Five Natural Active Ingredients Achieve Anti-fatigue Function by Synergistic Antioxidation and Regulating the Structure of Intestinal Flora

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Abstract In this study, the synergistic anti-fatigue effects of five natural active ingredients (anthocyanin, tea polyphenol, Lycium barbarum polysaccharide, ginsenoside, salidroside) and their effects on intestinal flora in mice were investigated. Synergistic antioxidant in vitro showed that when ginsenoside: Lycium barbarum polysaccharide: salidroside = 2:1:6 (4 mg/mL), the maximum experimental scavenging capacity (ESC) of the system was 124.73±5.32 μmol Trolox/L, the synergistic effect (SE) was 2.75. Natural active ingredients of different concentrations and formulations were orally administered to mice. The results showed that compared with control group, the group H (Ginsenoside/Lycium barbarum polysaccharide/Salidroside (2/1/6) 200 mg kg⁻¹ d⁻¹) can significantly increase the exhausting swimming time, decreasing the content of blood lactic acid (BLA), serum urea nitrogen (BUN), and malondialdehyde (MDA), which were accompanied by a corresponding increase in liver superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), liver glycogen content (LG) (p < 0.05). The relative abundances of Verrucomicrobiota and Rokubacteria in group H were significantly decreased (p < 0.05), and the relative abundances of Acidobacteria, Parasutterella and Deferribacteres were significantly increased (p < 0.05). These data suggest that ginsenoside, Lycium barbarum polysaccharide, and salidroside achieve synergistic anti-fatigue effects by regulating intestinal flora and oxidative stress.

Keywords: natural active ingredients, antioxidant, synergistic effect, anti-fatigue, intestinal flora


1. Introduction

Fatigue is a physiological phenomenon that occurs when the body consumes too much mental or physical labor, and it is a sub-health state [1]. Chronic Fatigue Syndrome (CFS) with a prevalence of 0.3% may be associated with damage to the intestinal barrier and altered intestinal flora [2,3,4]. Gut flora may play an important role in fatigue [5]. Some studies showed that improving gut microbiota composition by taking kefir, reduces physical fatigue, and enhances exercise performance in Mice [6]. In addition, some antioxidants can scavenge free radicals and improve fatigue showed that fatigue-related indicators, including metabolites and energy-related indicators, were significantly associated with antioxidant levels. Antioxidants can interact with each other, such as synergy, which makes the antioxidant activity of the complex higher than that of the single ingredient [9]. The synergistic anti-oxidation between ingredients tends to have more potent physiological effects and more negligible dosage effects, which can also reveal the actual situation of these ingredients in the biological body [10]. Some studies indicated that antioxidant interactions between Radix Astragali and Cimicifuga foetida [11]. Other studies have shown that the peptides obtained by enzymatic hydrolysis of egg white have anti-oxidant and anti-fatigue effects [12].

Some reports have confirmed that natural products from medicinal and edible plants, animals and marine plants have good anti-fatigue activity, Spirulina can improve people's ability to resist mental and physical fatigue [13]. Salidroside, ginsenoside, anthocyanin, tea polyphenol, and Lycium barbarum polysaccharide have anti-oxidation and anti-fatigue activities and can improve the function of intestinal flora [14-18]. However, the research on the above five substances mainly focuses on the single anti-fatigue effect at present, the synergistic antioxidant and anti-fatigue effects of these natural active ingredients remain unclear.

In this study, we used these five natural active ingredients to study their synergistic antioxidant and anti-fatigue effects and to explore their impact on the intestinal microbiota of mice.
2. Materials and Methods

2.1. Materials

Anthocyanin was purchased from Chengdu Aifa Biological Headquarters. Tea polyphenol was purchased from Shandong Freda Biotechnology Co., Ltd. Lycium barbarum polysaccharide was purchased from Xi’an Shengqing Biotechnology Co., Ltd. Both ginsenoside and salidroside were purchased from Xi’an Ruying Biological Technology Co., Ltd. Glutathione Peroxidase (GSH-PX) assay kit (Colorimetric method), liver glycogen assay kit (LG), urea assay kit (BUN), blood lactic acid assay kit (BLA), superoxide dismutase (SOD) kit (A001-3), malondialdehyde (MDA) kit (A003-1) and total protein determination kit (A045-4) were from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Other reagents were analytically pure.

