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Original Article

Herbal Formula-3 ameliorates OVA-induced food allergy in mice may via modulating the gut microbiota

Sheng Liu[1,2], Bo Yang[1], Pingchang Yang[1], Zhigang Liu[1]

[1]*Department of Respirology & Allergy, Third Affiliated Hospital of Shenzhen University, Shenzhen 518020, China;* [2]*BGI Education Center, University of Chinese Academy of Sciences, Shenzhen 518083, China*

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Abstract: Formula-3 is a Chinese herbal medicine formula that was shown to inhibit food allergy in rats by stabilizing mast cells. But whether Formula-3 ameliorates food allergy through modulating the composition of intestinal microbiota remains to be explored. Here, we aimed to determine whether gut microbiota mediate the anti-food allergic effects of Formula-3. Mouse model of food allergy (FA) was induced by intragastrically administered with ovalbumin and cholera toxin for two weeks, then these mice were orally administrated daily with 1 ml PBS (0.1 mmol/L) or 1 ml Formula-3 (100 mg/m1) for four weeks. The number and abundance of gut mircrobiota were measured with 16S rRNA gene sequencing. We found administration of Formula-3 significantly alleviated FA by decreasing the serum levels of specific IgE, and Th2 cytokine IL-4, IL-5, and IL-13. The dominant characteristics of gut microbiota in mice with FA was the increase in *Firmicutes* and decrease in *Bacteroidetes*, and the emergence of *Deferribacteres*. Formula-3 treatment partially reversed the gut bacterial dysbiosis via increasing *Bacteroidetes* and decreasing *Firmicutes*. Moreover, Formula-3 decreased the bacteria from *Prevotella, Moryella* and *Clostridium*, and increased *Rikenella.* Functional analysis indicated modules involved in phosphotransferase system and lipopolysaccharide biosynthesis were enriched in FA mice, while Formula-3 treatment enriched pathways of multiple transport system. Our study reveals that Formula-3 may ameliorate food allergy through modulating the bacterial dysbiosis.

Keywords: Formula-3, gut microbiota, 16S rRNA, food allergy, immune diseases

Introduction

The prevalence of food allergies and intolerances has risen exponentially within the past decades [1, 2]. Food antigens can result in either IgE-mediated reactions or non-IgE-mediated reactions, but typical food allergies are IgE-mediated [2, 3]. Allergen activation of mast cells and basophils additionally initiates transcription of a number of cytokines including IL-4, IL-5 and IL-13, and these cytokines promote T helper type 2 (Th2) cell-mediated immune responses [4, 5]. Emerging research indicates that intestinal microbiota is important in immunity and in the development of food allergy (FA) [6-8]. Dysbiosis of the commensal flora by antibiotic treatment of mice is associated with the development of Th2 cell-type allergic responses and higher serum IgE levels [9]. Commensal bacteria *Clostridia* could regulate innate lymphoid cell function and protect against food allergen sensitization [10]. A recent study identified a *clostridial* species, *Anaerostipes caccae*, which protected against an allergic response to food [11]. These studies raise the intriguing question of whether the gut microbiota can be manipulated for food-allergy prevention and therapy.

Traditional Chinese medicine (TCM) has been used in Asian countries for thousands of years [12, 13]. Some TCM and their active ingredients, such as cinnamaldehyde and ginseng, can prevent metabolic diseases [14, 15]. Halofuginone is a natural plant alkaloid, and it can prevent autoimmune arthritis in mice by regulating the balance Between Th17 and Treg Cells [16]. The analysis of the composition of microbiota in diseases and its interactions with the host have been intensively investigated in the

Table 1. The recipe of the modified Chinese herbal medicine Formula-3 [25]

	TCM materia medica (pinyin)	Equivalent pharmaceutical name	Amount (g)	Part used
1	Ling zhi	*Ganoderma lucidum*	30	Fruiting body
2	Wu mei	*Fructus pruni mume*	30	Fruit
3	Huang lian	*Rhizoma coptidis*	9	Root
4	Gan jiang	*Rhizoma zingiberis officinalis*	9	Root
5	Ren shen	*Radix ginseng*	9	Root
6	Dang gui	*Corpus radix angelicae sinensis*	9	Root
Total			96	

last few years [17]. Hesperidin, found in citrus fruits, has shown the immunomodulatory actions by regulating gut microbiota in rats [18]. TCM can serve as a new source of drugs for gut microbiota-targeted disease management [19, 20]. Researchers also found metformin and the Chinese herbal formula may ameliorate type 2 diabetes with hyperlipidemia via enriching beneficial bacteria [21].

Food Allergy Herbal Formula-1 was shown to reduce lymphocyte proliferation as well as IL-4, IL-5 and IL-13 synthesis, reversing an established IgE-mediated food allergy [22]. Moreover, the food allergy herbal formula-2 (FAHF-2) also had the potential for treating food allergies [23, 24]. Formula-3, a formula developed in our laboratory that includes *Ganoderma lucidum*, *Rhizoma coptidis*, ginseng, and other components, was reported to reduce IgE production by inhibiting Th2 cytokine release and reverse allergic symptoms in rats [25]. Probiotic supplementation was also shown to ameliorate food allergy by modulating specific genera of the gut microbiota [26]. Therefore, Formula-3 ameliorates food allergy may through manipulating the intestinal microbiota and contributing to the immune homeostasis.

In the present study, we used a well-established FA model, ovalbumin (OVA)-induced food allergy mouse model [27] to investigate the potential of administration of the herbal medicine Formula-3 for treatment of FA. The aim was to establish the influence of Formula-3 administration on the microbiota composition in FA mice. Our results hold considerable promise for intestinal microbiota targeted approa ches to treat and prevent allergic diseases.

Materials and methods

Formula-3 preparation

Formula-3 consists of six Chinese herbal medicines and was prepared in a specific proportion as previously reported (**Table 1**). Component 1, *ling zhi*, was boiled separately for 1 hour, and the remaining components were then added and boiled for an additional 1 hour. At last, the decoction was filtered and lyophilized. 150 mg per mouse of lyophilized decoction in 1 mL was administered to each mouse daily.

