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Metabolome and gut microbiota variation with long-term intake of *Panax ginseng* extracts on rats†

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Ginseng, a widely used functional food and food additive, has been proven to have promotion effects of health on the body. However, whether the long-term intake of Ginseng is beneficial or has side effects on an organism is still unclear. In this study, untargeted GC-TOFMS metabolomic analysis of serum, cecum and ileum intestinal contents was conducted to understand the effect of the long-term intake of Ginseng extracts. 16S rRNA microbial sequencing technology was applied to investigate the effect of Ginseng extracts on the structure of gut microbiota. Cytokines in spleen were detected to determine the effect of Ginseng extracts on the immune system. Compared to control groups, the metabolites in serum, cecum and ileum, such as amino acids, amines and other metabolites related to carbohydrate metabolism, significantly varied between the C and GS groups. Ginseng extracts affected the structure of gut microbiota with a decreased abundance of TM7, while the abundance of *Proteobacteria*, *Methylobacteriaceae*, *Parasutterella*, *Sutterella* increased in the GS group. The increased abundance of *Bifidobacterium* and *Lactobacillus* demonstrated that Ginseng extracts contribute to probiotic amplification. Highly correlated with *Bifidobacterium* and *Lactobacillus*, interleukin 4 (IL4), IL10 and immunoglobulin A (IgA) levels were significantly elevated after the long-term intake of Ginseng extracts. These results indicated that the long-term administration of Ginseng extracts positively affected the host-gut metabolism, immune system, the anti-inflammation process and the gut intestinal microbiota structure.

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Introduction

Ginseng is an ancient herb and has been used worldwide as a "medicinal food". Ginsenosides are the main active components of Ginseng, which has been proven to have metabolic manipulation effects and health benefits in the prevention and treatment of physiological and psychological diseases, such as metabolic syndrome, depression and cancer.[1–3] Our previous study reported that small molecule metabolites in rats were significantly changed by the oral administration of ginsenosides.[4] It was reported that Ginseng extracts have extensive effects on the immune system, and even on the gastrointestinal system and gut microbiota.[5,6]

Since previous toxicity research generally originated from traditional toxicology research, there is only equivocal evidence for the benefit or toxicity of the long-term administration of Ginseng extracts, except for drug interactions.[7,8] It was found that natural products often acted on the gut flora, which was reflected in the metabolic changes, and can't be adequately captured by classic toxicological tools.[6] Specific gut bacteria dominated the metabolism of *Panax ginseng* and endo-exogenous metabolic interaction was involved in ginsenosides metabolism.[6,9] Thus, enteric bacteria probably play an important role in the pharmacokinetics, metabolism and pharmacological effects of Ginseng components. What's more, it was reported that the contents of Ginseng could induce the enhancement of immune systems *via* cytokine activation.[10,11]

The emerging field of metabolomics and gut microbiomics technology now enable us to discover the subtle disturbances of drugs, nutrients and external stimuli on metabolites, immune factors and the gut flora of the body, to further evaluate their potential benefit or toxicity.[12,13] Our study was

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designed to discover the variation of systemic metabolism, immune and gut microbiota structure in rats with long-term Ginseng extract intake, using gas chromatography coupled to time-of-flight mass spectrometry (GC-TOFMS), enzyme-linked immunosorbent assay (ELISA) and 16S rRNA sequencing approaches.

Experimental section

Chemicals and reagents

The urease, analytical pure heptadecanoic acid, analytical pure *p*-chloroamphetamine acid, analytical pure vasoxine and analytical pure pyridine were purchased from Sigma-Aldrich. The chromatographic pure methanol was purchased from Merck. The silylating reagent (BSFTA) was purchased from Shanghai Ambrosia Pharmaceutical Company.

Instrumentation

The analytical machine used in this study was a GC-TOFMS (GC was a 6890N Agilent model, the time-of-flight mass spectrometer TOFMS was a Pegasus Leco HT); a new type of rat metabolic cage (Suzhou Feng Animal Equipment Company); a 5415R Eppendorf refrigerated centrifuge (Germany Company); a Milli-Q Advantage ultra-pure water system; a Forma −80 °C ultra-low temperature refrigerator (American Thermo Company); an XS105 precision weighing scale (American Toledo Mettler Company).

Animal handing and sampling

This study was conducted in conformity with the Chinese national legislation and local guidelines, as well as the Shanghai Province Experimental Animal Management Ordinance and the Shanghai Jiao Tong University Laboratory Animal Management Measures. The protocol was approved by the Laboratory Animals Ethics Committee, Shanghai Jiao Tong University, Shanghai, 200240, P. R. China. Experimental: 18 Wistar male rats were purchased from Shanghai SLAC Laboratory Animal Company. Each of the rats was 300 g and kept indoors under 12 h day/12 h night with standard feeding. The indoor environmental relative humidity was controlled at 40%. The total Ginseng extracts were purchased from Hunan Nuoze Biological Technology Company. The purity of the Ginseng extracts was more than 85%.

