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Combined effects of oxytetracycline concentration and organic loading rate on semi-continuous anaerobic digestion of swine wastewater

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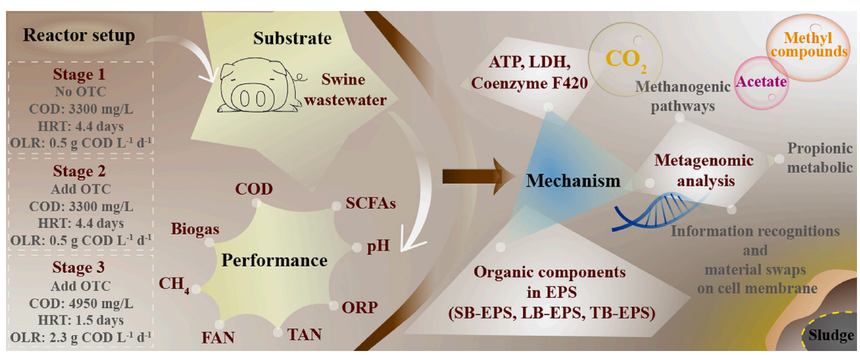
HIGHLIGHTS

- The large fluctuation of OLR of swine wastewater was simulated.
- 0.3 mg/L OTC boosted the biodegradability of the substrate.
- 8.0 mg/L OTC stimulated four methanogenic pathways (especially CO_2 => CH_4).
- 8.0 mg/L OTC reduced the metabolic functions of material swap on the cell membrane.
- 8.0 mg/L OTC reduced the functions of information recognition on the cell membrane.

GRAPHICAL ABSTRACT



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ABSTRACT

High concentrations of antibiotics in swine wastewater raises concerns about the potential adverse effects of anaerobic digestion (AD). Current studies mainly focused on the effects of various antibiotic concentrations. However, these studies didn't take into account the fluctuation of swine wastewater quality and the change of reactor operating conditions in practical engineering applications. In this study, it was found that in the operating systems with COD of 3300 mg/L and hydraulic retention time (HRT) of 4.4 days, the continuous addition of oxytetracycline for 30 days had no effect on the AD performance. Nevertheless, when COD and HRT were changed to 4950 mg/L and 1.5 days respectively, oxytetracycline at 2 and 8 mg/L increased the cumulative methane yield by 27% and 38% at the cost of destroying cell membrane, respectively, while oxytetracycline at 0.3 mg/L improved the performance and stability of AD. These results could be referred for practical engineering applications.

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1. Introduction

Pork accounts for one-third of the world's meat production (Cheng et al., 2018). As the largest pig-raising country in the world, China has raised>500 million pigs annually in the past decade (NBSC, 2022). The total amount of wastewater produced by a pig is equivalent to that of seven people. Therefore, the 671 million pigs raised in China in 2021 is equivalent to an increase in domestic sewage discharge of 4.7 billion people (NBSC, 2022; Sun et al., 2022). At present, the demand for pork is still growing. In order to boost pork production at a lower cost, traditional small pig farms are rapidly developing into intensive large ones (Hu et al., 2019; Tan et al., 2021b). Therefore, in the next few years, the quantity of swine wastewater will increase, and it could be expected that COD concentrations in swine wastewater and HRT of bioreactors will fluctuate considerably.

Anaerobic digestion (AD) has shown the advantages of not only working with high organic loading rate (OLR) and low operating cost but also recovering clean energy (methane) and reducing greenhouse gas emissions when treating organic wastes/wastewater, which is helpful to promote the goal of "carbon neutrality" (Liu et al., 2021a; Liu et al., 2021b; Nie et al., 2023; Tan et al., 2021a; Wen et al., 2016). Therefore, AD technology has been broadly applied in the treatment of swine wastewater (Liu et al., 2021a; Tan et al., 2021b). However, the treatment efficiency could drop greatly when AD systems were operated for a long time for the treatment of swine wastewater (Lourinho et al., 2020).

Antibiotics are considered to be one of the major reasons for the deterioration of treatment performance (He et al., 2020; Liu et al., 2021a). Antibiotics are usually used to treat and prevent animal diseases and improve the growth rate of animals (Hu et al., 2019; Li et al., 2022; Liu et al., 2021a). According to reports, the global consumption of veterinary antibiotics in 2010 was about 63,151 tons, which is anticipated to continue to augment to 105,596 tons by 2030 (Mushtaq et al., 2022). Among them, OTC has been widely used in pig feed because of its low cost and broad spectrum (Mushtaq et al., 2022). However, about 70% of OTC is excreted into urine and feces by pigs, which leads to the widespread detection of OTC in the liquid phase and suspended solids (solid phase) of swine wastewater at levels of 3.5–387 μg/L and 0.4–32 mg/kg, respectively (Mushtaq et al., 2022; Pan et al., 2011).

Studies have shown that OTC can inhibit AD. He et al. (2020) reported that when inoculum adapted to synthetic wastewater without OTC for a long time was used, OTC above 100 mg/L could inhibit COD removal and methane production in UASB reactors with continuous feed and constant OLR. However, Mushtaq et al. (2022) found that OTC only above 1.2 mg/L inhibited the removal of volatile solids and methane production in the AD system based on cow dung. This difference is attributed to the difference in the inoculum and operating conditions.

Nevertheless, some problems have been ignored in previous studies: (1) These reports focused on the effects of OTC on AD performance and microbial community, but did not explain how and why OTC inhibited AD performance (He et al., 2020; Huang et al., 2022; Mushtaq et al., 2022). Understanding the mechanism of OTC inhibiting AD performance is helpful for people to formulate management strategies to mitigate negative effects. (2) Most of the existing reports adopted batch experiment mode. However, the results of batch experiment and long-term continuous feeding experiment were quite different and even contradictory (Huang et al., 2022). Therefore, long-term semi-continuous/continuous experiment can get reliable and useful results and provide reference for practical engineering application. (3) These studies were only conducted on the separate level of OTC concentration, and did not consider the combined effect of OLR fluctuation of swine wastewater and OTC feeding on AD. However, the combined effect of these two factors has practical significance for the control of AD technology.

Yet the sudden increase of COD and the sudden decrease of HRT (a sharp increase of OLR) are unfavorable to AD technology (Lovato et al., 2012). However, due to the bioaccumulation effect of OTC, the bioreactor without OTC and the bioreactor with different levels of OTC may have different resistance to high OLR shock, which may have two changes: First, the AD systems with OTC show higher resistance to shock, which is beneficial to AD. Secondly, the AD systems with OTC have a weaker ability to resist adverse conditions, which directly leads to damage to AD system performances.

