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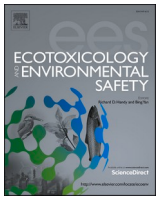

The beneficial or detrimental fluoride to gut microbiota depends on its dosages

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ABSTRACT

Fluoride, widely presented in drinking water and tea, may be detrimental or beneficial to the human health, depending on its dosages ingested. However, the relationship of different dosages of fluoride and gut microbiota is still unclear. In this work, the fermentation model using fecal samples provided by four volunteers was used to evaluate the effects of different dosages of fluoride (1, 2, 10 and 15 mg/L) on the gut microbiota in vitro. The result showed low dosages of fluoride (1 and 2 mg/L) had limited effect on the structure and functional Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of gut microbiota. Furthermore, the low dosage of fluoride could promote the growth of beneficial gut microbiota, including *Faecalibacterium* and *Lactobacillus*. Whereas, the high dosage of fluoride (10 and 15 mg/L) significantly changed the composition and functional KEGG pathway of gut microbiota. Moreover, the high dosage of fluoride could also reduce the beneficial gut microbiota, including *Faecalibacterium* and *Phascolarctobacterium*, and increase the harmful bacterium including Proteobacteria and Enterobacteriaceae. Both low and high dosages of fluoride showed limited effect on the productions of short-chain fatty acids (SCFAs). Thus, the beneficial or detrimental fluoride to gut microbiota depends on its dosages. The fluoride is expected to serve as a food additive in suitable dosage to improve human health through modulation of the gut microbiota. Moreover, more attention should be paid to toxicity of fluoride with high dosage to gut microbiota.

1. Introduction

Fluoride, one of important trace elements in the human body, could prevent dental cavities and promote the formation of strong bones with suitable dosage (Jagtap et al., 2012; Ferreira et al., 2021), whereas, intake of excessive fluoride over a long period of time may induce a serious health problem, such as damaging teeth and bones, disrupting the normal metabolism of phosphorus and calcium (Cai et al., 2015; X.C. Wang et al., 2019). A growing amount of evidence suggests that too much fluoride intake shows significantly destructive effects on the structure, metabolism, and function of testicles, liver, lung and kidney (Liang et al., 2020). Furthermore, fluoride may be related to the reduced intelligence quotient (IQ) in children and the increased risk of skeletal cancer (Fallahzadeh et al., 2018). The daily intake of fluoride from drinking water is the principal source for humans, thus, an acceptable fluoride concentration between 0.5 and 1.5 mg/L in drinking water has been presented by World Health Organization (WHO). Moreover, the brick tea containing high level of fluoride may increase the risk of fluorosis, which has become a public health challenge in the border regions of China (Peng et al., 2020; Xi et al., 2019).

Trillions of gut microbiota, a complex ecosystem of microorganisms, inhabit the human large intestine, which contributes to homeostatic regulation of the gastrointestinal (GI) tract (Oliphant and Allen-Vercoe, 2019). As a result, the gut microbiota plays a critical role in human health, such as eliminating exogenous toxins, metabolizing bile salts, biosynthesizing vitamins, providing a source of energy biogenesis, and protecting against pathogen overgrowth (Lynch and Pedersen, 2016). However, a growing number of studies highlight the fact that the dysbiosis of gut microbiota could lead to a series of diseases, such as obesity, inflammatory bowel disease, and diabetes (Makki et al., 2018; Peng

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et al., 2019). In recent decades, lots of food derived contaminants could induce the dysbiosis of gut microbiota, and thereby lead to detrimental effects on human health (Tsiaoussis et al., 2019; Zhai et al., 2017). Recently and more strikingly, the effect of fluoride on the gut microbiota was investigated. The result showed the excessive fluoride (50 and 100 mg/L) significantly altered the composition of gut microbiota in mice (L. Liu et al., 2019; J. Liu et al., 2019). Likewise, another similar work reported that fluoride (100 mg/L NaF for 60 days) reconstituted the composition of gut microbiota which may contribute to the intestinal dysfunction in mice (Fu et al., 2019). Miao et al., found the cecal microbiota of laying hens could be significantly changed after fluoride intervention at a level of 1237 mg/kg in diet (Miao et al., 2020a, 2020b). On the other hand, the fluoride with dosage of 4 mg/L or 4 mg/L with an additional dose of 2.25 μg of fluoride per day, could deplete acidogenic taxa in oral, but show limited effect on microbial community structure and function in mice (Yasuda et al., 2017). The different result of above reports may be due to the different dosages of fluoride used in the experiments. Nevertheless, there was limited information about the effects of different dosages of fluoride on the gut microbiota. Thus, the effects of different dosages of fluoride (1, 2, 10 and 15 mg/L) on the gut microbiota were investigated in vitro by fermentation model in this work. It is expected that this work could enhance our understanding of beneficial or detrimental fluoride to gut microbiota.

