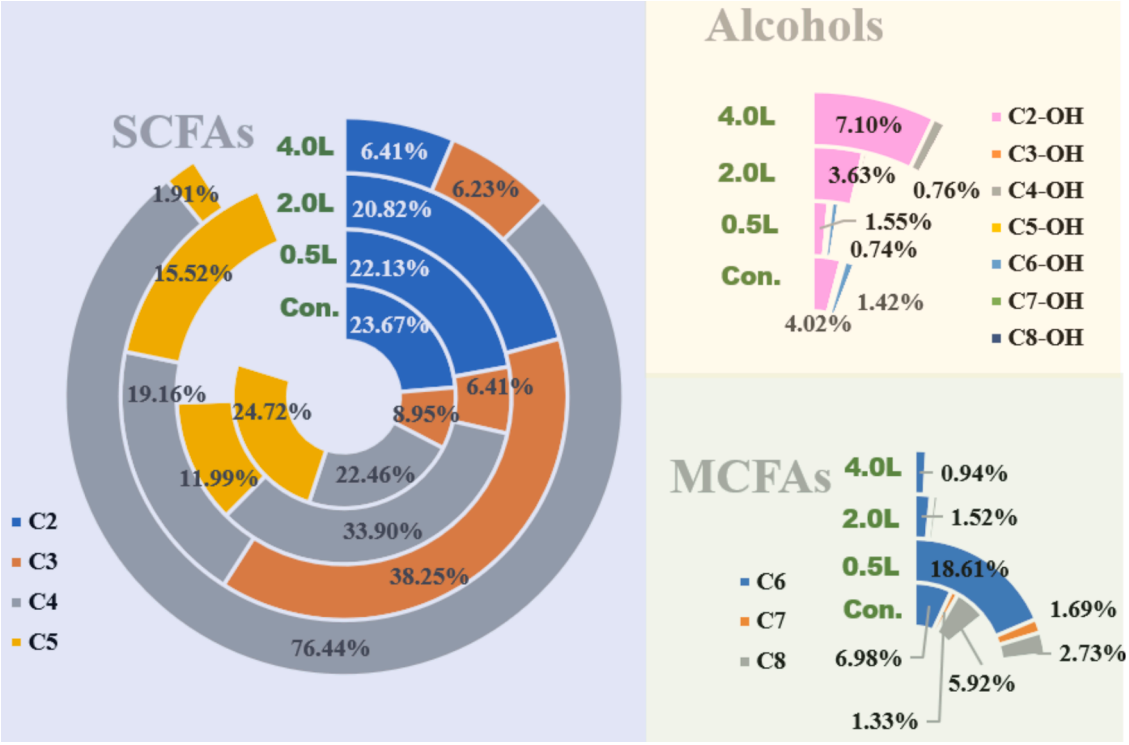

Fig. 2. Product distribution of the metabolites derived from anaerobic fermentation with or without *Lactobacillus* inoculation.

Lactobacillus inoculation. As for the 2.0L and 4.0L reactors, where 3.18 $\times 10^{11}$ and 6.35 $\times 10^{11}$ cell/L *Lactobacillus* were inoculated respectively, only 1.52 % and 0.94 % of the metabolites were composed of caproate, which was even less than that in the control. No obvious heptanoate and caprylate proportions were found in these two experimental systems with higher *Lactobacillus* inoculation, aligning with results in Fig. 1. In addition to the difficulty in elongating butyrate under low pH condition mentioned previously, the lower MCFAs distribution under higher *Lactobacillus* inoculation may also be due to the competition between alcohols producers and MCFAs producers.

Ethanol was the additional valuable fermentative products attained from the *Lactobacillus*-inoculated system. The highest proportion of ethanol was attained under the highest *Lactobacillus* inoculation, ensuring the ethanol provision for LCAs biosynthesis via MCFAs reduction (Richter et al., 2013; Wu et al., 2023a; Wu et al., 2018). Specifically, around 0.76 % of the metabolite consisted of butanol when high ethanol production was attained in 4.0L reactor.