The 18 male Wistar rats (34 weeks) were randomly divided into a control group (9 rats, group C) and a Ginseng extracts group (9 rats, group GS) after adapting for 1 week. Then the C group was fed with distilled water, while the group GS was given 100 mg kg^{-1} of Ginseng extracts in drinking water for 34 weeks. Then, 1 week later the rats were sacrificed to get whole blood samples and intestinal contents. All the serum samples were obtained from abdominal aorta sampling and centrifuged at 4 °C, 3000 rpm, for 20 min. Then the supernatant was transferred to vials and conserved at −80 °C in a refrigerator. The intestinal contents samples were also conserved in a −80 °C environment.

Sample preparation for the GC-TOFMS analysis

Serum. 50 μL serum and 300 μL mixed organic solvents (methanol : chloroform = 3 : 1) was added to a 1.5 mL centrifuge tube, then subjected to vortex oscillation for 30 s. Next, the mixed liquid is arranged in the −20 °C environment to precipitate for 10 min; then centrifuged for 10 min at 1000 rpm. Then 300 μL supernatant was transferred to a vial and 10 μL 0.1 mg mL^{-1} chlorobenzene alanine and 1 mg mL^{-1} heptadecanoic acid were added to the vial then dried under vacuum at indoor temperature. After the sample was dry, 15 mg mL^{-1} pyridine dissolved methoxamine 80 μL was added to the sample vial. Then the sample vial was subjected to vortex oscillation for 30 s and the sample vial was kept at 30 °C for 90 min. Finally, 50 μL BSTFA (1%TMCS) was added and the sample vial was kept at 70 °C for 60 min.

Ileum and cecum contents. 50 mg (ileum 50 mg, cecum 25 mg) was added to a 2 mL centrifuge tube, the sample was ground and 250 mL ultrapure water was added, then the sample was subjected to 2 min shaking and centrifuged for 10 min at 13 200 rpm. Then all the supernatant was transferred and 250 μL methanol was added to extract for a second round, which was the same process as the first round. Then 150 μL supernatant from the second extraction was added to the sampling-vial filled with the supernatant from the first round. Then all the samples were chemically derived before mass spectrometry analysis using trimethyl silylation (TMS) methodology to detect endogenous metabolites of rats.[14] The samples were vortex antigraded and centrifuged at 12 000 rpm for 10 min after thawing. A 50 μL aliquot of the supernatant was added into 10 μL urea and reacted for 15 min at 37 °C. After that, 10 μL 1 g L^{-1} seventeen acids (internal standard), and 10 μL 0.3 g L^{-1} *p*-chlorophenylalanine (internal standard) were added into the reaction system and centrifuged for 5 min. Then the supernatant was collected in a sampling vial and vacuum-dried at indoor temperature. Next, 80 μL of methoxyamine and BSFTA were added into the vial and the reaction was kept at 70 °C for 60 min. Then the vial was kept at indoor temperature for 1 h and mass spectrometry analysis followed.

Gut microbiota analysis based on 16S rRNA sequencing studies

A sample of the internal contents was removed from storage. Firstly, a QIAamp DNA Stool Mini Kit (no. 51504) was used in DNA extraction and the following steps were in accordance with a previous published study.[15]

Detection of immune factors in spleen

The immune factors in spleen were detected by an ELISA kit (IL-2, IL-4, IL-6, IL-10, IgA, IgM, IgG and NK cell ELISA Kit, Shanghai Jianglai Biotech, Shanghai, China). 6 rats in each group were selected for the detection of the immune factors, NK cells and immunoglobulins. Experimental procedures followed the manufacturer's instructions and the following steps were in accordance with a published study.[16]

Statistical analysis

The raw data were acquired from the GC-TOFMS after peak smoothing, peak picking and identification *etc*. using ChromaTOF software (v4.22; Leco Co.). Then the compounds name, peak area and retention time were included in the output type data. All the peak intensities were normalized to overall MS abundance to minimize the systematic biases caused by experiment. After that, TMS derivation and other peaks caused by noise were manually deleted. Then the raw data were input into SIMCA-P (v 13.0; Metrics) software to perform principle component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) to visualize the metabolic profiling and the trend of the scores plots. Also, PLS-DA was applied to select the discriminated metabolites with a threshold of variable importance in the projection (VIP) value >1.0. The following steps of significance identification were carried out by performing Student's *t* test ($p < 0.05$) and Fold Change (FC, compared to control group) with a range of >1.2 or <0.8. All the metabolites in the raw data were searched in the Human Metabolome Database (HMDB) and MetaboAnalyst 3.0. GraphPad Prism 5.0 and R were applied in the data statistical analysis. As for the sequencing data, we performed a LEFse analysis after acquiring raw data using Galaxy (https://huttenhower.sph.havard.edu/galaxy).