2. Materials and methods

2.1. Fermentation in vitro

The fermentation in vitro was carried out to investigate the effects of fluorides on gut microbiota according the previous work with some modifications (Chen et al., 2018c). Four healthy volunteers aged 20–30 years old who did not take any antibiotics, laxatives, probiotics or prebiotics, and had no gastrointestinal disorder for at least 3 months provided the fecal samples. The equal amount of fresh fecal samples from each volunteer were immediately mixed, and then homogenized by mixing with 9-fold of autoclaved modified physiological saline solution (cysteine-HCl 0.5 g/L, NaCl 8.5 g/L). The suspension was centrifuged at 500 g for 5 min at 4 °C to remove food residues obtaining 10% (w/v) fecal slurry. The basal nutrient growth medium contained yeast extract (2.0 g/L), peptone (2.0 g/L), KH_2PO_4 (0.04 g/L), $NaHCO_3$ (2.0 g/L), K_2HPO_4 (0.04 g/L), $CaCl_2$ (0.01 g/L), NaCl (0.1 g/L), $MgSO_4$ (0.01 g/L), Tween 80 (2.0 mL/L), bile salts (0.5 g/L), cysteine-HCl (0.5 g/L), vitamin K_1 (10 μL/L), resazurin (1.0 mg/L) and hemin (0.02 g/L), prepared in distilled water. The fermentation systems, containing different concentrations of fluoride (0, 1, 2, 10 and 15 mg/L), in vitro consisted of 18.0 mL of basal nutrient growth medium and 2.0 mL of the fecal slurry, which were named as C, F1, F2, F10 and F15 groups, respectively. The fermentation experiment was carried out at 37 °C for 24 h in an Anaero Pack System (Mitsubishi Gas Chemical Co., Inc., Tokyo of Japan). The fermentation samples were collected and stored in −80 °C for further study. The WHO guideline of fluoride in drinking water is no more than 1.5 mg/L (Bhatnagar et al., 2011). Thus, the low dosages of fluoride in this work were 1 and 2 mg/L. Furthermore, the excessive fluoride in drinking water with more than 10 mg/mL will lead to skeletal fluorosis (Guth et al., 2020), thus, the high dosages of fluoride in this work were set as 10 and 15 mg/L.

2.2. DNA extraction and high throughput sequencing

The total DNA of gut microbiota was extracted by a commercially available kit (TIANGEN Biotech, Beijing, China) according to the protocol of manufacturer. The 16S rRNA gene pyrosequencing was carried out by Center for Genetic & Genomic Analysis, personalbio. (Shanghai, China) using Illumina Miseq. The bacterial 16S rDNA hypervariable region 4 (V4) was amplified by high-fidelity polymerase chain reaction (PCR) using the primers 515F [F stands for forward] GTGCCAGCMGCCGCGGTAA and 907R [R stands for reverse] CCGTCAATTCMTTTRAGTTT, and specific sequencing labels were added to the library. After being quantified, mixed, and quality checked, the sequencing was carried out with the 2 × 300 bp paired-end method on the Illumina Miseq to obtain raw data. The raw reads were filtered and merged by several steps to obtain clean data, and then clustered into operational taxonomic units (OTUs) with the similarity of 97% by UCLUST. The taxonomical assignments were performed by Mothur at the 80% confidence level based on the Ribosomal Database Project (RDP) database. α-diversities was evaluated by Mothur. β-diversities was evaluated by R packages (V3.6.3, http://www.r-project.org/). Furthermore, the OTUs with 50 most relative abundance of gut microbiota were analyzed by linear discriminant analysis (LDA) effect size (LEfSe). The effect of fluoride on the functional profiles of microbial communities based on Kyoto Encyclopedia of Genes and Genomes (KEGG) were predicted by phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt).

2.3. Determination of pH and short-chain fatty acids (SCFAs)

A pH meter (FE20, Mettler-Toledo instruments Ltd., Shanghai, China) was used to detect the pH values before and after the fermentation. The levels of SCFAs in fermentation solutions, including acetic, propionic, n-butyric, isobutyric, nvaleric, and isovaleric acids, were evaluated using gas chromatography (GC) according to the previous work (Chen et al., 2017). Briefly, 0.4 mL of fermentation sample was mixed with 0.4 mL of internal standard solution containing 0.3 mg/mL of 2-ethylbutyric acid and 0.2 M of HCl. The levels of SCFAs in samples were measured by GC (7890 A, Agilent) equipped with a flame ionization detector (FID) and an HPINNOWAX column (30 m × 0.25 mm × 0.25 μm, Agilent). The initial oven temperature was set at 100 °C for 1 min, then increased to 180 °C at a speed of 5 °C/min, and further held for 4 min at 180 °C.

2.4. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Each fermentation experiment was performed in triplicate. Differences between different groups were analyzed using one-way analysis of variance (ANOVA) procedure by Tukey test. Statistical analyses of the obtained data were performed using SPSS 22 software (IBM, U.S.A.). Statistical analysis of KEGG pathway data was carried out by STAMP v2.1.3 with Welsh's *t*-test ($p < 0.05$). A p value of <0.05 was considered to be statistically significant.