Animals

All animal experiments were conducted according to the guidelines approved by the ethics committee of Shenzhen University. Twenty 8-week-old BALB/c mice were purchased from the Guangdong Experimental Animal Center and housed in a specific pathogen-free animal facility with a 12 h light-dark cycle and a temperature of 20±2°C. The mice were allowed 7 days to adapt to the laboratory environment before experiments were initiated. Mice were fed standard laboratory chow and randomly assigned to two groups: CK group (n = 7), and OVA-induced mice with food allergy (n = 13). Mice from CK group were orally administered 2 ml of sterile phosphate-buffered saline (PBS, 0.1 mmol/L) every day for two consecutive weeks. Mice from food allergy group were intragastrically administered with 1 ml OVA (1 mg/ml) plus 1 ml cholera toxin (0.2 mg/ml) in a final volume of 2 ml using a ball-end mouse feeding tube as previously described [26] in the first two weeks. At day 14, allergic responses were elicited in all of the mice through oral treatment with OVA at a dose of 100 mg/mouse dissolved in PBS. FA was evaluated by changes in rectal temperature for 1 h using a thermometer and by allergic shock. From third week, mouse model with FA were divided into FA (n = 6) and FAHerb (n = 7) group. Mice from CK and FA group were received PBS as controls, while FAHerb group were received Formula-3 (100 mg/ml × 1 ml daily per mouse) weekly (**Figure 1A**). After the last administra-

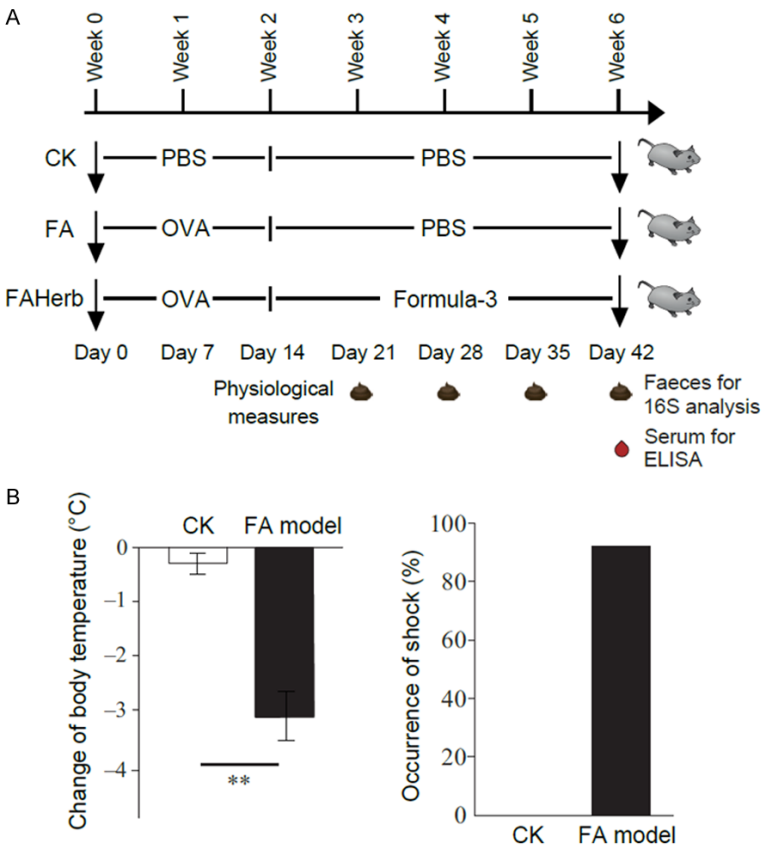

Figure 1. Experimental design and characterization of food allergy. A. Mouse experiment. CK group received PBS for six weeks as controls, while FA model (FA and FAHerb group) were sensitized with OVA plus CT intragastrically for two weeks. From week 3 to week 6, FA group received PBS as control, and FAHerb group received Formula-3. Physiological measures were collected at day 14. Faeces were collected at day 21, 28, 35, and 42 for 16S rRNA gene sequencing, and serum were collected at day 42 for ELISA. B. Characterization of FA model. Food allergy was estimated by changes in rectal temperature and shock in CK (n = 7) and FA model (n = 13) group at day 14.

tion at day 42, mice of FA and FAHerb group were stimulated by 100 mg/mouse OVA dissolved in PBS, while CK group was stimulated by 100 mg PBS at the same time. Then they were sacrificed for the following experiments.

Detection of cytokine and serum immunoglobulin levels

After treatment for six weeks, sera of the twenty mice at day 42 were collected. Then, we detected the contents of Th2 cytokine IL-4, IL-5 and IL-13, as well as OVA-specific IgE (sIgE) and specific IgG1 (sIgG1) by commercial enzyme-linked immunosorbent assay (ELISA) kits (Biolegend, USA) according to the manufacturer's instructions.

DNA extraction, amplification and sequencing

Feces samples from each group of mice at days 21, 28, 35, and 42, with a total of 80 samples were collected by Qiagen stool kit at nine o'clock in the morning, and immediately transferred to a -80°C freezer. The total DNA was extracted using Stool DNA Isolation Kit (Norgen Biotek, Thorold, Canada), following the manufacturer instructions. DNA concentrations were quantified in a Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and checked for size integrity by standard electrophoresis. The V4-V5 region of the bacterial 16S rRNA gene was amplified using specific PCR primers. DNA libraries were checked for size and concentration using a Bioanalyzer (Agilent Technologies, CA, USA). The sequencing was performed on Ion PGM™ platform according to the protocols of the BGI-Shenzhen laboratory.

Bioinformatics analysis

The 16S sequencing data were analyzed with pipeline based on Mothur v.1.33 [28]. The obtained high-quality sequences were clustered into operational taxonomic units (OTUs) at a 97% cutoff for sequence similarity. We conducted principal coordinate analysis (PCoA) according to the matrix of the UniFrac distance [29] of OTUs. Taxonomic classification of OTUs was assigned against the Greengenes database version 13_8 [30]. Alpha- and beta-diversity were calculated based on the microbial community results. We adopted PICRUSt [31] to produce predicted KEGG Ortholog (KO) classifications from v3.5 of IMG [32] with the 16S rRNA gene sequence data. The relative abundances of Level 1, 2, and 3 KEGG pathways were also predicted.