Based on the above assumptions, this study simulated the large fluctuation of COD and HRT in practical engineering applications. (1) By monitoring various indexes of OTC's influence on AD system performances, the dynamic change laws of AD system performances could be grasped. (2) By mastering the activities of lactate dehydrogenase (LDH) and coenzyme F420, the levels of adenosine triphosphate (ATP) and the releases of organic components in three layers of extracellular polymeric substances (EPS), the stress responses of anaerobic microorganisms exposed to OTC were studied at the cellular level. (3) Through metagenome analysis, the physiological state of anaerobic microorganisms at the gene level was provided. These analyses lead to the mechanisms of why and how OTC affects AD performances.

2. Materials and methods

2.1. Synthetic swine wastewater composition and inoculated sludge

Anaerobic sludge was got from a swine farm in Zhuzhou City, Hunan Province, China, where pigs had never been exposed to OTC. After the anaerobic sludge was screened by a 2 mm screen to eliminate other impurities, it was inoculated into a semi-continuous AD reactor with synthetic swine wastewater for domestication for more than one year. The granular sludge after domestication was used as inoculated sludge in this study.

The synthetic swine wastewater was synthesized based on authentic swine wastewater from a swine farm in Northeast China. The initial components of the synthetic swine wastewater were as follows (Wang et al., 2021): $C_6H_{12}O_6$, 2104 mg/L; $C_2H_3NaO_2$, 1403 mg/L; $K_2HPO_4{\cdot}3H_2O$, 78 mg/L; KH_2PO_4, 78 mg/L; NH_4Cl, 551 mg/L; $CaCl_2$, 75 mg/L; $MgSO_4{\cdot}7H_2O$, 23 mg/L; $FeCl_3{\cdot}6H_2O$, 8 mg/L. In addition, 10 ml trace element solution was introduced into the initial components of each liter of synthetic swine wastewater, and the components of trace element solution were as follows (Zhang et al., 2019): $MnSO_4{\cdot}H_2O$, 500 mg/L; $NiCl_2{\cdot}6H_2O$, 40 mg/L; $FeSO_4{\cdot}7H_2O$, 100 mg/L; $CoCl_2{\cdot}6H_2O$, 48 mg/L; H_3BO_3, 10 mg/L; $CuSO_4{\cdot}5H_2O$, 10 mg/L; $Na_2MoO_4{\cdot}2H_2O$, 25 mg/L; $AlK(SO_4)_2{\cdot}12H_2O$, 10 mg/L. The COD concentration of synthetic swine wastewater is about 3300 mg/L. More physicochemical characteristics of inoculated sludge and synthetic swine wastewater are shown in supplementary material.

2.2. Experimental setups and operating conditions of semi-continuous anaerobic reactors

Four AD reactors (serum bottles) were set up, including the control group without OTC (Oxytetracycline hydrochloride, Bomei Biotechnology Co., Ltd., China), and the experimental groups with 0.3 mg/L OTC, 2 mg/L OTC and 8 mg/L OTC respectively. Each AD reactor had a total volume of 500 ml and an effective volume of 220 ml (including 70 ml of inoculated sludge and 150 ml of synthetic swine wastewater). After each inoculation (or water change), each bottle was purged with high-purity nitrogen for 5 (or 2) minutes, then quickly covered with rubber stoppers and sealed with parafilm-PM996 to ensure a completely anaerobic environment of the reactors. The anaerobic reactors were operated at a constant temperature (35 ± 0.5 ℃) and a constant speed (130 ± 1 rpm) in a gas bath shaker under dark conditions. The running cycle of this study was divided into three stages: stage 1 (1–30 days), stage 2 (31–60 days) and stage 3 (61–70 days).

Stage 1: The operating conditions of Stage 1 were consistent with the domestication stage. The main purpose of Stage 1 was to select the anaerobic reactors with small errors as the reactors for this study. The details were as follows: the synthetic swine wastewater and the anaerobic sludge after domestication were added into the reactors in proportion, and the operating conditions were 4.4 days HRT and 0.5 g COD L^{-1} d^{-1} OLR. Stage 2 was entered under the condition of ensuring stable daily methane production, COD concentrations and short chain fatty acids (SCFAs) concentrations.

Stage 2: Adding OTC with corresponding concentrations to synthetic swine wastewater in each experimental group, other operating conditions were the same as those in stage 1.

In stage 3, the HRT was changed to 1.5 days, and the influent COD concentration was adjusted to 1.5 times (about 4950 mg/L) of stage 1 (or stage 2), so that the OLR was altered to 2.3 g COD L^{-1} d^{-1}, and other running conditions were the same as those of stage 2.

2.3. Analytical methods

The daily biogas volume was gauged by the water replacement method. A gas chromatograph (GC112A, China) furnished with a 4 mm × 2 m stainless steel column (with nitrogen as carrier gas at a flow rate of 30 ml/min) and a thermal conductivity detector was applied to analyze the levels of biogas components (including CH_4, H_2 and CO_2). The types and levels of SCFAs (including acetate, propionic, n-butyric, *iso*-butyric, n-valeric and *iso*-valeric) were gauged by a gas chromatograph (Agilent 6890, USA) fitted with a 30 m × 0.3 mm × 0.2 mm Rtx-waxDA column and a flame ionization detector.

The levels of COD, TSS and VSS were gauged in terms of the Standard Methods for the Examination of Water and Wastewater (APHA, 1998). The C/N ratio of AD sludge was analyzed by applying an organic element analyzer (Vario EL cube, Elementar Trading Co., Ltd., Germany). The oxidation–reduction potential (ORP) and pH of AD systems were gauged by a digital pH meter (PHS-3C, Leici Co., Ltd., China) fitted with 501 ORP composite electrode and E-201F pH composite electrode. The levels of total ammonia nitrogen (TAN) were gauged by Nessler's reagent spectrophotometry (Luo et al., 2016). The level of free ammonia (FAN) relative to TAN was gauged according to the calculation formula reported by Jiang et al. (2019), as displayed in Eq. (1).

$$FAN = TAN \times \left(\frac{10^{-pH}}{10^{-\left(0.09018+\frac{2729.92}{T(K)}\right)}} + 1 \right)^{-1} \tag{1}$$

where: FAN is the level of FAN (mg/L), TAN is the level of TAN (mg/L), and T (K) is the temperature (Kelvin).

EPS from sludge, including tightly bound EPS (TB-EPS), loosely bound EPS (LB-EPS) and soluble extracellular polymeric substances (SB-EPS), were extracted by heat-based extraction method on day 70 (Nie et al., 2023). Three dimension excitation-emission fluorescence matrix (3D-EEM, F-4600 FL, Japan) was used to distinguish the distribution of organic matter in three-layer EPS after filtration with 0.45 μm filter membrane. Proteins and polysaccharides concentrations were ascertained in accordance with standard methods on day 70 (Chen et al., 2021; Liu et al., 2021a).

The activity of coenzyme F420 was ascertained by ultraviolet spectrophotometry on day 70 (Cheng et al., 2007). The activity of LDH (Beyotime Biotechnology Co., Ltd., China) and the content of ATP (Shenzhen Zike Biotechnology Co., Ltd., China) was determined by the assay kits on day 70.