3.3. Microbial community composition and interaction network analysis

The abundant microbiome from the control and 0.5L system was specifically analyzed to better understand the possible network among microbial organisms and the mechanisms for producing value-added fermentative products including carboxylates and alcohols (Fig. 3). Specifically, the top genera were shared but exhibited different distributions in various samples (Fig. 3a). The changes in the microbial structure were in line with the alterations of end-products in response to various *Lactobacillus* inoculation. Fermentation system with *Lactobacillus* inoculation tended to build a stronger microbial network for recovering valuable carboxylates and alcohols based on the microbial analysis at phylum and genus levels.

Proteobacteria was the main phylum in the control system (40.83 %), which is commonly found in fermentation and anaerobic digestors (Jiang et al., 2020b). However, the abundance of this phylum dropped to 1.38 % upon the inoculation of *Lactobacillus*. *Firmicutes* became the dominant phylum in the 0.5L system, which accounted for 76.23 % of the microbial community (Fig. 3b). The dominance of *Firmicutes* was not a surprise, as the inoculated *Lactobacillus* species also belong to this phylum. As most of the chain elongators and ethanol/butanol/hexanol producers were belong to *Firmicutes* (Candry & Ganigue, 2021), the dominance of this phylum should be benefit for the following MCFAs and alcohols production. *Bacteroidetes* and *Actinobacteria* were the additional phyla detected at relative high abundance in the control and 0.5L systems. *Bacteroidetes* was reported to have anaerobic fermentation (Jiang et al., 2020a) and chain elongation relevance (Wu et al., 2023b). The abundance of this phylum was improved to 16.50 % following the inoculation of *Lactobacillus*, almost 5.1 times higher than that from the control.

The shift in the top microbial genus was in line with the changes of lactate and SCFAs concentrations in the control and 0.5L system. Given 1) *Lactobacillus* was externally inoculated in the 0.5L system, and 2) *Lactobacillus* attained higher growth rates than other anaerobic microorganisms (Rombouts et al., 2020), the abundance of *Lactobacillus* increased dramatically in the experimental group (20 %), making *Lactobacillus* the most prevalent organism for lactate production in the experimental group (Fig. 3c). *L. mucosae*, *L. panis*, *L. delbrueckii* were the dominant *Lactobacillus* species (see Supplementary Material). More lactate was expected to be formed following *Lactobacillus* inoculation compared to the control. Moreover, ethanol may also be partially produced by *Lactobacillus* species, as *L. mucosae* and *L. panis* are the facultative heterofermentative species. *Bacteroides*, a genus belongs to the phylum *Bacteroidetes*, was prevailed in the 0.5L system (1.06 %) and was also previously observed in the experimental system (Fig. 3d) (Zhang et al., 2023). *Bacteroides* might be the major functional contributor for substrate hydrolysis based on its increasing abundance upon *Lactobacillus* inoculation. *Bifidobacterium* and *Prevotella* are the key organisms involved in SCFAs production. Specifically, *Prevotella* was recently found with the capability of producing acetate via fermenting the polysaccharide contained in the wastes (She et al., 2020). The genes responsible for producing butyrate were harbored in *Bifidobacterium* (Crognale et al., 2021). The higher abundance of these two genera may therefore jointly lead to the higher yield of SCFAs in 0.5L system.