Results

Metabolic profiling of cecum, ileum and serum

To distinguish significantly changed metabolites induced by the long-term intake of Ginseng extracts among the GS and C groups, PCA and PLS-DA analytical procedures were performed in this study. The PCA score plot is shown in Fig. 1. There is a clear separation in the plot of the cecum samples between the GS group and C groups, suggesting that this significant variation may be induced by the Ginseng extracts intake. Additionally, there is significant separation observed in both PCA score plots of ileum and serum. All the PCA score plots are shown in Fig. 1. However, ileum and serum do not show very clear discrimination between the two groups. Besides this, PLS-DA score plots of cecum, ileum, and serum, shown in Fig. S1†, were conducted to separate the metabolites that contribute to the classification of the samples and obtain a more distinct separation among the GS and C groups.

In this study, a total of 77 significantly changed metabolites were screened. A total of 25 significantly changed metabolites were obtained from cecum metabolites and are summarized in Table S1.† Additionally, there were 35 ileum metabolites and 17 serum metabolites detected and these are summarized separately in Tables S2 and S3.† Amino acids, fatty acids, carbohydrates, carbohydrate metabolites and organic acids were significantly changed in the GS group. Besides this, Student's *t* test was applied in the metabolite analysis with the significance threshold at $p = 0.05$. Moreover, PLS-DA was conducted to get VIP values with the significance threshold at 1.0. All the results are shown in Tables S1–3.† For clarification, all significant variations are shown in a heat map by FC value in Fig. 2.

Overall structure changes of the gut microbiota

After the long-term intake of Ginseng extracts, the bacteria structure in ileum was significantly changed (Fig. 3 and 4). At first, the abundance of gut microbiota changed in the GS group much more than in the control group (Fig. 3A). It's obvious that the three metabolites have differentially abundant features in genus compared to the control group (Fig. 3B). There were four dominant phyla in the results. The abun-

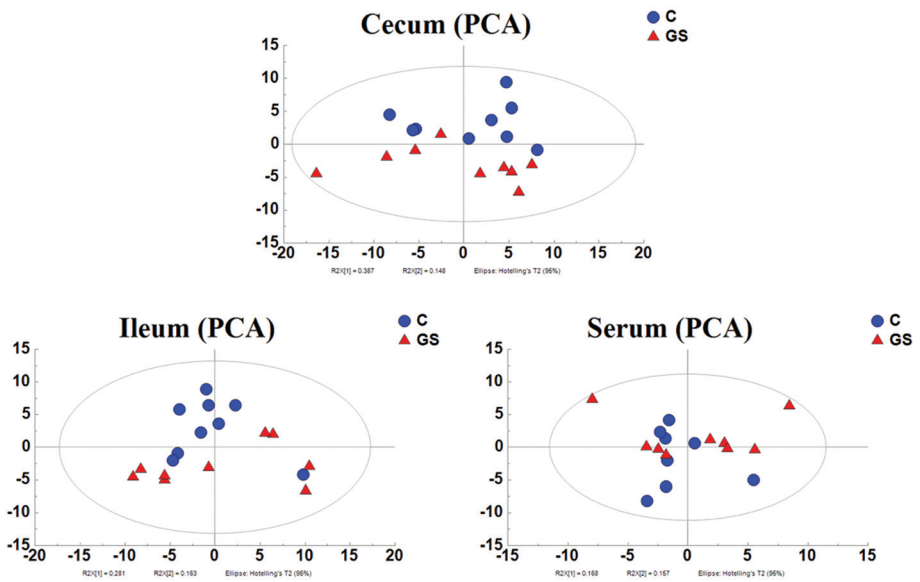

Fig. 1 PCA score plots based on the metabolic profiling of cecum, ileum and serum with long-term intake of Ginseng extracts among the C and GS groups. Cecum (R2X = 0.628, Q2 = 0.316), ileum (R2X = 0.674, Q2 = 0.239), and serum (R2X = 0.563, Q2 = −0.0355).