3. Results

3.1. Effect of fluoride on gut microbiota

The effects of different concentrations of fluoride (1, 2, 10, 15 mg/L) on gut microbiota were carried out by fermentation model in vitro. The gut microbiota was analyzed by high throughput sequencing analysis in this work. The Rarefaction measure of observed species tended to a plateau, and Rarefaction measure of Shannon had been stable with the increase of the sequencing depth for all 15 samples as showed in Fig. S1, indicated the data of sequencing have captured the most species in this work (Chen et al., 2018a). The α-diversity of gut microbiota including Chao1, Ace, Shannon and Simpson indices were evaluated. As showed in Fig. S2, the fluoride with different of concentration of fluoride showed limited effect on the α-diversity of gut microbiota ($p > 0.05$). The β-diversity, including principal component analysis (PCA), partial least squares discrimination analysis (PLS-DA) and Clustering analysis, were used to compare the overall structure of gut microbiota at the OTU level from different groups. As showed in Fig. 1(a), an obvious separation among the structure of gut microbiota was observed in different groups. Moreover, PC1 explained 56.89% of total variance, showed that fluoride

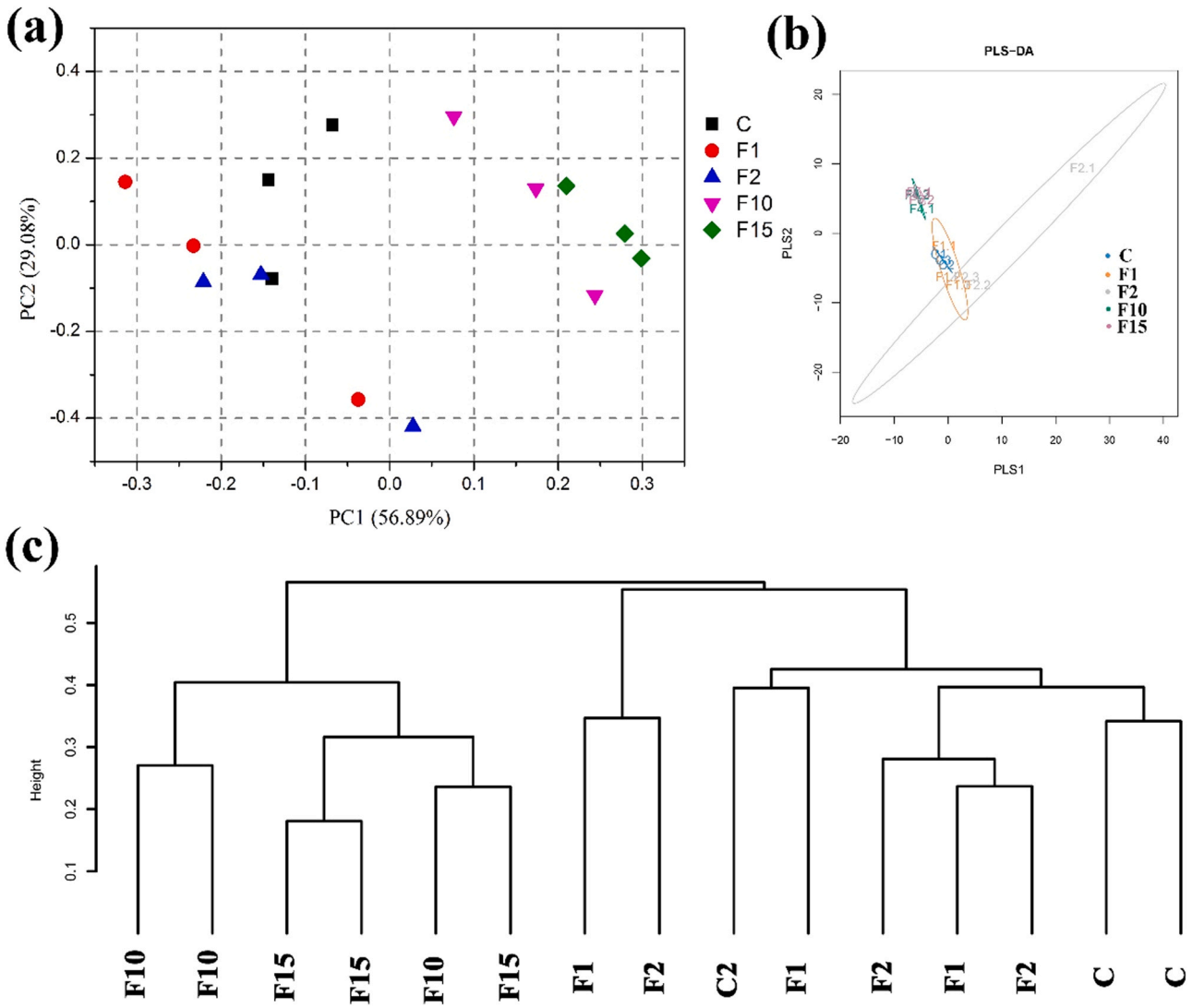

Fig. 1. The structure of gut microbiota. (a) Principal component analysis (PCA) and (b) Partial least squares discrimination analysis (PLS-DA) of gut microbiota at the OTU level, (c) Multivariate analysis of variance from matrix scores based on Jaccard method.

could shift the composition of gut microbiota in dose-dependent manner. Notably, the samples in C, F1 and F2 groups all sat together, whereas, the samples in F10 and F15 groups had kept away from them. As expected, the results of PLS-DA (Fig. 1b) and Multivariate analysis (Fig. 1c) suggested that all the samples in this work could be divided into two main groups, including the group with low dosages of fluoride (0, 1 and 2 mg/L) and the group with high dosages of fluoride (10 and 15 mg/L), which confirmed the result of PCA. Thus, low dosage of fluoride had limited effect on the structure of gut microbiota, whereas high dosage of fluoride could significantly change the composition of gut microbiota.

