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Anaerobic biodegradation of levofloxacin by enriched microbial consortia: Effect of electron acceptors and carbon source

Wenhui Shu [a], Yan Zhang [a,b,*], Donghui Wen [c], Qinyue Wu [a], He Liu [a,b,*], Min-hua Cui [a,b], Bo Fu [a,b], Jie Zhang [a], Ye Yao [a]

[a] Jiangsu Key Laboratory of Anaerobic Biotechnology, School of Environment and Civil Engineering, Jiangnan University, Wuxi 214122, China
[b] Jiangsu Collaborative Innovation Center of Water Treatment Technology and Material, Suzhou 215011, China
[c] College of Environmental Sciences and Engineering, Peking University, Beijing 100871, China

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ABSTRACT

For improving the understanding of anaerobic degradation mechanism of fluoroquinolone antibiotics (FQs), the degradation of a representative FQs, levofloxacin (LEV), by six enriched anaerobic consortia were explored in this study. The effect of sulfate and nitrate as the electron acceptor and glucose as the carbon source on LEV anaerobic degradation were investigated. Addition of glucose and nitrate alone deteriorated LEV removal from 36.5% to 32.7% and 29.1%, respectively. Addition of sulfate slightly improved LEV removal to 39.6%, while simultaneous addition of glucose and sulfate significantly enhanced LEV removal to 53.1%. Twelve biodegradation intermediates were identified, which indicated that cleavage of piperazine ring is prior to that of quinolone ring, and hydroxylation, defluorination, demethylation, and decarboxylation were the primary steps of LEV anaerobic degradation. *Lactobacillus*, unclassified _f_Enterobacteriaceae, and *Bacillus* were enriched by simultaneous addition of glucose and sulfate, with relative abundance of 63.5%, 32.7%, and 3.3%, respectively. The predicted high gene abundance of xenobiotics biodegradation & metabolism, carbohydrate metabolism, and assimilatory sulfate reduction in the consortium, indicated a co-metabolism between carbohydrate metabolism, sulfate metabolism, and LEV degradation under glucose and sulfate added condition. The study revealed that simultaneous addition of glucose and sulfate is the favorable condition for LEV anaerobic degradation.

1. Introduction

Fluoroquinolones (FQs), the third-generation of quinolone antibiotics, are a group of broad-spectrum antibiotics, which can inhibit the activity of two enzymes essential involved in DNA replication and transcription of microbes, and thus are extensively used in human and veterinary medicines (Van Doorslaer et al., 2014). Up to 75% of the used antibiotics are unaltered excreted in feces (Michael et al., 2013; Oberoi et al., 2019), resulting in wastewater treatment plants (WWTPs) as an important sink of FQs. The concentration of FQs in the influent of municipal WWTPs is about 2 ng/L to 8 μg/L (Michael et al., 2013; Hu et al., 2018; Zhang et al., 2017). Notably, FQs concentration in the influent of pharmaceutical WWTPs reached 0.32 mg/L to 7.91 mg/L (Guo et al., 2017; Hussain et al., 2016; Wajahat et al., 2019). For the health of environment, the FQs in wastewater are supposed to be effectively removed with wastewater treatment (Petrie et al., 2015; Luo et al., 2014). However, since WWTPs do not have specific treatment unit for antibiotics removal, the removal of FQs in WWTPs was insufficient. Consequently, the FQs have been detected in high concentrations in WWTP effluents and the effluents receiving environments (Klein et al., 2018; Fadario Frade et al., 2014; Hao et al., 2015; Xu et al., 2015). Long-term accumulation of antibiotics in environments will aggravate the occurrence and spread of antibiotic resistant bacteria and antibiotic resistance genes, which have caused a serious threat to human and animal health (Wess et al., 2020; Felis et al., 2020; Kovalakova et al., 2020). Therefore, it is necessary to strengthen the removal of FQs in wastewater treatment system.

Biological treatment is the core treatment process of WWTPs, and should be the key barrier preventing the spread of antibiotics to aquatic environments (Khan et al., 2020). However, doping of fluorine in molecules not only enhanced the antibacterial activity of quinolone antibiotics, but also made the compounds more recalcitrant to

* Corresponding authors at: Jiangsu Key Laboratory of Anaerobic Biotechnology, School of Environment and Civil Engineering, Jiangnan University, Wuxi 214122, China.
E-mail addresses: yanzhang@jiangnan.edu.cn (Y. Zhang), liuhe@jiangnan.edu.cn (H. Liu).

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biodegradation (Van Doorslaer et al., 2014; Feng et al., 2019; Amorim et al., 2014; Natarajan et al., 2005), thus only 9−22% of FQs were degraded by conventional biological treatment processes (Oberoi et al., 2019; Tiwari et al., 2017). Improving the capacity of biological treatment units for antibiotics degradation is a vital but challenging work for high FQs removal of WWTPs. Anaerobic biological treatment unit is an important unit of biological wastewater treatment process (Oberoi et al., 2019), which has shown the potential to break down recalcitrant compounds into simpler and less toxic products (Mitchell et al., 2013; Liu et al., 2018). How to enhance the degradation of FQs in anaerobic treatment unit is an important issue for improving FQs removal in WWTPs. However, previous studies mainly focused on aerobic degradation of FQs (Feng et al., 2019; Liu et al., 2013; Nguyen et al., 2018; Prieto et al., 2011), the mechanism of FQs anaerobic degradation is still unclear, which impeded the optimizing of anaerobic treatment unit for FQs removal.

Therefore, the general purpose of this study was to investigate the degradation of typical FQs under different anaerobic conditions, and to explore the anaerobic degradation mechanism of FQs. Under anaerobic condition, exogenous electron acceptors could significantly affect the degradation of organic pollutants (Martins et al., 2018). Besides, additional organic substrates as exogenous carbon source may enhance the degradation of refractory pollutants by not only promoting the growth of microbes, but also inducing some non-specific enzymes to participate in the co-metabolism of the refractory pollutants (Fischer and Majewsky, 2014; Dawas-Massalha et al., 2014). Thus, the impacts of exogenous electron acceptors and carbon source on FQs degradation under anaerobic conditions were investigated in this study. Furthermore, ofloxacin (OFL) is a typical fluoroquinolone antibiotic, that has strong antibacterial activity for treatment of serious bacterial infections (Van Doorslaer et al., 2014). With increased application in therapy, OFL has been extensively detected in wastewater, surface water, and even groundwater (Van Doorslaer et al., 2014; Jia et al., 2012; Mohapatra et al., 2016). Levofloxacin (LEV) is the L-body of ofloxacin, but exhibits up to 8–28 times more antibacterial potency against than the racemic ofloxacin (Zhao et al., 2020; Gong et al., 2000; Maia et al., 2018; Fujimoto and Mitsuhashi, 1990). High ecotoxicological effects of LEV on aquatic organisms, such as bacteria, algae, and invertebrates, have also been observed (Xiong et al., 2017; Robinson et al., 2005). Therefore, LEV was selected as a representative of FQs to study the mechanism of anaerobic biodegradation in this study. First, six anaerobic microbial consortia with LEV degradation ability were enriched under different electron acceptor and carbon source conditions. Subsequently, the degradation performance of LEV and other three typical FQs antibiotics, i.e. OFL, ciprofloxacin (CIP), and norfloxacin (NOR), of the six anaerobic microbial consortia were evaluated. Specifically, the intermediates of LEV anaerobic degradation under different electron acceptor and carbon source conditions were identified, and the degradation pathways were proposed. The community characteristics and predicted functions of the six anaerobic microbial consortia were also investigated. The impacts of exogenous electron acceptor and carbon source on LEV anaerobic degradation were revealed for the first time. This study could provide a new insight to the mechanism of FQs anaerobic degradation.