2.4. Metagenomic sequencing analyses

10 ml of anaerobic sludge was collected from the AD systems of the control group and the experimental group introduced with 8 mg/L OTC on day 70 for metagenomic analysis. According to the manufacturer's project, DNA was extracted applying the MagAttract® PowerSoil® Pro DNA Kit (IMB, QIAGEN, USA). NanoDrop2000 and TBS-380 were applied to inspect the purity and level of DNA, and 1 percent agarose gel electrophoresis (the voltage is 5 V/cm and the time was 20 min) was applied to check the quality of DNA. Covaris M220 (Gene Company Limited, China) was applied to fragment DNA, and about 400 bp fragment was selected for establishing a paired-end (PE) library. NEXT-FLEX® Rapid DNA-Seq Kit (Bioo Scientific, USA) was applied to establish the PE library. Metagenomic analyses were done on Illumina NovaSeq (Illumina, USA) sequencing platform. Use Prodigal/MetaGene (https://metagene.cb.k.u-tokyo.ac.jp/) for open reading frames (ORFs) forecast of contigs in the assembly outcomes. Genes with ORFs length ≥ 100 bp were designated and translated into amino acid sequences. High-quality reads were arrayed to the non-redundant gene catalogs to count gene abundance with 95 percent identity applying SOAPaligner. Raw sequencing data of metagenomes of sludge samples were deposited in NCBI Sequence Read Archive (SRA) with accession number PRJNA966784.

Diamond (version 0.8.35) was applied to contrast the amino acid sequences of the non-redundant gene catalog with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (the e-value of BLASTP alignment parameter was set to 10^{-5}), so as to obtain the KEGG function mapping to genes. The abundance of mapping functional classes was counted by the aggregate of gene abundance mapping to KO, Pathway, EC and Module.

2.5. Statistical analysis

All experiments were performed in triplicate, and all data were formulated as mean ± standard deviation. Origin 2021 (OriginLab Corporation, USA) was used to draw all graphs of this study. One-way analysis of variance of SPSS statistics 27 (IBM, USA) was applied to compare the significance of the experimental results, and $p < 0.05$ was regarded as statistically significant.

3. Results and discussion

3.1. Performances of semi-continuous anaerobic digestion systems

3.1.1. Changes of COD concentrations in effluents

Fig. 1 a displayed the dynamic changes of COD concentrations in effluents. In stage 1, the effluent COD concentrations of all anaerobic reactors dwindled from 3300 mg/L to 151 ± 31 mg/L, and the removal rates of COD were as high as 95%, which revealed that long-term domestication effectively enhanced the adaptability of anaerobic microorganisms to synthetic swine wastewater, and improved the degradation effect of organic matter and carbon conversion efficiency (Liu et al., 2021a; Wang et al., 2017).

In stage 2, the introduction of 0.3 mg/L OTC, 2 mg/L OTC and 8 mg/L OTC had no impacts on the effluent COD levels of AD systems, and the effluent COD levels of all anaerobic reactors remained at a low state and there were no significant differences. There were two possible reasons for this result: (1) because of the high adaptability of domesticated sludge, the reactors without or with different levels of OTC could reduce the COD concentration to a low level similar to that in the domestication stage within the agreed HRT. However, the rate of reducing COD concentration in each reactor might be different, and the rate from fast to slow or from slow to fast depended on the hormesis effect brought by different OTC concentrations to anaerobic microorganisms. Lovato et al. (2012) pointed out that at the high OLR level of 6 g COD L^{-1} d^{-1}, the organic matter removal rate was 71% at 2 h but reached 84% at 4 h and 6 h, which also indicated that a longer HRT was beneficial to the restoration of AD stability; (2) OTC has bioaccumulation effect in AD systems, and only when the OTC intake dose of semi-continuous anaerobic digesters reaches a certain level will it cause the promotion/inhibition effect of anaerobic microorganisms. He et al. (2021) proved that the environmental behavior of OTC in AD systems was mainly biosorption. Liu et al. (2021a) found similar results, that is,

