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Original Research Article

Enhanced fermentation by *Rhizopus oligosporus* promotes antioxidant capacity and intestinal development of Tibetan tea in mice

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Abstract

Fermentation of Tibetan tea is necessary during processing and its quality will be under the influence of microorganisms. In this study, *Rhizopus oligoporus* was inoculated in Tibetan tea for intensive fermentation to investigate its effect on the antioxidant function of Tibetan tea in vitro and in vivo and on the intestinal development of mice. After intensive fermentation of Tibetan tea by *R. oligoporus*, it was prepared as tea extract and the change of its antioxidant capacity was measured. Meanwhile, mice were administered the tea extracts. Antioxidant activity in liver and the effects on intestinal development of mice were determined. The results showed that the total antioxidant capacity (T-AOC), the ability to scavenge hydroxyl radicals and ABTS radical scavenging capacity of fermented tea were remarkably higher compared with non-fermented tea ($P < 0.05$). Fermented tea also increased T-AOC, catalase (CAT) and glutathione (GSH) activities and decreased malondialdehyde (MDA) content in the livers of mice ($P < 0.05$). The mRNA expression of SOD, CuZn-SOD, GSH and IκBα in the liver and intestinal barrier genes Mucin-1, Mucin-2, Claudin-1 and ZO-1 were upregulated ($P < 0.05$), whereas the mRNA expression of NF-κB and COX-2 were downregulated in the liver. In addition, fermented tea increased the numbers of l *Lactobacillus* spp. and Bifidobacterium spp. in the intestine of mice. The colonic muscle layer of mice thickened, and the crypt became shallower. After the intensified fermentation of *R. oligoporus*, the antioxidant capacity of Tibetan tea was strengthened, and it also enhanced the intestinal development of mice.

Keywords: Tibetan tea; *Rhizopus oligoporus*; antioxidant; fermentation; Probiotics; Intestinal barrier

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1. Introduction

Tibetan tea is a type of post-fermented tea, which is a representative Chinese black tea product with a history of more than 1300 years and is known as a tea for the livelihood of Tibetan compatriots. Relevant studies have shown that Tibetan tea is rich in nutrients such as tea polyphenols, theophylcin, flavonoids, amino acids, proteins, tea polysaccharides and other components (Xie et al., 2018, Liu et al., 2022). It is also known to regulate the intestinal flora, anti-inflammatory profile, and antioxidant properties. Balanced intestinal flora is vital for human health as it promotes metabolism, digestion, energy supply, nutrition, and defense mechanism (Wang et al., 2023) . Owing to its chemical profile it plays crucial role in improving the health of Tibetan people against alpine cold, hypoxia, strong radiation, and lipid/protein rich food (Xie et al., 2018, Liu et al., 2022).

In response to cellular responses, humans naturally produce reactive oxygen species (ROS) or free radicals, but excessive amount of ROS in body could be health/life threatening due to oxidation of macromolecules and degradation of cells (Qin et al., 2022, Vakifahmetoglu-Norberg et al., 2017). Some studies also reported that excessive number of free radicals in the host body promote the implication and progression of cancer, aging, and low immunity (Forman and Zhang, 2021). Nowadays, free radical scavenging compounds such as catalase (CAT), co-enzyme Q, vitamin A, glutathione (GSH), melatonin, polyphenols, ascorbic acid, α-linolic acid etc. are widely investigated to prevent and treat the ROS associated diseases (Ighodaro and Akinloye, 2017). It was found that improving the antioxidant system in the body can enhance the immunity of the body and promote intestinal development (Bernatoniene and Kopustinskiene, 2018).

Rhizopus oligoporus is a kind of fungus with wide distribution, strong reproduction ability and wide biological activity which is often used in fermented food to enhance food value (Lee et al., 2020). *R. oligoporus* can produce endocrine substances through the cell wall mainly including glycosidase, protease and amino acid molecules (Zhang et al., 2022). However, source of raw material, combination of fermenting microbes, and differences in processing technologies substantially influence the flavors and health function of Tibetan tea. At present, the role of microbial fermentation to improve the antioxidant capacity and to promote intestinal development is still little known.

