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Effects of traditional Chinese medicines on immunity and culturable gut microflora to *Oncorhynchus masou*

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ABSTRACT

The present study was conducted to evaluate the effect of dietary traditional Chinese medicines on the growth, immunity, and composition of culturable gut microflora in *Oncorhynchus masou*. Diets were formulated to contain no medicine (control), antitoxic decoction (A), general antiphlogistic decoction (B), or Herbae Artemisiae Capillariae decoction (C). Fish were manually fed twice daily till apparent satiation for 30 days. Compared with that in the control group, supplementation with the three kinds of Chinese herbal medicine enhanced fish growth significantly ($P < 0.05$). The activities of liver superoxide dismutase and glutathione peroxidase in the treatment groups were significantly higher compared with those in the control group ($P < 0.05$). The quantity of intestinal microflora was higher in the treatment groups compared with that in the control group. Moreover, there were some effects of dietary Chinese herbal medicine on the composition of intestinal microflora. Microflora of *Pseudomonas* sp., *Psychrobacter* sp., *Microbacterium* sp., *Macrococcus* sp., *Burkholderia* sp., and *Arthrobacter* sp. were found in the treatment groups, whereas there were none in the control group. There was a significant increase in their amounts in the treatment groups ($P < 0.05$). The three kinds of traditional Chinese medicines can improve the growth and immunity of *Oncorhynchus masou* and affect the quantity and composition of intestinal microflora.

1. Introduction

Masu salmon, *Oncorhynch*us masou (Brevoort, 1856), is a member of the family Salmonidae, ranging from the Kamchatka, Kuril Islands, Sakhalin, Primorsky Krai south through Korea, Taiwan, and Japan [1]. It was introduced to China in 1996, and has been an important species in coldwater fish aquaculture owing to its delicate meat and delicious taste [2]. At present, the artificial aquaculture of this fish is being practiced in more than ten provinces in China. However, diseases caused by bacterial and parasitic pathogens (e.g., *Aeromonas hydrophila*, *Gyrodactylus elegans*) are becoming severe and have resulted in a significant drop in growth and health, and increased morbidity and mortality. Severe disease outbreaks occurred in masu salmon fry in some farm with mortalities reaching a level of 50%–90% [3], thereby affecting the promotion and development of aquaculture and high-density culture. It is difficult to control the proliferation of these pathogens owing to inadequate basic research and inappropriate treatment. Therefore, exploring effective methods for preventing disease in *Oncorhynchus masou* culture has become urgent.

Traditional Chinese medicine (TCM), owing to its nature, vast resources, and low cost, has been used as medicine for thousands of years [4]. As food safety issues increasingly attract the attention of consumers to healthy aquaculture practices and the use of green fishery drugs, TCM as a feed additive is of great significance to improve the immune function and disease resistance in fish [5–7]. It has been confirmed that the application of TCM in aquaculture plays an important role in the Asian aquaculture industry [8,9]. TCM can be used as antibiotics or immune stimulants to prevent fish diseases and may be a potential alternative vaccine [10] and antibiotic [11,12]. In addition, TCM are also believed to improve growth, stimulate appetite, and relieve stress [13–18]. For instance, the survival rate of common carp (*Cyprinus carpio*) fed with Astragalus root extract was higher after challenged

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with *Aeromonas hydrophila* [19]. After feeding with *Achyranthes aspera*, the level of superoxide anion production, lysozyme, and plasma bactericidal activity of *Labeo rohita* increased [20]. Mortality owing to bacterial infections were significantly reduced in the *Vibrio harveyi*-fed grouper *Epinephelus tauvina* [21]. Moreover, different studies have shown that the use of a herbal mixture has better disease resistance effects than a single herbal medicine, e.g., a herbal formulation containing *Ocimum basilicum*, *Cinnamomum zeylanicum*, *Jugpans regia*, and *Mentha piperita* enhanced the non-specific immunity and disease resistance to *Aeromonas hydrophila* in the common carp [22]. TCM compound formulation involves a complex interaction between drugs. Compared with the use of single herb, it may have a better effect on immune stimulation or improved disease resistance in fish, and hence, is a more cost-effective approach [23]. So far, there is a lack of information on the use of compound preparations containing more than three types of TCM as feed supplements [23]. Thus, the study of TCM may be beneficial in the prevention and treatment of fish diseases in the aquaculture industry.