2.2. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was employed to assess the capacity of phenolic extracts to scavenge peroxyl radicals, and the adjusted method was outlined in Salvador et al. [19]. Briefly, aliquots of 30 μL of the samples, 60 μL of 508.25 mmol/L fluoresceins, and 110 μL of 76 mmol/L AAPH solution were blended and placed in microplates. Subsequently, the solutions were diluted with 75 mmol/L potassium phosphate buffer, pH 7.4, which was also employed as a blank. Finally, the reaction was carried out at 37 °C, and the measurements were recorded every min over a 2 h period, at excitation and emission wavelengths of 485 and 528 nm, respectively. The ORAC capacities of samples were expressed as μmol L⁻¹ Trolox.

2.3. Evaluation of the Antioxidant Synergistic Effect (SE) of Five Natural Active Ingredients in vitro

ORAC method was used to measure the antioxidant activity of each component of five natural active ingredients. Then they were mixed in pairings of 1:1, 1:2, 1:3, 2:1 and 3:1 (4 mg/ml) to determine the antioxidant synergistic effect (SE) of the mixture. SE was calculated according to the method of Fuhrman et al. [20]. Then select a group of optimal formulations to mix with the third active ingredient in pairings of 1:1, 1:2, 1:3, 2:1 and 3:1 (4 mg/ml) until have been selected an optimal formula for subsequent in vivo functional tests.

2.4. Animals and Care

Four-week-old male ICR mice (20 ± 2 g, specific pathogen-free (SPF), were purchased from Chengdu Dossy Experimental Animals Co., Ltd., the mice were all treated in strict accordance with Committee for Control and Supervision of Experiments on Animals (CPCSEA) and Institutional Animal Ethics Committee (No.030/2020) of Chengdu Dossy Experimental Animals Co., Ltd. They were housed 5-6 per cage (21.5 cm × 32 cm × 14 cm) under controlled temperature (25 ± 1 °C) and humidity (50 ± 10%), with a 12/12 h light/dark cycle. They were given free access to water and a standard laboratory murine diet.

After seven days of adaptive feeding, the mice were randomly divided into six groups (n = 16 per group, each group randomly divided into two subgroups, with 8 mice for the exhaustive swimming test and 8 mice for determining the biochemical parameters) and orally administered different treatments for four weeks (every day at 15:00-17:00 pm). The treatment groups were as follows: (1) Control group (Normal saline), (2) Ginsenoside (G) group (100 mg kg⁻¹ d⁻¹), (3) Ginsenoside/Lycium barbarum polysaccharide (2/1) (G+L) group (100 mg kg⁻¹ d⁻¹), (4) Ginsenoside/Lycium barbarum polysaccharide/Salidroside (2/1/6) (L) group (100 mg kg⁻¹ d⁻¹), (5) Ginsenoside/Lycium barbarum polysaccharide/Salidroside (2/1/6) (M) group (150 mg kg⁻¹ d⁻¹) and (6) Ginsenoside/Lycium barbarum polysaccharide/Salidroside (2/1/6) (H) group (200 mg kg⁻¹ d⁻¹).

2.5. Body Mass Change

The mice in six groups were administered treatments for 4 weeks, as mentioned above. Body weight was measured on days 0, 7, 14, 21 and 28.