The taxonomic analysis at the phylum level showed that the gut microbiota was mainly consisted of Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria (Fig. 2a), which was consistent with the previous report (Xie et al., 2017). The fluoride showed limited effect on the relative abundance of Firmicutes, Bacteroidetes and Actinobacteria (Fig. 2b, d and e). Specifically, the relative abundances of Proteobacteria in F10 and F15 groups were significantly higher than those in F1 and F2 groups (Fig. 2c), suggested that high dosage of fluoride could promote the growth of Proteobacteria. Furthermore, fluoride could reduce the relative abundance of Fusobacteria in dose-dependent manner. The 20 most relative abundances of gut microbiota at the genus level were showed in Fig. 3a, it was obvious that fluoride especially high dosage of fluoride could significantly affect the composition of gut microbiota at the genus level. Low dosage of fluoride (1 and 2 mg/L) had limited effect on the gut microbiota except Unclassified_Lachnospiraceae. However, high dosage of fluoride groups (F10 and F15) showed significantly lower relative abundance of *Clostridium*, Unclassified_Ruminococcaceae, *Faecalibacterium*, *Fusobacterium*, *Phascolarctobacterium*, Unclassified_Clostridiales, Unclassified_Lachnospiraceae and classified_Veillonellaceae compared with C, F1 or F2 group ($p < 0.05$). Importantly, fluoride seem to inhibit the growth of *Lactobacillus* in dose-dependent manner, although there was no significant difference among these groups ($p > 0.05$).

The different gut microbiota species in the same genus may exhibit different responses to fluoride intervention, thus, it is important to investigate the difference at OTU level among these group after fluoride treatment. The 50 most relative abundances of gut microbiota at the OTU level were analyzed by LEfSe. The comparative analysis of gut microbiota among C, F1, F2, F10 and F15 groups was showed in Fig. 4. Thereinto, 20 OTUs were significantly different ($p < 0.05$), including 3 OTUs in C group, 5 OTUs in F1 group, 2 OTUs in F2 group, 1 OTUs in F10 group and 9 OTUs in F15 group, respectively, which were significantly higher than other groups based on the LDA scores (log 10). Especially, the relative abundance of *Lactobacillus* in F1 group, including OTU541, OTU5326, OTU1515 and OTU54, was more abundant than those in C,