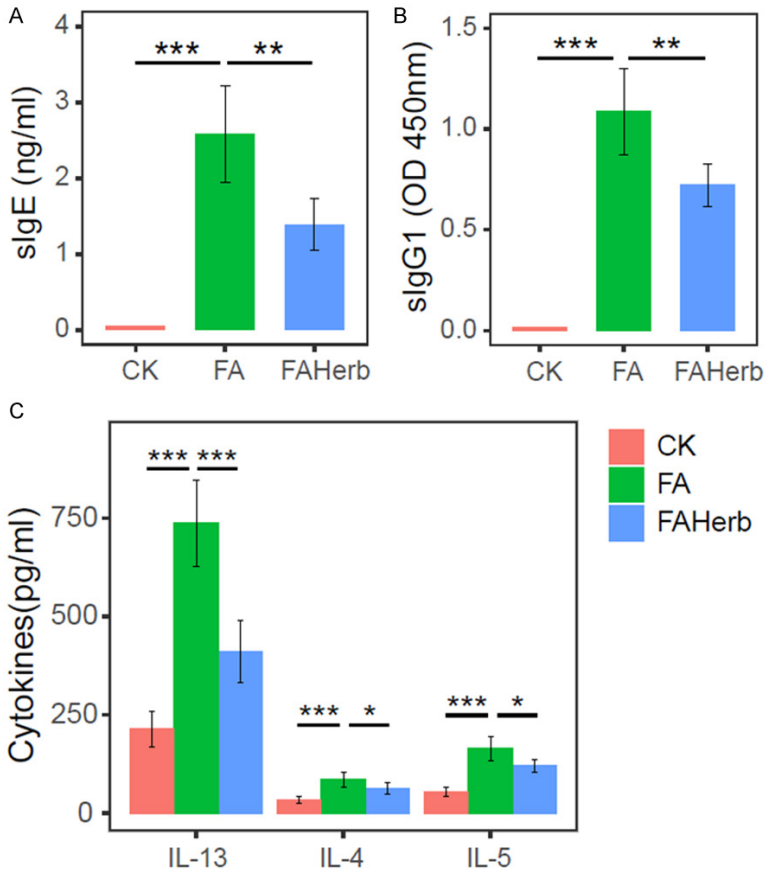

Figure 2. OVA-specific antibodies IgE, IgG1, and cytokines IL-4, IL-5, IL-13 in mice at the day 42. CK, n = 7; FA, n = 6; FAHerb, n = 7. Different groups were shown in different colours. Data represent the mean ± standard error. Two tailed t-test was used to identify significant difference, *denotes $p < 0.05$, **denotes $p < 0.01$, and ***denotes $p < 0.001$.

Statistical analyses

Student's t test was applied to assess whether any differences of immune parameters occurred in different groups. Permutational multivariate analysis of variance (PERMANOVA) was used to assess effects of different phenotypes on OTUs profiles. OTUs or bacteria taxa that exhibited significant differences between two groups were identified by two-tailed Wilcoxon rank-sum tests with $P < 0.05$ and BH adjusted FDR < 0.1. Differentially enriched KEGG modules [33] were identified according to their reporter score [34] from the *Z*-scores of individual KOs. A reporter score of $Z = 1.96$ or $Z = -1.96$ was used as a detection threshold for significantly different modules.

Association between the species and KEGG pathways

Spearman's correlation coefficient was calculated between the relative abundance of the bacteria and the KEGG pathways. We determined the strength of association to be strong when the correlation coefficient is greater than +0.5 or less than -0.5, and the corresponding *p*-value is less than 0.05.

Results

Formula-3 alleviated the allergic responses in food allergy mice

At day 14, we detected the physiological measures in control mice and OVA sensitized mice. A gradual decrease in body temperature and an increase in shock (12 out of 13) were induced in OVA sensitized mice (**Figure 1B**), which were the typical symptoms of FA. At day 28, the levels of sIgE and IgG1 were high in FA group, indicating that the mice have been sensitized to the allergen. As expected, the levels of sIgE and IgG1 decreased significantly in FAHerb group compared to FA group ($P < 0.01$, Table S1; **Figure 2**). The results suggested that administration of Formula-3 suppresses OVA-induced food allergy in therapeutic ways. IL-4, IL-5 and IL-13 levels were significantly elevated in food allergic mice compared to CK (control group) mice, and then these interleukins were significantly decreased in the FAHerb group (**Figure 2**), suggesting that Formula-3 could effectively suppress Th2 cytokine secretion and alleviate IgE-mediated allergy.

Structural and diversity changes of intestinal microbiota in FA mice and the response to Formula-3 treatment

A total of 4,542,839 reads were obtained from 80 samples and after filtering, they were classified into OTUs with a 97% global similarity. We removed OTUs detected in less than 10% of the samples and finally retained 1368 OTUs. The most abundant phyla included *Firmicutes* (839 OTUs) and *Bacteroidetes* (402 OTUs), which is consistent with the conclusion that the

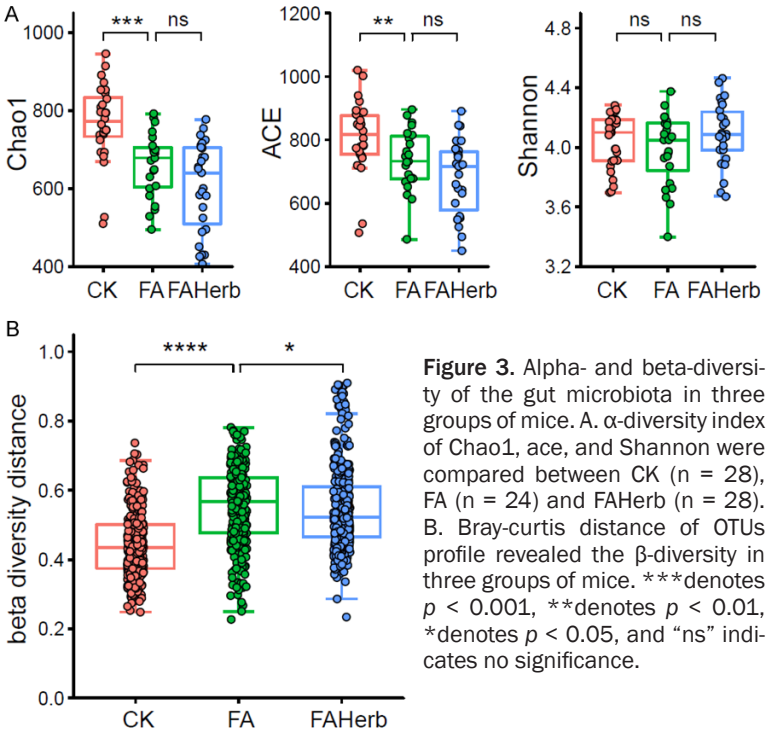

Figure 3. Alpha- and beta-diversity of the gut microbiota in three groups of mice. A. α-diversity index of Chao1, ace, and Shannon were compared between CK (n = 28), FA (n = 24) and FAHerb (n = 28). B. Bray-curtis distance of OTUs profile revealed the β-diversity in three groups of mice. ***denotes $p < 0.001$, **denotes $p < 0.01$, *denotes $p < 0.05$, and "ns" indicates no significance.

mouse gut metagenome is dominated by *Firmicutes* and *Bacteroidetes* [35].