Many microorganisms parasitize or live in symbiosis with the digestive tract of an healthy animal. They form a micro-ecosystem, which has an impact on the growth and health of animals. Moreover, it is affected by individual traits, environmental conditions, and type of food [24]. It has been proved that TCM can enhance individual immunity and adjust the micro-ecosystem in the micro-ecosystem of the digestive tract, and thus, enhance disease resistance in cultured fish [25]. Thus, it might have a positive implication for *Oncorhynchus masou* farming.

In this study, three kinds of TCM, recorded in many Chinese herbal monographs, were investigated. Antitoxic decoction can enhance immunity to protect the body from being infected [26]. General antiphlogistic decoction was initially recorded in Dong-Yuan-Shi-Yao-Fang, and used for reducing inflammation, clearing away toxic materials, and preventing epidemic diseases [27]. Herbae Artemisiae Capillariae decoction is traditionally used for protecting the liver and cholagogue action, which are important for the maintenance of health and regulation of body functions [28]. In this study, we aimed to estimate the effects of dietary TCM on the growth, immunity, and intestinal microflora in *Oncorhynchus masou*, and provide a theoretical basis for the practice in salmonid aquaculture.

2. Materials and methods

2.1. Feed preparation

The herbal medicines were mixed in specific proportions as shown in Table 1. The herbal powder was immersed in sterilized water (molecular biology grade) for 30 min (1:10 *w/v*) and then boiled for 30 min. The extract was removed, another portion of sterilized water (1:10 *w/v*) added, and boiled again for 30 min. The extracts were filtered, pooled, and prepared into three kinds of TCM: Group A (antitoxic decoction), Group B (general antiphlogistic decoction), and Group C (Herbae Artemisiae Capillariae decoction). All decoctions were stored at 4 °C for further use.

Diet formulation and composition are shown in Table 2. Before mixing, the ingredients were finely ground (< 250 μm) using a laboratory grinder and mixed with other ingredients and micronutrients (vitamins and minerals). The fish feed containing no TCM was used as the control. The extracts were mixed thoroughly with the other feed ingredients. The dry ingredients were mixed thoroughly for 15 min, then the lipid source was added and mixed in a mixer for 10 min. Dietary mixtures were extruded using a double-screw extruder (G-250; Machine Factory of Muyang, China) to form 2.0 mm pellets. The feed was dried (moisture approximately 120 g kg^{-1}) in an air-convection dryer at 55 °C, sealed in plastic bags, and stored at −20 °C for further use.

Table 2
Nutrients and ingredients used to prepare the basal diets (air-dry basis, %).

Ingredients	Composition
White fish meal	48.0
Soybean meal	15.0
Wheat middings	20.5
Blood meal	4.0
Soybean lecithin	2.0
Fish oil	5.0
Soybean oil	4.0
Premix*	1.5
Nutritional level	
Gross energy (MJ kg^{-1})	19.46
Moisture	12.03
Crude protein	44.14
Crude lipid	12.26

Note: Premix 1.50%, include: Choline 0.2%; Magnesia 0.2%; Antimildew 0.05%; Antioxidant 0.025%; Betain 0.10%; Vitamin premix 0.3%; Mineral premix 0.2%; Zeolite 0.425%. 2 Vitamin and mineral mixture provide the following (kg^{-1} of the diet): V_C 100 mg; V_A 15000 IU; V_{D3} 3000 IU; V_E 60 mg; V_{K3} 5 mg; V_{B1} 15 mg; V_{B2} 30 mg; V_{B6} 15 mg; V_{B12} 0.5 mg; Nicotinic acid 175 mg; Folic acid 5 mg; Pantothenic acid 50 mg; Biotin 2.5 mg; Inositol 300 mg; Fe 25 mg; Zn 30 mg; Cu 3 mg; Mn 15 mg; I 0.6 mg.

