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Metagenomics exploring the effect of recombinant rice based on lotus seed starch-broken rice flour on intestinal flora in rats

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Abstract

High fat diet may cause obesity, diabetes, atherosclerosis, hypertension, hyperlipidemia and other diseases, and even mortality. The recombinant rice based on mixing lotus seed starch and broken rice flour was used to feed high-fat diet rats, and its effects on body weight and organ index were determined. The changes of intestinal flora in rats were studied by macrogenomic technology, and the effects of lotus seed starch-broken rice flour recombinant rice (LSBR) on intestinal flora in rats were discussed. The results showed that adding enough LSBR could effectively inhibit the weight gain of rats, reduce the liver index from 0.3498 to 0.2836, and the kidney index from 0.0079 to 0.0072. The reduction of intestinal flora abundance and diversity caused by high-fat diet were improved, the relative abundance ratio of Firmicutes/Bacteroidete was reduced from 11.04 to 2.17, while the relative abundance of beneficial bacteria was increased.

Keywords: lotus seed starch; recombinant rice; organ index; macrogenomic; intestinal microflora.

Practical Application: Effects of recombinant rice on body weight, organ index and structure of intestinal microflora in rats.

1 Introduction

Starch is the main carbohydrate source in human diet, which can be digested and hydrolyzed to produce maltodextrin, glucose and other energy substances, and it is an important raw material for the body to supply energy. Starch is rich in sources, which can be extracted from corn, wheat, potatoes, rice and other plant grains. Besides being edible, it can be widely used in food industry as thickener, stabilizer and filler. Lotus seed is a food has high starch content. More than 60% of the dry basis of lotus seeds is starch, of which amylose accounts for 40%, which is conductive to the formation of resistant starch type 3 (RS3). Many modification methods can be used to improve the processing characteristics of lotus seed starch to broaden its application range. At present, the methods of starch modification mainly include chemical modification (Yıldırım-Yalçın et al., 2019), physical modification (Das & Sit, 2021), biological modification (Yang et al., 2021) and composite modification (Reddy et al., 2021). The content of resistant starch for lotus seed starch is greatly increased after modification (Zhang et al., 2013).

Resistant starch (RS), also known as enzyme resistant starch or indigestible starch, can not be enzymatically hydrolyzed in the small intestine, but can almost completely reach the large intestine. In the large intestine, it can be fermented to produce SCFAs and a small amount of gas, which can produce beneficial physiological effects. Studies have shown that resistant starch has significant effects on physiological functions of rats, such as weight loss, reducing blood glucose and cholesterol, preventing gastrointestinal diseases and promoting mineral absorption (Bede & Zaixiang, 2021). Non-digestible carbohydrates (such as RS, etc.) can be used as fermentation substrate by intestinal flora and induce various changes in the intestine (Bang et al., 2019). Relevant studies have found that the acids and enzymes produced by intestinal flora using RS will erode and hydrolyze the structure of starch particles, resulting in cracks, holes, flakes and other structures of starch particles. These structures can protect beneficial bacteria in the intestine and promote their proliferation (Reyes et al., 2018), such as promoting the proliferation of beneficial bacteria in the intestine (such as *Bacteroides, Bifidobacterium longum* (Kałużna-Czaplińska et al., 2017), *Clostridium algidicarnis, Lactobacillus* (Cao et al., 2020), etc.) and inhibiting the growth of harmful bacteria (He et al., 2017). The balance of intestinal flora was regulated, thus preventing intestinal diseases, and consequently, improved intestinal health and metabolic diseases.

Reconstituted rice, also known as engineering rice, takes starch as raw material, adds an appropriate amount of nutrients, and makes rice grains similar to natural rice through extrusion, ripening, cutting and drying. At present, the research of recombinant rice mainly focuses on the preparation of recombinant rice by adding protein, fat and dietary fiber with starch from different sources, so as to improve its physicochemical, nutritional and *in*

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vitro digestion characteristics (Na-Nakorn et al., 2021; Saadat et al., 2019; Yogeshwari et al., 2018). However, there are few studies on the *in vivo* digestive characteristics of recombinant rice and its effect on intestinal flora.

The composition of intestinal flora mainly includes Firmicutes, Bacteroidetes, Actinomycetes and Proteus (Cao et al., 2020). After high-fat diet (HFD) in rats, Firmicutes and Proteobacteria increased, while Verrucomicrobia and Bacteroidetes decreased (Tomas et al., 2016). The abundance of Bacteroidetes and Firmicutes in the intestine of lean and obese mice was different, and their ratio was positively correlated with the diet induced obesity phenotype. These flora changes can be completely reversed after returning to normal diet, indicating that diet is the main contributing factor to the changes of intestinal flora in obesity (Grigor'eva, 2020). Studies have shown that intestinal bacteria were transplanted into aseptic rats from sibling fetal rats with different obesity, the body weight and fat content of rats transplanted with intestinal microflora of obese rats were significantly higher than that of rats transplanted with intestinal microflora of lean rats (Ridaura et al., 2013). This shows that diet can not only cause changes in intestinal flora (Abdelbasset et al., 2022), but also cause metabolic diseases such as obesity.

Macrogenomic technology is widely used in the study of microbial communities in complex environments, including the study of intestinal flora, as a way to study microbial diversity, population structure, and evolutionary relationships through genetic screening and/or sequencing analysis using the genomes of microbial communities in environmental samples as the object of study (Biçer et al., 2021; Dimov, 2022; Grigor'eva, 2020; Hua et al., 2020).

In this study, broken rice powder and lotus seed starch were mixed to prepare LSBR. The effects of LSBR on body weight and organ index of rats fed with HFD and its effects on intestinal flora of rats were explored. The broken rice was further processed and improved its added value and avoid waste. Moreover, the effect of LSBR intervention on the structure, abundance and changes of intestinal flora was analyzed to provide a new idea for designing a healthy diet to prevent diabetes.