To further clarify the α-diversity in the gut microbiota, we performed PERMANOVA using the means of Chao1, ACE and Shannon index. There did not exist significant difference over these four weeks (Figure S1). The organismal diversity remained steady within the individual, so we combined the samples from different weeks into one group. Chao1 and ACE indices were significantly lower in FA than CK group (**Figure 3A**), indicating that the community richness in food allergy mice is considerably lower. Formula-3 didn't significantly change the richness or diversity of the gut microbiota in FA mice. For β diversity analysis, we found a significant difference between CK and FA group (Bray-curtis distance, $P < 0.001$, **Figure 3B**). Food allergic mice had a higher β diversity, and notably, Formula-3 down-regulated it ($P < 0.05$, **Figure 3B**). We next sought to determine whether the composition of gut microbiota changed by food allergy and the treatment of Formula-3. PERMANOVA showed there were no significant difference for the OTUs profile between week 3 to week 6 in CK, FA or FAHerb group (Table S2). We merged samples from these four weeks, and found there was a significant difference between CK and FA. Moreover, PCoA of the unweighted UniFrac distance based on OTUs profile showed substantial changes in the overall structure of the intestinal microbiota between CK and FA (**Figure 4A**, Adonis $P < 0.001$). A clustering pattern was also observed in FA and FAHerb along the PC2 ($P < 0.05$ for PC2). Food allergy brought dramatic changes to the gut microbiota in mice, and Formula-3 could regulate it to some extent.

Formula-3 treatment changed the intestinal microbiota in food allergic mice

To explore the underlying mechanism by which Formula-3 modulate OVA-induced FA, we next examined the variation of the compositional intestinal microbiota by analyzing 16S rRNA gene. The most prevalent phyla in healthy mice (CK) were *Bacteroidetes* and *Firmicutes*, followed by *Proteobacteria*, *Tenericutes* and *Actinobacteria*. *Firmicutes* and *Bacteroidetes* accounted for > 90% of the bacterial population. The relative abundance of *Firmicutes* increased significantly in FA compared to CK mice, while *Bacteroidetes* significantly decreased in FA (**Figure 4B**). Moreover, the abundance of *Proteobacteria*, and *Tenericutes* were altered, and became more abundant in FA mice than CK mice. Notably, *Deferribacteres* did not appear (relative abundance zero) in CK group, but appeared in FA and FAHerb groups, which suggests the bacteria from *Deferribacteres* may play a crucial role in food allergy. A decrease in the relative abundance of *Firmicutes* and an increase in *Bacteroidetes* were observed in FAHerb group compared to FA group, although not reaching significant level (**Figure 4B**). Meanwhile, Formula-3 also slightly down-regulated the abundance of *Deferribacteres*, *Proteobacteria* and *Tenericutes* (**Figure 4C**). Therefore, Formula-3 alleviated food allergy in mice may through reversing the intestinal disorders.

The proportions of gut microbiota at the family level in healthy mice were also very distinct from that in food allergic mice (**Figure 5**). Kru-