2.2. Feeding experiment

Oncorhynchus masou (body weight 51.75 ± 1.06 g) were obtained from Bohai Coldwater Fish Base, Chinese Academy of Fishery Sciences, with 100 fish per tank (600 L, 12 tanks in total). The fish were acclimatized in laboratory conditions for 14 days and adapted to the experimental control feed prior to the experiment. Each treatment was performed in triplicate. A treatment without TCM added in fish feed was used as the control. The TCM compositions of the treatment feeds were as follows: Group A: 0.08% extract; Group B: 0.05% extract; and Group C: 0.04% extract. During the feeding trial, fish were manually fed twice daily till apparent satiation, and it was ensured that no feed was left over. The fish were cultured in a flow-through system with spring water (water flow rate: approximately 2.0 L/s) for 30 days. Water quality was measured (using YSI 6600 V2-2, Ohio State, USA) daily during the experimental period, and data were as follows: temperature, 11.50–11.75 °C; dissolved oxygen, 7.5–9.8 mg/L; pH, 7.2–7.5; and ammoniacal nitrogen, < 0.2 mg/L. Fish were weighed both at the start and end of the feeding trial.

Table 1
The decoction composition of TCM.

Group	Composition
A	*Cyrtomium fortunei* 9 g, *Folium isatidis* 15 g, *Radix isatidis* 15 g, *Radix shikonin* root 15 g, *Radix sophorae* 9 g, *Capillary artemisia* 9 g, *Platycodon grandiflorum* 6 g, *Radix liquiritiae* 6 g
B	*Scutellaria baicalensis* 15 g, *Coptis chinensis* 15 g, *Citrus reticulata* peel 8 g, *Radix liquiritia* 10 g, *Radix scrophulariae* 12 g, *Platycodon grandiflorum* 6 g, *Forsythia suspense* 20 g, *Radix isatidis* 30 g, *Radix bupleuri* 30 g, *Lasiosphaera fenzlii* 10 g, *Bombyx batryticatus* 20 g, *Cimicifugae racemosae* 6 g, *Mentha haplocalyx* 3 g, *Fructus arctii* 12 g
C	*Capillary artemisia* 8 g, *Rheum officinale* 6 g, *Scutellaria baicalensis* 6 g, *Coptis chinensis* 6 g, *Rehmannia glutinosa* root 6 g, *Flos lonicerae* 6 g, *Forsythia suspensa* 4 g, *Radix isatidis* 4 g, *Radix curcumae* 3 g, *Radix liquiritiae* 4 g

2.3. Sample collection

Ten fish were selected randomly from each tank. Blood from the caudal vein of five fish was collected into heparin tubes and centrifuged at 3000 *g* for 10 min at 4 °C, to obtain plasma samples. Samples were obtained from dissected liver and spleen. All samples were immediately stored at −80 °C in a freezer for immunity determination. Another 5 fish were sampled randomly from each tank. The surfaces of the fish were disinfected with 75% alcohol before being taken into a bio-clean room. After further disinfection, the abdominal cavity was opened, the intestine wiped with 75% alcohol and washed four times with sterile water. Tissue samples including the foregut, midgut, and hindgut were collected with an aseptic scissor, washed with aseptic physiological saline, weighed, and then prepared into a homogenate with aseptic physiological saline (1:4).

2.4. Immunity determination

Before measuring the immunity indices, the pooled livers and spleens were manually homogenized in a glass homogenizer with 0.86% NaCl (*w/v*) to obtain 10% homogenate. Liver and spleen lysozyme activity was measured spectrophotometrically following the method of Sankaran and Gurnani (1972) [29]. Activities of superoxide dismutase (SOD) in the liver and plasma, glutathione peroxidase (GSH-px) in the liver, and nitric oxide (NO) content in the plasma and spleen were analyzed spectrophotometrically using diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute, China).

2.5. Culture and count of bacteria

The intestinal contents (1 g) and aseptic physiological saline (9 mL) were mixed and made into a homogenate (10^{-1}); then gradient dilution (1:10) was continued. Diluents (0.1 mL) were coated in freshwater fish agar medium (FWA) [30]. The bacteria were grown on nutrient FWA for 2 d at 37 °C. Single colonies were inoculated into FWA media with the spread plate method [30] and were then grown on nutrient FWA for 18–24 h at 25 °C. The number of bacteria (CFU·g^{-1}) were calculated.