Therefore, the present study was designed to evaluate the effects of *R. oligoporus* fermentation on the antioxidant capacity of Tibetan tea (both In vitro and In vivo) and on intestinal development in mice. T-AOC activity, hydroxyl radical scavenging capacity, and ABTS radicals were determined from water extract of fermented tea. Meanwhile, mice were gavage by tea extract, detection of the activities of T-AOC, CAT and GSH and the mRNA expression of Cu/Zn-SOD, SOD, GSH, IκBα, NF-κB and COX-2 in the liver of mice was performed. Additionally, mRNA expression of Mucin-1, Mucin-2, Claudin-1 and ZO-1, intestinal histological changes of mice, and the number of beneficial bacteria in the intestine were observed. This research explored the effect of fermentation of *R. oligoporus* on the health function of Tibetan tea, and the results can provide a reference for further development and application of Tibetan tea.

2. Materials and Methods

2.1. *R. oligoporus* culture and fermentation design

Thermotolerant *R. oligoporus* (BNCC 353555) was purchased from Beijing Beina Chuanglian Biotechnology Research Institute and cultivated at 37 ℃ (180 rpm) on the rotary shaker (Radobio, Shanghai, China) for 24 h and then inoculated into YPD broth. Prior to its use in fermentation, *R. oligoporus* was diluted with sterile water to a concentration of 10^6 colony forming units (CFU)/mL. Tibetan tea (one bud with five to seven leaves) was provided by Sichuan Youyi Tea Co., Ltd. (Ya'an, China). 15 g of Tibetan tea was inoculated with 1 mL bacterial suspension in the experimental group while, the same volume of sterile water was added to the control group. Mixture of Tibetan tea was fermented in a thermo-hygrostat incubator with gradient temperature for 7 days. Temperature profile during fermentation was as follows: 1-2 days at 37 ℃, 3-4 days at 40 ℃, and 5-7days at 45 ℃. Samples were stored at -20 ℃ for further analysis.

2.2. Preparation of extract

Fermented Tibetan tea was extracted in distilled water (1:15, w/v) by boiling for 30 min. The filtered tea leaves were extracted again for 30 min as described above. The extracts were combined and freeze-dried. The dry infusion was stored at -80°C.

2.3. In vitro determination of antioxidant capacity of Tibetan

The antioxidant capacity of 0.6 g unfermented/fermented tea was determined by dissolving it in 100 mL distilled water. T-AOC activity, hydroxyl radical scavenging and ABTS radical scavenging ability of Tibetan tea were measured according to the instructions (Solarbio, Beijing, China).

2.4. Ethical statement

Six-week-old C57BL/6J mice (male, weight：22-26 g) were obtained from SPF Biotechnology Co., Ltd. (Beijing, China). Animal experiments were conducted in accordance with the Southwest Medical University Guidelines and approved by the Ethical Committee on Laboratory Animals (Approval No.: swmu20220137).

2.5. Animal experimentation design for *In vivo* analyses

All mice had been raised in a set of pathogen-free conditions with controlled humidity levels of 50–55% and temperatures of 20–25°C, at the same time the light conditions were 12h light and 12h dark. Animals were fed and watered ad libitum and acclimatized for 1 week. Eighteen male mice were distributed randomly into three groups as follows: control (C) group, unfermented Tibetan tea (T) group, and R. oligoporus fermented tea (R) group. Tea extract was dissolved by adding water to make a concentration of 12 mg/mL. Mice in groups T and R were treated with 0.2 mL of tea extract by gavage daily, while the C group accepted the same volume of double distilled water for a total of 13 days. The mice were euthanized 2 weeks after gavage. middle of the colon (2~3cm) was washed with phosphate buffer solution (PBS) and then stabilized in 4 % paraformaldehyde for the purpose of follow-up histological analysis. Liver and intestinal tissues as well as contents of the intestine were collected rapidly and stored at -80°C for further analysis.

2.6. Measurement of antioxidant activity of liver

Liver tissue from mice was collected, washed with sterile saline and a 10% (w/v) liver homogenate was prepared. According to the manufacturer's instructions (Beijing Solarbio), kits were used to measure total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and malondialdehyde (MDA) activity respectively.

2.7. RNA extraction and RT-PCR

Total RNA was extracted from the liver and colon of each group of mice by the one-step method using Trizol. 2% agarose gel electrophoresis and Nanodrop were used to determine integrity and purity of the extracted nucleic acid. RNA was immediately reverse transcribed into cDNA. The expression of antioxidant-related genes in the liver and intestinal barrier-related genes in the colon was quantified using real-time fluorescence PCR. β-actin gene (Actb) was used as the housekeeping gene and three replicates of each sample were used to calculate the relative expression of target genes using the 2-ΔΔCt method.