2. Materials and methods

2.1. Six LEV-degradation anaerobic consortia

Microorganisms in the effluent of a Up-flow Anaerobic Sludge Bed (UASB) treating pharmaceutical wastewater was collected by centrifugation and used as the inoculum for enriching LEV-degradation anaerobic consortia. Anaerobic enrichment experiments were conducted using 250 mL serum bottles filled with 150 mL of N_2-purged medium. Inoculation and medium replacement were conducted in an anaerobic glovebox. The serum bottles were sealed with Teflon coated rubber stoppers and plastic caps to ensure anaerobic condition. All the microbial consortia were enriched with mineral salt medium (MSM) as the basic medium, and supplemented with LEV as the selection pressure. The composition of MSM are shown in Text S1. For exploring the effect of exogenous electron acceptors and carbon source on LEV degradation, glucose, nitrate, and sulfate were added into different enrichment groups as shown in Table 1. The enrichment of LEV-degradation consortia was conducted with an initial LEV concentration of 10 mg/L, which was gradually increased to 50 mg/L (as shown in Table S1) achieving a high selection pressure for eliminating antibiotic sensitive microbes. Subsequently, the LEV concentration was gradually decreased back to 10 mg/L, to form stable microbial communities. At the end of each cycle, LEV degradation performance of the enriched consortia was evaluated by detecting residual LEV concentration in the culture medium. Total six LEV-degradation anaerobic consortia were obtained after almost 6 months enrichment, which were (1) consortium M, without glucose and electron acceptor addition, (2) consortium M-G, with glucose addition, but without electron acceptor addition, (3) consortium M-N, with nitrate addition, (4) consortium M-S, with sulfate addition, (5) consortium M-G-N, with glucose and nitrate addition, and (6) consortium M-G-S, with glucose and sulfate addition.

2.2. Anaerobic degradation of LEV and other FQs

LEV biodegradation capability of the six microbial consortia were investigated after 6 months enrichment. The degradation performance of the six consortia were evaluated with the corresponding media as shown in Table 1. Considering that FQs exist in influents of pharmaceutical WWTPs with high concentrations that up to mg/L (Guo et al., 2017; Hussain et al., 2016; Wajahat et al., 2019), initial LEV concentration of 10 mg/L was adopted in this study. The degradation experiments were conducted in 150 mL serum bottles filled with 60 mL culture medium. Enriched consortia were added into the serum bottles achieving initial optical density of the culture at 600 nm (OD_{600}) of approximate 0.15, which could ensure sufficient microorganisms for LEV degradation and suitable biomass density for further growth of the consortia. For distinguishing abiotic degradation and microbial adsorption, abiotic control and inactive cell control were also conducted as shown in Table 2. In inactive cell controls, 0.1% (wt/vol) sodium azide (NaN_3) was added in the bottles to inhibit the activity of the consortia. All experiments were carried out for 10 days at 35 °C in dark. Anaerobic condition in the bottles were achieved by purging the medium with nitrogen gas for 20 min. Each degradation experiment was carried out in triplicate. The OD_{600}, the concentration of LEV as well as glucose, nitrate, and sulfate were measured on day 0, 1, 3, 5, 8, and 10. The metabolic intermediates of LEV anaerobic degradation were analyzed at the end of the degradation experiments. The ability of the six microbial consortia for OFL, CIP, and NOR degradation were also evaluated in corresponding media containing 10 m/L each antibiotic.

2.3. Analytical methods

OD_{600} was measured with a UV-1100 ultraviolet spectrophotometer (MAPADA China). Prior to chemical analysis, biomass was removed by centrifugation at 8000 rpm and 4 °C for 15 min followed by filtration through a 0.22 μm membrane filter. Nitrate and sulfate were analyzed

Table 1
Electron acceptor and carbon source conditions of the six consortia.

Consortium	Glucose	Nitrate	Sulfate
M	–	–	–
M-G	20 mM	–	–
M-N	–	10 mM	–
M-S	–	–	10 mM
M-G-N	20 mM	10 mM	–
M-G-S	20 mM	–	10 mM

Table 2
Experimental design for degradation tests.

Groups	Medium	Bacterial consortia	10 mg/L LEV	0.1% NaN_3
Active cell	+	+	+	−
Inactive cell	+	+	+	+
Abiotic control	+	−	+	−

0.1% NaN_3: inhibitor of microorganisms, for inhibiting the LEV degradation; "+", indicates "presence"; "−", indicates "absence"

using an ion chromatograph (Metrohm 883, Switzerland). For FQs analysis, solid-phase extraction (SPE) was conducted with Oasis HLB cartridges (3 mL/60 mg; Waters, USA). The cartridges were first conditioned with 10 mL methanol, and washed 10 mL ultra-pure water, subsequently loaded with 1 mL sample and again washed with 5 mL of ultra-pure water. Finally, the cartridges were loaded with 10 mL methanol to elute the extracted organic matters. The collected elutes were blown with nitrogen gas to dryness, then re-dissolved with 1 mL methanol. The solutions after filtering through a 0.22 μm filter were loaded into a HPLC (U3000, Thermo Fisher Scientific, USA) equipped with an Extend-C18 column (250 mm, 4.6 mm, 5 μm; Shimadzu, Japan) for FQs measurement. The column temperature was controlled at 30 °C. The four FQs (LEV, OFL, CIP, and NOR) were detected using 0.1% of formic acid and acetonitrile (80:20, vol/vol; pH adjusted to 3 with triethylamine) as the mobile phase with a flow rate of 1.0 mL/min, under detection wavelength of 295 nm, 293 nm, 279 nm, and 281 nm, respectively.

2.4. Identification of degradation products

The intermediates of LEV anaerobic degradation by the six consortia were analyzed by a liquid chromatography-time of flight mass spectrometry (LC-TOF-MS/MS) (Triple TOF X500R, AB SCIEX, MA, USA). Chromatographic separation was achieved using a Kinetex 2.6μ C18 100A column (100 × 2.1 mm; Phenomenex) under flow rate of 0.3 mL/min with a gradient method of Phase A acetonitrile and Phase B Milli-Q water (with 0.1% formic acid). The gradient was as follows: 95% of B within 1 min; decreasing from 95% to 5% of B within 13 min; continuing from 5% to 95% of B within 1 min; constant 95% of B for 3 min. Full scan was used in positive and negative ionization modes to determine any biodegradation products.

2.5. Microbial community analysis of the enriched consortia

The consortia were collected by centrifugation at the end of LEV degradation experiment. DNA was extracted using a PowerSoil®DNA Isolation Kit (MP Biomedicals, USA) following the manufacturer's instruction. DNA quality and quantity were assessed by 1% agarose gel electrophoresis and a NanoDrop spectrophotometer. Amplicons of the 16S rRNA gene was prepared by Polymerase Chain Reaction (PCR) using the forward primer of 515FmodF (5′-GTGYCAGCMGCCGCGGTAA-3′) and the reverse primer of 806RmodR (5′-GGACTACNVGGGTWTCTAAT-3′). The amplification products were purified and sequenced with Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Low quality reads were filtered with QIIME (Caporaso et al., 2010) as described in a previous publication (Zhu et al., 2018). Operational taxonomic units (OTUs) were clustered with a similarity cutoff of 97% by Usearch (Edgar, 2010) based on the remained high quality reads. The taxonomy of the sequences were assigned by comparing against with the SILVA database by Ribosomal Database Project (RDP) Classifier with a confidence threshold of 70%. The raw sequencing data was deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA695419).

2.6. Functional classification prediction

The prediction of functional classification of the consortia was performed by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) based on 16S rRNA gene sequences. The 16S rRNA gene sequencing libraries were first subsampled to achieve an even sequencing depth prior to the PICRUSt analysis. Subsequently, the OTU table was used as the input file for metagenome imputation of each microbial community. The functional genes probably contained in the samples were predicted with PICRUSt, and functional classification of the predicted gene were analyzed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (Kanehisa and Goto, 2000). The predicted functional composition profiles were compared at functional categories (level 1), subcategories (level 2), and individual pathways (level 3).