2.6. Purification and identification of bacteria

Different colonies were inoculated into FWA medium with streak culture according to bacterial morphology, color, and wettability. This process was repeated thrice. After purification, 30–40 single colonies were selected randomly from each culture plate to inoculate into the FWA slant medium for further use. The bacteria were identified using the 16S rRNA PCR method [31] described below. The samples were thawed on ice, and then genomic DNA were separately extracted using the Ezup Stool DNA kit (Shenggong, Shanghai, China) based on the manufacturer's protocol and stored at −80 °C. The integrity of the DNA samples was assessed visually using agarose gel electrophoresis on 1.0% and quantified using a Qubit v2.0 fluorometer (Life Technologies, Darmstadt, Germany). The DNA concentration was determined by using a fluorescence spectrophotometer (Lumina, Thermo Fisher, USA). To identify bacterial isolates, amplification of 16S rRNA from positions 28 to 1492 was performed according to Espejo et al. (1998) [32] using a universal primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCRs were performed in triplicate using 15 μL reaction system with 0.5 μL each primer (μmol), 1.0 μL DNA template (20 ng μL^{-1}), 1.5 μL 10 × PCR ExTaq Buffer, 1.0 μL dNTPs (2.5 mmol), 0.15 μL 10 × ExTaq polymerase (5 U μL^{-1}). The PCR amplification conditions were 1 cycle of 98 °C for 3 min (initial denaturation), followed by 25 cycles of 98 °C for 15 s (denaturing), 50 °C for 30 s (annealing) and 72 °C for 30 s (extension), and finally 1 cycle of 72 °C for 5 min (final extension). Te amplified PCR products were examined by 2% gel electrophoresis, purified by using the MinElute Gel Extraction Kit (Qiagen) to remove the unspecific DNA fragments and quantitated by using Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The products were pooled together with equal amount and sequenced on the Illumina HiSeq platform (Hiseq 2500 PE250, Illumina, USA).

Table 3
Effects of dietary TCM on weight gain rate and specific growth rate (n = 3).

Treatment	WGR (%)	SGR (% day^{-1})
Control	16.99 ± 0.87[a]	0.75 ± 0.03[a]
Group A	21.13 ± 1.52[b]	0.91 ± 0.06[b]
Group B	26.77 ± 2.16[c]	1.13 ± 0.09[c]
Group C	20.62 ± 1.79[b]	0.89 ± 0.07[b]

Note: No significant differences with the same subscript in the same column ($P > 0.05$).
Weight gain rate (WGR) = [(Final body weight - initial body weight)/initial body weight] × 100%.
Specific growth rate SGR = (Ln final weight - Ln initial weight) × 100%/day.

2.7. Statistical analysis

All statistics were performed using the SPSS package (version 23.0). All data were expressed as a mean ± standard deviation of replicates (n = 3) and subjected to one-way ANOVA and Duncan's multiple range test. Difference was considered significant at $P < 0.05$. The results of 16S rRNA PCR were analyzed with BLAST.

3. Results

3.1. Effects of dietary TCM on growth

As shown in Table 3, the weight gain rate and specific growth rate of the treatment groups were significantly increased compared with that of the control group ($P < 0.05$). No mortality was observed during the 30 days of experimentation.

3.2. Effects of dietary TCM on immunity

Effects of dietary TCM on immunity are shown in Table 4. The liver SOD and GSH-px activities in the treatment groups were significantly higher compared with that in the control group ($P < 0.05$). There were no significant differences in plasma SOD, plasma NO, spleen NO, liver lysozyme, and spleen lysozyme among the groups ($P > 0.05$).

3.3. Effects of dietary TCM on quantity of intestinal microflora

As shown in Fig. 1, the level of culturable gut microflora in the treatment groups showed an increasing trend. The levels were significantly ($P < 0.05$) higher in all the treatment groups compared with that in the control group, especially for group A, which was over ten times higher. In addition, the intestinal microflora level in group A showed a decreasing trend from the foregut to the hindgut.

3.4. Effects of dietary TCM on the composition of intestinal microflora

As shown in Table 5, culturable gut bacteria belong to 12 genera: *Chryseobacterium* sp., *Pantoea* sp., *Enterobacter* sp., *Kluyvera* sp., Bacillus sp., *Pseudomonas* sp., *Ralstonia* sp., *Psychrobacter* sp., *Microbacterium* sp., *Macrococcus* sp., *Burkholderia* sp. and *Arthrobacter* sp. respectively. All the genera belong to 4 phyla. *Pantoea* sp., *Kluyvera* sp., *Enterobacter* sp., *Psychrobacter* sp., *Pseudomonas* sp., *Ralstonia* sp. and *Burkholderia* sp. belong to Proteobacteria. *Bacillus* sp. and *Macrococcus* sp. belong to Firmicutes. *Chryseobacterium* sp. belong to Bacteroidetes. *Microbacterium* sp. and *Arthrobacter* sp. belong to Actinobacteria.