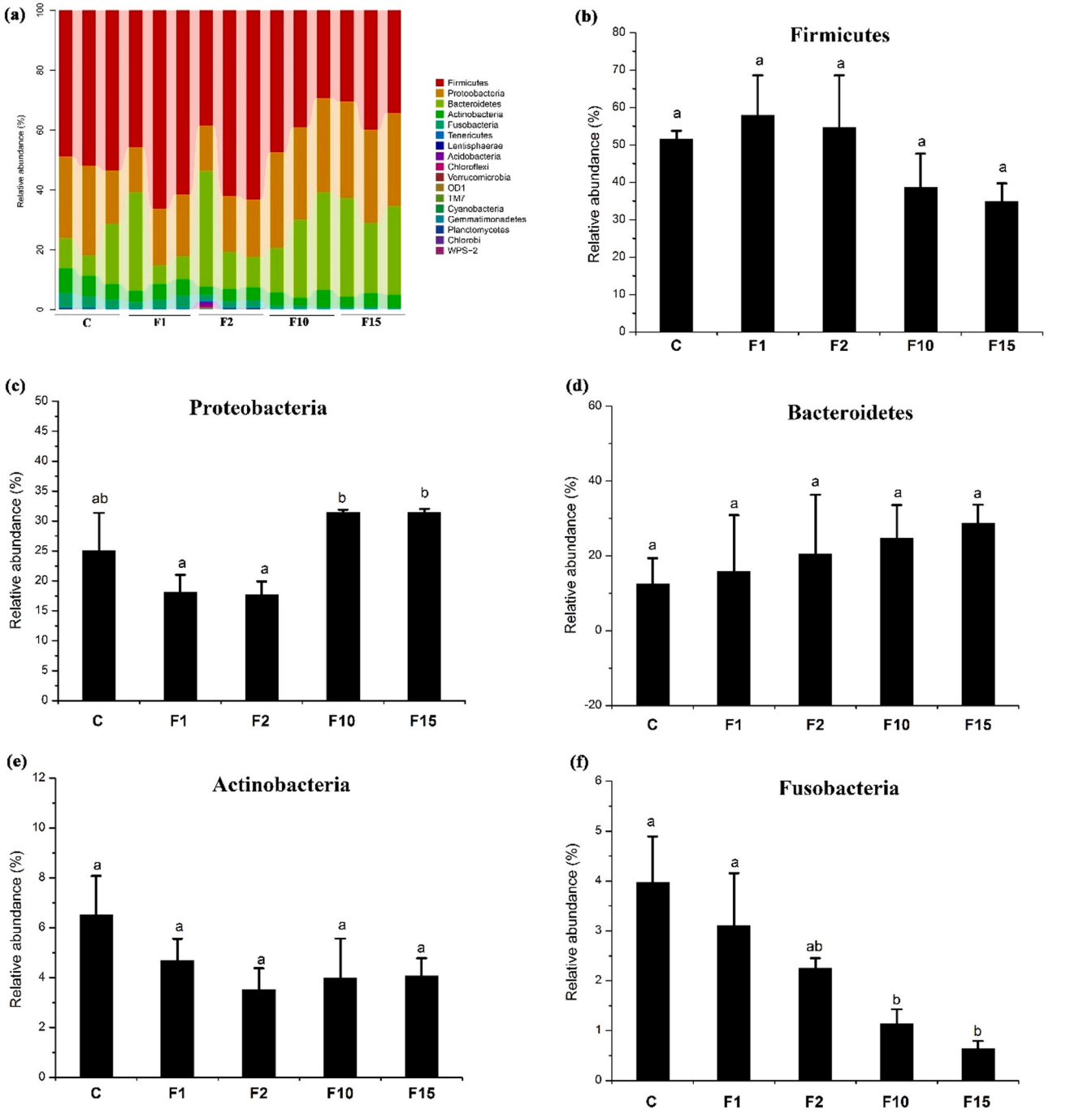

Fig. 2. Compositions of gut microbiota at the phylum level. (a) Bacterial taxonomic profiling of gut microbiota at the phylum level, and the relative abundances of (b) Firmicutes, (c) Proteobacteria, (d) Bacteroidetes, (e) Actinobacteria and (f) Fusobacteria. Data are expressed as the mean ± SD. n = 3. Differences between different groups were analyzed using ANOVA procedure by Tukey test. The different letters represent significant differences between different groups ($p < 0.05$).

F2, F10 and F15 groups. For F2 group, 2 OTUs (OTU4087 and OTU3325), that belong to *Faecalibacterium*, were significantly higher than other groups. However, high dosage of fluoride groups including F10 and F15 increased the relative abundance of OTU1588, OTU3023, OTU2415, OTU739, OTU358, OTU2267 and OTU3528, which belonged to Enterobacteriaceae. The effect of different dosages of fluoride on the microbial community functions was predicted by PICRUSt. As showed in Fig. S3, the low dosage of fluoride (F1 and F2) had no effect on KEGG pathways ($p > 0.05$). Whereas, 6 (4 enriched and 2 depleted) and 9 (7 enriched, 2 depleted) functional modules were significantly changed in F10 and F15 groups compared with those in C group ($p < 0.05$) in Fig. 5, respectively. High dosage of fluoride could reduce the xenobiotics biodegradation and metabolism, and enrich the functional modules of cancers and neurodegenerative diseases.

3.2. Effect of fluoride on pH and productions of SCFAs

The pH is one of the most important indices for fermentation by gut microbiota. The changes of pH were monitored in this work. As showed in Fig. S4, there was no significant difference among these group after fermentation, suggest fluoride had limited effect on pH of fermentation by gut microbiota. A growing body of work implicates SCFAs, considered as the main metabolite produced by gut microbiota, have many potential health-promoting functions (Koh et al., 2016). In this work, the