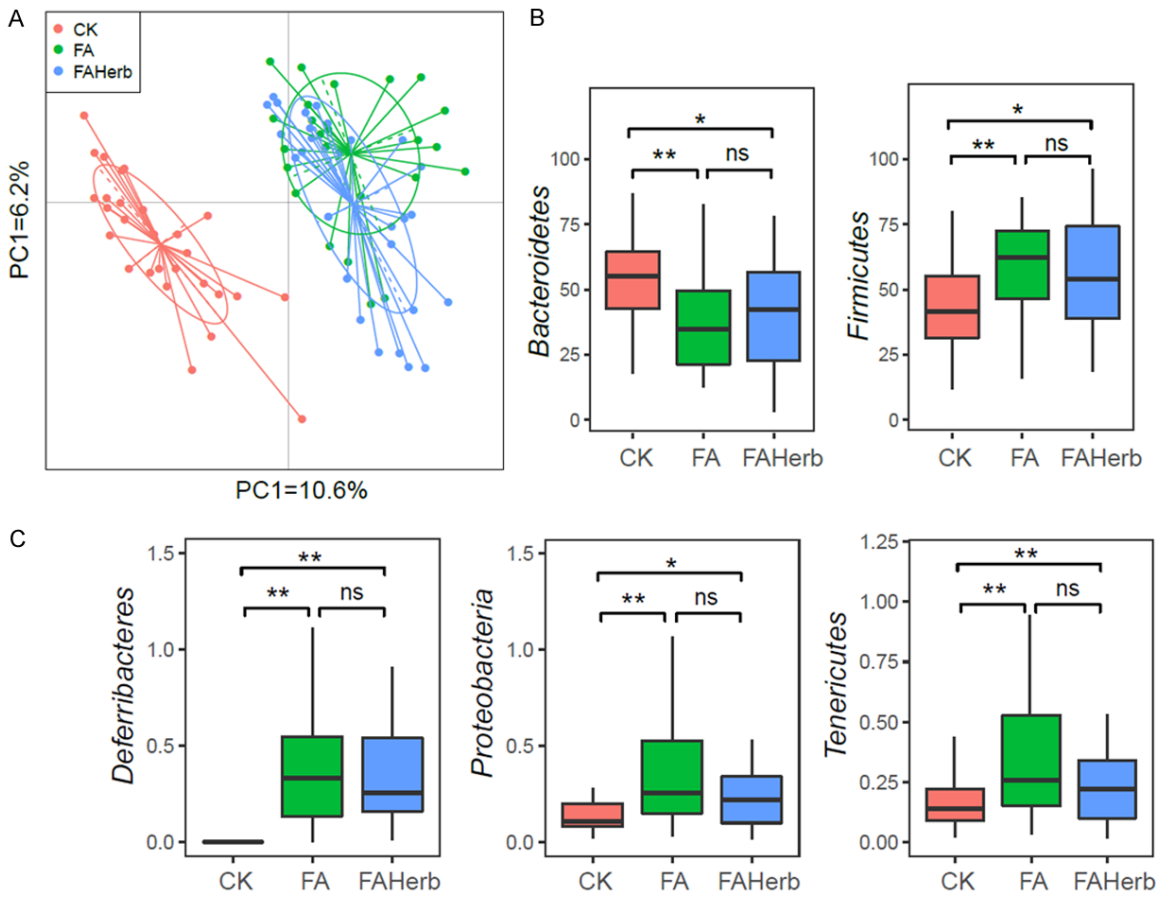

Figure 4. Structure of gut microbiota in three groups of mice. A. The PCoA based on unweighted UniFrac distance of the OTUs abundance. The contributions of principal coordinate 1 (PC1) is on the X-axis and 2 (PC2) is on the Y-axis. B. Boxplots of the relative abundance of two dominant phyla *Firmicutes* and *Bacteroidetes* in three groups of samples. C. Boxplots of the relative abundance of *Deferribacteres*, *Proteobacteria*, *Tenericutes* in three groups of samples. Samples from CK (n = 28), FA (n = 24), and FAHerb (n = 28) are in red, green, and blue, respectively.

skal-Wallis test was applied to assess whether any differences occurred between four weeks. We found the Family-level bacterial composition remained stable among three groups during week 3 to week 6. Wilcoxon rank-sum tests further showed that bacteria from of *Rikenellaceae*, *Porphyromonadaceae*, *Sphingomonadaceae* and *Burkholderiaceae* were deficient in FA mice than CK mice, while *Odoribacteraceae*, *Helicobacteraceae*, *Deferribacteraceae*, and *Coriobacteriaceae* were abundant in FA mice. Formula-3 treatment significantly reduced *Prevotellaceae* in FA mice (FDR < 0.1).

At the genus level, the abundances of *Tannerella*, *Prevotella*, *Paraprevotella*, and *Sphingomonas* were significantly higher in CK group (FDR < 0.05, **Table 2**). By contrast, *Odoribacter*, *Enterococcus*, *Clostridium*, *Moryella*, *Coprobacillus*, *Flexispira*, and *Anaeroplasma* were significantly higher in FA group. The FAHerb group had lower abundances of *Prevotella*, *Moryella*, *Clostridium* and *Eubacterium*, and a considerably higher abundance of *Rikenella* and *Dorea* than FA group (**Table 2**). From the changing trend of microbial compositions, we could see Formula-3 partly reversed the gut dysbiosis induced by FA. The average abundance of *Bacteroides* was reduced from 3.2% in the CK group to 2.5% in the FA group, and rised to 3.8% in the FAHerb group. Besides, *Lactobacillus* up-regulated in FA group and then down-regulated in FAHerb group, but they were not statistically significant. Detailed analysis indicated that enriched species in CK were *Sphingomonas asaccharolytica* and *Burkholderia glathei*, while *Mucispirillum schaedleri*, *Lactobacillus brevis*, *Streptococcus anginosus*,

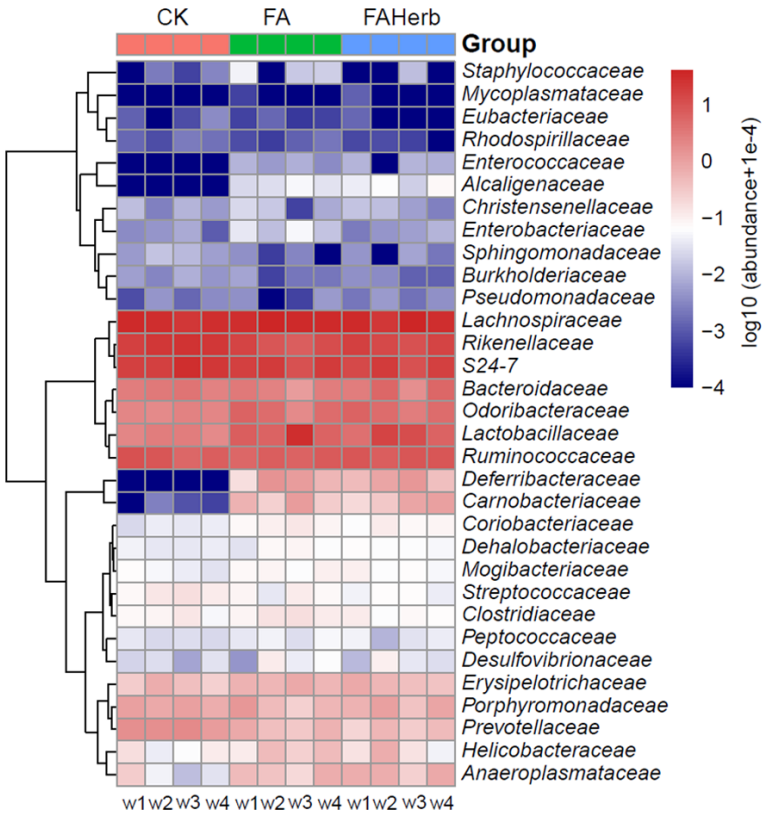

Figure 5. Heatmap showing the abundance of family-level bacteria in the intestinal bacteria from three groups of mice. Samples from CK, FA, and FA-Herb are shown in different color on the top of the figure, while w1 to w4 on the bottom denote week 1 to week 4.

Coprobacillus cateniformis, and *Flexispira rappini* were enriched in FA group. Mice in FA-Herb group had a significantly lower proportion of *Eubacterium dolichum* and *Streptococcus anginosus,* comparing to the FA group. Collectively, these results show that intestinal microbiota in mice experimental model of food allergy are very distinct from normal ones, and Formula-3 treatment could regulate specific bacteria.