2 Materials and methods

2.1 Materials and chemicals

Resistant starch and broken rice were food grade and purchased from Wuchang Baoxin rice planting professional cooperative (Harbin, Heilongjiang, China). Lotus seed (food grade) was purchased from Wubaitang Ecological Agriculture Co., Ltd. (Changsha, Hu'nan, China).

2.2 Preparation of LSBR

The 30% lotus seed starch and 70% broken rice flour were mixed and used as raw materials. The water content of the fixed material was 30%, the extrusion temperature was 120 °C, and the screw speed was 180 r/min. LSBR was dried at 50 °C for 48 hours.

2.3 Animal feeding and grouping

Male Sprague-Dawley rats were purchased from Changchun Yisi Experimental Animal Technology Co., Ltd. (Changchun, Jilin, China). All animal experiments met the welfare and ethical requirements of medical laboratory animals and were approved by the medical and scientific research ethics committee of Harbin University of Commerce. The environmental living conditions of the animals used in the experiment are controlled as follows: the temperature is 20-22 °C, the relative humidity is 40-60%, and the lighting time is 12 h, alternating day and night. The rats were fed adaptively for seven days, and the diet was free during the feeding period.

After there was no abnormality in adaptive feeding, each rat was numbered and weighed with an ear beater. According to the test, the rats were randomly divided into 7 groups with 12 rats in each group (Figure 1). During the dietary intervention, the animals drank freely, observed the mental activity status, hair color and activity of the rats every day, and measured their body weight once a week. The overall test was lasted for 6 weeks.

2.4 Determination of organ indexes

After 8 weeks of intervention, fasting weight was recorded. The organ indexes were determined according to the previous method with slightly modifications (Zhang et al., 2020). The liver, kidney and other organs and tissues of rats were dissected, the tissues were washed in normal saline to remove blood, and then sucked dry with filter paper, weighed and record. The weight of each organ is obtained, and the organ index is calculated according to Equation 1.

$$\text{Organ index} = (\text{organ mass}) / (\text{rat mass}) \times 10 \quad (1)$$

DNA extraction and barcoded pyrosequencing

Total genome DNA from samples was extracted according to manufacturer's protocols. DNA concentration was monitored by Equalbit dsDNA HS Assay Kit. Using 20-30 ng DNA as template, two highly variable regions of V3 and V4 of DNA were amplified by upstream primers containing "CCTACGRRBGCAGCAGKVRVGAAT" sequence and downstream primers containing "GGACTACNVGGGTWTCTAATCC" sequence. The library was quantified to 10 nM, and PE250/FE300 paired-end sequencing was performed according to the Illumina MiSeq/Novaseq (Illumina, San Diego, CA, USA) instrument manual.

After quality filter, purify chimeric sequences, the resulting sequence for OTU clustering, use VSEARCH clustering (1.9.6) sequence (sequence similarity is set to 97%), then the 16 S rRNA reference database is Silva, 132. Then RDP classifier (Ribosomal Database Program) with bayesian algorithm of OTU species taxonomy was used to analysis representative sequences, and count community composition of each sample under different species classification level.

2.5 Statistical analysis

The tests were repeated for 3 times. Origin 2021 software was used for drawing, spssstatistics 26.0 software was used for