2.6. Exhaustive Swimming Test

After 28 days, 8 mice in each group were taken out for an exhaustive swimming test. Briefly, 30 min after the last intragastric administration with G, G+L, L, M, H, or normal saline, each animal was equipped with a lead block (10% of body weight) on the tail root, and then placed in the swimming pool (50 cm × 50 cm × 40 cm) filled with 30 cm deep of freshwater maintained at 25 ± 1°C. The mice were assessed to be exhausted when they failed to rise to the water surface within a 7 s period, and the swimming time was immediately recorded.

2.7. Analysis of Biochemical Parameters

After four weeks, biochemical parameters were analyzed in the other 8 mice from each group. 30 min after the last administration of natural active ingredients, the mice were forced to swim for 90 min without loads. After resting for one hour, the blood was collected through eyeballs, and serum was prepared by centrifugation at 4000 rpm at 4 °C for 15 min. Levels of BLA and BUN were determined according to the recommended procedures provided by the kits. Then the liver was sacrificed and the liver was homogenized with normal saline to a 10 % solution at 4 °C. The activity of liver glycogen, MDA, SOD and GSH-Px was tested following the recommended procedures provided by the manufacturers of the respective kits. At the same time, the spleen and kidneys of the mice were collected, the blood stains were washed with normal saline, and the filter paper was blotted dry and weighed to calculate the index of each organ.

2.8. Sequencing of Fecal Microbiota

At the end of 4-week of experiments, a total of 18 samples fresh fecal samples with an average of 3 in each group were randomly selected for sequencing. Briefly,
DNA was amplified for the V3 and V4 variable regions of 16S rRNA genes using primer pairs: 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with dual-index barcodes to tag each sample. Eighteen samples of PCR products were examined by Beijing Biomarker Technologies Co., Ltd.

2.9. Statistical Analysis

All the tests were conducted in triplicate. The experimental data are expressed as the mean ± standard deviation. The results were subjected to a one-way analysis of variance (ANOVA). Using SPSS 20.0 software, LSD and Dunnett’s T3 tests were performed to determine the significant difference between samples within a 95% confidence interval.

3. Results and Discussion

3.1. Antioxidant Synergistic Effect (SE) of Five Natural Active Ingredients in vitro

ORAC method is often used to determine the antioxidant capacity of active plant ingredients. This method can directly reflect the dynamic information of peroxyl radicals participating in the chain interruption process and quantify it. It is a chemical evaluation method closest to the real physiological system of the human body. In addition, the ORAC method is also a new antioxidant evaluation method recommended internationally [21].

The ORAC test results showed that all the components had antioxidant capacity in the selected concentration range. Except ginsenoside, the other four natural products showed no concentration dependence in vitro antioxidant capacity (Table 1).

Table 2 displayed that there were significant differences between experimental scavenging capacity (ESC) and the theoretical scavenging capacity (TSC) in the pairwise compound system (p<0.05). When the ratio of ginsenoside: Lycium barbarum polysaccharide = 1:1, the synergistic antioxidant effect of the compound system was the best at 4 mg/mL, the antioxidant value was 118.59±4.82 μmol Trolox/L, and the synergistic effect (SE) was 2.14.

Table 3 showed that when ginsenoside: Lycium barbarum polysaccharide: salidroside = 2:1:6 at 4 mg/mL, the maximum ESC of the system was 124.73±5.32 μmol Trolox/L, and the SE was 2.75. The result is consistent with Zhang et al., who reported synergistic antioxidant effects between the typical functional components of hydroethanolic leaf extracts [22]. However, our research is mainly on the synergistic antioxidant effect between five natural active substances, which provides a basis for the synergistic antioxidant effect among different substances. Therefore, it was selected for a subsequent in vivo test.