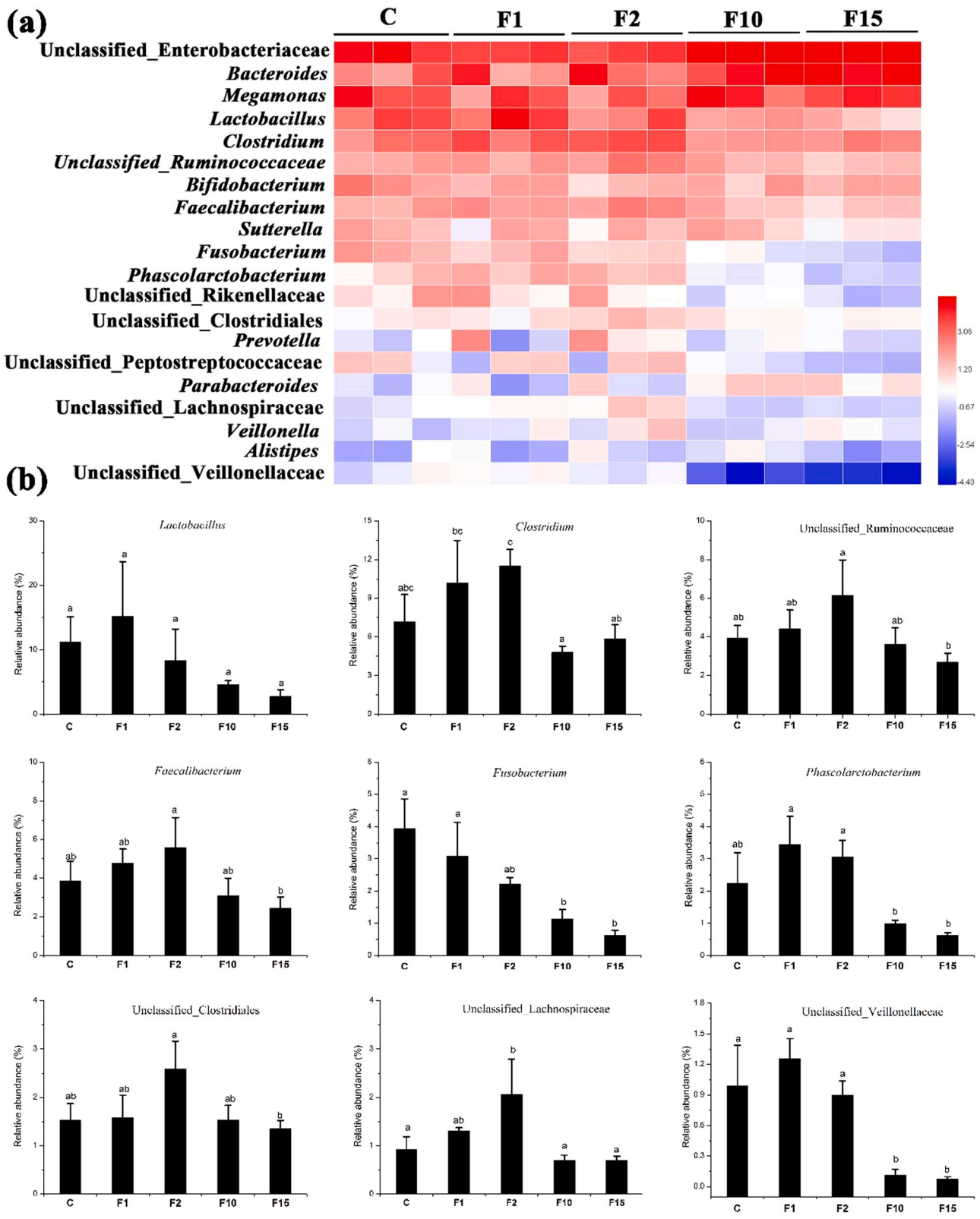

Fig. 3. Compositions of gut microbiota at the genus level. (a) Bacterial taxonomic profiling of 20 most relative abundance of gut microbiota at the genus level, and (b) the relative abundances of *Lactobacillus*, *Clostridium*, Unclassified_Ruminococcaceae, *Faecalibacterium*, *Fusobacterium*, *Phascolarctobacterium*, Unclassified_Clostridiales, Unclassified_Lachnospiraceae and classified_Veillonellaceae. Data are expressed as the mean ± SD. n = 3. Differences between different groups were analyzed using ANOVA procedure by Tukey test. The different letters represent significant differences between different groups ($p < 0.05$).

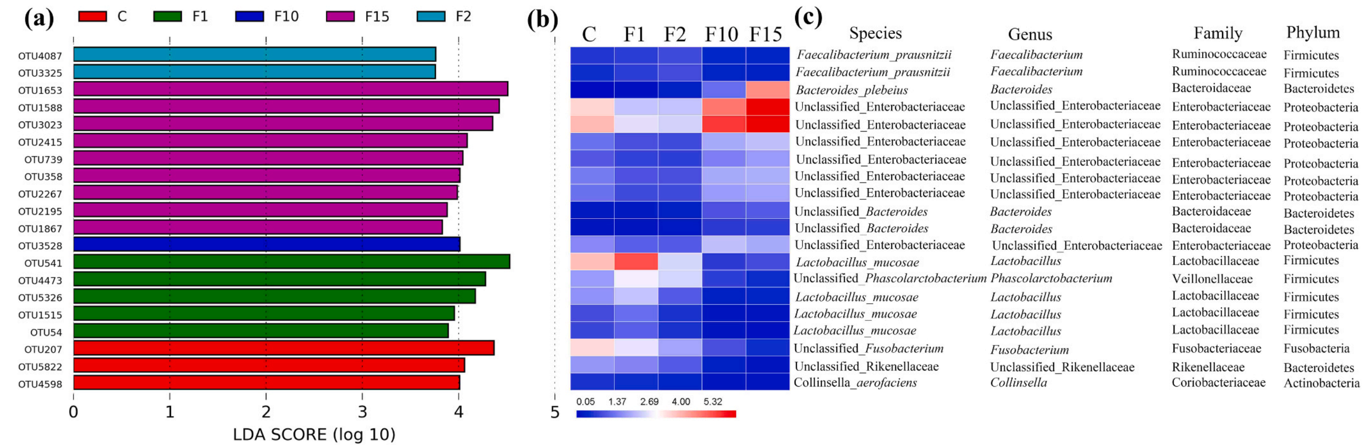

Fig. 4. The comparative analysis of gut microbiota using LEfSe among C, F1, F2, F10 and F15 groups at OTUs level. (a) Histogram of linear discriminant analysis (LDA). (b) Heatmaps of gut microbiota with the relative abundance of OTUs based on result of LEfSe. (c) Represented bacterial taxa information including species, genus, family and phylum.