2.7. Statistical analysis

The differences of LEV removal by the six enriched consortia were analyzed by analysis of variance (ANOVA) with Student-Newman-Keulstest method using SPSS 17.0.

3. Results and discussion

3.1. Biodegradation of LEV by the enriched consortia

LEV degradation performance of the six enriched microbial consortia are shown in Fig. 1. The LEV concentration was decreased by 36.5% in consortium M, 32.7% in consortium M-G, 29.1% in consortium M-N, 39.6% in consortium M-S, 43.2% in consortium M-G-N, and 53.1% in consortium M-G-S. For distinguishing abiotic degradation and microbial adsorption, the decrease of LEV concentration was also evaluated in abiotic and inactive cell controls. In inactive cell controls, the LEV concentration decreased on the day 1, then quickly rebounded, which indicated a sequence process combined by adsorption and desorption. The concentration of LEV at the end of the experiments in inactive cell controls were only slightly lower (1.6−6.0%) than the initial concentration, indicated the tiny effect of adsorption on LEV removal. Meanwhile, there was almost no variation of LEV concentration in abiotic controls. It can be concluded that the decrease of LEV concentration in the six consortia groups was owing to the degradation by enriched microorganisms. Based on ANOVA analysis (Fig. S1), the LEV degradation performance of M-G-S was significantly higher than that of other consortia. In addition, the OD_{600} increased during LEV degradation in all the six consortia groups, indicated that the enriched consortia could grow with degradation of LEV. The high increment of OD_{600} in glucose added groups indicated the growth promotion of carbon source addition.

The consumptions of glucose, nitrate, and sulfate during LEV degradation are shown in Fig. 2. Glucose concentration in the M-G group dropped dramatically, indicated the utilization of glucose by consortium M-G. It was consistent with the higher increment of OD_{600} in M-G than that in M. However, the LEV removal of M-G was slightly lower than that of M, which indicated that degradation enhancement based on co-metabolism was not achieved. Similar phenomenon has been reported, such as Zhang (Zhang et al., 2020) found that the removal of chloramphenicol by enriched microbial consortia with glucose or NaAc addition was lower than that without external carbon source addition. This could be attributed to that some microbes in consortium M-G may have a preference for more bioavailable glucose over the refractory LEV.

In M-N group, nitrate did not decrease during LEV degradation, indicated that consortium M-N almost could not use LEV as the electron donor to reduce nitrate, thus nitrate can hardly be used as an electron acceptor of LEV degradation. It was consistent with the lower LEV decrement in M-N than that in M. However, in the M-G-N, almost all the nitrate was consumed, revealed that glucose addition promoted the reduction of nitrate. Moreover, the LEV removal (43.2%) of the M-G-N

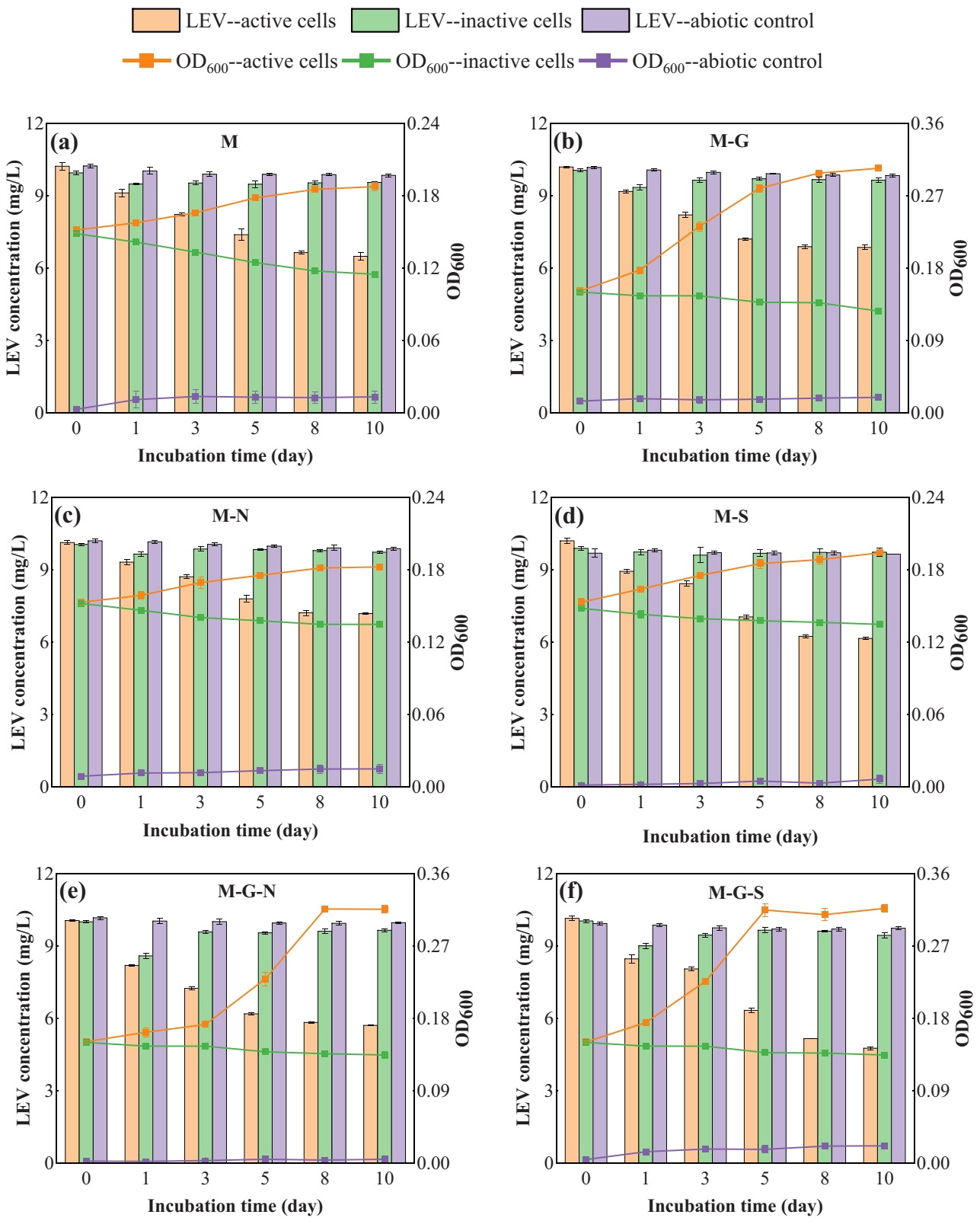

Fig. 1. Degradation of LEV by the six enriched consortia. (a) Consortium M; (b) Consortium M-G; (c) Consortium M-N; (d) Consortium M-S; (e) Consortium M-G-N; (f) Consortium M-G-S. Error bars indicate the standard deviations of triplicate experiments.

group was significantly higher than that of the M-N group (29.1%) and the M-G group (32.7%), indicating that the addition of glucose promoted the degradation of LEV along with the enhanced nitrate reduction.