Clostridium, the well-known multi-functional genus within *Firmicutes* (Abo et al., 2019), became the major and core contributor for synthesizing highly valuable fermentative products in this study (Fig. 3e). Specifically, no abundant *Clostridium* was observed in the control, with

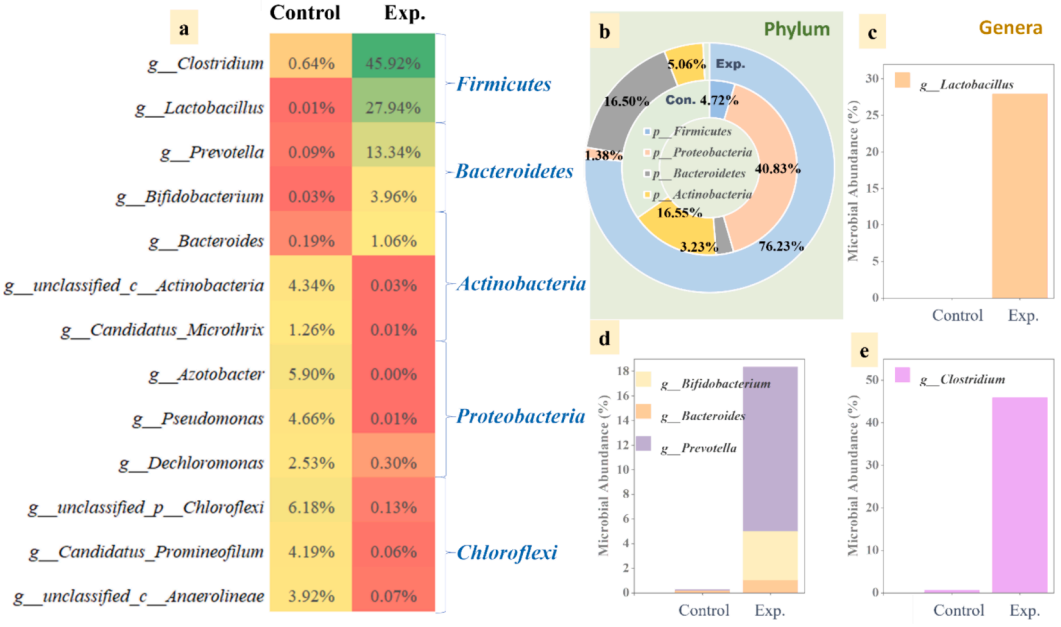

Fig. 3. Microbial analysis in the samples attained from the control and 0.5L experimental system: a) the top microbial genera; b) the percent of microbial abundance on phylum level; c) the abundance of the microbial genus involved in lactate production; d) the abundance of the microbial genus involved in hydrolysis and SCFAs production; and e) the abundance of the microbial genus involved in chain elongation and LCAs production.

only 0.65 % of the microbial community ascribed to this genus. However, this microbial genus was enriched significantly in the 0.5L system (45.92 %). The high abundance of *Clostridium* developed in the 0.5L system should facilitate the production of MCFAs and other valuable chemicals, including ethanol, butanol and hexanol (Kundiyana et al., 2011). Similar findings were also reported previously, wherein the dominance of *Clostridium* can expand substrate spectrum and induce higher yield of valuable alcoholic compounds via anaerobic fermentation (Cui et al., 2021).

Overall, *Lactobacillus* inoculation affected the microbiota structure and microbial interactions in the fermentation systems heavily, steering the metabolite structure and the carbon recovery of waste in return. The improving abundance of the organisms involved in hydrolysis, SCFAs production, and lactate synthesis laid the foundation for smooth proceeding of MCFAs, ethanol, butanol, and hexanol productions under *Lactobacillus* inoculation. The absolute predominance of *Clostridium* in the experimental group ensured the microbial structure for improving carbon recovery efficiency and value via WAS fermentation.

3.4. Potential pathways for carboxylates and alcohols production

The functional genes contained in the control and 0.5L system were annotated towards KEGG database to construct the pathways for MCFAs and valuable alcohols productions via WAS anaerobic fermentation. The key genes and associated enzymes involved in various pathways were presented in Fig. 4, along with the changes in their abundance. More details can be found in Supplementary Material.