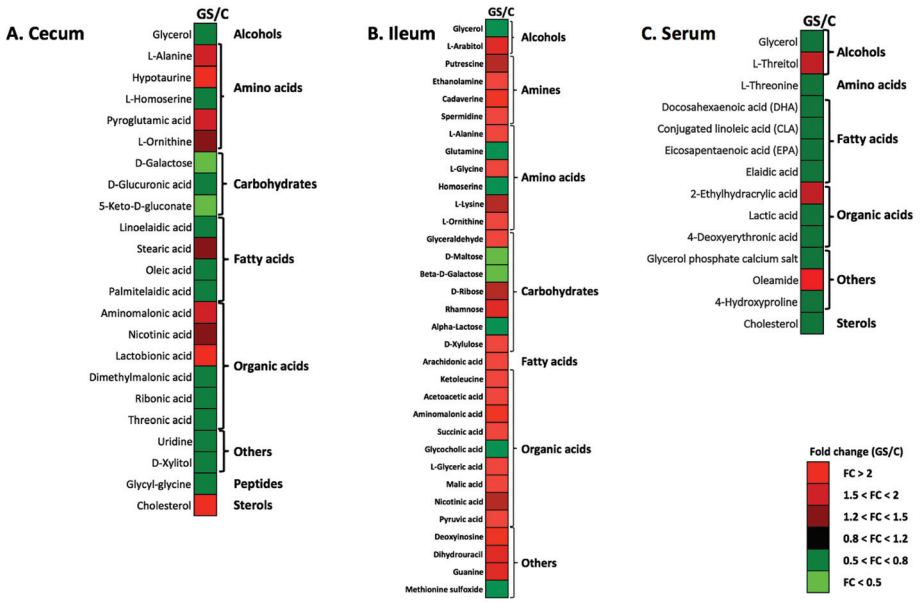

Fig. 2 Heat map of the metabolites in cecum (A), ileum (B) and serum (C) groups. Note: GS/C means the Fold Change of the GS *vs.* the C group. All the outcomes were selected by a threshold of $p < 0.05$.

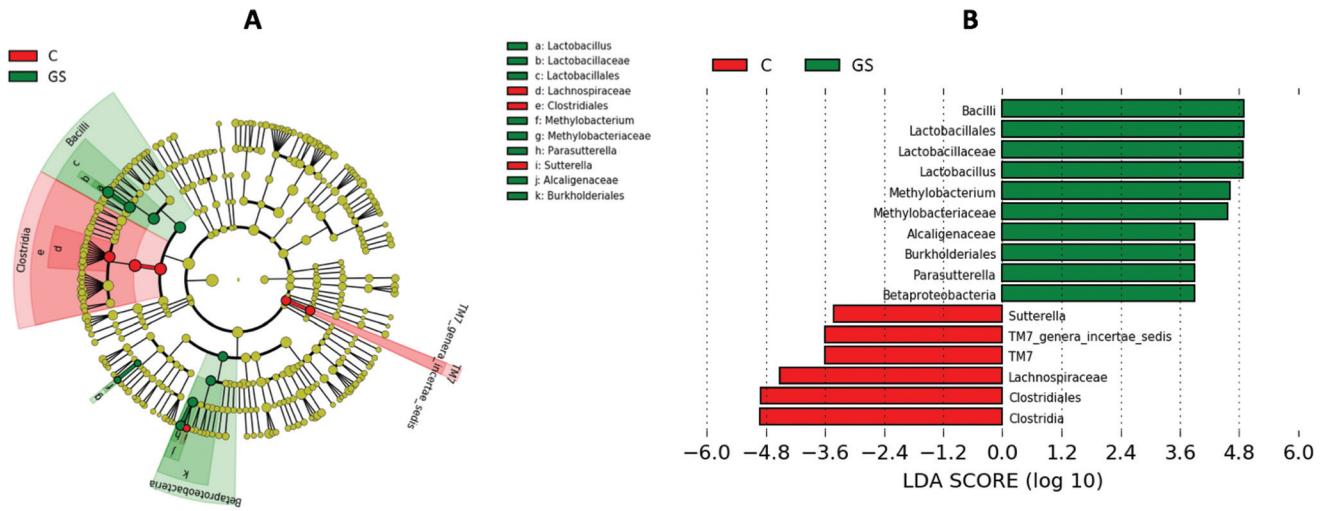

Fig. 3 (A) LEFse analysis of gut microbiota ($n = 9$), from outside to inside, the circles indicate genus, family, order, class, phylum, and kingdom; (B) LEFse analysis of differentially abundant features in the genus ($n = 9$).

dances of *Bacilli*, *Betaproteobacteria*, *Clostridia* and *TM7* were different. First of all, *Bifidobacterium* was an important genus and is significantly elevated in the GS group compared to the C group (Fig. 4A). In the phylum of *TM7*, a decreasing trend can be found in the GS group when compared with the C group (Fig. 4B). In the phylum of *Proteobacteria*, *Methylobacteriaceae*, *Parasutterella* and *Sutterella* were the three dominant genera. *Methylobacteriaceae* and *Parasutterella* were increased largely in the GS group, while *Sutterella* could only be found in the control group (Fig. 4C). *Lactobacillus* dominated in *Firmicutes*. In this phylum, *Lactobacillus* was largely increased in the GS group (Fig. 4D). Furthermore, Pearson correlation analysis was conducted to evaluate the effect of the gut microbiota on metabolites in Fig. S2 and S3.†

Alteration of immune factors in the GS and C groups

The results of immune factor detection show that IL-4, IL-10, and IgA were significantly elevated in the spleen in the GS group (Fig. 5). On the contrary, IL-2, IL-6, IgG, IgM, and NK decreased to a certain degree in the GS group compared to the C group. All of the results show that the long-term administration of Ginseng extracts could induce alterations in the immune factors in the spleen.