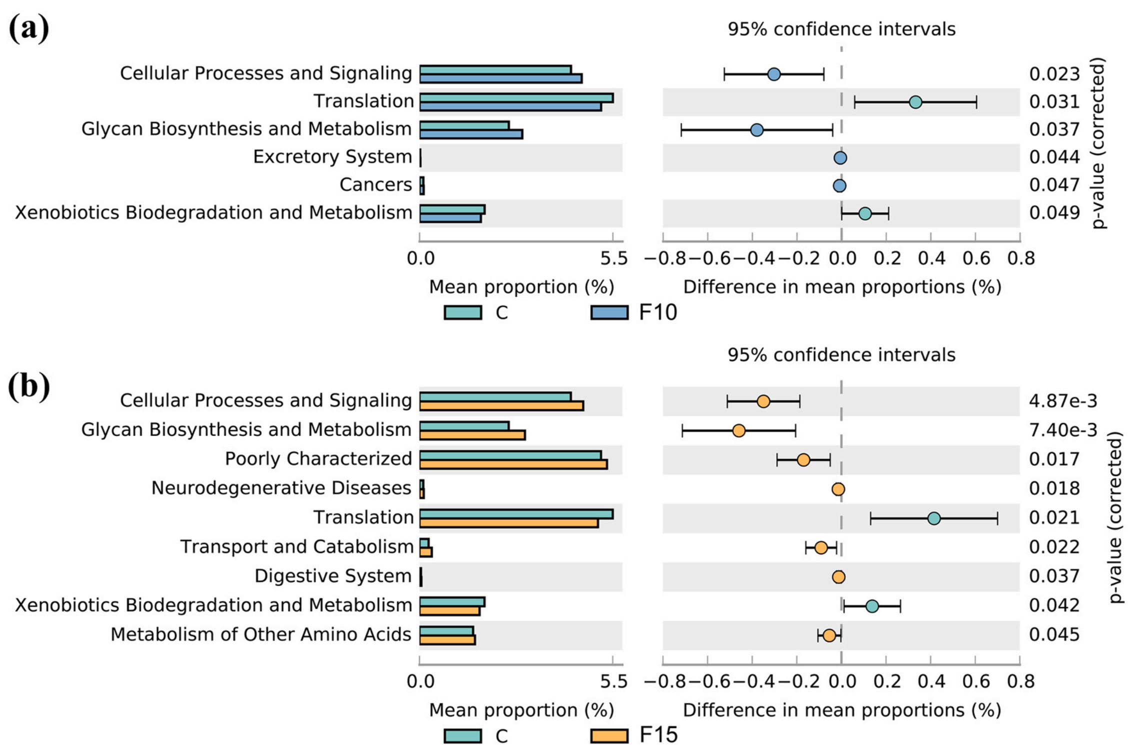

Fig. 5. Differentially abundant KEGG pathways between C and F10 (a) and C and F15 (b) groups. Statistical analysis was carried out by STAMP v2.1.3 with Welsh's *t*-test. All pathways with a $p < 0.05$ are shown.

levels of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid were measured to investigate the potential effect of fluoride on metabolite of gut microbiota (Table S1). As expected, the SCFAs in fermentation solution mainly consisted of acetic acid, propionic acid and butyric acid. However, both low and high dosages of fluoride showed limited effect on the productions of SCFAs.

4. Discussion

Fluoride, regarded as essential component in the human body, is beneficial for body health in appropriate dosage, whereas excessive fluoride showed obviously health hazard, which can result in endemic dental and skeletal fluorosis (Miao et al., 2020a). Thus, the beneficial or detrimental fluoride to host health mainly depends on its ingested dosages. The gut microbiota, regarded as the forgotten organ of human body, may act as an important modulator of the crosstalk between diet and our health status (Chen et al., 2018b). Recently and more strikingly, it has been reported that fluoride with high dosages ranged from 45 to 100 mg/L could also modulate the gut microbiota (Fu et al., 2019; J. Liu et al., 2019). However, another similar work showed that low dosage of fluoride showed limited effect on gut microbial communities in mice (Yasuda et al., 2017). There was limited information about the effects of different dosages of fluoride on the gut microbiota. Thus, the different dosages of fluoride (1, 2, 10 and 15 mg/L) on the gut microbiota were investigated in vitro by fermentation model in this work. As expected, the fluoride showed significantly different effects on the gut microbiota in dose-dependent manner. Especially, the high dosage of fluoride could obviously change the structure of gut microbiota, which was consistent

with the previous reports (Fu et al., 2019; J. Liu et al., 2019). Likewise, the high dosage of fluoride could significantly alter the functional modules, such as reducing the xenobiotics biodegradation and metabolism, and increasing cancers and neurodegenerative diseases, which may contribute to potential risk of diseases.