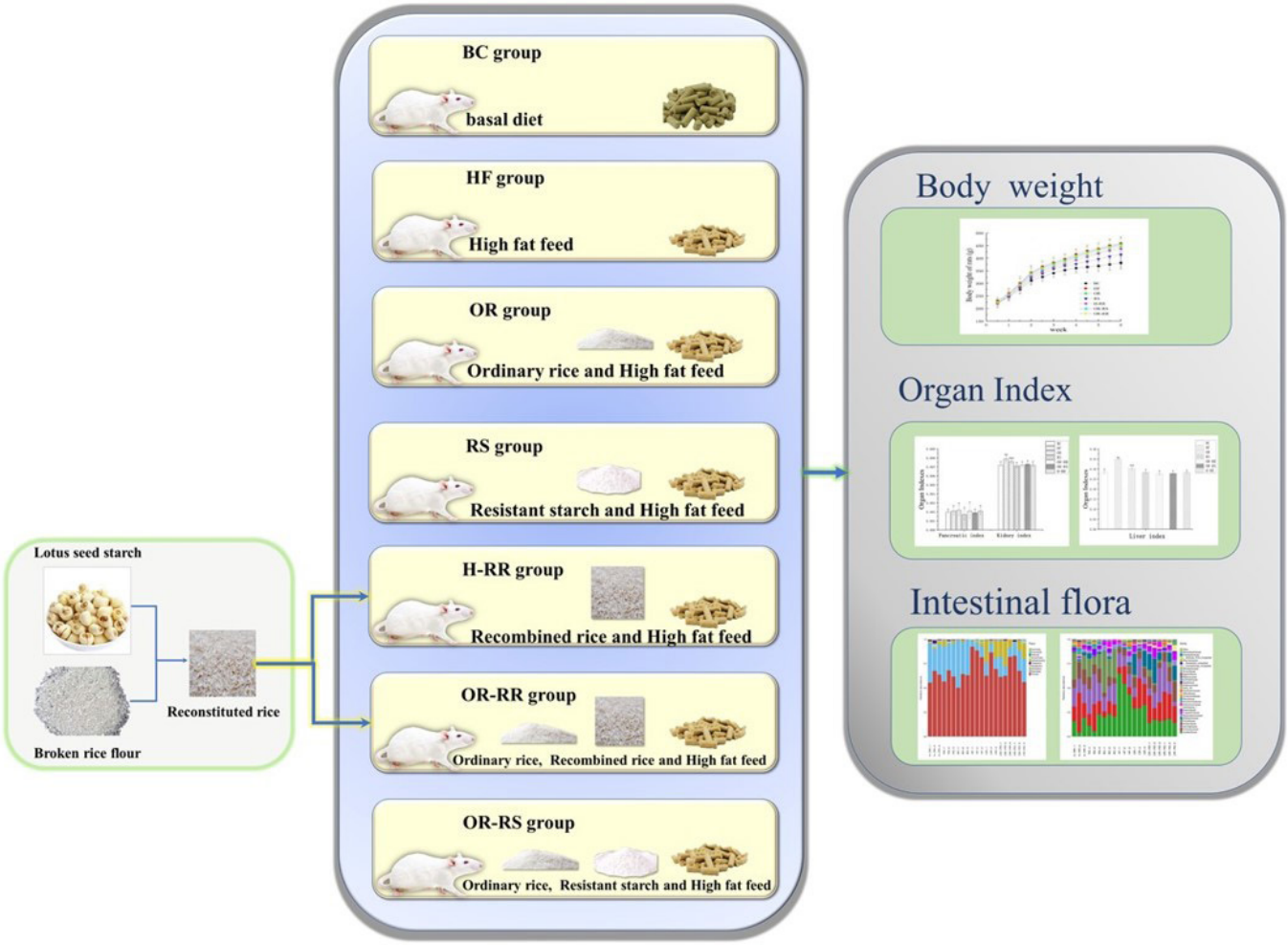

Figure 1. Experimental design. BC group (blank control), HF group (high fat feed), OR group [15 g/(kg bw/d) ordinary rice and high fat feed], RS group [15 g/(kg bw/d) resistant starch and high fat feed], H-RR group [15 g/(kg bw/d) recombined rice and high fat feed], OR-RR group [10 g/(kg bw/d) ordinary rice, 5 g/(kg bw/d) recombined rice and high fat feed], OR-RS group [10 g/(kg bw/d) ordinary rice, 5 g/(kg bw/d) resistant starch and high fat feed] groups.

data processing, and ANOVA was used for significance analysis. $P < 0.05$ means significant difference.

3 Results

3.1 Effect of LSBR on body weight and organ indexes of rats

It can be seen from Figure 2 that the weight of rats in each group was close at the beginning of the test, and there was no significant difference. However, with the progress of the test, the weight of rats in each group showed different changes. After dietary intervention, the body weight of rats in BC, RS and H-RR group increased by only 159.01 g, 192.43 g and 209.14 g, while that of rats in HF group increased by 240.18 g. At the completion of the experiment, the body weight of rats in HF group > OR group > OR-RR group > OR-RS group > H-RR group > RS group > BC group. The minimum weight of BC group was 381.75 g and the maximum weight of HF group was 459.33 g. There was significant difference among H-RR and RS groups and other groups ($P < 0.05$), but there was no significant difference between OR, OR-RR and OR-RS and HF group. The above results showed that dietary intervention in H-RR and RS groups can significantly improve the weight gain caused by HFD.

It can be seen from Figure 2b-2c that after feeding for 8 weeks, the liver weight and kidney index of HF group were significantly higher than those of other groups ($P < 0.05$), while the liver and kidney index of OR group were significantly different from those of other groups ($P < 0.05$) and higher than those of other groups except HF group. There was no significant difference between RS, OR-RR, OR-RS, H-RR group and BC group. The data in the table showed that the growth and development of rats were in good condition during the test. OR-RR, OR-RS, H-RR and RS groups can improve the adverse effects of HFD on liver and kidney.

3.2 OTU cluster analysis

After high-throughput sequencing using Illumina MiSeq sequencing platform, a total of 1688390 valid sequences were