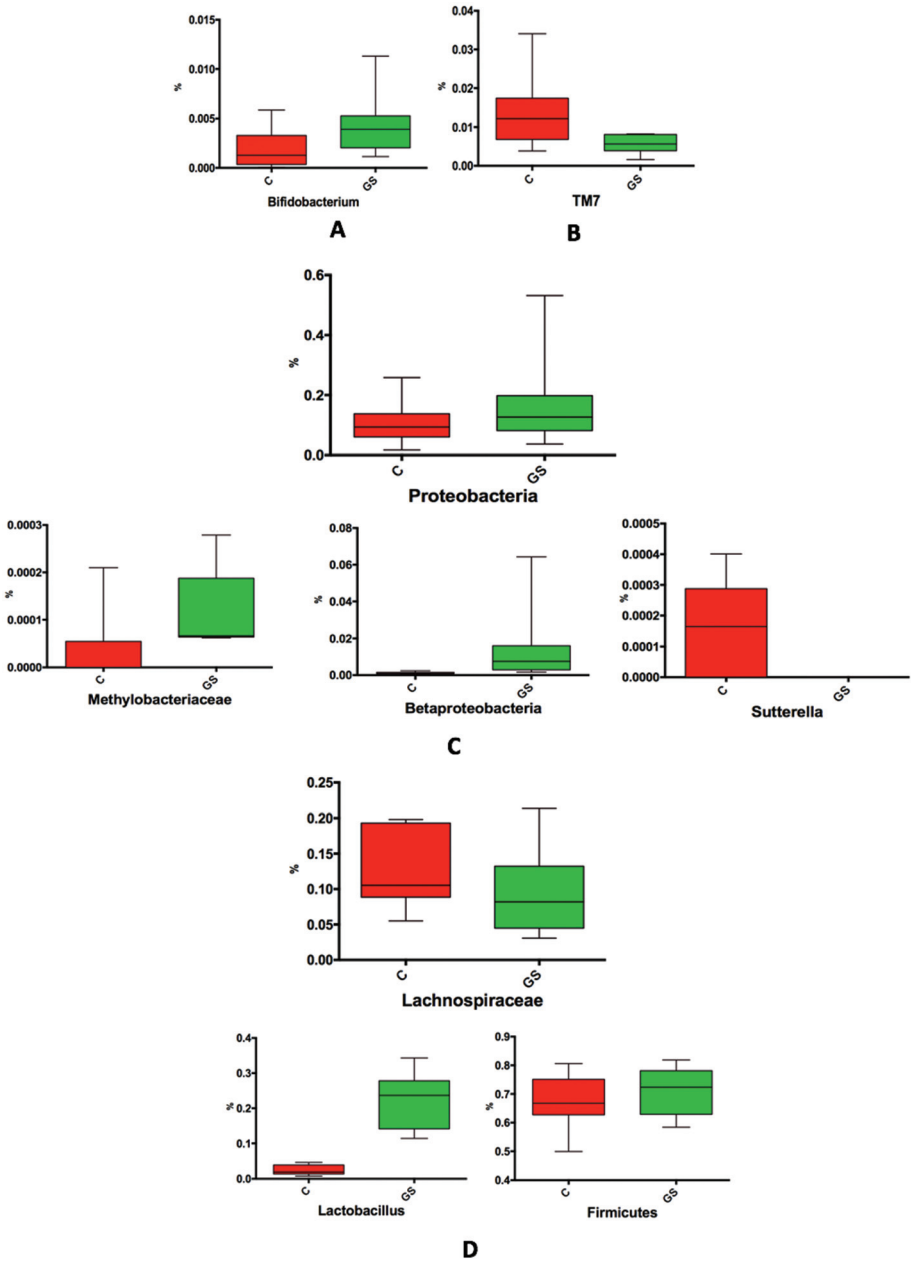

Fig. 4 The significantly changed phylum and genera of the gut microbiota (n = 9).

Discussion

The present study indicates that the long-term oral administration of GS extracts had little effect on the serum metabolites detected using GC-TOFMS based metabonomics, which indirectly confirms the results of the traditional toxicity study.[7] The absorption of Ginseng ingredients generally happens in the small intestine, and its action on the gut flora is mainly in cecum.[17] Therefore, we measured the metabolites of ileum and cecum, and analyzed the cecum microbiota structure flora. A one week interval after GS administration eliminated the direct impact of Ginseng components on the body, reflecting the carryover effects of Ginseng. In spite of this, when compared to the control group, long-term administration significantly changed the gut metabolites of rats in the GS group, as well as the gut microbiota structure.

The effect of Ginseng extract intake on metabolomic profiling

From the PCA score plots (Fig. 1), much more overlap space was found in serum than in cecum and ileum, in which the metabolic clusters of the C and GS groups were far away from each other, indicating the metabolic effect in cecum and ileum could be persistent after long-term oral administration of GS. It is interesting that the GS associated variations in ileum and serum is generally down-regulated, while in cecum it is up-regulated, as seen in the heatmap in Fig. 2.