Metabolic pathways altered by Formula-3 in the gut microbiome

PERMANOVA analysis indicated that KOs exhibited similar overall results between different weeks, and there was a significant distinction between CK and FA ($P = 0.02$). 24 second-level (L2) metabolic pathways are enriched in CK group (**Figure 6**). In comparison, 11 L2 pathways are up-regulated in FA group compared with CK group, such as membrane transport, xenobiotics biodegradation and metabolism, cell motility, immune system diseases, and signal transduction. Meanwhile, immune system diseases, and signal transduction are down-regulated in FAHerb group compared with FA group (**Figure 6**; Table S3). Therefore, Formula-3 ameliorated metabolic pathways of immune system diseases in mice with food allergy.

Furthermore, 36 KEGG modules varied significantly between CK and FA. Modules involved in phosphotransferase (PTS) system, Glutamate transport system, Lipopolysaccharide biosynthesis and Manganese/zinc/iron transport system were more abundant in FA than CK group. Fifty-five modules were altered between FA and FAHerb. Transport system, Complex I (NADH dehydrogenase) and Type II secretion system were elevated in FAHerb group. These results suggest that functionally, the microbiota could be perturbed in food allergic mice and Formula-3 could partly regulate it.

Association analysis between the abundance of bacteria and predicted KEGG pathways showed that some genera were closely associated with microbial metabolic pathways. *Bacteroides*, *Parabacteroides*, *Tannerella*, and *Prevotella* had a significant positive correlation with Cellular antigens ($P < 0.001$). Formula-3 decreased the abundances of *Prevotella*, which is possibly helpful to alleviate food allergy. These findings could explain how altered bacteria affect the functional metabolism in mice.

Discussion

We constructed the mouse model of food allergy, and found that administration of Formula-3 suppresses food allergy in therapeutic ways. We present a comparative structural analysis of the gut microbiome from healthy mice and food allergic mice with or without Formula-3

Table 2. The comparison of significantly different genera in CK, FA and FAHerb

	CK	FA*	FAHerb#	*p* value*	FDR*	*p* value#	FDR#
Tannerella	-	↓	↑	**0.0002**	0.001	1.0000	1.000
Paraprevotella	-	↓	↑	**0.0000**	0.000	0.9209	0.975
Prevotella	-	↓	↓	**0.0001**	0.000	**0.0054**	0.133
Sphingomonas	-	↓	↑	**0.0002**	0.001	0.2980	0.769
Burkholderia	-	↓	↓	**0.0137**	0.035	0.8693	0.975
Adlercreutzia	-	↑	↓	**0.0064**	0.020	0.4068	0.825
Flexispira	-	↑	↓	**0.0022**	0.008	0.0860	0.393
Mucispirillum	-	↑	↓	**0.0000**	0.000	0.9059	0.975
Jeotgalicoccus	-	↑	↓	**0.0275**	0.067	0.6931	0.970
Enterococcus	-	↑	↓	**0.0005**	0.002	0.4587	0.825
Clostridium	-	↑	↓	**0.0021**	0.008	**0.0248**	0.203
Moryella	-	↑	↓	**0.0116**	0.032	**0.0102**	0.166
Anaerovorax	-	↑	↓	**0.0001**	0.000	0.1672	0.630
Eubacterium	-	↑	↓	0.3382	0.535	**0.0017**	0.046
Ethanoligenens	-	↑	↑	**0.0110**	0.032	0.2746	0.748
Carnobacterium	-	↑	↑	**0.0000**	0.000	0.3928	0.825
Coprobacillus	-	↑	↑	**0.0000**	0.000	0.2748	0.748
Odoribacter	-	↑	↑	**0.0035**	0.011	0.6556	0.970
Sutterella	-	↑	↑	**0.0000**	0.000	0.4473	0.825
Anaeroplasma	-	↑	↑	**0.0000**	0.000	0.5819	0.891
Rikenella	-	↓	↑	0.1974	0.370	**0.0149**	0.082
Dorea	-	↓	↑	0.5188	0.687	**0.0214**	0.093

Note: *denotes FA versus CK group, and #denotes FAHerb versus FA group. ↑ Indicates increase, and ↓ indicates decrease. Genera those with *P* value < 0.05 and FDR < 0.1 are shown. *P* values < 0.05 are in bold text.

treatment. The vast majority of the gut microbial community is composed of only five phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Tenericutes* and *Actinobacteria*), which is similar with reports that *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* are dominant in human [36]. *Deferribacteres* was elevated in FA mice compared to healthy mice, and it was dominated by *Deferribacteraceae* that had increased in dextran sodium sulfate (DSS)-induced colitis [37]. Patients with allergic inflammation of the gastrointestinal tract are at risk of iron deficiency [38]. Thus, we inferred that iron metabolic deficiencies were involved in food allergy induced by bacteria from *Deferribacteres*.

Previous reports have shown that a high *Firmicutes*-to-*Bacteroidetes* (*F/B*) ratio can induce the development of obesity [35], systemic lupus erythematosus [39] and rheumatoid arthritis [40]. The *F/B* ratio increased in the gut of FA mice compared with healthy mice, and Formula-3 contributed to decreasing it. Formula-3 regulated the balance in *Firmicutes* and *Bacteroidetes*, which could one of the potential mechanisms for its alleviation of FA. Furthermore, we discovered that bacteria from *S24-7* family and *Rikenellaceae* were decreased in FA mice. Some members of the *S24-7* can induce T-dependent responses [41] that are targeted by the IgA. A study had suggested a potential positive role for *Rikenellaceae,* a *Bacteroidales* family significantly enhanced by probiotic treatment with strain *B. bifidum Bb* [42]. Specifically, *Rikenellaceae* family members were suppressed in IBD patients relative to healthy controls [43]. The gut microbiota in FA mice is so different from normal ones that it just be the medicine targets for Formula-3.