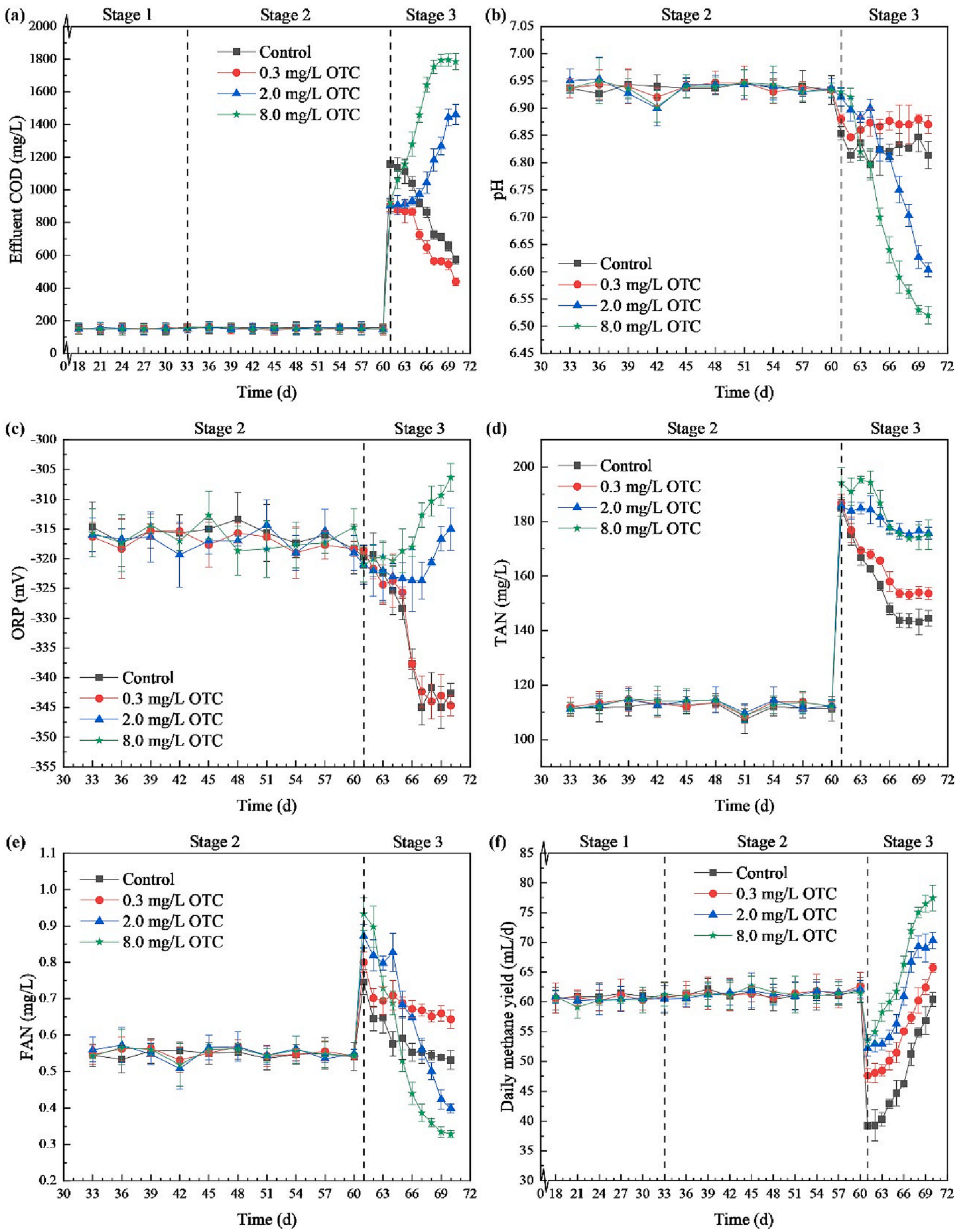

Fig. 1. Dynamic changes of (a) COD concentrations in effluents, (b) pH values, (c) ORP values, (d) TAN concentrations in effluents, (e) FAN concentrations in effluents, and (f) daily CH_4 yield in semi-continuous AD systems with different OTC concentrations.

tetracycline had a bioaccumulation effect in semi-continuous AD reactors, and after 100 days, 2–4 mg/L tetracycline caused the change of effluent COD, which was attributed to the environmental behavior of tetracycline in AD systems.

In stage 3, when the OLR raised sharply to 2.3 g COD L^{-1} d^{-1} due to the alterations of HRT and influent COD concentration, the original balance of the four AD systems was broken, and different COD removal trends appeared, which should be attributed to the different resistance of different reactors to high OLR impact after 30 days of OTC feeding. The effluent COD level of the control group was 1159 ± 20 mg/L on day 61, while the effluent COD levels of the three experimental groups introducing OTC were about 900 mg/L on day 61, which was well below that of the control group, and there existed no prominent differences between the effluent COD levels of the three experimental groups on day 61. This meant that in the first few days of sudden changes in operating conditions, anaerobic reactors fed with OTC were more adaptable to high OLR stimulation. The difference was that the COD concentrations of the control group and the 0.3 mg/L OTC experimental group gradually decreased, and they gradually adapted to the adverse impacts of high OLR shock on AD systems, which should be ascribed to the high adaptability of acclimated sludge (Liu et al., 2021a; Wang et al., 2017). In addition, 0.3 mg/L OTC conspicuously boosted the removal of COD. On day 70, the COD levels (and removal rates) of the control group and 0.3 mg/L OTC experimental group were 572 ± 25 mg/L (88%) and 439 ± 24 mg/L (91%), respectively. On the contrary, the inhibition of COD removal by 2 mg/L OTC and 8 mg/L OTC increased gradually with time, and the higher the concentrations, the stronger the inhibition. On day 70, the COD concentrations (and removal rates) of effluents with 2 mg/L OTC and 8 mg/L OTC were 1461 ± 60 mg/L (71%) and 1784 ± 49 mg/L (64%), respectively. The mechanisms for the effects of OTC on COD removal were presented in sections 3.2, 3.3, and 3.4.

3.1.2. Changes of short chain fatty acids concentrations in effluents

SCFAs are necessary intermediates for methanogenesis, and the core of the high efficiency of the AD methanogenesis process lies in the balance between the production and consumption of SCFAs (Huang et al., 2022). The concentrations of SCFAs in effluents were monitored every three days in the first and second stages (days 18–60). However, the accumulations of SCFAs in all anaerobic reactors were zero, which meant that the acclimated inoculated sludge had strong methanogenic performance, and whether OTC was added or not, it could not prevent SCFAs produced in the acidification stage from being rapidly utilized by the methanogenic bacteria and converted into methane.

As shown in Fig. 2, the concentrations of SCFAs in the effluents were monitored every two days in the third stage (days 61–70). Interestingly, the concentrations of SCFAs were consistent with the trend of COD concentrations in the effluents. 0.3 mg/L OTC prominently facilitated the consumption of SCFAs. On the contrary, SCFAs accumulated gradually in the AD reactors introduced with 2 mg/L OTC and 8 mg/L OTC, particularly in the reactors introduced with 8 mg/L OTC on day 68 (566 ± 27 mg/L). On day 70, SCFAs levels in the reactors introduced with 2 mg/L OTC and 8 mg/L OTC were 406 ± 14 mg/L and 461 ± 25 mg/L, respectively, which were far below the fault thresholds (1000–1500 mg/L) of AD systems (Huang et al., 2022).

Among the types of SCFAs, propionic accumulated the most in each reactor, while other acids were at a lower level. Qiao et al. (2016) found a similar result that propionic accumulated significantly when OLR increased sharply. Besides, Liu et al. (2021a) reported that tetracycline at 4 and 8 mg/L could also cause significant accumulations of propionic. Due to the sensitivity of propionic acid oxidation bacterial to the change of reactor operating conditions and the slow growth of propionic acid oxidation bacterial, the degradation of propionate takes longer than other acids, which explained the accumulation of propionic when HRT was shortened (OLR was increased) (Qiao et al., 2016). Moreover, the standard Gibbs free energy of propionic degradation ($\Delta G^{\circ} = +76$ kJ/mol) is much higher than other SCFAs, which is very unfavorable for methane generation (Qiao et al., 2016). Nevertheless, the depletion of acetate and butyric can be considered as a signal for the recovery of propionate-oxidizing bacteria activity (Huang et al., 2022). In this study, the concentrations of acetate in the control group and the reactors introduced with 0.3 mg/L OTC continued to drop, the levels of acetate in the reactors introduced with 2 mg/L OTC and 8 mg/L OTC remained at a very low state (<30 mg/L), and the butyric levels in the four AD systems fluctuated in the range of 4.4–22 mg/L, so the levels of propionic should