Lactate is the primary fermentative metabolite abundantly produced in the 0.5L system, with *Lactobacillus* identified as the main and robust producer of lactic acid as discussed in Section 3.3 (Rombouts et al., 2020; Xie et al., 2021). The abundantly produced lactate then underwent the conversion to acetyl-CoA, with pyruvate acting as the key intermediate. The improving overall abundance (1.07-fold higher than the control) of genes annotating this process may indicate that the transmembrane transport and intracellular metabolism of pyruvate and acetyl-CoA were likely to be more active in the 0.5L system (Liu et al., 2022). Pyruvate and acetyl-CoA are the important by-products and intersections in the network of metabolic pathways, which would be directed to produce other metabolites, including MCFAs, alcohols, and SCFAs.

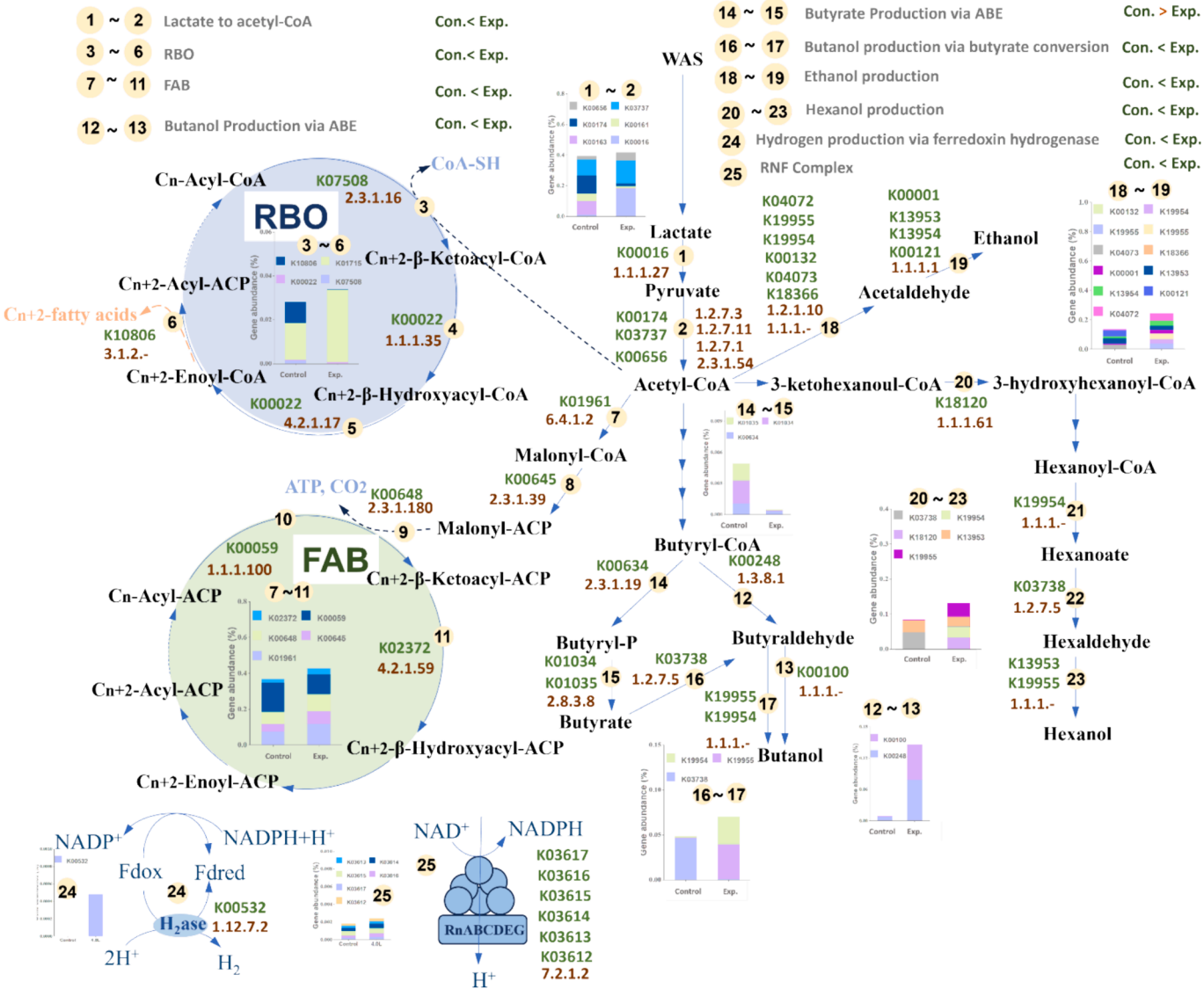

Fig. 4. MCFAs and valuable alcohols production pathways constructed according to KEGG database together with relative abundance of functional genes responsible for MCFAs/alcohols production from WAS fermentation with or without *Lactobacillus* inoculation.

RBO and FAB were identified as the two CE pathways, according to the identification of key genes involved in these cycles (Fig. 4). Genes controlling these two cyclic pathways were generally promoted upon *Lactobacillus* inoculation. Specifically, in the RBO cycle, the lactate-derived acetyl-CoA worked as the central cyclic substrate, entering this cycle to start MCFAs synthesis through elongating SCFAs with 2 carbon atoms every cycle (Cavalcante et al., 2017). Most of the genes controlling the bioreaction steps involved in RBO were more abundant in the *Lactobacillus*-inoculated system (see Supplementary Material), implying a smoother proceeding of MCFAs production via this cyclic pathway (Wu et al., 2023a). As for FAB pathway, more biological steps were required to form MCFAs compared to its counterpart platform, as acetyl-CoA needs to be transformed into malonyl-CAP before initiating the cyclic process (Lian & Zhao, 2015). The enzymes (E.C. 6.4.1.2, E.C. 2.3.1.39, & E.C. 2.3.1.180) catalyzing the conversion of acetyl-CoA to malonyl-ACP were likely upregulated, as indicated by their increased gene abundance. The FAB pathway might be the core platform for MCFAs synthesis, as the overall gene abundance of this pathway was higher than that of RBO (Fig. 