More specifically, Formula-3 decreased the abundance of bacteria from *Moryella, Prevotella, Clostridium* and *Eubacterium,* and increased the abundances of *Bacteroides*, *Dorea* and *Rikenella* in FA mice. *Rikenella* was reported to be less abundant in mice with an autoimmune disease [44]. Therefore, Formula-3 contributed to increasing *Rikenella* that may have positive effects on the immune regulation. Remarkably, allergic disease has been associated with a considerably lower prevalence of *Bacteroides*, since a lack of *Bacteroides* colonization may be associated with a poor Th1 response [45]. Furthermore, a previous study shows the genus *Dorea* was tightly negatively correlated with Th2/Treg ratio [46], assuming *Dorea* may contribute to induction of Tregs and suppress allergic reaction. Particular species including *Clostridium* species promote the development of regulatory T cells and allergic sensitization in the gastrointestinal tract [47], so Formula-3 may protect immune balance of the gastrointestinal tract. Notably, a previous study showed

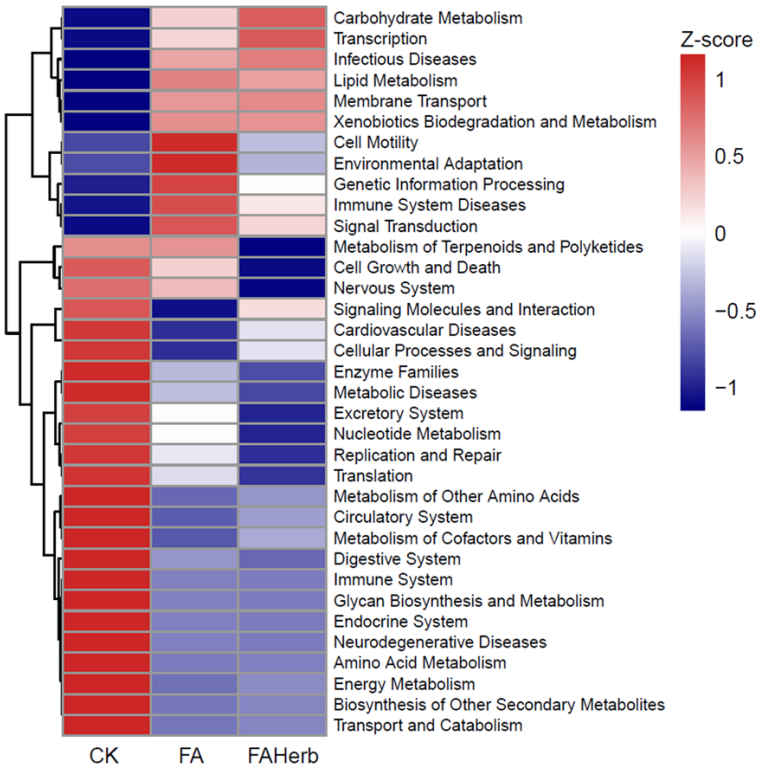

Figure 6. Heatmap for second-level (L2) metabolic pathways of gut microbiota in CK, FA and FAHerb group. The KEGG pathways are ordered by unsupervised hierarchical clustering. Blue color represents relatively low level, while red color represents relatively high level.

that the presence of *Prevotella copri* was strongly correlated with disease in rheumatoid arthritis patients [48]. Some bacteria from *Prevotella* may exacerbate food allergy in FA samples.

Functional analysis of inferred metagenomes revealed Formula-3 down-regulated the metabolic pathways of immune system diseases, and signal transduction in FA mice. Furthermore, modules of phosphotransferase (PTS) system, Glutamate transport system, Lipopolysaccharide biosynthesis, and Manganese/zinc/iron transport system were enriched in FA. Lipopolysaccharide contributes to the structural integrity of the bacteria and induces strong immune responses in animals [49, 50]. And meanwhile, iron, zinc, and manganese uptake systems significantly contribute to the virulence of many pathogenic bacteria [51]. The results indicated that food allergy may trigger immune defenses and induce the gut microbiota to cause virulence. Formula-3 mainly increased pathways involved in Complex I (NADH dehydrogenase) that is used in the electron transport chain for generation of ATP, and ABC transporters that are responsible for the ATP powered translocation of many substrates across membranes [52]. Formula-3 also enriched pathways related to Type II secretion system. These finding suggest Formula-3 regulate the energy metabolism and promote the membrane transport in the gut.

Gut microbiome could be a promising target for innovative therapeutic and preventive strategies against FA [53]. Currently, many studies have suggested that TCM are potent treatments for food allergy [54, 55]. Notably, Formula-3 significantly inhibited Th2 cytokine release and stabilized mast cells to moderate food allergy in our previous studies [25]. In this study, the serum levels of specific IgE and IL-4, IL-5, IL-13 of Th2 cytokine, were significantly decreased in the FAHerb group compared to the FA group (**Figure 1B**), confirming the effectivity of Formula-3 to alleviate allergy diseases. High molecular weight polysaccharides (HMWP) in *Ganoderma lucidum* can prevent gut dysbiosis and obesity-related metabolic disorders [20]. Ginsenoside Rb1 may effectively ameliorate the progression of allergic asthma through relegating Th1/Th2 [56]. Thus, some active ingredients of Formula-3, such as HMWP and Ginsenoside Rb1, may improve immune homeostasis and alleviate food allergy in our study. Our findings are encouraging, but more data are needed to better validate the potential of modulating the Formula-3-gut microbiome-immune system axis. Dissecting how herbal medicines influence gut bacteria communities and the immune system will contribute to building up a precision medicine approach for FA care.

To conclude, this study comprehensively examined the structure and function of intestinal microbiota in healthy mice and FA mice. For-

mula-3 could decrease the Th2 cytokine levels and alleviate food allergy, which are associated with the regulation of intestinal dysbacteriosis. The possible molecular mechanisms were the alteration of phyla *Firmicutes*, *Bacteroidetes*, *Deferribacteres*, *Proteobacteria*, and genera *Rikenella* and *Dorea*, together with the regulation of functional pathways related to signal transduction and immune system diseases. Finding the dysbiotic food allergy signature will offer windows of opportunity for the interventional trials in prevention or treatment of food allergy.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhigang Liu, Department of Respirology & Allergy, Third Affiliated Hospital of Shenzhen University, Shenzhen 518020, China. Tel: +86-755-86671907; Fax: +86-755-866-71906; E-mail: lzg195910@126.com

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Table S1. Specific serum immunoglobulin IgE and Th2 cytokines in mice (CK, n = 7; FA, n = 6; FAHerb, n = 7) at the day 42