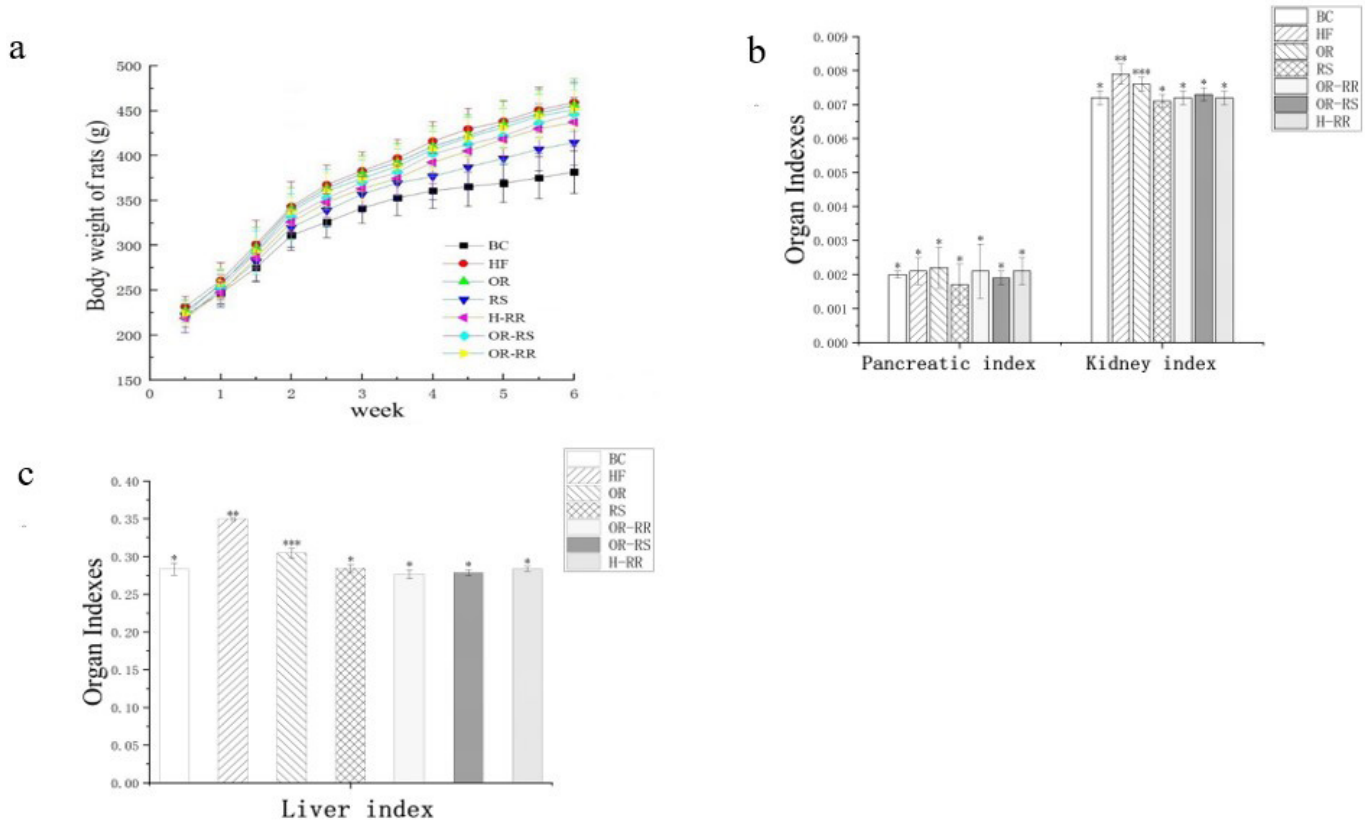

Figure 2. Analysis of body weight and organ index in rats. Error bars indicate mean values ± standard deviations. Different characters (*) on the top of each bar indicate significant different ($p < 0.05$) between bars for each organ index tested.

obtained from 7 groups of 21 samples after sequencing data quality optimization. Among them, 223696 were in BC group, 217771 in HF group, 241390 in RS group, 257824 in H-RR group, 234594 in OR-RS group, 191565 in OR-RR group and 321550 in OR group.

3.3 Grade abundance curve

Rank abundance curves are a way to analyze diversity. Rank abundance curve can reflect both species abundance and evenness. Species abundance is reflected by the length of the curve on the horizontal axis. The larger the range of the curve on the horizontal axis, the higher the species abundance; Species evenness is reflected by the shape (smoothness) of the curve. The flatter the curve, the higher the species evenness (Wu et al., 2022). The rarefaction curve is suitable for describing the change of species that can be detected with the increase of the sample size.

As shown in Figure 3a, the intestinal flora of rats changed after HF, OR, RS, OR-RR, OR-RS and H-RR intervention. Compared with the intestinal flora of HF group, the rank abundance curves of other groups have a larger span and more gentle. H-RR and RS groups are similar to BC group. The results showed that under the intervention of H-RR, RS, or, OR-RR and OR-RS, the change of intestinal flora caused by HFD is inhibited, and the effect of H-RR and RS groups is the most obvious.

According to the rarefaction curve of Figure 3b, with the increase of sequencing quantity, the number of OTUs increased significantly and then slowly. Then, with the deepening of sequencing quantity, the number of OTUs did not change significantly. The amount of sequencing in this experiment is sufficient to reflect the basic situation of changes in intestinal flora.

3.4 Alpha diversity and PCoA analysis of intestinal flora in the rat

The α-diversity is related to two main factors, namely species richness and species uniformity of individual distribution. These two factors are used to describe the relative abundance or proportion of individuals in a species. The effects of different diets on community diversity index (expressed as Shannon) and community abundance (expressed as Chao1) were showed in Figure 4a. It can be seen from Figure 4a that the addition of resistant starch can improve Chao1, while HFD will lead to the decrease of Chao1, indicating that the intestinal community abundance of rats under the intervention of resistant starch and LSBR diet has been improved. The Chao1 index of OR group and H-RR group was similar to that of BC group. Through the observation of Figure 4b, HFD will lead to the decrease of Shannon index, while the addition of resistant starch and LSBR will improve Shannon index and increase community diversity.

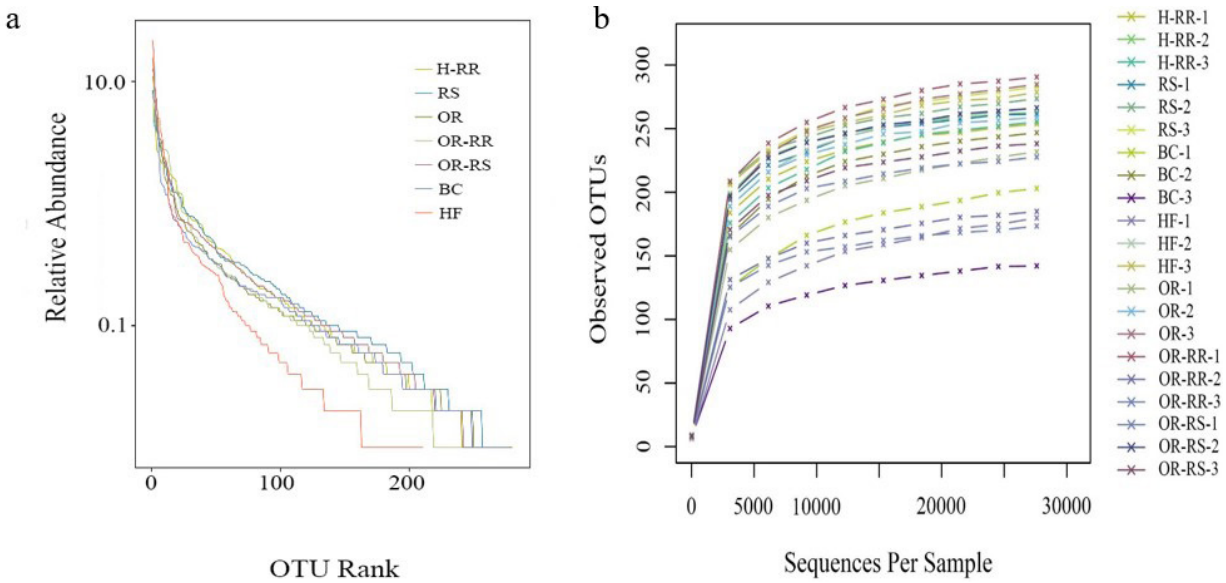

Figure 3. Rank abundance curves (a) and rarefaction curve (b) of intestinal flora when rats fed with different diets.