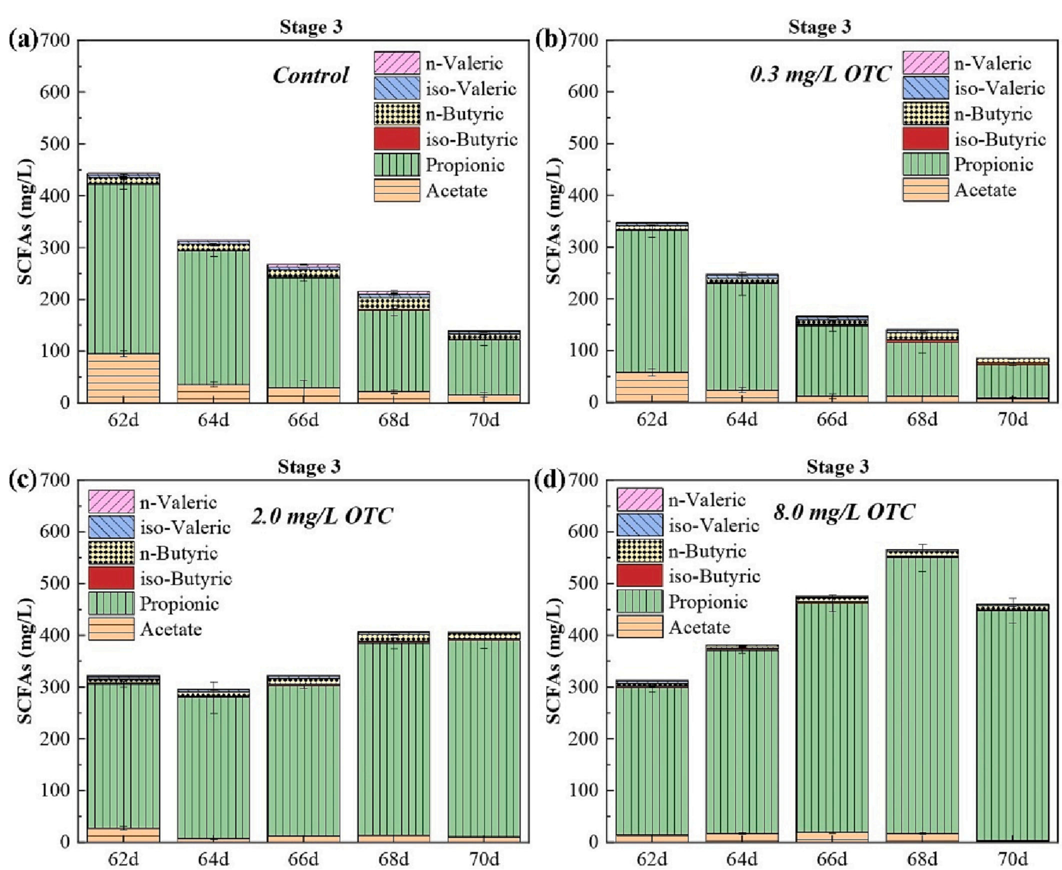

Fig. 2. Dynamic changes of SCFAs concentrations in effluents of semi-continuous AD systems with various OTC concentrations in the third stage. (a) Control, (b) 0.3 mg/L OTC, (c) 2 mg/L OTC, and (d) 8 mg/L OTC.

be dropped. In fact, in the AD system introduced with 8 mg/L OTC, the level of propionic reached the highest state (533 ± 26 mg/L) on day 68 and declined to 445 ± 24 mg/L on day 70, which meant that the accumulations of propionic in the anaerobic reactors fed with OTC should not have substantial impacts on the AD systems.

3.1.3. Changes of pH and oxidation–reduction potential values in anaerobic digestion systems

In order to further clarify the influences of different OTC concentrations on the anaerobic microenvironment, the change rules of pH and ORP values were discussed (Fig. 1 b and c). In the second stage, the pH and ORP values of all reactors were stable at around 6.94 and -317 mV, respectively. When OLR increased sharply in stage 3, the pH and ORP values of each reactor began to change, and the change laws of pH and ORP values were highly consistent with the COD concentrations in the effluents of the corresponding reactors, whether in stage 2 or stage 3.

Once the third stage was started, the pH values of all reactors decreased, which was attributed to the accumulations of SCFAs caused by the impact of high OLR (Qiao et al., 2016; Wang et al., 2017). The disparity was that the pH values of the control group and the reactor introduced with 0.3 mg/L OTC were maintained around 6.82 (control group) and 6.88 (0.3 mg/L OTC) after several days. However, the pH values in the reactors with 2 and 8 mg/L OTC decreased continuously until 6.60 ± 0.01 (2 mg/L OTC) and 6.52 ± 0.02 (8 mg/L OTC) on day 70, which revealed that the high levels of OTC could not borne the accumulations of SCFAs and led to the growth of acidity in AD systems. Nevertheless, the pH values of all reactors within 70 days were within the suitable range of AD (6–8) (Huang et al., 2022), which could not cause the operation obstacles of AD reactors.

For the third stage, the ORP values of the control group and the AD reactor with 0.3 mg/L OTC were almost the same ($p > 0.05$), and they dropped gradually from -317 mV to -344 mV, and then remained stable. On the contrary, high concentrations of OTC (2 and 8 mg/L) significantly increased the ORP values and weakened the reduction environments of anaerobic systems, and the change range of 8 mg/L OTC was greater than that of 2 mg/L OTC. It has been pointed out that the best range of ORP values suitable for methanogenesis in AD is -300 ~ -360 mV (Vongvichiankul et al., 2017). Therefore, from the perspective of ORP values, AD systems were still carried out with high efficiencies.

3.1.4. Changes of total ammonia nitrogen concentrations in effluents

Ammonia is an essential nutrient to maintain microbial life activities, but an inappropriate C/N ratio in swine wastewater can easily cause TAN (i.e., FAN and NH_4^+) concentrations to augment (Jiang et al., 2019). When the concentrations of TAN exceed the inhibition thresholds, the activities of methanogens are inhibited, which leads to the operation obstacles of anaerobic reactors and even the failures of the whole AD. However, at present, the toxicity mechanisms of TAN are still controversial, and there is a lack of experience on inhibition threshold (Jiang et al., 2019). Nevertheless, in stage 2 of this study (Fig. 1 d), long-term acclimated anaerobic sludge did not show any impacts on TAN when stimulated by OTC, and TAN concentrations fluctuated around 110 mg/L in all AD systems. These phenomena should be attributed to the high adaptability of anaerobic sludge after long-term domestication (Jiang et al., 2019; Liu et al., 2021a; Wang et al., 2017).

In stage 3 (Fig. 1 d), TAN concentrations in all reactors increased to 185 ± 3 mg/L ~ 194 ± 6 mg/L on day 61 and then continued to decrease until they reached a stable level. Among them, the decline rate of the control group was the fastest. The decrease of TAN concentrations could be attributed to the increased activities of anaerobic microorganisms, which led to stronger nitrogen assimilation (Huang et al., 2019).

As displayed in Fig. 1 e, the concentration of FAN in the reactor fed with 2/8 mg/L OTC was higher than that in the control group ($p < 0.05$) on day 61, while the concentration of FAN in the reactor fed with 0.3 mg/L OTC was not significantly different from that in the control group ($p > 0.05$) on day 61. In the following days, the concentrations of FAN in the control group and the reactor fed with 0.3 mg/L OTC remained basically stable, and the concentrations of FAN in the reactor fed with 0.3 mg/L OTC were higher than that in the control group. However, the concentration of FAN in the reactors fed with 2 and 8 mg/L OTC continued to decrease until it was lower than that in the control group ($p < 0.05$). It has been reported that in mesophilic anaerobic digesters, FAN is generally considered as the main reason for the inhibition of methanogens by TAN (Jiang et al., 2019). FAN is easily diffused into methanogens cells through cell membranes, while NH_4^+ is difficult to diffuse through cell membranes (Jiang et al., 2019). Nevertheless, a previous study showed that TAN was a more important factor to inhibit the methanogenic activity of AD than FAN (Lay et al., 1998). Therefore, the essence of TAN inhibiting AD is still controversial, and the mechanisms of TAN inhibiting methanogens are still unclear. Happily, TAN concentrations in all reactors were beneficial to the AD at all stages of the semi-continuous operations of the reactors. According to McCarty. (1964), TAN in the range of 50 ~ 200 mg/L is beneficial to AD. Methane yields in section 3.1.5 has also proved this point.