In M-S group, LEV removal (39.6%) was higher than that of M group (36.5%) with sulfate consumed by 4 mM, indicated that the consortium M-S could use sulfate as an electron acceptor to enhance LEV degradation. Higher LEV removal under sulfate condition than that under nitrate condition was revealed. In previous CIP degradation study (Martins

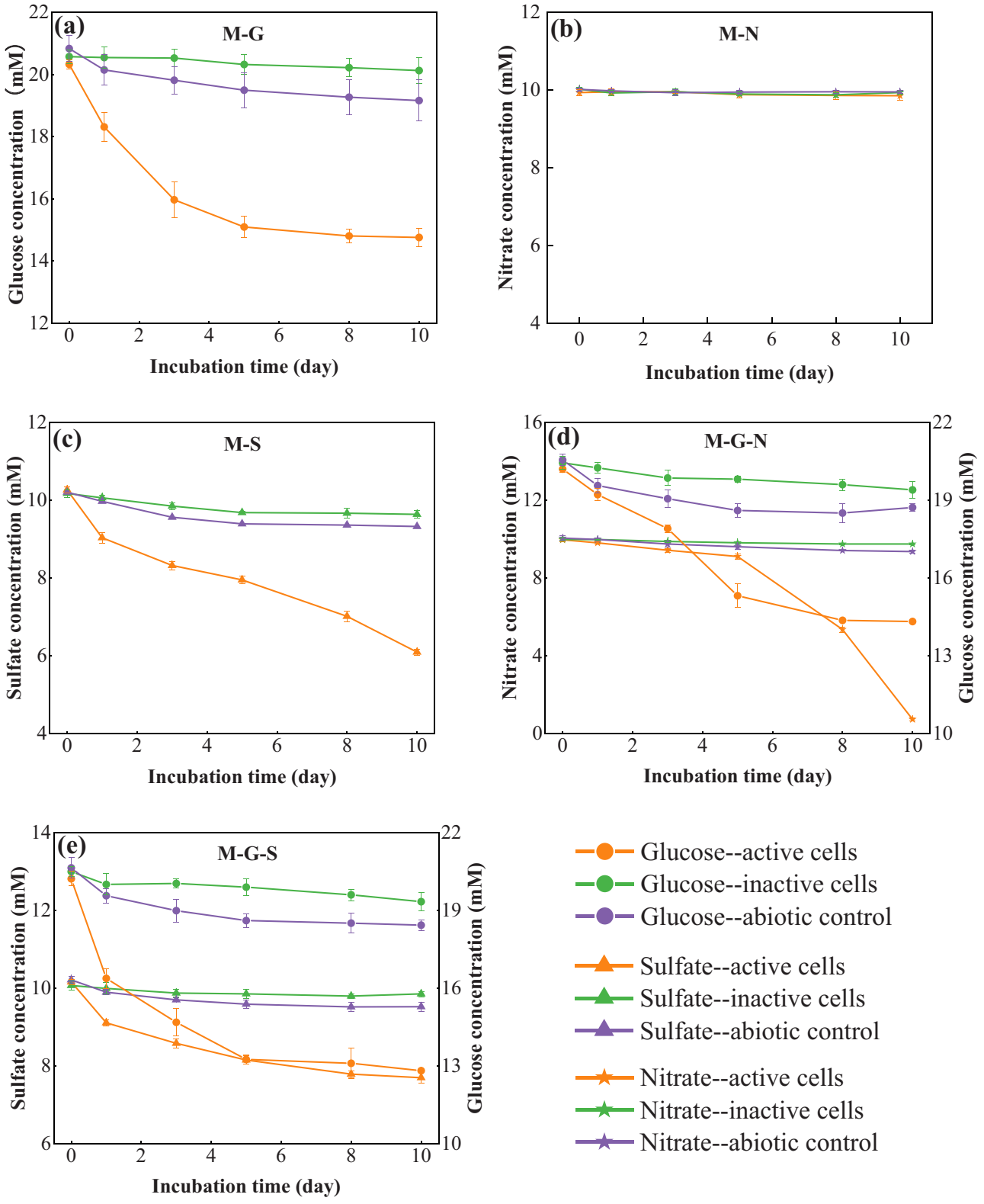

Fig. 2. Consumption of glucose, nitrate, and sulfate during LEV degradation. (a) Consortium M-G; (b) Consortium M-N; (c) Consortium M-S; (d) Consortium M-G-N; (e) Consortium M-G-S. Error bars indicate the standard deviations of triplicate experiments.

et al., 2018), CIP removal under sulfate condition was also higher than that under nitrate condition. Considering the similar molecular structure of LEV and CIP, the consistent results indicated that sulfate rather than nitrate might be a better electron acceptor for FQs anaerobic degradation. M-G-S further increased LEV removal to 53.1%, however, the consumption of sulfate was not increased. It indicated that the promoted degradation of LEV by glucose addition in the case of sulfate as the electron acceptor was not owing to the enhanced sulfate reduction, but might be owing to co-metabolism of glucose and LEV. Nonetheless, highest LEV degradation was achieved in M-G-S, which revealed that

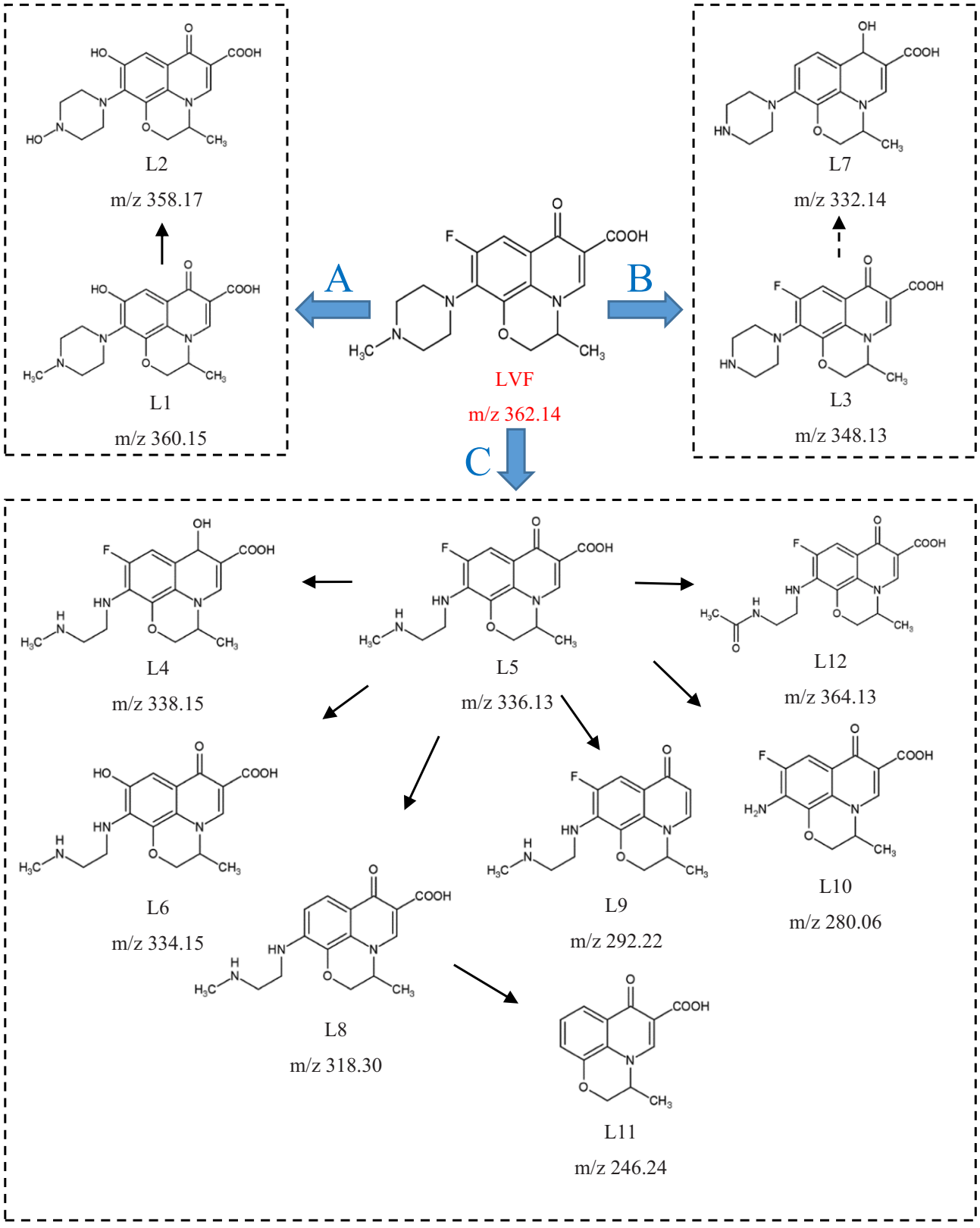

Fig. 3. Biodegradation products and possible pathways of LEV biodegradation in the six enriched consortia.

simultaneous addition of glucose and sulfate is conducive to enhance LEV degradation. Co-metabolism of LEV might be achieved in M-G-S with simultaneous addition of glucose and sulfate.