2.8. Histological analysis

Colons were embedded in paraffin, sectioned and stained with hematoxylin and eosin（HE）for histological analysis, and small intestinal cells were detected with Alcian blue-periodic acid Schiff (AB-PAS) staining. The sections were observed using a microscope (BX53M; Olympus, Tokyo, Japan).

2.9. Determination of intestinal probiotics in mice

Total DNA of intestinal contents was extracted by DNA extraction reagent. DNA integrity and purity were detected by 1% agarose gel electrophoresis and Nanodrop respectively. RT-qPCR was used to count intestinal microorganisms using log copies/mL, as described previously (Wang et al., 2021). Primer sequences were shown in Online Resource 1.

2.10. Statistical analyses

All data are displayed as the mean ± SEM.

Statistical analysis of all data was performed using GraphPad Prism 9.0, and p-values less than 0.05 were considered statistically significant.

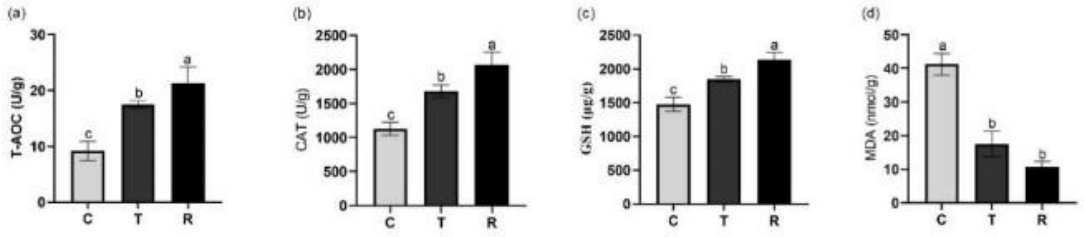

Fig. 1. Effect of antioxidant enzyme (a) T-AOC, (b) CAT, (c) GSH, (d) MDA of fermented tea on liver cells.

3. Results

3.1. *In vitro* determination of antioxidant capacity of fermented tea

Significant T-AOC, hydroxyl radical scavenging ability, and ABTS radical scavenging ability were detected in the water extract of tea samples. Overall, the results indicated an increasing trend in antioxidant capacity of fermented Tibetan tea as compared to unfermented formulation (**Table 1**). The T-AOC activities of T group and R group were 51.18 ±2.00 U/g and 56.22 ±1.17U/g, respectively. The T-AOC of fermented tea was remarkably high In vitro than that of unfermented tea ($p < 0.05$). Compared with that of T group (6.78±0.92%), the scavenging ability of hydroxyl free radical of R group (9.38±1.15%) was significantly increased ($p < 0.05$). The ABTS scavenging ability values of T and R group were 25.90±2.58 mmol/ (0.02 g/mL) and 33.24±1.65 mmol/ (0.02 g/mL)), respectively, with a significant difference ($p < 0.05$).

Table 1. *In vitro* comparison of antioxidant capacity between unfermented and fermented Tibetan tea.

Feature	T group	R group
T-AOC (U/g)	51.18 ±2.00^{b}	56.22 ±1.17^{a}
Hydroxyl radical scavenging power (%)	6.78±0.92%^{b}	9.38±1.15%^{a}
ABTS free radical scavenging ability (mmol/ (0.02 g/mL))	25.90±2.58^{b}	33.24±1.65^{a}

Notes: The data are expressed as the mean ± SEM. The different lowercase letters indicate significant differences ($p < 0.05$). The a and b represent significant differences among two groups, the same letter represents no significant difference between two groups ($p > 0.05$), and different letters represent significant differences between two groups ($p < 0.05$)

3.2. *In vivo* determination of antioxidant capacity of fermented tea

3.1.1. *Effects of fermented tea on antioxidant enzymes in the liver*

The results of oxidase activity (**Fig. 1**) in mouse liver showed that concentrations of T-AOC, CAT and GSH were higher in both T and R groups as compared to group C. Notably, R group was statistically more significant than T group ($P<0.05$). The MDA levels was significantly lower in groups R and T compared with C group ($P<0.05$)

3.2.2. *Effect of fermented Tibetan tea on expression of antioxidant genes in liver*

The results of *In vivo* analysis to determine the expression level of antioxidant genes on the liver of mouse gavage with *R. oligoporus* fermented Tibetan tea showed that R group remarkably enhanced the mRNA expression levels of SOD, CuZn-SOD, GSH and IκBα, and considerably down-regulated those of NF-κB and COX-2 ($p <0.05$) as compared ti C group. Similar trend was observed upon comparing the results of fermented tea and non-fermented tea where mRNA expression levels of SOD, CuZn SOD and IκBα were obviously up-

regulated in R- group while NF-κB was significantly lower ($p < 0.05$) as compared to T group (**Fig. 2**).