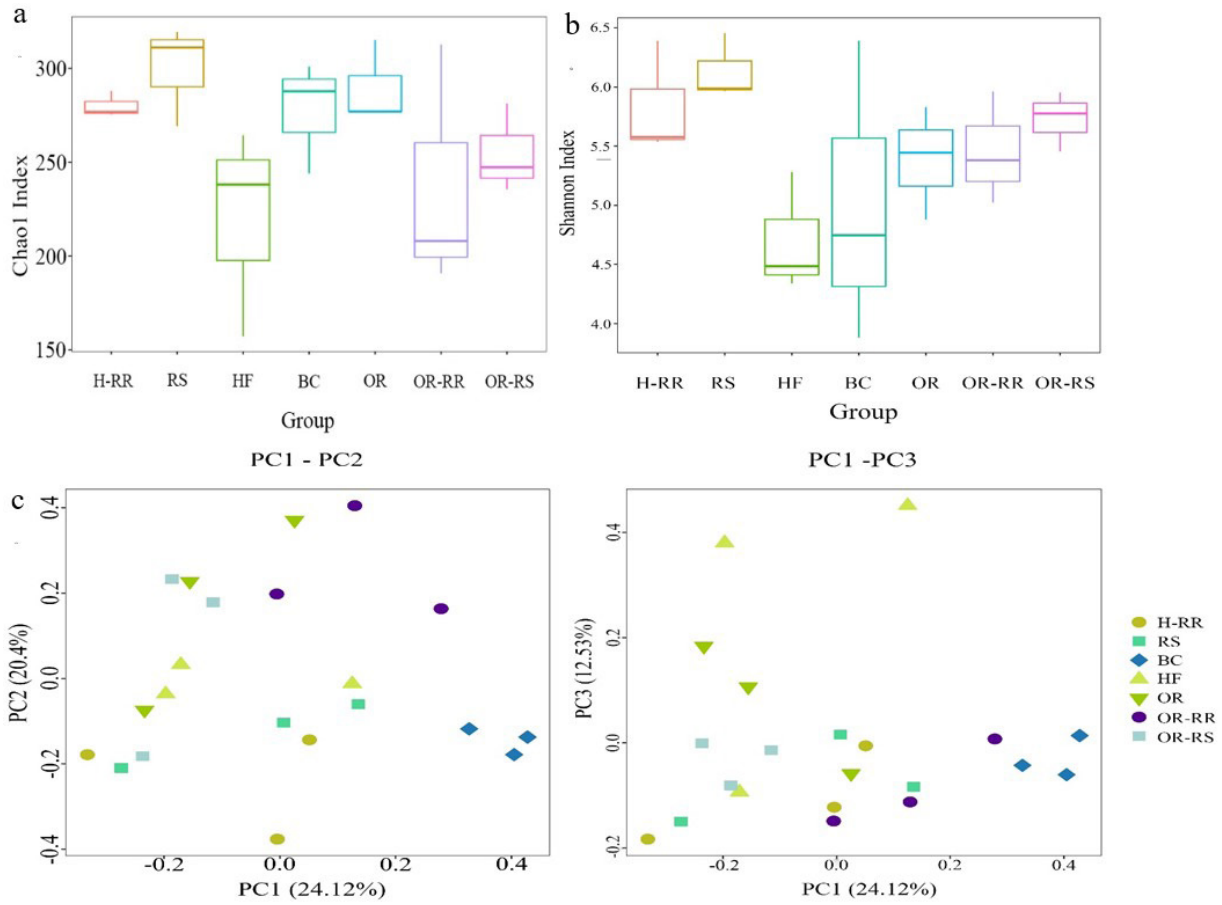

Figure 4. Alpha diversity and PCoA analysis of intestinal flora in rats. (a) Chao Index; (b) Shannon Index; (c) PCoA analysis

PCoA is a visual method to study the similarity or difference of data, which is similar to PCA. After sorting through a series of eigenvalues and eigenvectors, select the eigenvalues mainly in the first few places to find the most important coordinates in the distance matrix (Figure 4c). The results showed that the first three factors, PC1, PC2 and PC3, accounted for 24.12%, 20.4% and 12.53% of the variation, respectively. The results reflected the significant difference of microflora structure between BC

group and other groups. The microflora structure of H-RR group is similar to that of RS group, while the microflora structure of OR group, OR-RR group and OR-RS group is similar due to the addition of ordinary rice starch, which indicates that the changes of intestinal microbiota are regulated by different diets in different ways. Through the observation of PC1- PC3, it can be found that the microflora structure of other groups is similar except HF group, indicating that other recombinant rice has a certain regulatory effect on the intestinal flora of rats.

3.5 The difference of intestinal flora distribution at the level of phylum and family

The distribution of intestinal flora in fecal samples of 21 rats in seven groups at the phylum classification level was analyzed in Figure 5a. The intestinal flora of different phyla was marked with different colors, and the ordinate represented the abundance of flora. It can be seen from the all samples flora in the figure, Firmicutes and Bacteroidetes respectively account for 68.82 ± 12.06% and 22.32 ± 12.50%, which are the dominant flora. The abundance of Firmicutes in HF group was significantly higher than that in other groups except OR group ($P < 0.05$). The abundance of Bacteroidetes in BC group, H-RR group and RS group was significantly higher than that of HF control group, OR group, OR-RS group and OR-RR group ($P < 0.05$).