### Table 1. ORAC of five natural active ingredients in vitro

<table>
<thead>
<tr>
<th>Active ingredients</th>
<th>Concentration (mg/mL)</th>
<th>ORAC value (μmol/L Trolox)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.00</td>
<td>104.29±0.36</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>99.54±0.73</td>
</tr>
<tr>
<td>Salidroside</td>
<td>6.00</td>
<td>101.68±1.10</td>
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<tr>
<td></td>
<td>8.00</td>
<td>98.96±2.77</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>101.20±0.55</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>102.32±2.42</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>114.39±1.15</td>
</tr>
<tr>
<td>Ginsenoside</td>
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<td>118.73±3.95</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>119.48±0.49</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>121.07±0.79</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>79.33±0.52</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>80.63±0.64</td>
</tr>
<tr>
<td>Lycium barbarum polysaccharide</td>
<td>3.00</td>
<td>81.11±0.64</td>
</tr>
<tr>
<td>Tea polyphenol</td>
<td>0.06</td>
<td>103.51±4.22</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>105.53±0.77</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>101.19±2.04</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>86.76±2.04</td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>0.30</td>
<td>105.40±2.06</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>110.12±0.97</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>107.15±1.72</td>
</tr>
</tbody>
</table>

Table 2. The theoretical and experimental scavenging capacity of the combination of two natural ingredients in different proportions calculated by ORAC assay

<table>
<thead>
<tr>
<th>combination of materials</th>
<th>1.00</th>
<th>2.00</th>
<th>3.00</th>
<th>4.00</th>
<th>5.00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSC</td>
<td>ESC</td>
<td>SE</td>
<td>TSC</td>
<td>ESC</td>
</tr>
<tr>
<td>1:1</td>
<td>55.10±0.40</td>
<td>85.48±3.50</td>
<td>87.38±1.55</td>
<td>104.86±1.83</td>
<td>58.20±0.47</td>
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<tr>
<td>Ginsenoside:</td>
<td>60.92±0.25</td>
<td>91.01±7.54</td>
<td>62.57±1.49</td>
<td>107.33±1.72</td>
<td>63.16±0.36</td>
</tr>
<tr>
<td>Lycium barbarum</td>
<td>64.68±0.19</td>
<td>89.83±4.25</td>
<td>66.12±1.39</td>
<td>107.89±0.56</td>
<td>66.65±0.44</td>
</tr>
<tr>
<td>polysaccharide: 1:2</td>
<td>51.53±0.62</td>
<td>87.96±2.26</td>
<td>54.76±1.71</td>
<td>108.19±2.02</td>
<td>55.91±0.87</td>
</tr>
<tr>
<td></td>
<td>50.59±0.74</td>
<td>91.65±6.33</td>
<td>54.41±1.81</td>
<td>108.75±1.70</td>
<td>55.77±2.00</td>
</tr>
</tbody>
</table>

SE stands for synergistic effect; TSC refers to the theoretical scavenging capacity of the composite antioxidants; ESC refers to the experimental scavenging capacity of the composite antioxidants.

The ORAC values of the interaction of other natural ingredients are omitted.
synergistic anti-fatigue in mice, few studies have reported active ingredients to mice has no effect on the weight gain index. Our research show that treating different natural ingredients to mice may improve the material metabolism by regulating the effect on body mass, the cause of weight changes in mice along with feeding time. However, in the mice in group L on the 28th day of feeding, their body mass showed that the exercise capacity of mice was enhanced in exhaustive swimming. The increase in exercise tolerance is a direct measure of the anti-fatigue effect; the exhaustive swimming experimental model is a common and widely used method for assessing fatigue resistance [26]. In the exhaustive swimming test, the prolongation of swimming time showed that these several natural active ingredients can improve exercise tolerance in mice.

3.3. Exhaustion Swimming Time in Mice

The changes in body mass of mice treated with natural active ingredients were measured once weekly over 28 days. As shown in Table 4, there was an increase in body mass along with feeding time. In the current research on synergistic anti-fatigue in mice, and at the same time, it has no damage to the organs of mice and may have a protective effect.