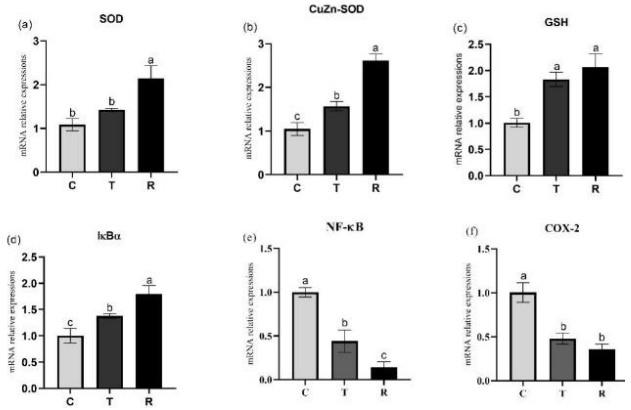

Fig. 2. Expression level of the antioxidant-related target gene（a） SOD, (b) Cu/Zn-SOD, (c) GSH, (d) IκBα, (e) NF-κB, and (f), COX-2 in the liver cells of mouse models gavage with R. oligoporus fermented Tibetan tea.

3.2. Effects of fermented tea on intestinal development and probiotics in mice

Histological analysis revealed that the mice consuming *R. oligoporus* fermented Tibetan tea exhibited improved intestinal development (**Fig. 3**). This indicates that although histological structure of the colon remained intact and the goblet cells were arranged in order in the recesses in all groups, the intestinal muscle layers were thicker in the two groups of mice fed Tibetan tea. The edge of crypt was regular, and the depth of the crypt was shallower, particularly in the R group.

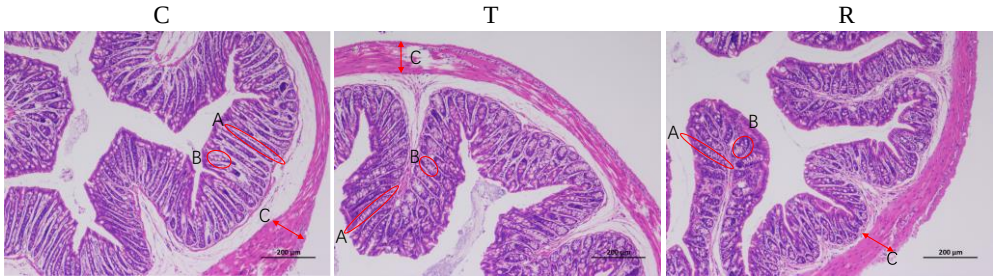

Fig. 3. Results of H&E staining (100×) to determine the effects of control (C), unfermented tea (T), and fermented tea (R) on intestinal development at (A) muscle layer, (B)goblet cell, (C) crypt in mice.

3.3. Effect of fermented Tibetan on expression levels of genes related to intestinal barrier functions

Enhancing the barrier of the intestine is beneficial for the development of intestine. After 13 days of gavage of *R. oligoporus* intensive fermented Tibetan tea, the mRNA expression levels of Mucin-1, Mucin-2, Claudin-1, and ZO-1 were significantly increased in the colon of the mouse as compared to those who were fed with C and T group ($p < 0.05$; **Fig. 4**).

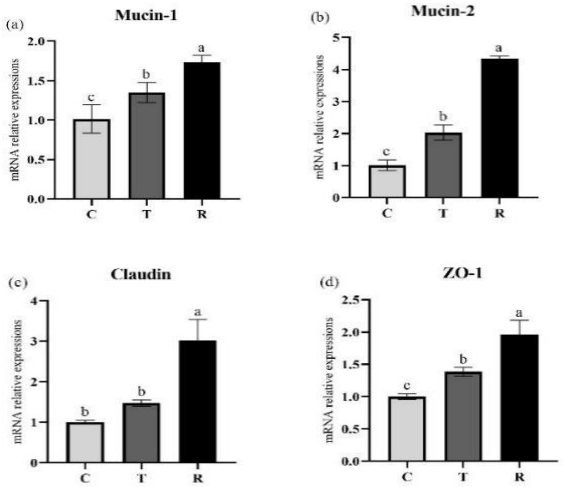

Fig. 4. Levels of mRNA expression correlated with intestinal barrier. (a) Mucin-1, (b) Mucin-2, (c) Claudin-1, and (d) ZO-1.