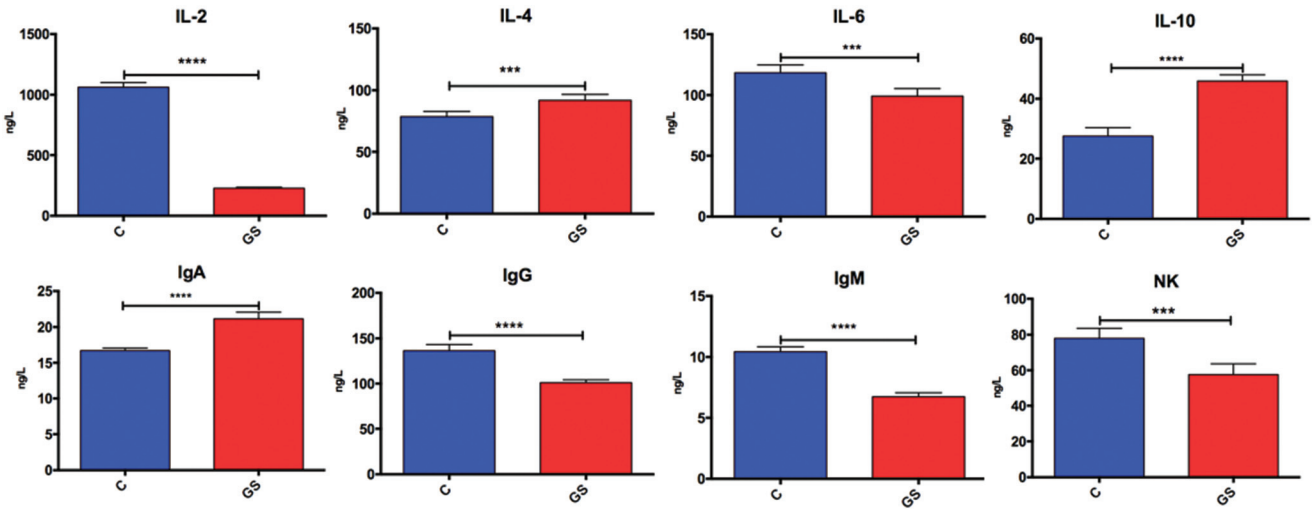

Fig. 5 Alterations of immune factors, immune globulin and NK cell in the GS and C groups (n = 6). (Note: Student's t test was applied in the statistical analysis, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.)

Firstly, many amino acids, such as alanine, hypotaurine, glycine, lysine and ornithine, were significantly up-regulated by GS intake in the cecum, ileum and serum samples. In particular, the fold changes of hypotaurine in cecum, and alanine, glycine and ornithine in ileum were much greater than the other amino acids. Hypotaurine has been shown to act as an antioxidant and provide anti-inflammatory effects to cells.[18] Alanine is an important amino acid in regulating glucose metabolism.[19] Ornithine, one of the most important amino acids, has been shown to possess a determinate effect on polymorphonuclear leucocyte (PMN), which can influence and modulate the PMN host defence capability.[20] Glycine is a key amino acid involved in DNA production, phospholipids and collagen production, and energy metabolism, usually acting as an effective inducer participating in anti-inflammation effects and enhancing immune function.[21] Lysine is one of the essential amino acids and has been reported to participate in histone demethylation and regulation of NO synthesis in immune responses.[22] In addition, lysine has been proven to prevent the development of Alzheimer's dementia with adequate doses of supplementation.[23] As discussed above, it was discovered that long-term oral GS administration showed beneficial influences on the immune function of rats and enhanced anti-inflammation capability.

Secondly, polyunsaturated fatty acids (PUFAs) changed a lot in the GS group as compared with the control group. PUFAs have been reported to have an efficient contribution to chronic inflammation and can enhance immune response.[24] Arachidonic acid (ARA) in ileum and cecum was up-regulated after stopping the Ginseng extracts intake. ARA is one of the polyunsaturated and essential fatty acids, which is recognized as a constituent of animal phosphatides and involved in the coagulation process, and might be a linkage to Ginseng's antiplatelet functions.[25] Meanwhile, other polyunsaturated fatty acids, such as linoelaidic acid, palmtelaidic acid and oleic acid, were down-regulated in cecum. Linoelaidic acid was reported to enhance adipogenic differentiation, playing a significant role in the development of adipose tissue.[26] Besides this, linoelaidic acid could also have an effect on the metabolism of oleic acid and linoleic acid.[27] Palmtelaidic acid has been shown to be beneficial to human health in reducing adiposity.[28] What's more, for PUFAs in serum, the levels of DHA, CLA and EPA were down-regulated. DHA and EPA have been shown to modify the immune cell fatty acid composition and have an anti-inflammatory effect.[29] Accordingly, it's much more possible that the long-term intake of Ginseng extract could induce an increasing level of anti-inflammation factors in serum.