### 3.2. Effects on Body Mass and Organ Index in Mice

The changes in body mass of mice treated with natural active ingredients were measured once weekly over 28 days. As shown in Table 4, there was an increase in body mass along with feeding time. However, in the mice in group L on the 28th day of feeding, their body mass decreased significantly compared with groups M, H, G+L and the control group (p < 0.05). The results obtained in this study for body mass analysis are similar to those published by Cai et al. [23]. The possible reason may be that salidroside in the low-dose group has a more significant effect on body mass, the cause of weight changes in mice may improve the material metabolism by regulating the neuromodulation center, and the results are consistent with those of Zhao [24,25]. At the same time, as shown in Table 5, there was no significant difference in the indexes of the liver, kidney, and spleen of the mice in each experimental group (p > 0.05). In the current research on synergistic anti-fatigue in mice, few studies have reported the effect of multiple natural ingredients on the organ index. Our research show that treating different natural active ingredients to mice has no effect on the weight gain in mice, and at the same time, it has no damage to the organs of mice and may have a protective effect.

### Table 3. The theoretical and experimental scavenging capacity of the combination of three natural ingredients in different proportions calculated by ORAC assay

<table>
<thead>
<tr>
<th>Concentration (mg/mL)</th>
<th>TSC</th>
<th>ESC</th>
<th>SE</th>
<th>TSC</th>
<th>ESC</th>
<th>SE</th>
<th>TSC</th>
<th>ESC</th>
<th>SE</th>
<th>TSC</th>
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<th>TSC</th>
<th>ESC</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>45.12 ± 0.29</td>
<td>45.45 ± 0.32</td>
<td>46.27 ± 0.05</td>
<td>123.88 ± 3.97</td>
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<tr>
<td>2.00</td>
<td>45.97 ± 0.23</td>
<td>45.38 ± 0.22</td>
<td>46.21 ± 0.47</td>
<td>123.34 ± 1.48</td>
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</tr>
<tr>
<td>3.00</td>
<td>46.95 ± 0.21</td>
<td>45.91 ± 0.26</td>
<td>46.78 ± 0.47</td>
<td>123.03 ± 0.63</td>
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<tr>
<td>4.00</td>
<td>41.13 ± 0.39</td>
<td>42.77 ± 0.11</td>
<td>43.59 ± 0.58</td>
<td>13.01 ± 1.39</td>
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<tr>
<td>5.00</td>
<td>43.40 ± 0.44</td>
<td>43.43 ± 0.11</td>
<td>43.62 ± 0.64</td>
<td>13.20 ± 1.32</td>
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</tr>
</tbody>
</table>

3.2. Effects on Body Mass and Organ Index in Mice

The changes in body mass of mice treated with natural active ingredients were measured once weekly over 28 days. As shown in Table 4, there was an increase in body mass along with feeding time. However, in the mice in group L on the 28th day of feeding, their body mass showed that the exercise capacity of mice was enhanced in exhaustive swimming. These results were consistent with Kang et al. [24,25]. The possible reason may be that salidroside in the low-dose group has a more significant effect on body mass, the cause of weight changes in mice may improve the material metabolism by regulating the neuromodulation center, and the results are consistent with those of Zhao [24,25]. At the same time, as shown in Table 5, there was no significant difference in the indexes of the liver, kidney, and spleen of the mice in each experimental group (p > 0.05). In the current research on synergistic anti-fatigue in mice, few studies have reported the effect of multiple natural ingredients on the organ index. Our research show that treating different natural active ingredients to mice has no effect on the weight gain in mice, and at the same time, it has no damage to the organs of mice and may have a protective effect.