The number of *Lactobacillus* spp. and *Bifidobacterium* spp. in intestinal tract of mice in group R was significantly increased than that in group C (p<0.05). There were no significant changes in *Enterococcus spp*. and *Streptococcus spp*. in the intestines of mice in the three groups (**Fig. 5**). The numbers of *Lactobacillus spp*. in the intestine of group C, T, and R were 8.28±0.45 log (copies/mL), 8.478±0.32 log (copies/mL) and 9.88±0.45 log (copies/mL), respectively. *Bifidobacterium spp*. were 8.49±0.41 log copies (copies/mL), 8.87±0.35 log copies/mL and 9.40±0.21 log copies/mL in the C, T, and R groups respectively.

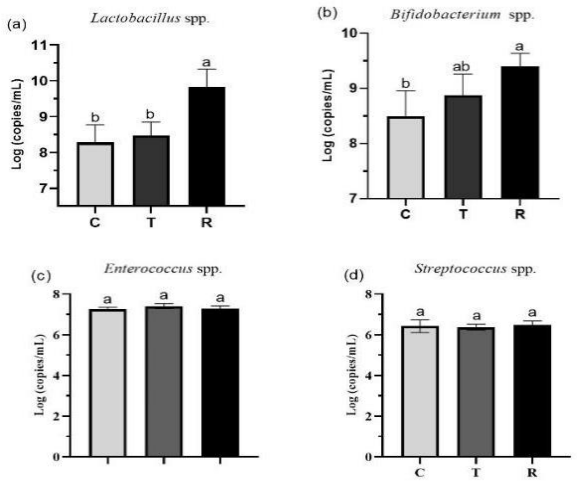

Fig. 5. Effect of fermented Tibetan tea on the concentration of intestinal probiotics (a) *Lactobacillus spp*., (b) *Bifidobacterium spp*., (c) *Enterococcus spp*., (d) *Streptococcus spp*. in the mice.

4. Discussion

Tibetan tea is the oldest dark tea in China that is prepared by the fermentation therefore, its quality is closely related to microorganisms. During the pile- fermentation process, microorganisms and their extracellular enzymes can initiate various biochemical reactions such as degradation, polymerization, oxidation, methylation and glycosylation (Zhang et al., 2022) that enriches Tibetan tea with a variety of bioactive substances such as tea polyphenols, tea polysaccharides, theanine, theophylcin and caffeine. Presence of these compounds in the Tibetan tea draws attention to its antioxidant related health functions. Many bioactive substances (e.g., tea polyphenols) are rich in hydroxyl and 2-phenylbenzopyrane structures, which form the basis of antioxidant activity of Tibetan tea (Lee et al., 2020). The active ingredients of Tibetan tea can be directly used as reducing oxidants to remove free radicals, as well as chelate metal ions, thus improving the activity of antioxidant enzymes (e.g., CAT and GSH) by regulating antioxidant-related genes in the host, so as to prevent and treat some chronic diseases related to oxidative stress (Lee et al., 2020).

R. oligoporus is often use in fermented food. It was found that solid fermentation of *R. oligoporus* can help transform nutrient quality and improve antioxidant capacity of food and beverages. The mycelium growth of *R. oligoporus* can hydrolyze proteins into peptides, degrade oligosaccharides into monosaccharides, and increase the content of soluble polyphenols (Qiang et al., 2022). T-AOC, ABTS and hydroxyl radical scavenging ability are commonly used indexes in the In vitro determination of antioxidant ability (Forman and Zhang, 2021). The stronger the free radical scavenging ability, the stronger the antioxidant ability of Tibetan tea. In this study, the antioxidant capacity of Tibetan tea after intensive fermentation by *R. oligoporus* was significantly enhanced (p <0.05). Additionally, studies have confirmed that microbial fermentation can affect the metabolism of tea components and improve the bioavailability (Ighodaro and Akinloye, 2017).