Besides amino acids and fatty acids, there were several kinds of metabolites that were significantly changed in our results. First of all, glycerol was down-regulated in each sample of the administration groups, indicating the possible fat digestion regulation action of GS, probably from the gut. L-Arabitol was significantly up-regulated in ileum. It's remarkable that amines, such as putrescine, ethanolamine, cadaverine and spermidine were all up-regulated in ileum, and were all reported to play an important role in cell growth, cell differentiation, and immune system enhancement.[30] Polyamines were also reported to be critical in many physiological and pathological processes and can even provide drug targets.[31] Putrescine and spermidine were found to participate in the polyamine pathway in gut microbiota.[32] Ethanolamine was reported to participate in intestinal inflammation.[33] Furthermore, organic acids in cecum, ileum and serum were significantly changed at the same time. Especially in ileum, succinic acid, malic acid and pyruvic acid, which play a crucial role in carbohydrate metabolism, were significantly elevated in the results. These results indicate that the long-term intake of Ginseng extracts may enhance the carbohydrate metabolism intensity.

Finally, other metabolites in the results were also changed a lot. A significantly elevated metabolite in serum, oleamide, was reported to participate in the anti-inflammation effect in an Alzheimer's disease study.[34] Cholesterol was one kind of sterol and was reported to enhance the inflammation reaction.[35] Moreover, there were metabolites that were down-regulated in the results. Metabolites related to carbohydrate metabolism were significantly decreased in cecum, such as D-galactose and 5-keto-D-gluconate. In addition, D-maltose and beta-D-galactose in ileum were significantly down-regulated.

The effect of Ginseng extracts on gut microbiota structure

The structure of gut microbiota plays an important role in mammal gastrointestinal tract systems, including participating in digestion, metabolism and immunomodulation.[36] From the result of the 16S rRNA gut microbiota analysis, the gut microbiota structure of the C and GS groups were very different from each other (Fig. 3A). The dominant class and genera of the gut microbiota were shown to have different abundant features in the result (Fig. 3B). On the whole, administration of Ginseng extracts could lead to a structure change of the gut microbiota. The significantly changed phylum, family and genera were selected to be discussed (Fig. 4) as followed.

Bifidobacterium and *Lactobacillus* have been known as probiotics and were reported to positively control gastrointestinal infections, improve lactose metabolism, anticancer properties, mutagenicity, and reduce serum cholesterol and anti-inflammation.[37,38] In our results, *Bifidobacterium* and *Lactobacillus* were increased significantly in the GS group compared to the control group, indicating that the long-term intake of Ginseng extracts could improve the dominance of probiotics in gut flora and enhance gut immune function (Fig. 4A and D). Next, *TM7* abundance was decreased in the GS group (Fig. 4B). *TM7* was reported to be a potential pathogen and participates in inflammatory mucosal diseases.[39] Also, *Proteobacteria* abundance increased in the GS group and so did *Methylobacteriaceae* and *Parasutterella*. Most interestingly, *Sutterella* only appeared in the C group (Fig. 4C). *Proteobacteria* is a kind of Gram-negative bacteria and is reported to participate in inflammatory progression and enhance the diseases progression.[40] As a subclass of *Proteobacteria*, *Parasutterella* was reported to be down-regulated in the obesity group induced by a high-fat diet, which can be used as a health index of intestinal flora.[41] In the Pearson correlation of the results of gut flora and metabolites (Fig. S2 and 3†), *Parasutterella* is positively correlated with nicotinic acid and aminomalonic acid. Nicotinic acid is a regulator of lipid metabolism, inhibiting the mobilization of adipose tissue to reduce plasma free fatty acids, which can decrease cardiovascular disease incidence.[42] *Lactobacillus* was also positively related to nicotinic acid and aminomalonic acid changes both in cecum and ileum.

In addition, tryptophan was also positively correlated with *Johnsonella* and *Oscillibacter* in the results of ileum microbiota and metabolites correlation analysis (Fig. S3†). Tryptophan has been shown to participate in important metabolic pathways such as in cancer and the immune system.[43] In the analysis of the correlation between metabolites and gut bacteria in cecum, a variety of metabolites such as pyroglutamic acid, stearic acid, ribonic acid, uridine, D-xylitol and enteric bacteria such as *Ruminococcus*, *Streptococcus* and *Escherichia/Shigella* were positively related. These results suggest that the long-term intake of Ginseng extracts could induce beneficial changes in the gut flora structure of rats, thereby affecting the gut microbiota metabolism process in rats.