Group	ID	sIgE (ng/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)
CK	1	0	27.286	42.206	167.100
CK	2	0	39.413	49.264	228.972
CK	3	0	42.301	70.813	278.315
CK	4	0	28.206	61.130	207.861
CK	5	0	41.156	63.004	237.092
CK	6	0	26.081	41.156	162.452
CK	7	0	34.073	54.596	213.632
FA	1	2.135	78.121	145.290	678.110
FA	2	3.099	100.342	187.302	808.032
FA	3	2.976	95.210	178.031	775.270
FA	4	1.990	66.310	135.217	663.102
FA	5	1.923	60.211	130.014	602.111
FA	6	3.359	110.912	208.012	898.034
FAHerb	1	1.451	52.312	110.356	465.323
FAHerb	2	1.244	69.357	134.546	376.348
FAHerb	3	0.987	50.921	99.657	287.486
FAHerb	4	1.893	76.132	112.342	402.316
FAHerb	5	1.643	80.123	128.640	521.212
FAHerb	6	1.137	45.352	136.464	416.487
FAHerb	7	1.393	62.366	120.344	411.529

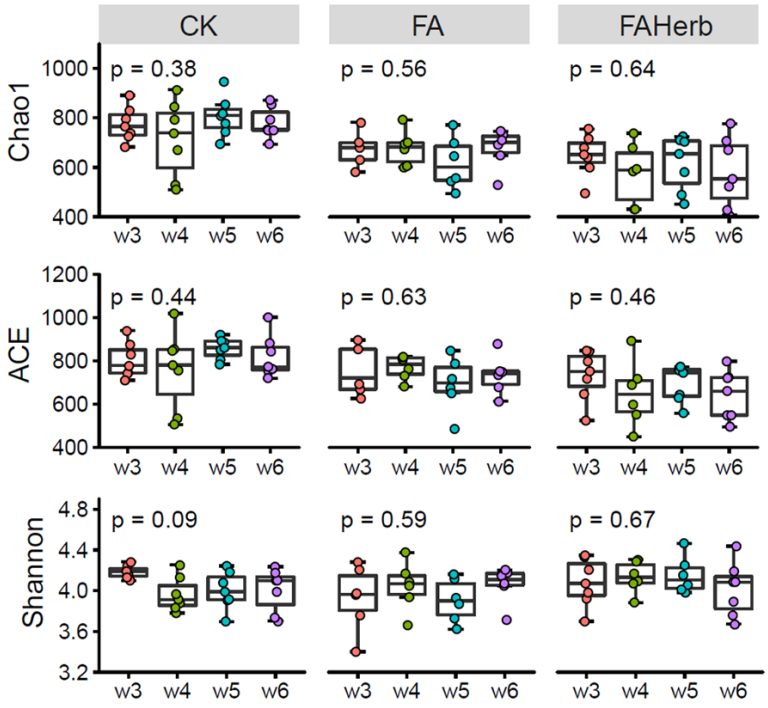

Figure S1. Alpha-diversity evaluated by Chao1, ACE and Shannon index at the time of week 3 to week 6 (at day 21, 28, 35, 42) in three groups of mice. The *p* value at the top left of the legend were calculated by ANOVA.

Table S2. Permanova based on the abundance of OTUs in five different situations

1. Permanova based on the OTUs in CK samples from week 3 to week 6.						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr (> F)
Group	3	0.34647	0.11549	1.1524	0.12591	0.2392
Residuals	24	2.40526	0.10022		0.87409	
Total	27	2.75172			1	
2. Permanova based on the OTUs in FA samples from week 3 to week 6.						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr (> F)
Group	3	0.6698	0.22326	1.4829	0.18196	0.0823
Residuals	20	3.0111	0.15055		0.81804	
Total	23	3.6809			1	
3. Permanova based on the OTUs in FAHerb samples from week 3 to week 6.						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr (> F)
Group	3	0.4923	0.16409	1.0488	0.1159	0.3739
Residuals	24	3.755	0.15646		0.8841	
Total	27	4.2472			1	
4. Permanova based on the OTUs in CK and FA samples from all 4 weeks.						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr (> F)
Group	1	1.1456	1.14557	8.9044	0.15117	1.00E-04
Residuals	50	6.4326	0.12865		0.84883	
Total	51	7.5782			1	
5. Permanova based on the OTUs in FA and FAHerb samples from all 4 weeks.						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr (> F)
Group	1	0.2628	0.26278	1.6573	0.03208	0.0812
Residuals	50	7.9281	0.15856		0.96792	
Total	51	8.1909			1	

Table S3. The average abundance of KEGG pathways in samples from CK, FA, and FAHerb group

	CK	FA	FAHerb
Amino Acid Metabolism	11.11564	10.82565	10.83223
Biosynthesis of Other Secondary Metabolites	1.16549	1.10426	1.10689
Carbohydrate Metabolism	12.06657	12.16036	12.22147
Cardiovascular Diseases	0.00004	0.00002	0.00003
Cell Growth and Death	0.61186	0.61025	0.60682
Cell Motility	2.56331	2.70266	2.60123
Cellular Processes and Signaling	4.06270	3.98619	4.01697
Circulatory System	0.00882	0.00697	0.00726
Digestive System	0.05252	0.04835	0.04785
Endocrine System	0.33020	0.31551	0.31535
Energy Metabolism	7.28687	7.10806	7.11893
Environmental Adaptation	0.16877	0.17184	0.16951
Enzyme Families	2.44597	2.42396	2.41666
Excretory System	0.02344	0.02239	0.02139
Genetic Information Processing	2.86840	2.90225	2.88575
Glycan Biosynthesis and Metabolism	3.02245	2.82194	2.82192
Immune System	0.09193	0.08770	0.08761
Immune System Diseases	0.05613	0.05909	0.05790
Infectious Diseases	0.39222	0.39409	0.39435
Lipid Metabolism	3.00415	3.06995	3.06443
Membrane Transport	11.27961	11.92417	11.95194
Metabolic Diseases	0.11574	0.11323	0.11224
Metabolism of Cofactors and Vitamins	4.82271	4.65426	4.68707
Metabolism of Other Amino Acids	1.67508	1.64263	1.64630
Metabolism of Terpenoids and Polyketides	1.99326	1.99304	1.97666
Nervous System	0.10864	0.10853	0.10809
Neurodegenerative Diseases	0.15541	0.14333	0.14320
Nucleotide Metabolism	4.71666	4.71012	4.70401
Replication and Repair	10.39647	10.36393	10.34102
Signal Transduction	1.61396	1.64766	1.63618
Signaling Molecules and Interaction	0.19181	0.18731	0.19019
Transcription	2.86349	2.92949	2.96157
Translation	6.49159	6.46994	6.45590
Transport and Catabolism	0.44449	0.39065	0.39289
Xenobiotics Biodegradation and Metabolism	1.79358	1.90021	1.89820