4). However, this assumption needs further validation. The detection of the gene involved in ferredoxin hydrogenase production suggested that in-situ synthesized H_2 may contribute to MCFA production. This H_2 was likely utilized more effectively in the presence of *Lactobacillus* compared to the control, due to the higher abundance of the gene associated with hydrogenase production.

Butanol was the main LCAs synthesized in the 0.5L reactor. Three potential mechanisms for the butanol production were depicted in Fig. 4, with associated genes and enzymes marked. *First* is the classic acetone-butanol-ethanol (ABE) pathway, wherein acetyl-CoA was converted to butyryl-CoA, subsequently leading to the formation of butyraldehyde and butanol as the end-product. Enzyme (E.C. 1.2.7.5) involved in this process may use electrons from reduced ferredoxin to form butyraldehyde (Nissen & Basen, 2019). ABE is a widely observed pathway for butanol synthesis when fermenting carbon-rich substrates (Poe et al., 2020). As key genes annotating this pathway were more abundant upon *Lactobacillus* inoculation, more butanol should be formed theoretically. Also, the enriched *Clostridium* in *Lactobacillus*-inoculated system might be the promising candidate for butanol synthesis via ABE platform (Poe et al., 2020; Zhang et al., 2020), further ensuring the dominant role of ABE in forming butanol. *Second* is the less butyrate production through butyryl-CoA conversion via ABE platform based on the downregulated gene abundance, ensuring more butyryl-CoA was converted to butanol rather than wasted as butyrate. A *third* mechanism contributing to butanol production involves butyrate reduction while utilizing on-site formed ethanol (Lou et al., 2024). Genes and enzymes (E.C. 1.2.7.5 & E.C. 1.1.1-) governing this potential mechanism were identified and tended to be enhanced slightly after *Lactobacillus* inoculation. Genes for ethanol production was enriched upon *Lactobacillus* inoculation, laying the foundation for higher ethanol yield in the experimental system. Most of the ethanol was likely converted to NADH, providing reducing force for butanol synthesis through the enriched *Clostridium* (Contreras-Davila et al., 2020). This process reduced organic acids to aldehydes via an Fd-dependent reaction, which are subsequently converted to alcohols by NAD(P)H-dependent alcohol dehydrogenase (E.C. 1.1.1-).