The effect of Ginseng extracts on immune factors

The immunoregulatory cytokines and immunoglobulins results showed that IL4, IL10 and IgA were significantly increased in the GS group compared to the C group, while the level of IL2, IL6, IgG, IgM and NK were decreased in the GS group. IL4 was reported to participate in the regulation of cognitive function and the immune system.[44] IL4, IL6 and IL10 were secreted by T helper (TH) 2 cells and act as stimulating factors for the secretion of IgG from the B cell, which was

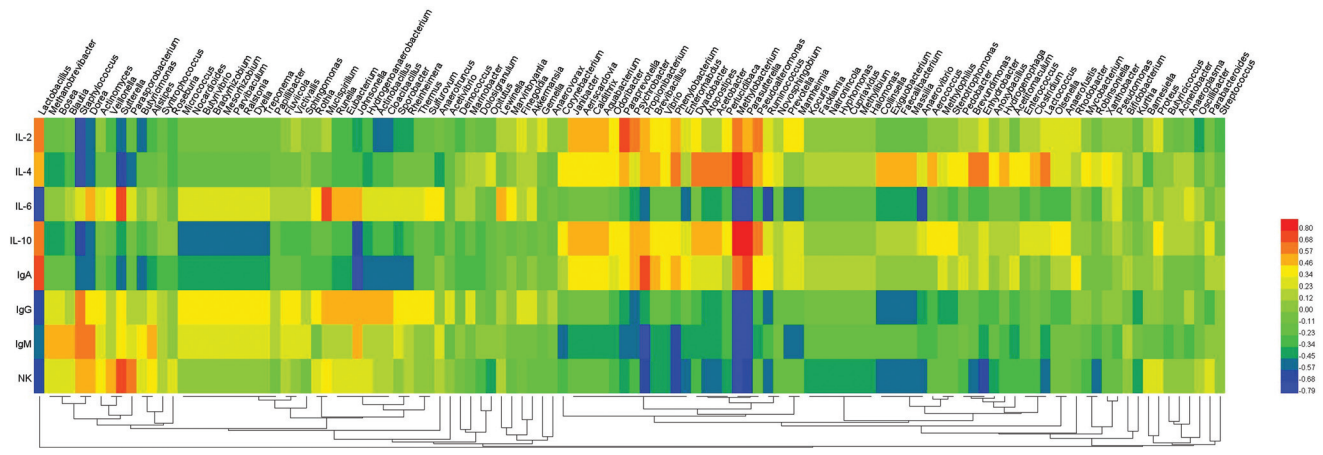

Fig. 6 Spearman correlation coefficient analysis of cecum microbiota and metabolites. Note: The Spearman correlation coefficient was calculated using *R*. A value >0 indicates a positive correlation between the gut microbiota and the metabolites, while a value <0 indicates a negative correlation.

shown to enhance the function of humoral immunity. IL10 is considered as an immunosuppressive and a broad anti-inflammatory cytokine.[45] IL10 can also enhance the function of NK cells and the activity of the mast cell.[46] In the blood, IgA interacts with immune effector cells to initiate inflammatory reactions.[47] It's remarkable that IgA has an intergrowth relationship with gut flora.[48] This finding indicated that an increased amount of probiotics might induce enhancement secretion of IgA in our results. What's more, IL2, IL6, IgG and IgM were all reported to enhance anti-inflammatory properties and innate immunity.[49] All of these results demonstrate that the long-term administration of Ginseng extracts changed the secretion level of cytokines and immunoglobulins.

It was reported that there are bidirectional communications between the microbiota and the immune system of the host. The immune cells could regulate the abundance of microbiota in the gut, and in turn, the effectors induced by the microbiota can influence the function and development of the immune cells.[50] Correlation coefficient analysis was used to identify the systematic effects of Ginsing on the microbiota and the immune system of the spleen (Fig. 6). The results indicate that the interactions between the immune factors and the microbiota were varied and may participate in different metabolic pathways, and most of the interactions were not reported before. The immune factors had a similar trend of correlation variation across *Lactobacillus*, *Methylobaterium* and *Parasutterella*, and were high with IL-2, IL-4, IL-10 and IgA and the opposite with the others. A high level of lactobacillus could stimulate the immune cells which induced the regulatory cytokines IL-2, IL-10 and IgA.[51,52]

Conclusion

In summary, this study illustrated the effects of the long-term intake of Ginseng extracts on host-gut metabolism, the gut microbiota structure and the immune cytokines of rats. Our results indicated that amino acids, polyunsaturated fatty acids and amines were significantly changed, and many gut metabolites were highly correlated with bacteria metabolism. Potential probiotics including *Parasutterella* and *Lactobicillus* were up-regulated while harmful *TM7* was inhibited. The analysis of the content of immune factors in the spleen demonstrated that immune factors related to the enhancement of immune function were elevated. What's more, the abundance of *Lactobicillus* and *Lactobicillus* were positively correlated with IL2, IL4, IL10 and IgA. Many of these changes were highly correlated with immune changes and the anti-inflammation process, indicating the potential long-term beneficial influence of Ginseng extracts on rats.

Author contributions

X.W. and W.J. designed the study, revised the manuscript, and had primary responsibility for the final content; Y.S. designed the study, performed the animal study, collected the samples, acquired the data for analysis, analyzed the data and drafted the manuscript; S.C. performed the animal study and analyzed the data; R.W. performed the animal study and collected the samples; X.X. performed the animal study and collected the samples; C.W. collected the samples; S.F. performed the animal study; X.Z. revised the manuscript; J.S. performed the animal study; J.L. acquired the data for analysis. All authors read and approved the final manuscript and declared no conflicts of interest.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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