### Table 4. Effects of polysaccharide fractions on body mass in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>0 d</th>
<th>7 d</th>
<th>14 d</th>
<th>21 d</th>
<th>28 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.44±5.25*</td>
<td>28.43±3.33*</td>
<td>34.09±3.12*</td>
<td>37.11±3.22*</td>
<td>37.62±3.46*</td>
</tr>
<tr>
<td>G</td>
<td>24.46±2.46*</td>
<td>28.89±2.28*</td>
<td>33.81±2.70*</td>
<td>36.14±3.25*</td>
<td>35.46±3.78*</td>
</tr>
<tr>
<td>G+L</td>
<td>25.99±2.45*</td>
<td>29.92±2.68*</td>
<td>32.59±3.70*</td>
<td>35.09±4.21*</td>
<td>36.32±3.86*</td>
</tr>
<tr>
<td>L</td>
<td>25.66±2.00*</td>
<td>28.2±3.94*</td>
<td>31.75±4.55*</td>
<td>34.09±4.40*</td>
<td>32.92±1.86*</td>
</tr>
<tr>
<td>M</td>
<td>25.81±1.80*</td>
<td>30.32±1.92*</td>
<td>34.57±1.66*</td>
<td>36.81±1.75*</td>
<td>36.47±2.07*</td>
</tr>
<tr>
<td>H</td>
<td>25.26±1.92*</td>
<td>29.3±2.78*</td>
<td>33.39±3.10*</td>
<td>35.68±3.02*</td>
<td>36.1±2.61*</td>
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</tbody>
</table>

The results were expressed as mean ± standard deviation. Groups: Control group (Normal saline), Ginsenoside (G) group, Ginsenoside/Lycium barbarum polysaccharide (2/1) (G+L) group, Ginsenoside/Lycium barbarum polysaccharide/Salidroside (2/1/0) (L, M, H) group.

Different letters in the same column indicate a significant difference (p < 0.05).
Table 5. The effect of natural active ingredients on the organ index in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Organ index</td>
<td>Organ index</td>
<td>Organ index</td>
</tr>
<tr>
<td>Control</td>
<td>0.0525±0.0073&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0151±0.0022&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0030±0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G</td>
<td>0.0486±0.0049&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0147±0.0016&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0035±0.0006&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G+L</td>
<td>0.0541±0.0055&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0139±0.0014&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0046±0.0030&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L</td>
<td>0.0518±0.0022&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0148±0.0013&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0031±0.0004&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>M</td>
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<td>0.0167±0.0019&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0027±0.0005&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>H</td>
<td>0.0547±0.0040&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0148±0.0002&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0042±0.0016&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

The results were expressed as mean ± standard deviation.
Groups: Control group (Normal saline), Ginsenoside (G) group, Ginsenoside/Lycium barbarum polysaccharide (2/1) (G+L) group, Ginsenoside/Lycium barbarum polysaccharide/Salidroside (2/1/6) (L, M, H) group.
Different letters in the same column indicate a significant difference (<i>p</i> < 0.05).

3.4. Analysis of Liver Glycogen in Mice

Glycogen is an essential source of energy during exercise, and plays a vital role in prolonging exercise time [28]. An increase in liver glycogen is advantageous to enhance the endurance of the exercise [29]. Fatigue will occur when the glycogen is largely consumed. Thus, liver glycogen is a sensitive parameter related to fatigue, and should always be determined in the study of anti-fatigue effects [30].

The effect of improving exercise fatigue by increasing muscle glycogen concentration has been reported previously. Our present data are similar to those published by Zeng et al. [31], who analysed glucose supplementation can improve exercise fatigue. In our study, the liver glycogen contents after 90 min swimming for all groups are shown in Figure 1B. The liver glycogen content of each experimental group was higher than that of the control group. Compared with the control group, the liver glycogen content in groups G+L, M and H increased significantly (<i>p</i> < 0.05). At the same time, compared with other experimental groups, the content of liver glycogen in the groups M and H increased significantly (<i>p</i> < 0.05). This shows that the experimental group of high doses can improve the liver glycogen content of mice. This suggests that these five natural active substances may improve fatigue symptoms by increasing liver glycogen content and energy reserves.
3.5. Analysis of Blood Urea Nitrogen and Blood Lactic Acid in Mice