The production of hexanol occurred through the conversion of acetyl-CoA, with hexanoate as the key intermedial metabolite (Fig. 4). Specifically, acetyl-CoA was first transformed into hexanoyl-CoA via a series of enzymatic reactions. The produced hexanoyl-CoA was then converted to hexanoate, ultimately resulting in the formation of hexanol (Daniell et al., 2012). The in-situ produced ethanol would assist the hexanol production from hexanoate by providing reducing force. Be consistent with the fluctuating but enhanced hexanol yield in the 0.5L system than the control, the genes annotating the enzymes responsible for these biological reactions were generally promoted, with a higher overall genetic abundance observed in the *Lactobacillus*-inoculated system.

In general, the MCFAs and valuable alcohols production is a complex and interconnected process, closely tied to the interactions among various pathways within the anaerobic fermentation system. This study comprehensively mapped a pathway for synthesizing valuable carboxylates and alcohols via *Lactobacillus*-mediated WAS fermentation systems, employing detailed analysis of genes and enzymes. The pathways controlling the formation of these value-added products depended on the amount of on-site lactate and other available fermentative compounds. The genes for producing carboxylates and alcohols appeared to be enhanced in the 0.5L system.

3.5. Implication and future perspectives

With the aim of building a carbon–neutral industry, microbial production of valuable MCFAs or alcohols from WAS via anaerobic fermentation is becoming increasingly attractive due to the higher monetary value contained in these fermentative products. The value of the carbon recovery from anaerobic fermentation system can then be enhanced through extending the product portfolio from WAS. However, though the MCFAs production offers an alternative product for anaerobic fermentation, using WAS directly to perform CE process is still limiting currently. This is because EDs were used to be externally added into the systems to facilitate CE reaction (Li et al., 2023), increasing the capital cost of waste valorization, and lowering the net benefits of the produced MCFAs thereafter. Lactate is one of the common ED to initiate MCFAs production (Asunis et al., 2019), which can be easily attained from anaerobic fermentation and is compatible with most of the reactor microbiome (Kucek et al., 2016). Therefore, one novel strategy to encourage MCFAs production via enhancing in-situ lactate production through *Lactobacillus* inoculation was performed and optimized in this study.

In the one-pot fermentation system, the bioreactions are complex and may occur simultaneously. The metabolite structure was controlled by not only the reactor microbiome but also the fermentative products, especially the level of in-situ lactate produced during operation. Therefore, the MCFAs yield varied with the change of *Lactobacillus* inoculation. Specifically, the peak of MCFAs production (2000 mg COD/L) was achieved under the lowest *Lactobacillus* inoculation via lactate-based CE process. Nevertheless, the higher in-situ lactate production resulting from higher *Lactobacillus* inoculations were not favorable to MCFAs production. The dispersion of the carbon to form other fermentative products might be the reason for the low MCFAs yield under higher *Lactobacillus* inoculations (Fig. 5).

High monetary value is still able to be recovered by forming valuable fermentative products when high amount of lactate was formed on-site after inoculating high amount of *Lactobacillus*. Specifically, besides MCFAs, ethanol, butanol, and hexanol were abundantly synthesized in the 4.0L system. Up to 2663 mg COD/L ethanol was produced upon high *Lactobacillus* inoculation. Butanol emerged as the primary synthesized alcoholic compound, reaching ~277 mg COD/L in 4.0L system. As *Lactobacillus* inoculation affected the microbial structure heavily, the microbial genus (i.e., *Clostridium*) with the trait of converting carboxylates to alcohols became the dominant organism, facilitating the alcohol production via carboxylates reduction substantially. The fermentative spectrum was expanded more intensively in response to the change of internal lactate yield.