As can be seen from Figure 5b, the relative abundance of Lactobacillaceae in H-RR and RS group was significantly higher than that in other groups except HF group. The relative abundance of Bacteroidaceae in BC, RS and H-RR group was significantly higher than that of other groups. The highest relative abundance of Prevotellaceae in BC group was 30.24 and the lowest in HF group was 0.59. There were significant differences between the above two groups and other groups. The relative abundance of Prevotellaceae in RS group and H-RR group was 13.96-14.74, while that in the other three groups was only 1.50-3.92. The abundance of Prevotellaceae in normal rats' intestinal flora was significantly higher than that of non-normal rats (Liu et al., 2022), indicating that recombinant rice could effectively restore rats' disordered intestinal flora.

3.6 The difference of intestinal flora distribution at genus and species level

The composition and changes of intestinal flora in rats under different dietary intervention were shown in Figure 6, using the cluster heat map analysis of the top 30 genera and species of relative abundance. After the intervention of seven different diets, there are similarities and differences in the composition of intestinal flora at the genus and species levels, and there are great differences in the composition of some main intestinal flora. To be specific, there were significant differences in the composition of intestinal flora at the genus and species level between RS group, H-RR group and HF group, while there were similarities and differences between OR-RS and OR-RR and HF group.

The relative abundance of *Lactobacillus* in HF group was significantly higher than that in other groups ($P < 0.05$). The relative abundance of Lactobacillus in RS group and H-RR group decreased. The highest relative abundance of *f_Prevotellaceae _ Unclassified* in the BC group was 22.3767 and the lowest in the HF group was 0.4500. The relative abundance of *f_Prevotellaceae _ Unclassified* could only be increased to 8.6933 in the RS group. There was no significant difference in the relative abundance of *Alloprevotella* among BC, H-RR and RS groups, but it was significantly different in other groups.

3.7 Comparison of relative abundance of the five strains with the largest difference between H-RR and HF group

The horizontal coordinate is the classification name of the five strains with the largest difference between the two groups of samples, and the vertical coordinate is the relative abundance of strains in Figure 7. The relative abundance of *Bacteroides, [Eubacterium]_xylanophilum_group, Romboutsia,*

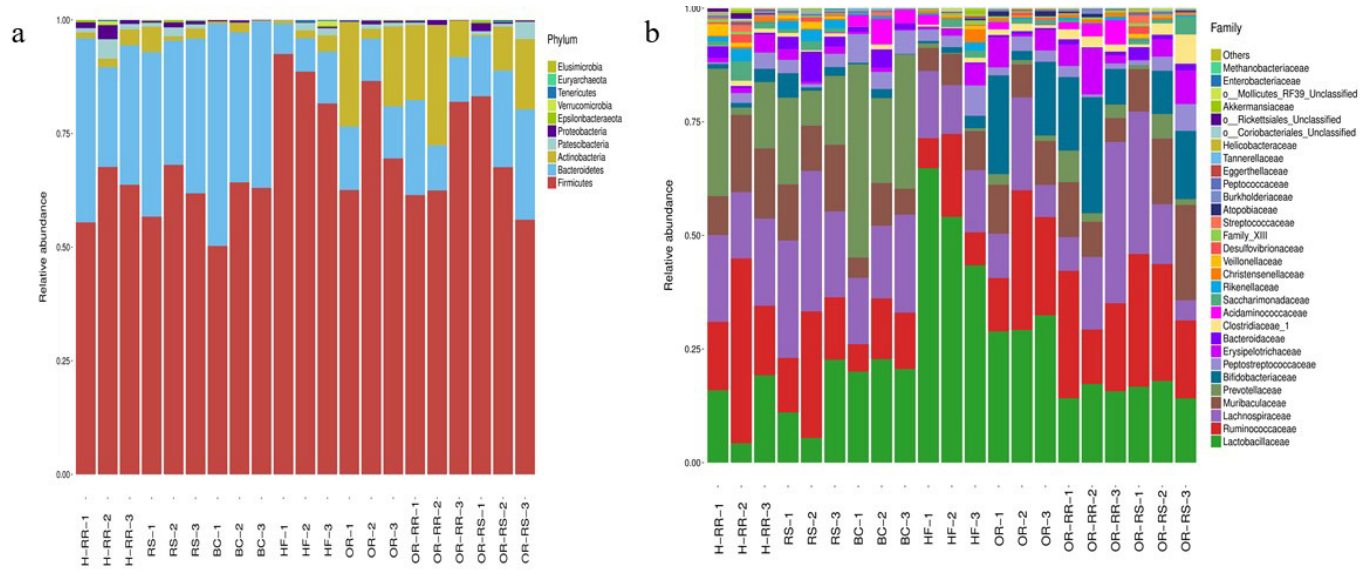

Figure 5. Relative abundance of major intestinal flora at phyla level (a)/family level (b).