3.2. Identification of biodegradation products and pathways

The biodegradation intermediates of LEV obtained from all the six consortia were identified with LC-TOF-MS/MS. The protonated molecules $[M+H]^+$ and the proposed structure of the metabolites are shown

in Table S2. Three possible primary biodegradation pathways of LEV were proposed as elucidated in Fig. 3. In pathway A, L1 was generated by replacing fluorine with hydroxyl, and subsequently transformed into L2 via substituting methyl on piperazinyl with hydroxyl. Hydroxylation is an important step for FQs degradation, which could happen at different sites of quinolone ring during CIP and NOR aerobic degradation by bacterial strains (Amorim et al., 2014; Kim et al., 2011), enrichments (Jia et al., 2018; Liao et al., 2016), and fungi (Prieto et al., 2011; Wetzstein et al., 1999). During anaerobic degradation in this study, hydroxylation was found only at fluorine-substituted site on quinolone ring besides at N-methyl site on piperazine ring. In pathway B, L3 was formed by removing methyl from the piperazinyl of LEV, then transformed into L7 via removing fluorine and reducing ketone to hydroxyl. L3, generated by N-demethylation reaction on piperazine ring, has been reported during OFL aerobic degradation by isolated strains of *Labrys portucalensis* F11 (Maia et al., 2018) and ligninolytic fungi (Cvancarova et al., 2015), while L7 was not reported in the corresponding studies. In pathway C, L5 were produced from LEV by piperazine ring opening and shedding a -C_2H_2-. Subsequently, the L5 was transformed into L4, L6, L9 and L10 by reducing ketone to hydroxyl, replacing fluorine with hydroxyl, decarboxylation, and shedding piperazinyl residues, respectively. L8 was also formed via removing fluorine from L5, which was further transformed into L11. In addition, L12 was generated by acetylation on the fragmented piperazine ring, which might be catalyzed by enzymes with acetyl transfer activity, such as glutamine synthetase (Kim et al., 2013) and aminoglycoside acetyltransferase (Robicsek et al., 2006). Among all the metabolic intermediates, L3, L5, and L10 have been reported in LEV aerobic degradation (Amorim et al., 2014; Cvancarova et al., 2015), while other intermediates were detected for the first time. The anaerobic degradation of LEV was mainly happened on piperazine ring, which also was found during aerobic degradation of CIP, NOR, and OFL (Feng et al., 2019; Liao et al., 2016; Adjei et al., 2007; Jung et al., 2009). Therefore, consistent with that of aerobic degradation, it is considered that rupture of piperazine ring is prior to the breakage of quinolone ring during FQs anaerobic degradation.

The metabolic intermediates detected in the six consortia under different carbon sources and electron acceptor were compared, as shown in Fig. S2. For pathway A, L1 was detected in all the six consortia, while, L2 was not detected in consortium M-N and M-G-N, which indicated that nitrate addition might has adverse effect on substitution of a methyl by a hydroxyl. For pathway B, L3 was detected in all the six consortia, however, L7 was not detected in consortium M-G, M-G-N, and M-G-S, which indicated that glucose supplementation might be not conducive to bioconversion from L3 to L7. For pathway C, L4, L5, L6, L8, L9, L10, and L11 were detected in consortium M as well as in M-S and M-G-S, while L9 and L11 were not detected in M-G, M-N, and M-G-N, which indicated that addition of sulfate as the electron acceptor might be beneficial to deep degradation of LEV along with high removal. As adding a fluorine atom in quinolone antibiotics improves the DNA gyrase binding and cell penetration potency (Ling et al., 2018), FQs have a higher antibacterial activity and a broader antibacterial spectrum than the first two generations of quinolone antibiotics. Defluorination will undoubtedly reduce the antibacterial activity of FQs. The first common way of biological defluorination is hydroxylated defluorination (Alexandrino et al., 2017). Hydroxylated defluorination intermediates of LEV, such as L1 and L6, were detected in all the six consortia. The second proposed biological defluorination way is direct defluorination from the parent compound, which represents one of the most effective way to reduce the antibacterial activity of FQs (Liao et al., 2016). However, direct defluorinated intermediates were rarely found in previous study of FQs biological degradation (Gao et al., 2018). Suspected direct defluorination intermediates of LEV, i.e. L7 and L8, were detected in this study, which indicated the good potential of LEV degradation and antibacterial activity reduction of the enriched consortia.

In general, pathways of LEV degradation under different anaerobic conditions were investigated for the first time. Addition of carbon source and electron acceptor did not affect the primary degradation pathways, however, might impact on the followed degradation steps to a certain extent. A series of anaerobic degradation intermediates of LEV were found in this study, which could improve the understanding of the mechanism of LEV anaerobic degradation.

3.3. Degradation of other fluoroquinolones by the enriched consortia

In order to further understand whether the six LEV-degradation consortia able to degrade other fluoroquinolones antibiotics, OFL, CIP, and NOR degradation performance of the consortia were investigated. As shown in Fig. 4, 30.5−59.1% OFL were removed by the six consortia. OFL and LEV are chiral molecules, it is understandable that the consortia could degrade both LEV and OFL. CIP and NOR were also degraded by the six consortia with removal of 29.6−63.9% and 29.9−56.7%, respectively. The ability of the six consortia for diverse FQs degradation might be explained by the similar chemical structures of FQs. From the degradation pathway analysis, it can be found that the main degradation sites of LEV were on the piperazine ring. The degradation of OFL, CIP, and NOR by the enriched consortia mainly attacked the common piperazine ring structure. The OFL, CIP, and NOR degradation performance of the different consortia were consistent with that of LEV degradation, in which the consortium M-G-S showed the highest removal of the three FQs. The removal of CIP was higher than that of OFL and NOR in four consortia including M-G-S, and the degradation of CIP, OFL, and NOR were all slightly higher than that of LEV. The different degradation capacity for the four different FQs might be owing

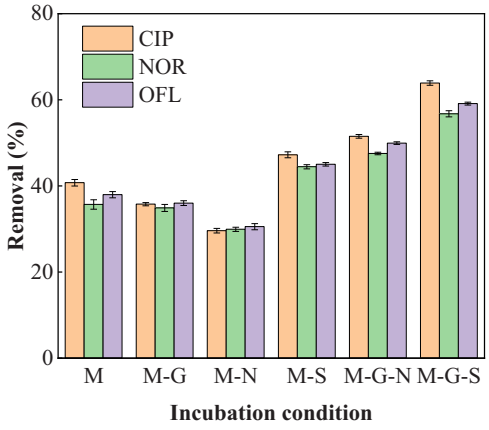

Fig. 4. Degradation of three other fluoroquinolones by six enriched consortia. Error bars indicate the standard deviations of triplicate experiments.