Overall, the type and the amount of the valuable products recovered from WAS fermentation system were controlled by the interconversion reactions among various metabolites. The microbial production of a mixture of MCFAs/alcohols through a *Lactobacillus*-inoculated fermentation system suggests the potential development of a more profitable market compared to traditional ED-fed systems. Utilizing these end products as sustainable and economically viable platform chemicals could be a promising avenue. This *Lactobacillus* inoculation technology can be directly applied in the current wastewater facilities, as no big alternation is required for the treatment units. Also, this technology can

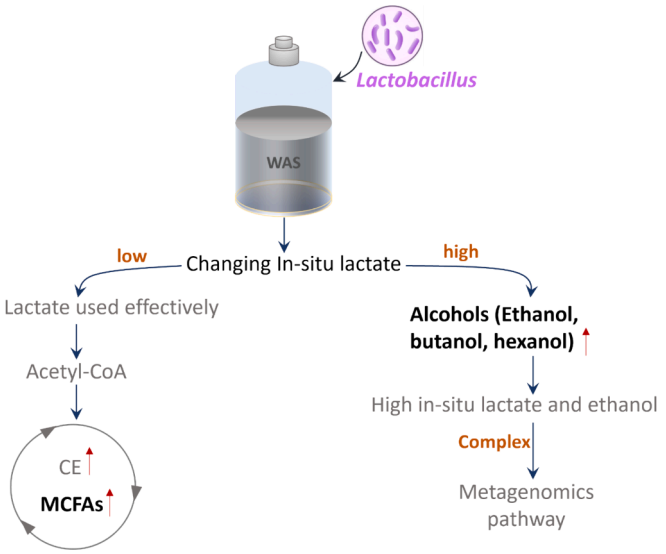

Fig. 5. Schematic diagram of how alternations in *Lactobacillus* inoculation controlled the metabolites structure and diversified the metabolic reactions in the WAS fermentation system. The "↑" marked in the figure indicated the positive effect of *Lactobacillus* inoculation on the biological step or the fermentative products.

also be utilized to treat other organic biosolids to assist the achievement of carbon neutral society. For the next stage and beyond, further studies are needed to test the viability of adopting this novel fermentation paradigm on a larger scale and in a long-term continuous mode. Moreover, future studies should also focus on producing high value-added products with higher purification by better steering the bioreactions within fermentative systems, thereby facilitating subsequent commercialization and practical application.

4. Conclusion

Lactobacillus inoculation effectively improved MCFA synthesis from WAS fermentation, with optimal carbon recovery achieved by adjusting inoculation levels. At the lowest inoculation (7.94×10^{10} cells/L), MCFAs reached 2000 mg COD/L, nearly triple the control. Higher inoculations (3.18×10^{11} and 6.35×10^{11} cells/L) increased alcohol production instead, with ethanol and butanol peaking at 2663 mg COD/L and 277 mg COD/L, respectively. These metabolic shifts were driven by sufficient in-situ lactate production for MCFA synthesis and higher ethanol production facilitating alcohol generation via enriched *Clostridium*. Adjusting *Lactobacillus* levels tailors the fermentation products, favoring either MCFAs or valuable alcohols.

CRediT authorship contribution statement

Lan Wu: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Huu Hao Ngo:** Writing – review & editing, Formal analysis. **Chen Wang:** Writing – review & editing, Investigation. **Yanan Hou:** Writing – review & editing. **Xueming Chen:** Writing – review & editing. **Wenshan Guo:** Writing – review & editing. **Haoran Duan:** Writing – review & editing. **Bing-Jie Ni:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition. **Wei Wei:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, 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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131191.

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