The changes in SCFAs, pH, ORP, and TAN discussed in sections 3.1.2 ~ 3.1.4 reflected the stability of all AD systems (Huang et al., 2022). The results of 70-day semi-continuous experiment showed that all AD systems were in a stable state.

3.1.5. Changes of biogas yields in anaerobic digestion systems

At all stages of the study, the percentages of H_2, CO_2 and CH_4 in biogas did not differ significantly in all reactors ($p > 0.05$) and remained stable at around 0.8% (H_2), 15% (CO_2) and 23% (CH_4). The low methane content and methane generation potential were mainly related to the wastewater components used in this study. Methane content and methane generation potential in carbohydrate-based AD reactors is generally low (Li et al., 2019b).

The dynamic changes of daily methane yields are displayed in Fig. 1 f. The results revealed that the introduction of OTC had no impacts on methane yields in the second stage with constant OLR, and the daily methane yields were stable at about 61 ml/d. However, in the third stage, when OLR increased sharply, the daily methane yields of all reactors changed. Obviously, the addition of low (0.3 mg/L) and high concentrations (2 and 8 mg/L) of OTC promoted methane yields, and with the growth of OTC concentrations, the facilitate process of methane yields was stronger. Nevertheless, Mushtaq et al. (2022) found that OTC above 3 mg/L significantly reduced methane yields in the AD systems based on cow dung. Li et al. (2021) reported that OTC had little effect on methane yields in AD systems based on acidic wastewater. These different results should be attributed to the different substrate properties and operating conditions. In the continuous experiments of Li et al. (2021), it was found that *Methanosaeta* was enriched by adding OTC under acidic conditions, so *Methanosaeta* showed high tolerance to OTC in acidic wastewater in methanogenic archaea community. In the study of Mushtaq et al. (2022), cow dung increased the ratio of SCFAs to total alkalinity under the impact of OTC concentration higher than 3 mg/L, which confirmed the decrease of biogas production. In addition, Mushtaq et al. (2022) adopted batch experiments. Due to the different feeding methods of batch experiment and continuous experiment, the results of batch experiment and continuous experiment are quite different (Huang et al., 2022).

However, as described in sections 3.1.1 and 3.1.2, OTC at a low concentration (0.3 mg/L) promoted the removals of COD and SCFAs. OTC at high concentrations (2 and 8 mg/L) inhibited the removals of COD and SCFAs, and 8 mg/L OTC had the strongest inhibitory impacts on the removals of COD and SCFAs. This is different from most research results. Taking the results reported by Wang et al. (2017) as an example, with the increase in COD removal rates, biogas yields increased and SCFAs accumulations decreased. According to previous studies, COD in influent is mainly converted into biomass, SCFAs and biogas in AD systems (Xiong et al., 2017). According to the principle of energy conservation, reduced COD removal rates and increased SCFAs accumulations and methane yields should reduce substrate utilization of biomass (Cetecioglu et al., 2013). More evidence and detailed analyses of OTC inhibiting COD removals but promoting methane yields will be presented in the following sections.

3.2. Impacts of oxytetracycline on releases of organic components in extracellular polymeric substances of sludge

EPS chelate plenty of organic matter and act as a vital defense system to prevent foreign substances from invading anaerobic microbial cells (Xiang et al., 2023). Proteins (PN) and polysaccharides (PS) compose the principal parts of EPS, accounting for 70%~80% (Liu et al., 2021a; Wang et al., 2017). In addition, EPS also contain a small number of lipids, humus and other polymers (Wang et al., 2017). According to the location of EPS in anaerobic sludge, EPS are clearly divided into three layers (Wang et al., 2017), and each layer of EPS has specific biological functions. TB-EPS are situated in the innermost tier of anaerobic sludge and closely adhered to the cell surfaces, in which the entangled organic matters are difficult to decompose (Zhou et al., 2015). As the basic structure of EPS, TB-EPS determine the stability of anaerobic sludge structures (Wang et al., 2017), and the contents of PN and PS in TB-EPS can reflect whether the stable structure of sludge EPS is destroyed (Wang et al., 2023b). In addition, TB-EPS also act as a vital part to the sedimentation and flocculation abilities of anaerobic sludge (Wang et al., 2017). SB-EPS and LB-EPS have loose structures and rheological properties (Wang et al., 2017; Zhou et al., 2015), in which SB-EPS are more likely to shed into more soluble microbial products and become liquid (Wang et al., 2017). Therefore, if LB-EPS and SB-EPS fall off, additional organic matter will be provided for the substrate to be used by anaerobic microorganisms (Zhou et al., 2015). The content of organic components (including PN, PS and other organic compounds) in three EPS can reflect the migration and transformation of these organic components (Wang et al., 2023b). Studying these three kinds of EPS can help people to deeply understand the response of sludge structure to OTC addition.

3.2.1. Contents of proteins and polysaccharides in three kinds of extracellular polymeric substances

As shown in supplementary material, on the last day of this study (day 70), all concentrations of OTC increased the levels of PN and PS in EPS. The boosted EPS production is considered as the stress response of anaerobic microorganisms to resist the damage of foreign substances (Vongvichiankul et al., 2017). But it may also mean that microbial cells rupture, resulting in the release of a large quantity of organic substances (Maqbool et al., 2019).

As displayed in supplementary material, contrasted with the PN levels in the control group, the PN levels in LB-EPS and TB-EPS boosted with the growth of OTC levels, and the PN levels in SB-EPS had very little difference among the groups. It is reported that the main component of PN in EPS is exoenzymes, which contribute to the electron transfer between methanogens and syntrophic bacteria, thereby boosting methane yield (Li et al., 2019a). In addition, the increase of PN contents in TB-EPS is considered to be the enhancement of the flocculation ability and stability of sludge (Li et al., 2019a; Wang et al., 2017).

Because the PS in EPS have the structure of the complex network and fine chain, the boost of PS secretion (supplementary material) helps microorganisms to block OTC and avoid direct contact between OTC and cells (Li et al., 2019a).

It is reported that the destruction of EPS can improve the speed and degree of organic degradation by anaerobic microorganisms (Zhou et al., 2015). According to the information shown in supplementary material, the reasons why the COD levels in the effluents gradually boosted under the feeding of 2 and 8 mg/L OTC in the third stage might be that SB-EPS and LB-EPS were destroyed under the stimulation of high levels of OTC, and plenty of soluble PS and PN were produced, which provided abundant substrates for SCFAs and methane production.