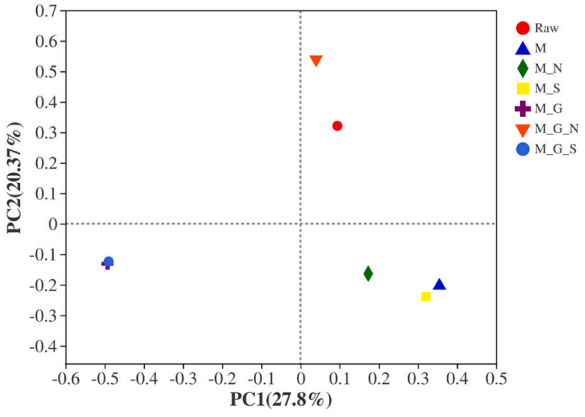

Fig. 5. PCoA analysis of the microbial communities according to Bray-Curtis distances of OTUs.

to the toxicity level of the FQs antibiotics, as the antibacterial efficacy of LEV was higher than OFL, CIP and NOR (Fujimoto and Mitsuhashi, 1990; Jung et al., 2009; Pan et al., 2018; Sulochana et al., 2005; Sato et al., 1986). Considering the piperazine ring is the common structure of FQs antibiotics (Van Doorslaer et al., 2014), the six enriched consortia are supposed to have the universally capacity for most FQs degradation. Studies have found that main detected metabolites have less antimicrobial activity for target bacteria than the original fluoroquinolone antibiotics (Pan et al., 2018; Wetzstein et al., 1997, 2007). For instance, N-acetylation of fluoroquinolone inactivate the antibiotic by making it negatively charged at physiological pH and thus less able to enter a bacterial cell (Jung et al., 2009; Adjei et al., 2006; Parshikov et al., 2001). Therefore, the enriched consortia could be used for bioaugmentation of FQs removal in anaerobic processes and toxicity reducing of the wastewater.

3.4. Microbial community of the six enriched consortia

Microbial communities in the six enriched consortia as well as in the source inoculum (named as Raw) were characterized based on 16S rRNA gene sequencing. The estimation of microbial communities based on Illumina sequencing are shown in Table S3. The inoculum, Raw, showed the highest microbial diversity, with shannon index and chao 1 index of 2.67 and 155.00 respectively, while the six enriched consortia showed lower diversity than the Raw inoculum. LEV degradation microbes were supposed to be enriched under antibiotic selection pressure, thus resulting in the relative low diversity. The diversity of consortium M-G, M-G-N, and M-G-S that added with glucose were much lower than other consortia, which was consistent with previous reported that artificial culture with a single carbon source like glucose, citrate, and leucine would simplify the microbial community (Goldford et al., 2018). The addition of nitrate and sulfate as the electron acceptor also simplified the

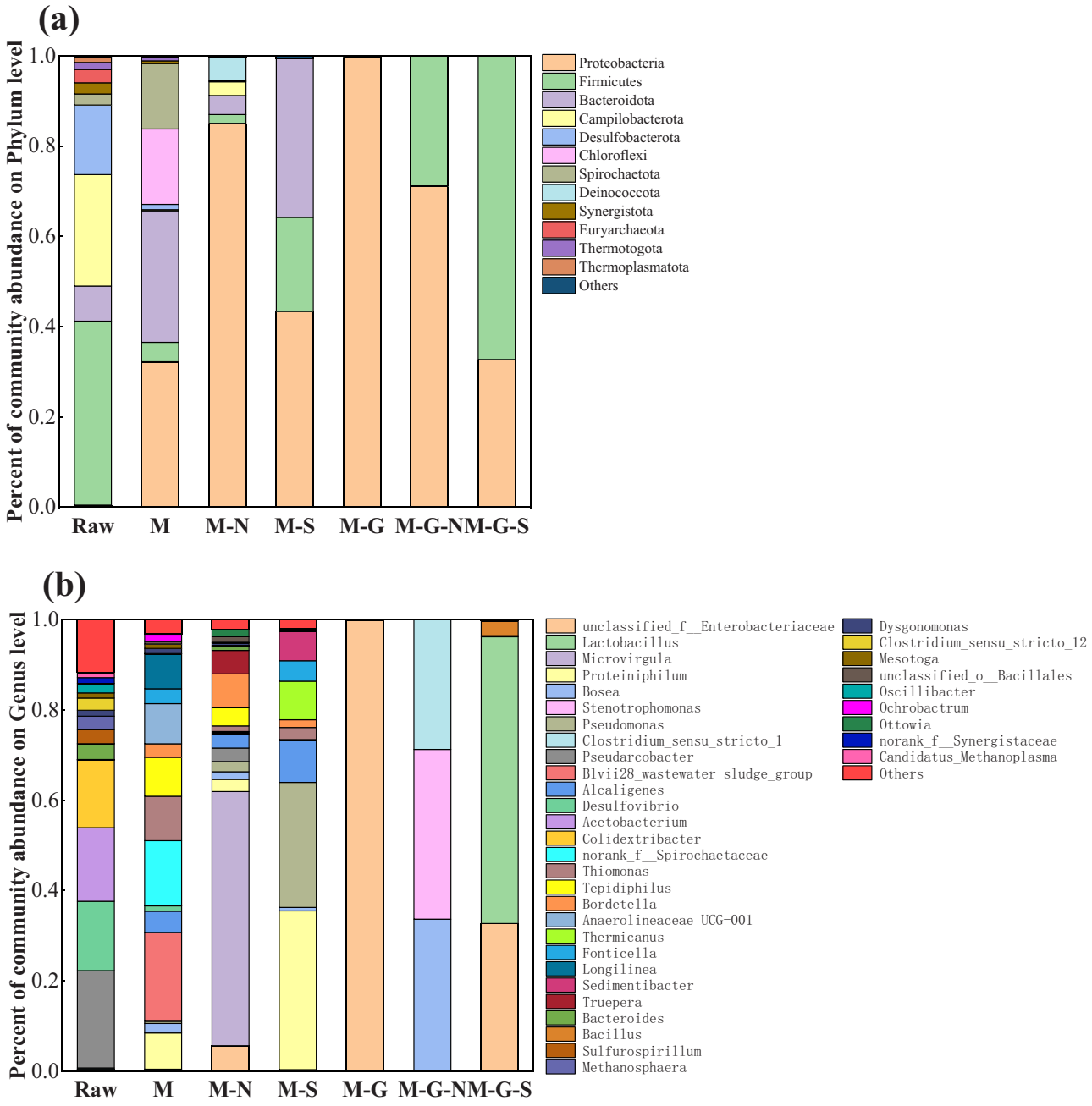

Fig. 6. Microbial community composition of the enriched consortia. (a) Phylum level; (b) Genus level.

microbial community during enrichment, however, the decrease of microbial diversity caused by electron acceptor addition was much lower than that caused by glucose addition. Furthermore, though enrichment decreased the microbial diversity, a part of the detected OTUs were shared by different consortia (Fig. S3). The similarity of microbial communities in the six consortia were further analyzed with PCoA analysis. As shown in Fig. 5, the M was far away from the Raw, indicated the variation of microbial community after enrichment under LEV selection pressure. Two kinds of electron acceptor respectively addition, especially sulfate addition, impacted little on microbial community, while glucose addition significantly shifted the microbial community. Almost the same location of M-G and M-G-S further indicated the relatively low impact of sulfate on microbial community, even glucose was added. In general, glucose has a greater impact on microbial community than the two kinds of electron acceptor.

The composition of microbial communities at phylum and genus level are shown in Fig. 6. Although the consortia were enriched from the same inoculum, different enriching conditions resulted in different microbial composition of the six LEV degradation consortia. At phylum level, the predominant bacteria of the Raw inoculum were Firmicutes (40.7%), Campilobacterota (24.7%), Desulfobacterota (14.4%) and Bacteroidota (7.7%). After a long enrichment, Proteobacteria became the main phylum in the consortia with relative abundance of 32.2−99.8%. Firmicutes was another main phylum in consortium M-S, M-G-N, and M-G-S, and was the most abundant one (67.2%) in M-G-S. Bacteroidota was detected in relative high abundance of 29.1% and 7.8% in consortium M and M-S, respectively. Consistent with high shannon index of consortium M, more phyla, i.e. Spirochaetota and Chloroflexi were detected in relative high proportion.