BUN is the result of protein and amino acid metabolism and can serve as a susceptible index for evaluating the endurance ability of the body [32]. If the body cannot obtain enough energy from sugar or fat metabolism, protein and amino acids will be metabolized to produce energy to maintain physiological functions. BUN is formed in the liver as a product of protein and amino acid metabolism and then carried by the blood to the kidneys. There is a remarkable inverse correlation between BUN level and the degree of fatigue [32]. Wang et al. [33] reported that different doses of hairtail peptide may reduce the BUN level of mice after swimming, thus reducing the protein and amino acid in metabolism and relieving fatigue; this research supports our results. In our study, the mice were placed in the swimming tank and forced to swim for 90 min, and blood samples were collected to determine the levels of BUN. As shown in Figure 1C, groups L, M, and H were significantly lower than the control group (p < 0.05).

Lactate is produced by glycolysis under anaerobic conditions during high-intensity exercise. Therefore, blood lactate is an essential parameter for determining the degree of fatigue after exercise [34]. Zeng et al. [31] inhibits the accumulation of lactate through oral arctigenin, caffeine, and glucose, thus providing the possibility of delayed fatigue, which is consistent with our test results. As shown in Fig.1D, compared with the control group, the blood lactic acid content of the experimental group was significantly reduced, and the difference was significant (p < 0.05). At the same time, in groups M and H compared with group L, the blood lactic acid content was significantly reduced (p < 0.05). This shows these natural active substances may reduce the accumulation of harmful substances by increasing liver glycogen and energy reserves.

3.6. Effects on Hepatic SOD, GSH-Px Activities, and MDA Content after the Mice Swimming

Although reactive oxygen species are produced during normal metabolic processes and perform various physiological functions, excessive production of reactive oxygen by strenuous exercise causes peroxidation of the membrane lipids, inducing tissue damage and DNA damage in cells [25]. Defensive systems for oxidative damage include the anti-oxidative enzymes SOD and GSH-Px [25]. Malondialdehyde (MDA) is the final product of lipid peroxidation caused by free radicals generated by enzymatic and non-enzymatic systems and then acts on unsaturated fatty acids in biofilms. The quantitative measurement of MDA can be used to assess the damage to the membrane system and the oxidative damage to cells [35]. Some studies have shown that the increase of SOD and GSH-Px levels in mice indirectly protects the cell membrane structure and resists oxidative damage, thus shows an anti-fatigue effect [36].

It can be seen from Table 6 that after the mice swam without load, the SOD value of the experimental group was higher than that of the control group, and the SOD activity of the groups M and H was significantly higher than that of the control group (p < 0.05). At the same time, the GSH-Px activity of the experimental group was higher than that of the control group. The GSH-Px activity of the groups G+L, L, M, and H was significantly higher than in the control group (p < 0.05). The data in our study are similar to those published by Lee et al. [36], who analysed after *Pinus koraiensis* leaf treatment, SOD and GSH-Px levels increased in the mice, and enhanced antioxidant capacity, and improved symptoms of fatigue in mice. In contrast, the content of MDA in the experimental group was significantly lower than the control group (p < 0.05). This result is in agreement with Xu et al., who found that the anti-exercise fatigue effect in mice is improved when MDA content is decreased significantly (p < 0.05) [37]. This suggests that these several natural actives may protect body tissues from damage by reducing the production of free radicals and increasing the activity of related enzymes.