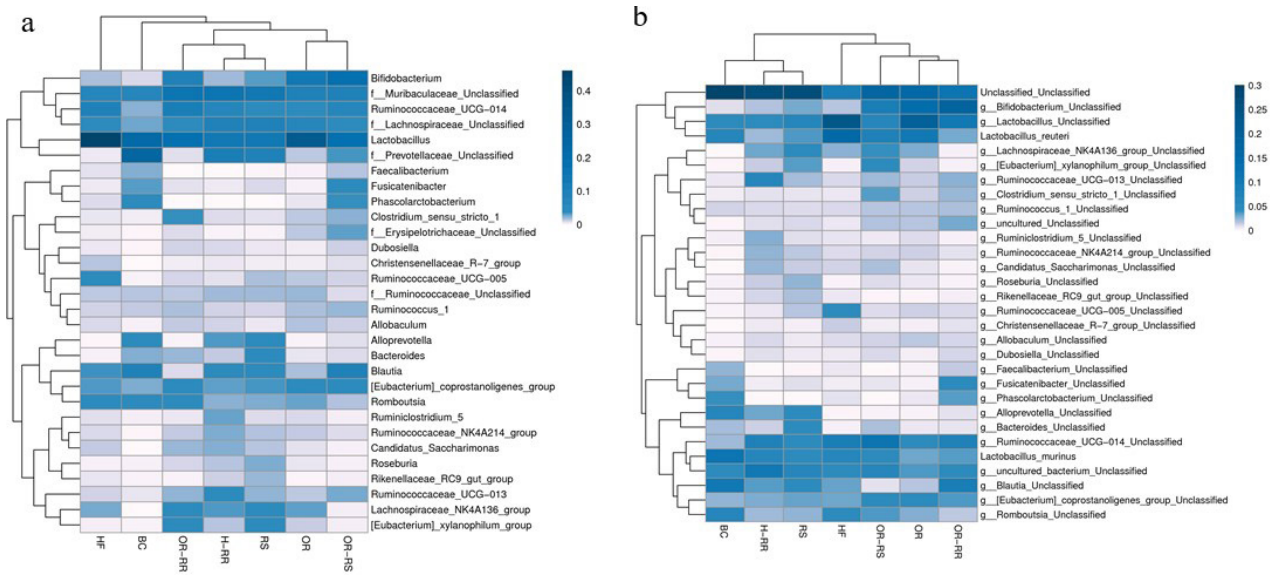

Figure 6. Heat map of species distribution at genus (a)/species (b) level.

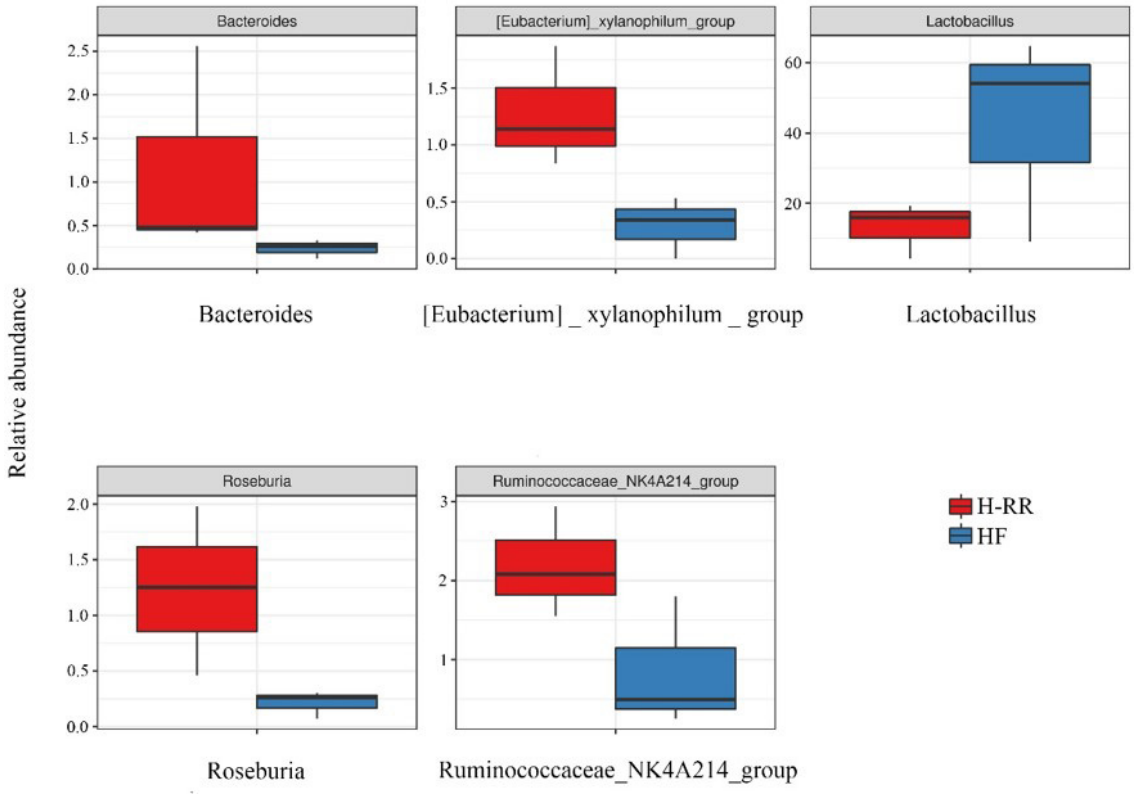

Figure 7. Relative abundance distribution of the five strains with the largest difference between H-RR and HF group.

Ruminococcaceae_NK4A214 in the H-RR group was higher than that in the HF group ($P < 0.05$), while the relative abundance of *Lactobacillus* was lower than that in the HF group ($P < 0.05$).

4 Discussion

Long term HFD can lead to obesity, and obesity can cause cardiovascular diseases, type II diabetes and non-alcoholic fatty liver disease (Fruh, 2017). Feeding high fat diet will aggravate liver and kidney burden in rats, resulting in a high organ index (Shang et al., 2017). In this study, LSBR was added to the HFD to reduce the body weight and organ index of rats to inhibit obesity.

Dietary environment has a huge impact on the formation of intestinal flora, which leads to increased risk of metabolic syndrome and other common diseases, such as obesity, enteritis,

diabetes and Alzheimer's disease (Hang et al., 2022; Kang et al., 2019). Intestinal microorganisms with special composition in the intestine may be the main regulator of host metabolism, promoting the interaction between functional foods and host health (Hang et al., 2022). The study on the composition of intestinal flora by metagenomic technology found that there were significant differences in the composition of intestinal flora between obese and non-obese individuals, and the low abundance of intestinal microbiota was associated with obesity and hyperlipidemia (Chatelier et al., 2013).