At genus level, the composition of the main genera in the enriched consortia were different from each other, and distinct from the Raw inoculum. After enrichment with LEV as the selective pressure, the consortium M still showed high diversity with Blvii28_wastewater-sludge_group (19.5%), norank_f_Spirochaetaceae (14.4%), *Thiomonas* (9.9%), and Anaerolineaceae_UCG-001 (8.9%) as the main genera. M-N and M-S also were found having a few genera, with *Microvirgula* (56.3%), and *Proteiniphilum* (35.1%) as the dominant ones, respectively. However, with decreased diversity caused by glucose addition, only several genera were detected in M-G, M-G-N, and M-G-S. The consortium M-G-S, which has the highest LEV degradation performance (removal of 53.1%), was composed of *Lactobacillus* (63.5%), unclassified _f_Enterobacteriaceae (32.7%), and *Bacillus* (3.3%). The consortium M-G-N, which also achieved good LEV removal (43.2%), consisted of *Stenotrophomonas* (37.6%), *Bosea* (33.5%) and *Clostridium* (28.7%). While the consortium M-G was mainly unclassified _f_Enterobacteriaceae (99.7%). Enterobacteriaceae is a group of facultative anaerobes with strong carbohydrate metabolism ability (Durica-Mitic et al., 2018). In addition, certain species belongs to Enterobacteriaceae has been reported as a FQs degrader (Liyanage and Manage, 2018). Combined carbohydrate metabolism and FQs degradation abilities resulted in the predominance of Enterobacteriaceae in the consortium M-G. The dominant genus in consortium M-G-S, *Lactobacillus*, is a homofermenter

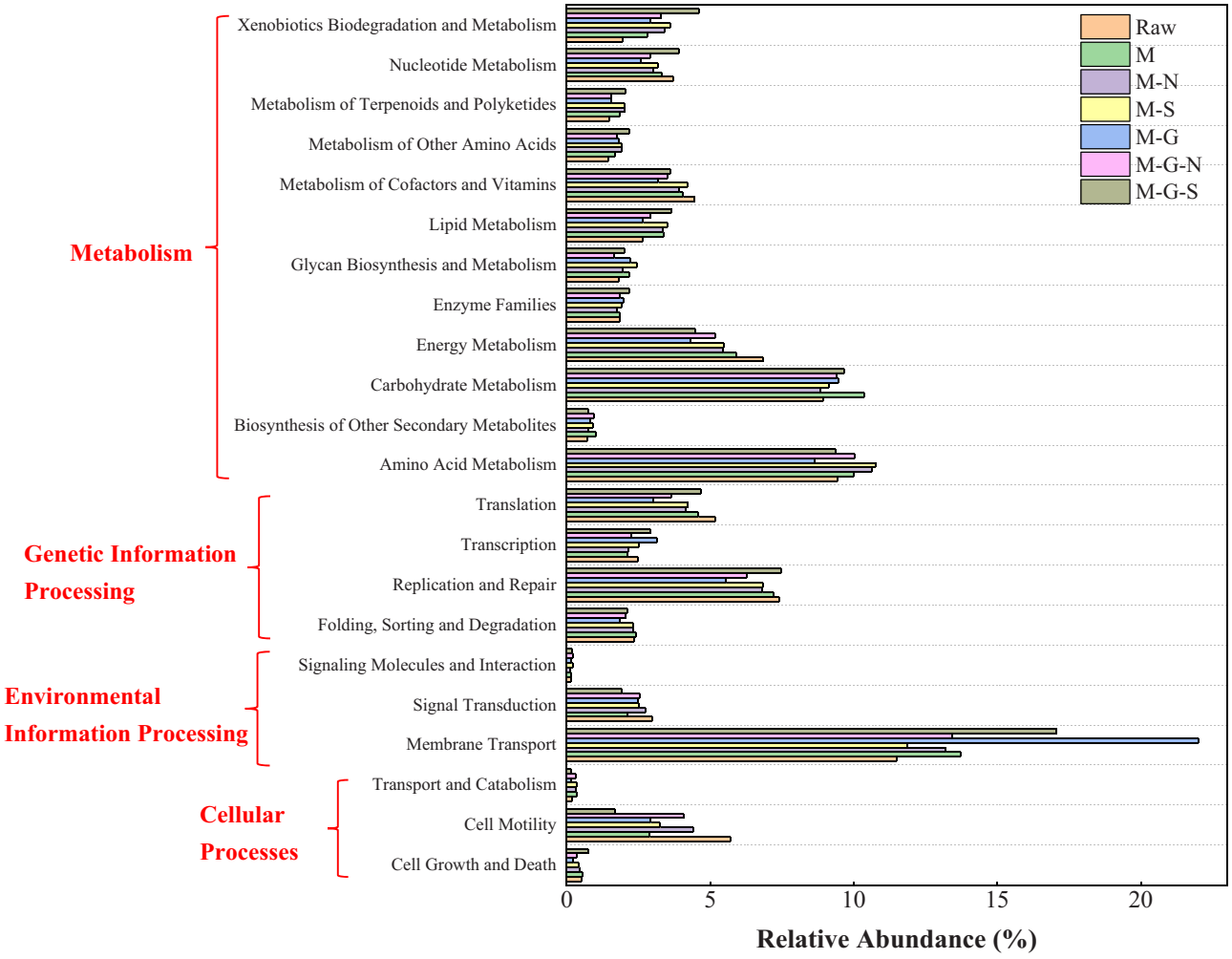

Fig. 7. Relative abundance of KEGG-assigned functional categories in the samples. The percentage of matched gene numbers was assigned to specific KEGG functional categories.

that ferments sugars releasing by-product of lactic acid. Owing to the similar metabolic function with *Lactococcus*, *Lactobacillus* could contribute to provide nutrients and growth factors to other LEV-degraders in the consortium, as the considered role of *Lactococcus* in CIP degradation enrichment (Feng et al., 2019). It could be reasonably inferred that *Lactobacillus* in consortium M-G-S may play a co-metabolism interaction with Enterobacteriaceae. In addition, *Lactobacillus* has been previously described as multi-drug resistant bacteria (Campedelli et al., 2019), in which the *Lactobacillus gasseri* isolated from hospital effluent showed high CIP degradation ability (Liyanage and Manage, 2018). Therefore, *Lactobacillus* in M-G-S may also participate in LEV degradation. Considering *Lactobacillus* was the main different between the microbial community of M-G and M-G-S, the *Lactobacillus* might be the key role for the high LEV ability of M-G-S. In addition, *Bacillus*, with relative abundance of 3.3%, may also play an important role for LEV degradation in M-G-S, as a lot of *Bacillus* strains, such as *B. pumilus*, *B. licheniformis*, and *B. amyloliquefaciens*, can produce laccases (Loncar et al., 2013; Lu et al., 2013; Reiss et al., 2011). While laccases, a group of oxidoreductive enzymes, are able to catalyze the degradation of many organic pollutants including some FQs (Prieto et al., 2011; Pan et al., 2018; Ding et al., 2016; Yang et al., 2017). The dominant genus in M-G-N, *Stenotrophomonas*, is also a group of microorganisms that could degrade organic pollutants (Liao et al., 2016; Leng et al., 2016), such as pesticide (Shen et al., 2010), insecticide (Tang et al., 2012), monoaromatic hydrocarbons (Zhao et al., 2009), polycyclic aromatic hydrocarbons (Lee et al., 2002), tetracycline (Leng et al., 2016) and steroid hormones (Juhasz et al., 2002). It is reasonable that *Stenotrophomonas* may have the ability to degrade LEV and contribute to LEV degradation in M-G-N. The results revealed that electron acceptors and glucose addition shifted the microbial community in enriched consortia, which resulted in the different LEV degradation capacity.

3.5. Predicted functions of the six enriched consortia

The function profile of the six enriched consortia were analyzed with PICRUSt by compared with the KEGG database. The majority of predicted functional genes in the enriched consortia were assigned into Metabolism (42.16−48.87%), Environmental Information Processing (14.59–24.64%), Genetic Information Processing (13.54−17.43%), Cellular Processes (2.18−6.41%), Human Diseases (0.68−1.34%), and Organismal Systems (0.38−0.85%), with 13.83−15.24% predicted genes assigned in unclassified pathways (Fig. S4). The relative abundance of predicted genes assigned in Metabolism, Environmental Information Processing, Genetic Information Processing, and Cellular Processes showed certain differences between the six consortia.