3.7. Effects of Diets on the Intestinal Flora in Mice

The influence of diet on the intestinal flora of mice is shown in Figure 2. In Figure 2A, the Shannon index indicated that the microbial diversity of the groups G, G+L, L, H, and M was higher than the control group. In Figure 2B, Jaccard-based PCoA showed the six groups of microbiota were not significantly aggregated (p > 0.05). Inflammatory bowel disease (IBD) patients usually show inflammation. The coincidence of IBD and fatigue is caused by chronic intestinal inflammation [38]. Fatigue caused by exercise can also lead to intestinal inflammation. Therefore, inflammation may also result from fatigue [39]. In Figure 2C, at the phylum level, *Verrucomicrobiota* and *Rokubacteria* of group H decreased significantly compared to the control group (p < 0.05), and *Acidobacteria* and *Deferribacteres* of group H increased significantly compared to the control group (p < 0.05). In Figure 2D, at the genus level, the relative abundance of *Parasutterella* in the experimental group was significantly higher than that in the control group (p < 0.05). Correlation analysis showed that the relative abundance of *Parasutterella* in anti-inflammatory intestinal bacteria increased after taking anti-fatigue food ginseng extracts, which was consistent with the significant increase of *Parasutterella* in the experimental group [40].

The composition of intestinal flora can be changed by aging, physiological state, drugs, various diseases, diet, stress and other factors, which impact intestinal metabolism, nutrition, efficacy and physiological function. Intestinal flora is closely related to the health and disease of the host [41]. Corn peptides (CPs) can increase the abundance of anti-fatigue related strains such as *Lactobacillus, Agrobacterium* and *Ackermann* bacteria, to enhance the body's ability to resist fatigue [42]. Natural plant bioactive compounds have the functions of oxidation resistance, growth promotion and fatigue resistance. Natural plant bioactive compounds can improve intestinal tissue morphology, digestion and absorption capacity, and optimize intestinal colony structure and internal environment, to promote beneficial bacteria or inhibit harmful bacteria.
Table 6. Effects of natural active ingredients on SOD, MDA and GSH-Px in mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Doses (mg/kg)</th>
<th>SOD (U/mg prot)</th>
<th>GSH-Px (U/mg prot)</th>
<th>MDA (nmol/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>36.55±1.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.37±6.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.93±3.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G</td>
<td>100</td>
<td>41.33±2.40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>58.97±4.27&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.53±0.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G+L</td>
<td>100</td>
<td>44.54±1.76&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>64.58±2.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.62±0.91&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>L</td>
<td>100</td>
<td>44.14±5.41&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>61.34±1.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.30±1.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>M</td>
<td>150</td>
<td>47.09±5.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.06±3.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.56±2.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>H</td>
<td>200</td>
<td>47.64±5.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.32±2.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.20±0.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The results were expressed as mean ± standard deviation.
Groups: Control group (Normal saline), Ginsenoside (G) group, Ginsenoside / Lycium barbarum polysaccharide (2/1) (G+L) group, Ginsenoside/Lycium barbarum polysaccharide/Salidroside (2/1/6) (L, M, H) group.
Different letters in the same column indicate a significant difference (<i>p</i> < 0.05).

Figure 2. Shannon diversity indexes (A) and principal component analysis (PCoA) (B) at OTU level of fecal microbiota. Bacterial taxonomic profiling at the phylum level of fecal microbiota(C). Bacterial taxonomic profiling at the genus level of fecal microbiota (D). The results were expressed as mean ± standard deviation. Groups: Control group (Normal saline), Ginsenoside (G) group, Ginsenoside/Lycium barbarum polysaccharide (2/1) (G+L) group, Ginsenoside/Lycium barbarum polysaccharide/Salidroside (2/1/6) (L, M, H) group (Different letters in the same column indicate a significant difference (<i>p</i> < 0.05))

4. Conclusions

In our study, Lycium barbarum polysaccharide, ginsenoside, and salidroside have synergistic anti-fatigue and intestinal flora improvement functions. These natural active ingredients can increase swimming time, LG content, SOD, and GSH-Px activities, reduce BLA, BUN and MDA content, and increase the abundance of bacteria such as Parasutterella. The possible mechanism of improving fatigue symptoms is to increase the activity of related metabolic enzymes, increase energy reserves, reduce the accumulation of harmful substances, and enhance the abundance of intestinal flora. The current results may provide a background for these five naturally active substances to become fatigue-improving diets. However, further study is needed to elucidate the exact mechanism of the effect of these five natural active ingredients on fatigue.

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