3.2.2. Releases of other organic components in three kinds of extracellular polymeric substances

3D-EEM is usually used to judge the biodegradability of substrates (Yang et al., 2019). It is worth noting that higher (lower) fluorescence intensity indicates more (less) organic matter content. As displayed in supplementary material, because the fluorescence intensity (FI) of undiluted TB-EPS was too high and exceeded the detection range, the 3D-EEM profiles of TB-EPS diluted ten times were displayed. In contrast to the control group, high levels of OTC (2 and 8 mg/L) dropped the FI of regions I and IV in TB-EPS and SB-EPS but boosted the FI of regions I and IV in LB-EPS. Substances in regions I (tyrosine protein-like substances) and IV (soluble microbial by-product-like substances) are pointed out to be biodegradable, whereas substances in regions II (tryptophan protein-like substances), III (fulvic acid-like substances) and V (humic acid-like substances) are pointed out to be non-biodegradable (Chen et al., 2021; Wu et al., 2022). It could be inferred that high levels of OTC could convert biodegradable substances in TB-EPS into SB-EPS and LB-EPS, and the destruction of SB-EPS (also possibly SB-EPS and LB-EPS) was used to generate more methane and SCFAs. In addition, all concentrations of OTC converted non-biodegradable humic acids in SB-EPS into biodegradable tyrosine-like proteins and microbial by-products, and at the same time, some SB-EPS fell off, which provided abundant substrates for hydrolysis, acidogenesis (acetogenesis) and methanogenesis, and then produced extremely high SCFAs and methane generation potential (Chen et al., 2021; Wang et al., 2017; Zhou et al., 2015).

It was worth noting that the rates of hydrolysis and acidogenesis (acetogenesis) in the AD system introduced with 0.3 mg/L OTC were relatively balanced with those of methanogenesis, which interpreted that 0.3 mg/L OTC boosted the removals of COD and SCFAs and the production of CH_4 as explained in previous chapters. Nevertheless, due to excessive substrates for hydrolysis, acidogenesis (acetogenesis) and methanogenesis in the AD systems fed with 2 and 8 mg/L OTC, the rates of hydrolysis and acidogenesis (acetogenesis) were higher than that of methanogenesis, which led to significant accumulations of COD and SCFAs.

3.3. Impacts of oxytetracycline on lactate dehydrogenase, coenzyme F420 activities and ATP content of sludge

LDH level is an important index to reveal the toxicity mechanism of AD. Only when the cell membrane is seriously damaged, will LDH be released from the cell to the extracellular matrix (Yue et al., 2020). Therefore, LDH level is a marker of cell membrane integrity and cell membrane damage. As shown in supplementary material, LDH levels were measured on the last day (day 70) of this study. The results revealed that there existed no prominent difference in LDH levels between the control group and the experimental group introduced with 0.3 mg/L OTC ($p > 0.05$). However, 2 and 8 mg/L OTC significantly increased LDH activity ($p < 0.05$), which indicated that cells were destroyed after exposure to high concentrations of OTC. Once the cell membrane is destroyed, a large number of PN, PS and other biodegradable organic matter are released and dissolved in the substrates, which provides a large number of substrates for AD.

Coenzyme F420 is an important auxiliary component of most methanogens (Wang et al., 2023a). Therefore, the level of coenzyme F420 can basically reflect the activity of methanogens in AD systems (Dolfing and Mulder, 1985; Wang et al., 2023a). As displayed in supplementary material, with the growth of OTC concentrations, the activity of coenzyme F420 also increased ($p < 0.05$), and all concentrations of OTC increased the activity of methanogens, which also confirmed the results of daily methane yields described in section 3.1.5.

The activity of anaerobic microorganisms is the key to determining the AD performance (Zhang et al., 2016). As a carrier of bioavailable energy, ATP reflects the activity of anaerobic microorganisms and is considered as an indicator of cell metabolic activity (Liu et al., 2021a; Zhang et al., 2016). As displayed in supplementary material, OTC boosted the activity of anaerobic microorganisms from low to high levels, and the higher the level of OTC, the stronger the activity of ATP ($p < 0.05$), which explained the boost in daily methane yields observed in Fig. 1 f.

3.4. Impacts of oxytetracycline on functional traits of anaerobic sludge

3.4.1. Metabolic functions of propionic metabolic pathways

8 mg/L OTC reduced the metabolic function of propionic (ko00640). The absolute abundance of ko00640 in the AD reactor introduced with 8