At subcategory level (shown in Fig. 7), relatively significant differences between the six enriched consortia were found in cell motility, membrane transport, amino acid metabolism, energy metabolism, and xenobiotics biodegradation & metabolism. Consortia enriching reduced the motility of the microorganisms, in which sulfate and glucose addition decreased the abundance of predicted genes in cell motility more than that of nitrate addition. The predicted genes in membrane transport and amino acid metabolism in most of the consortia were higher than that of the inoculum Raw, however, anaerobic accumulation decreased the abundance of predicted genes in energy metabolism. Xenobiotics biodegradation & metabolism is the subcategory of predicted functional genes that directly related to LEV degradation. As shown in Fig. 7, the abundance of xenobiotics biodegradation & metabolism in the six enriched consortia were all higher than that in the inoculum Raw, indicated that acclimation under LEV pressure enhanced the capacity of xenobiotics biodegradation. Thereinto, M-G-S showed the highest abundance in xenobiotics biodegradation & metabolism, with M-S followed. The abundance in the six consortia was consistent with their LEV degradation capacity.

Twenty individual pathways in xenobiotics biodegradation & metabolism were further compared as shown in Fig. 8. The six enriched consortia were significantly higher than that of the inoculum Raw both in pathway types and abundances of xenobiotics biodegradation & metabolism. The abundances of benzoate degradation, aminobenzoate degradation, chloroalkane and chloroalkene degradation, and naphthalene degradation were significantly improved in the six consortia compared to that in the inoculum Raw. As LEV is a benzene and nitrogen-heterocyclic compound, the functional genes of the above xenobiotics degradation pathways could play a role in LEV degradation. In

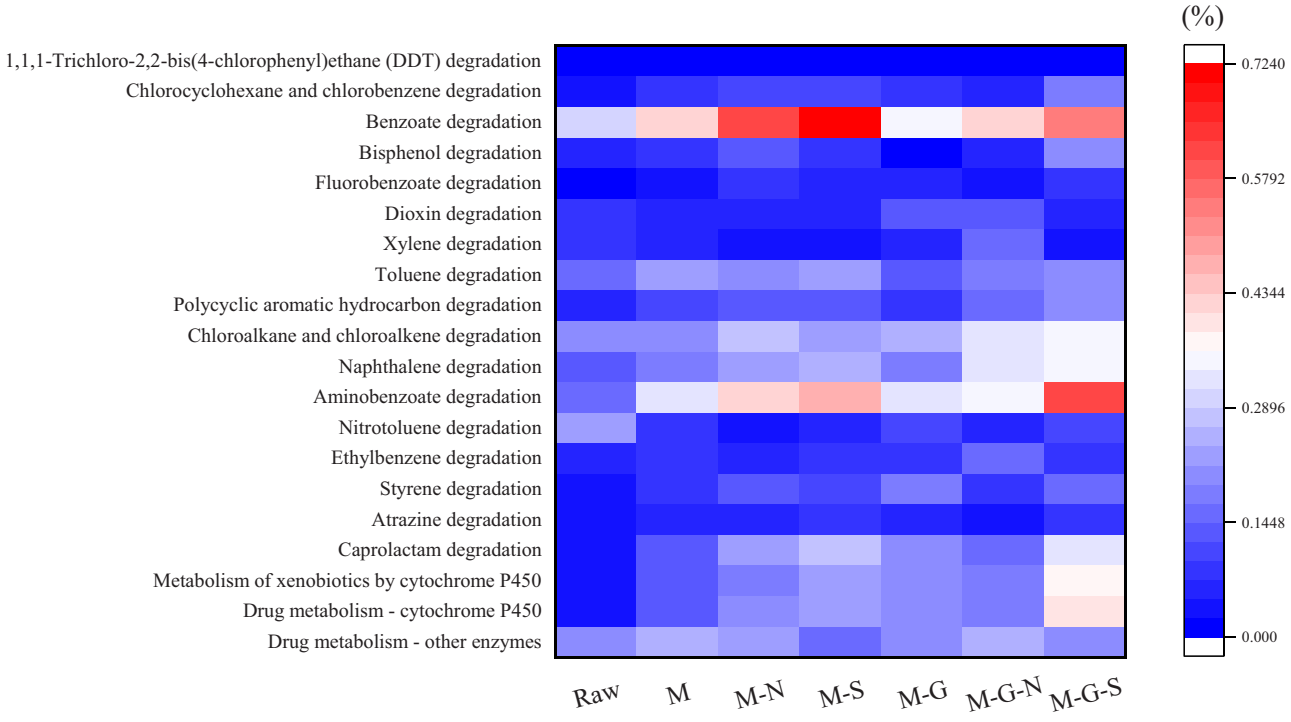

Fig. 8. Relative abundance of various predictive functions assigned in xenobiotics degradation and metabolism.

addition, cytochrome P450, which was not showed in the inoculum Raw, was revealed in all of the six consortia, with the highest abundance found in M-G-S. It has been reported that CIP and NOR could be degraded via demethylation and hydroxylation of piperazine ring under the catalysis of cytochrome P450 (Prieto et al., 2011; Jia et al., 2018; Gao et al., 2018). The high predicted functional gene abundance of cytochrome P450 and other xenobiotics degradation pathways in M-G-S provided the reason for the best LEV degradation performance of M-G-S among the six enriched consortia. The results also further confirmed that simultaneous addition of glucose and sulfate is conducive to enhance LEV degradation capacity of the microbial consortia. Besides the improved abundances in xenobiotics biodegradation & metabolism, the predicted abundances of genes related to carbohydrate metabolism, such as glycolysis/gluconeogenesis, pentose phosphate pathway, and pentose & glucuronate interconversions, in M-G-S were also higher than that of M-S and the inoculum Raw (Fig. S5). The relative abundance of the genes for assimilatory sulfate reduction in M-G-S were also significantly higher than other consortia. It indicated that the addition of glucose and sulfate may have formed a co-metabolism effect between carbohydrate metabolism, assimilatory sulfate reduction, and LEV degradation.

4. Conclusions

This study investigated the anaerobic degradation performance and pathways of LEV, especially the effect of electron acceptors and carbon source. LEV anaerobic degradation performance was not improved with addition of glucose or nitrate alone, and only slightly improved by adding sulfate as the electron acceptor, but significantly enhanced with simultaneous addition of glucose and sulfate. Cleavage of piperazine ring is prior to that of quinolone ring during LEV anaerobic degradation. Hydroxylation, defluorination, demethylation, and decarboxylation are the main primary steps of LEV anaerobic degradation. *Lactobacillus*, unclassified _f_Enterobacteriaceae, and *Bacillus* were enriched with simultaneous glucose and sulfate addition. The predicted high gene abundance of xenobiotics biodegradation & metabolism, carbohydrate metabolism, and assimilatory sulfate reduction in the consortium M-G-S, indicated a co-metabolism between carbohydrate metabolism, sulfate metabolism, and LEV degradation under glucose and sulfate added condition. Simultaneous addition of glucose and sulfate is the favorable condition for enhanced degradation of LEV. This study provided a new insight to the mechanism of LEV anaerobic degradation.

CRediT authorship contribution statement

Wenhui Shu: Investigation, Methodology, Data curation, Formal analysis, Writing - original draft. **Yan Zhang**: Conceptualization, Writing - review & editing, Data curation, Formal analysis, Funding acquisition. **Donghui Wen**: Funding acquisition, Conceptualization. **Qinyue Wu**: Investigation, Methodology. **He Liu**: Project administration, Methodology, Supervision. **Min-hua Cui**: Methodology, Data curation. **Bo Fu**: Supervision. **Jie Zhang**: Methodology. **Ye Yao**: Methodology.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125520.

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