Dietary intervention can affect the composition of intestinal microbiota and unhealthy conditions caused by microbial imbalance (Rogers & Aronoff, 2016). Several studies have profoundly confirmed the link between intestinal flora imbalance and HFD, including that HFD can lead to the reduction of intestinal flora richness and diversity (Bang et al., 2019). In this study, metagenomic technology was used to observe the effect of LSBR on the changes of intestinal flora in rats caused by HFD. The results showed that HFD induced intestinal flora imbalance, which was consistent with the findings of Hua et al. (2020). The intake of LSBR led to significant changes in the overall intestinal microbial community structure, which partially improved the structural and ecological imbalance induced by HFD. By observing the span of the rank abundance curves on the horizontal axis, it is found that the span of the rank abundance curves in RS group is the largest on the horizontal axis, indicating that the addition of resistant starch improves the species abundance of rat intestinal flora, while the species abundance of H-RR and BC is similar. At the same time, Chao1 index of each group is RS > OR > H-RR > BC > OR-RS > OR-RR > HF, and the Shannon index is RS > H-RR > OR-RS > OR-RR > OR > BC > HF, it showed that the intake of resistant starch or LSBR could improve the abundance and diversity of intestinal flora in rats, and restore the intestinal flora intervented by HFD to the level of normal diet rats.

Compared with lean rats, obese rats and obese rats induced by HFD, an ecological imbalance characterized by an increase in the relative abundance of Firmicutes: Bacteroidetes (F/B) has been identified (Grigor'eva, 2020). 16S rDNA sequencing studies of obese human fecal samples, including a study of 154 twins, revealed that human obesity was also associated with decreased diversity and proportion of Bacteroides in feces, and weight loss was associated with increased proportion of Bacteroides (Wan et al., 2020). Firmicutes can effectively break down those indigestible carbohydrates and convert them into absorbable short chain fatty acids, so as to obtain more energy. Bacteroides are involved in many important metabolic activities in the human colon, including the fermentation of carbohydrates, the utilization of nitrogen-containing substances and the biotransformation of bile acids and other steroids.. Bacteroides can produce bile salt hydrolase (BSH). Under the action of BSH, bound bile acids will be transformed into free bile acids. As a key enzyme to catalyze the hydrolysis of amide bonds on bound bile acids, BSH promotes cholesterol or bile to enter the microbial membrane. The increase of bacteroidete abundance can increase the activity of BSH and reduce the production of secondary bile acids- α/β. The content of steroid dehydrogenation rate limiting enzyme (Gu et al., 2017). In this study, the analysis found that the F/B of HF group reached 11.04 and was significantly higher than that of other groups ($P < 0.05$), while the F/B of BC, H-RR and RS group was only 1.56, 2.17 and 1.96 respectively, which was significantly different from other groups ($P < 0.05$). This result is consistent with the research result of Xu et al. (2020). In addition, the relative abundance of Proteobacteria in BC, RS and H-RR group was significantly different from that in other groups ($P < 0.05$). The increase of Proteobacteria may lead to obesity or Alzheimer's disease (Sun et al., 2020). Interestingly, the intestinal flora of patients with moderate and severe covid-19 was characterized by lower proportion of F/B and higher abundance of Proteobacteria (Moreira-Rosário et al., 2021). Studies have shown that HFD can reduce the abundance of Prevotella and Alloprevotella (Kong et al., 2019), while the abundance of Prevotella and Alloprevotella increased significantly after adding resistant starch or LSBR ($P < 0.05$). In conclusion, LSBR and resistant starch can reduce the relative abundance of Proteobacteria and increase the relative abundance of Prevotella and Alloprevotella by adjusting F/B.

Bacteroides can metabolize polysaccharides and oligosaccharides to provide nutrition for the host and other intestinal microbial residents. High fat diet can reduce the abundance of Bacteroides; however, the study found increasing the abundance of Bacteroides can reduce the body weight of rats (Wang et al., 2020). *[Eubacterium]_ xylanophilum_group*, *Ruminococcaceae_ Nk4a214* is an effective butyric acid producing bacterium in the intestine. As an important short chain fatty acid produced by the fermentation of "non-digestible carbohydrates" by intestinal flora, butyric acid can provide energy for intestinal epithelial cells, so as to maintain the function of intestinal mucosal barrier (Liu et al., 2018). Previous studies have shown that dietary fiber can promote the growth of *[Eubacterium]_xylanophilum_Group* to stimulate butyric acid production by intestinal microorganisms (Mukherjee et al., 2020). *[Eubacterium]_xylanophilum_group* was negatively correlated with body weight and serum total cholesterol level (Wei et al., 2021a). Studies have shown that *Ruminococcaceae* is significantly negatively correlated with overall fat and regional fat, while *Romboutsia* is positively correlated with body fat measurement, which is different from the results of this experiment (Wei et al., 2021b). This study shows that LSBR can inhibit obesity and adjust organ index by increasing the relative abundance of beneficial bacteria such as *Bacteroides, [Eubacterium]_xylanophilum_group, Romboutsia,* and *Ruminococcaceae_NK4A214*.

5 Conclusion

H-RR can significantly reduce body weight and reduce the increase of organ index related to obesity, and the effect of high-dose intake of H-RR is the best. Although our results should be interpreted carefully, the beneficial effect of H-RR may be related to the interaction of host intestinal microflora, mainly by restoring the abundance, diversity and composition of host intestinal microflora. In particular, *Bacteroides, [Eubacterium]_ xylanophilum_group, Ruminococcaceae_NK4A214* mediates changes in host metabolism. As H-RR helps to restore the intestinal microbiota damaged by HFD, it can play an important role in inhibiting obesity as an auxiliary diet.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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