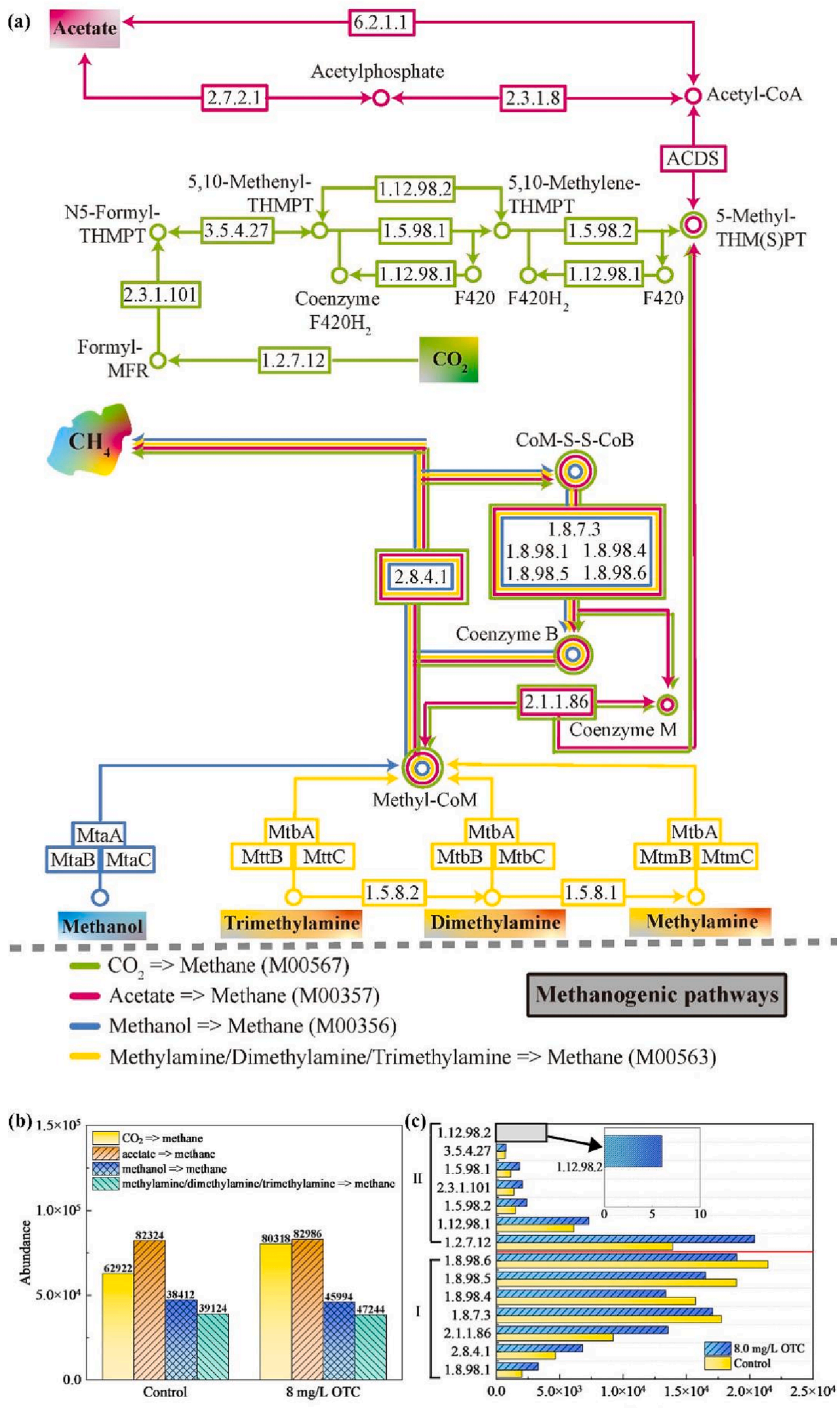

Fig. 3. Different methanogenic pathways based on KEGG (Map: 00680). (a) Metabolic pathways, (b) Gene abundance encoding key enzymes, and (c) Gene abundance encoding key enzymes (further subdivided).

mg/L OTC was 0.6 times that in the control group, which was compatible with the results acquired in section 3.1.2 that 8 mg/L OTC resulted in a large quantity of propionic accumulations.

3.4.2. Metabolic functions of methanogenic pathways

In order to further understand the reasons for the change of the methanogenic performance of anaerobic reactors introduced with OTC, the abundance of genes encoding key enzymes (Fig. 3b) in four methanogenic pathways (Fig. 3 a) was analyzed. As displayed in Fig. 3 b, 8 mg/L OTC boosted the abundance of genes encoding key enzymes in the four methanogenic pathways by 28% (CO_2 => CH_4), 0.8% (acetate => CH_4), 20% (methanol => CH_4) and 21% (methylamine/dimethylamine/trimethylamine => CH_4), respectively, contrasted with the control group, which was compatible with the results acquired in section 3.1.5. Among them, the abundance of genes encoding key enzymes in the carbon dioxide reduction methanogenesis pathway of two AD systems changed the most.

As displayed in Fig. 3 c, the genes encoding key enzymes in the carbon dioxide reduction methanogenesis pathway were categorized. The enzymes included in part I not only have a hand in the carbon dioxide reduction methanogenesis pathway but also are accountable for the other three methanogenesis pathways. Among them, EC: 2.1.1.86 is an enzyme that plays a role in two methanogenic pathways (carbon dioxide reduction and acetotrophic reaction), and is responsible for the synthesis of Coenzyme M, Methyl-CoM and 5-Methyl-THM (S) PT. The other six enzymes play a role in all four methanogenic pathways, among which EC: 2.8.4.1 is the last enzyme in the process of converting the four substrates into methane. The other five enzymes (EC: 1.8.7.3, EC: 1.8.98.6, EC: 1.8.98.5, EC: 1.8.98.4 and EC: 1.8.98.1) are accountable for the reduction of CoM-S-S-CoM to Coenzyme B and Coenzyme M. The gene abundance of these enzymes did not alter regularly, and on the whole, 8 mg/L OTC had almost no impact on the gene abundance of the enzymes encoding in part I.

Part II is the enzymes that only act in the carbon dioxide reduction process, including EC: 3.5.4.27, EC: 2.3.1.101, EC: 1.5.98.1, EC: 1.12.98.2, EC: 1.2.7.12, EC: 1.5.98.2 and EC: 1.12.98.1. Contrasted with the control group, the abundance of genes encoding these enzymes was boosted when introduced with 8 mg/L OTC. It is reported that methanogens usually reduce H_2 in AD systems during carbon dioxide reduction (Li and Zhou, 2020). In section 3.1.5 of this research, it was discovered that the control group and the experimental group introduced with 8 mg/L OTC had the same CO_2 content and low H_2 content, which meant that the AD system fed with 8 mg/L OTC had the potential to produce more CO_2 and H_2, but the produced CO_2 and H_2 could be utilized quickly.

3.4.3. Metabolic functions of information recognitions and material swaps on cell membrane

In the AD system fed with 8 mg/L OTC, the abundance of these genes was 0.9 times (ko02010), 0.8 times (ko02024), 0.9 times (ko00540), 0.9 times (ko02020) and 0.7 times (ko03010) of the control group, respectively. Among them, ABC transporters (ko02010) can transfer nutrients needed by cells from the external environment to the inside of cells, and can also transfer antibiotics, fatty acids and other substances that are not conducive to cell growth out of cells (Jiang et al., 2021). Quorum sensing (ko02024) is a regulatory system which can regulate different group behaviors of AD microorganisms, such as virulence factor production, biofilm formation and antibiotic synthesis (Jiang et al., 2021). Lipopolysaccharide biosynthesis (ko00540) is involved in the synthesis of the outer membrane of gram-negative bacteria, and lipopolysaccharide is an indispensable outer membrane substance for the normal survival of gram-negative bacteria (Bos and Tommassen, 2004). Two-component systems (ko02020) can regulate the formation of biofilm and are signaling pathways to regulate plenty of bacterial characteristics (Zhang et al., 2022). The principal biological function of ribosomes (ko03010) is to convert genetic code into construct protein polymers and amino acid sequences (Zhang et al., 2022). These results revealed that the feeding of 8 mg/L OTC weakened the ability of material swap and information recognition on the cell membrane, which had adverse impacts on the life activities of anaerobic microorganisms.

4. Conclusions

Due to the different resistances of different AD systems fed semi-continuously to high OLR shock, the mechanisms of methane production boosted by low and high levels of OTC were different: (1) 0.3 mg/L OTC mainly improved the biodegradability of SB-EPS; (2) 2 and 8 mg/L OTC mainly destroyed the cell membrane and transformed biodegradable organic substances from TB-EPS into SB-EPS and LB-EPS, which led to the release of numerous biodegradable organic substances into liquid. Generally speaking, 0.3 mg/L OTC was beneficial to AD, while 2 and 8 mg/L OTC could promote methane production at the expense of cell destruction.

CRediT authorship contribution statement

Zhiwei Huang: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Qiuya Niu:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision. **Wenkai Nie:** Investigation, Writing – original draft, Writing – review & editing. **Yan Lin:** Methodology, Writing – original draft, Writing – review & editing. **Shaohua Wu:** Validation, Writing – review & editing. **Xiang Li:** Validation, Writing – original draft, Writing – review & editing. **Jay J. Cheng:** Methodology, Writing – original draft, Writing – review & editing. **Chunping Yang:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

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