Proteobacteria, one of the most abundant phyla in gut microbiota, includes many common human pathogens, such as *Escherichia*, *Yersinia*, *Rickettsia*, *Shigella*, *Brucella* and *Salmonella*, which are related to a lot of human diseases (Rizzatti et al., 2017). A growing amount of evidence suggests that Proteobacteria may be served as a potential microbial signature of dysbiosis of disease (Mukhopadhya et al., 2012; Shin et al., 2015). In this work, the high dosage of fluoride could increase the relative abundance of Proteobacteria, which may result in the increasing risk of disease. The high dosage of fluoride also exhibit obvious effect on the gut microbiota at the genus level. *Faecalibacterium*, a most abundant bacterium in the healthy human gut microbiota, plays an important role in human health, thereinto, *F. prausnitzii* is expected as a potentially novel probiotic bacterium for human health (Miquel et al., 2013; Quevrain et al., 2016). *Phascolarctobacterium* has been reported to be positively associated with success of weight loss (Pedrogo et al., 2018). The high dosage of fluoride showed significantly lower relative abundances of *Faecalibacterium* and *Phascolarctobacterium*, which may potentially lead to the risk of disease. The decreased relative of *Faecalibaculum* was also reported in mice after treatment with high dosage of fluoride (Fu et al., 2019).

At OTU level, the low dosage of fluoride could increase the relative abundances of *Lactobacillus* (OTU541, OTU5326, OTU1515 and OTU54) and *Faecalibacterium* (OTU4087 and OTU3325). *Lactobacillus* species, considered as superior probiotics, play an important role in the prevention and treatment of many diseases (Nowak et al., 2019). Furthermore, many species belonging to *Lactobacillus* have been used as commercial probiotics widely consumed by humans according to US Food and Drug Administration reports (Das et al., 2020). Thus, the low dosage of fluoride could promote the growth of beneficial gut microbiota, which may improve the host health. Enterobacteriaceae, a large family of intestinal and systemic pathogens such as *Escherichia coli*, *Shigella*, *Klebsiella* and *Salmonella*, are related to many human diseases, such as chronic inflammation and colorectal cancer (Little et al., 2018). Whereas, the high dosage of fluoride mainly increased the relative abundance of Enterobacteriaceae (OTU1588, OTU3023, OTU2415, OTU739, OTU358, OTU2267 and OTU3528). Miao et al. found dietary high sodium fluoride could alter the cecum microbial community of laying hens by increasing the relative abundances of Gammaproteobacteria, *Escherichia-Shigella*, Streptococcaceae, and Enterobacteriaceae and decreasing relative abundance of *Lactobacillus*, which was consistent with the result in this work (Miao et al., 2020b). The information about food diets of volunteers was not recorded in this work. As we known, the effect of the fluoride treatment may vary with different individuals due to different structures of gut microbiota. Furthermore, the relationship between fluoride and gut microbiota was complicated. In this work, we only found that fluoride could affect the composition of gut microbiota. However, the potential mechanism is still unknown. Thus, the more work should be carried out to further demonstrate these results.

An increasing amount of evidence suggests that chronic fluoride exposure could result in various diseases, however, the potential mechanisms are still unknown (L. Liu et al., 2019; Xie et al., 2020). The gut microbiota has been regarded as an important modulator of the crosstalk between diet and many health (Fan and Pedersen, 2020; Lynch and Pedersen, 2016). In recent years, the toxicities of food derived compounds have been reported to be related to gut microbiota (W. Wang et al., 2019; Chassaing et al., 2015). Thus, gut microbiota could been put forward as a new target in the fluoride-induced diseases, which should be our next work.

5. Conclusion

In the present work, the different dosages of fluoride on the gut microbiota and productions of SCFAs were investigated in vitro. Both low and high dosages of fluoride showed limited effect on the α-diversity of gut microbiota. The low dosage did not affect the structure and functional KEGG pathway of gut microbiota, whereas, the high dosage of fluoride significantly changed the structure and functional KEGG pathway of gut microbiota. Furthermore, the low dosage of fluoride showed health-promoting function on gut microbiota by promoting the growth of beneficial gut microbiota, including *Faecalibacterium* and *Lactobacillus*. However, the high dosage of fluoride can induce the gut microbiota dysbiosis, though increasing the harmful bacterium including Proteobacteria and Enterobacteriaceae, and reducing the beneficial gut microbiota, including *Faecalibacterium* and *Phascolarctobacterium*.

CRediT authorship contribution statement

Guijie Chen: Software, Formal analysis, Writing - original draft. **Pengcheng Hu:** Methodology, Data Curation, Investigation. **Zhichao Xu:** Investigation. **Chuanyi Peng:** Writing - review & editing. **Yijun Wang**: Writing - review & editing. **Huimei Cai:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition. **Xiaochun Wan:** Resources, Writing - review & editing, Supervision, Project administration.

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.ecoenv.2020.111732.

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