The liver is the main organ for synthesizing various antioxidant enzymes. The mouse liver's total antioxidant capacity (T-AOC) is made up of diverse antioxidant compounds and antioxidant enzymes. CAT and GSH are important antioxidant enzymes in mice (Bernatoniene and Kopustinskiene, 2018). Antioxidant enzymes inhibit the production of free radicals, maintain normal cell function and metabolic health, and enable cells to survive and develop without free radical damage (Zhou et al., 2018). The improvement of antioxidant capacity can protect a variety of biochemical organs in the body and protect the body cells from free radical damage, thus reducing the level of inflammatory stress in the internal body. The level of MDA in mice reflect oxidative stress levels. A number of studies

have confirmed that Tibetan tea can improve the activities of T-AOC, CAT and GSH in the liver of mice, reduce MDA levels, and improve the antioxidant capacity of mouse liver (Cheng et al., 2021). The effectiveness of fermented tea was more powerful than that of unfermented Tibetan tea. SOD is the initial key link in the regulation of superoxide anion free radicals in mice, and CuZn-SOD is another expression form of SOD. GSH can remove peroxide and inhibit the formation of hydroxyl free radicals (Yin et al., 2021). COX-2 is a speed-limiting enzyme which is involved in the arachidonic acid metabolic pathway, which regulates inflammation and oxidative stress in mice. IκBα acts as inhibitory protein, which sequesters the transcription factor NF-κB in the cytoplasm as an inactive complex (Tang et al., 2019b). Our results showed that compared with unfermented tea, fermented tea significantly increased the enzyme activity and antioxidant gene expression in mouse liver. At the same time stimulate the IκBα/NF-κB pathway that improves the antioxidant ability of mice. It was confirmed that *R. oligoporus* could improve the antioxidant capacity of Tibetan tea.

Similar to liver, intestinal tract also plays essential role in intestinal function and intestinal microbiology. The crypt depth can reflect the formation rate of intestinal epithelial cells. The increased rate of cryptogenic cell formation in shallow crypt is beneficial to enhance the intestinal damage repair effect (Hariyanto et al., 2022), and the shallow crypt increases the area of intestinal food absorption, which is conducive to enhancing the absorption and utilization of Tibetan tea active substances in mice, thus enhancing the intestinal shielding function of mice.

Results of present study indicated that *R. oligoporus* fermented Tibetan tea could regulate intestinal microorganisms in mice and promote the abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. The findings are in accordance with previously reported studies (Li et al., 2021). *Bifidobacterium* spp. and *Lactobacillus* spp. are the most important probiotics in intestinal microbiota, and they are also considered as new sources of natural antioxidants (Tang et al., 2019a). The host's antioxidant system can respond to changes brought about by oxidative stress with the help of the metabolites (e.g. short-chain fatty acids) they produce. In addition, they can produce antioxidant enzymes such as SOD and CAT in the host to enhance the host's antioxidant system. Meanwhile, the increase of *Bifidobacterium* spp. and *Lactobacillus* spp. numbers is beneficial to optimize intestinal microflora and promote intestinal shielding function. They can affect epithelial growth and survival, development and regulation of innate and adaptive immunity, and competitive rejection of pathogens(Wang et al., 2018). It also antagonizes and competes with opportunistic pathogens, ameliorates the function of digestion, participates in the maturement of the immune system early in life and maintains immune homeostasis, neuro-regulation, and synthesis of vitamins and also other beneficial compounds during life (Sun, 1990). At the same time, the mouse intestinal barrier can also resist the invasion of microorganisms and other foreign pathogens into the body and prevent intestinal metabolites from being discharged through lymphatics. Mucin proteins (Mucin 1 and Mucin 2) are the main components of intestinal mucus (Yu et al., 2021). They perform a central role in upholding the gut barrier. ZO-1 and Claudin-1 are key members of the tight junction proteins which are essential in keeping the functional integrity of the intestinal epithelial barrier. (Wang et al., 2017). In our study, fermented Tibetan tea significantly promote the mRNA expression of Mucin-1 and Mucin-2 and tight link (Claudin-1 and ZO-1). The results indicates that fermented tea can enhance the physical and chemical barrier of mouse intestinal tract. Therefore, *R. oligoporus* fermentation of Tibetan tea can strengthen the oxidation resistance of Tibetan tea In vivo and In vitro as well as can promote the intestinal development of mice.

5. Conclusion

Fortified fermentation of *R. oligoporus* could improve the antioxidant capacity of Tibetan tea in both In vitro and In vivo (P<0.05) setups. It has also been confirmed that fermented tea can promote intestinal development, enhance intestinal shielding function and facilitate the growth of probiotics (*Bifidobacterium* spp. and *Lactobacillus* spp.), thus optimizing the intestinal environment.

Therefore, this study revealed that the potential of Tibetan tea as a functional food was enhanced by the intensive fermentation of *R. oligoporus* because its health functions were stronger after the intensive fermentation.

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Conflict of Interest

It is declared that authors have no conflict of interest.

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