In the control group, the dominant bacterium was *Chryseobacterium* sp. (36.4%), belonging to phylum Bacteroidetes, family

Table 4
Effects of dietary TCM on non-specific immunity of *Oncorhyrchus masou*.

Index	Control	Group A	Group B	Group C
Liver T-SOD (U/mgprot)	22.40 ± 5.08[a]	30.41 ± 4.07[b]	34.07 ± 6.60[b]	28.75 ± 4.94[b]
Liver GSH-px (U/mgprot)	88.38 ± 80.15[a]	235.32 ± 111.01[b]	266.84 ± 144.13[b]	267.49 ± 120.54[b]
Plasma T-SOD (U/mL)	214.79 ± 3.78	214.98 ± 20.55	183.17 ± 42.38	197.45 ± 14.62
Plasma NO (μmol/L)	12.98 ± 4.37	13.37 ± 3.04	12.88 ± 2.86	12.39 ± 2.20
Spleen NO (μmol/L)	0.88 ± 0.10	0.86 ± 0.16	0.76 ± 0.19	0.80 ± 0.11
Liver lysozyme (U/mL)	569.58 ± 149.62	427.97 ± 44.84	447.55 ± 201.01	475.52 ± 58.05
Spleen lysozyme (U/mL)	7791.90 ± 2113.74	7213.87 ± 2317.45	7791.91 ± 2110.58	8624.28 ± 1452.23

Note: No significant differences with the same subscript in the same row ($P > 0.05$).

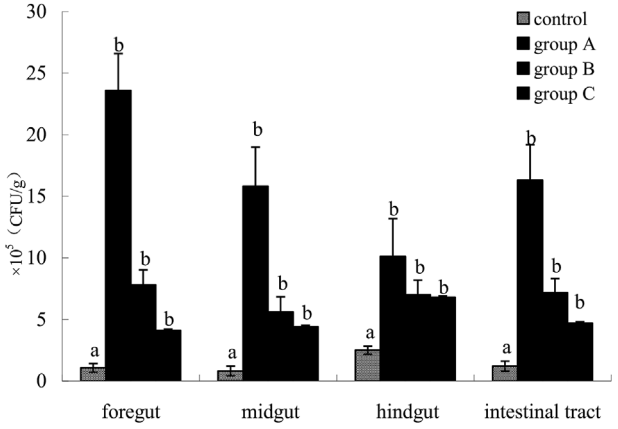

Fig. 1. Effects of dietary TCM on quantity of culturable gut microflora.

Flavobacteriaceae; in group A, the dominant bacterium was *Pantoea* sp. (29.1%), belonging to phylum Proteobacteria, class Gammaproteobacteria, and family Enterobacteriaceae; the dominant bacterium in group B was same. The dominant bacteria in group C were not obvious as in the other groups. They were *Bacillus* sp. (19.5%), *Enterobacter* sp. (18.8%), and *Chryseobacterium* sp. (16.2%) which belong to the phyla Firmicutes, Proteobacteria, and Bacteroidetes, respectively.

4. Discussion

4.1. Effects of dietary TCM on growth

TCM can enhance growth performance and the utilization of feed [33]. Grass carp (*Ctenopharyngodon idella*) fed on 10–40 g kg^{-1} honeysuckle extract (*Lonicera japonica*) diet had a significantly higher weight gain rate [34]. A basal feed containing 10–40 mg kg^{-1} TCM (*Allium mongolicum* Regel) had a beneficial effect on the growth of the juvenile northern snakehead fish (*Channa argus*) [35]. Gabriel (2019) found that dietary *Aloe vera* polysaccharides supplementation significantly increased the growth and feed utilization in African catfish (*Clarias gariepinus*) [36]. In this study, the obtained results revealed that dietary TCM enhanced the growth performance. The improvement of fish growth and feed utilization may be owing to the palatability or attractiveness of the diets, which leads to the increase in feed intake and the improvement in growth performance [37]. In addition, dietary TCM supplementation might inhibit potential pathogens in the digestive tract, increase the number of beneficial microorganisms, and/or enhance the activity of microbial enzymes, thereby improving feed digestibility and nutrient absorption.

4.2. Effects of dietary TCM on immunity

It is important to evaluate the protective effects of immune stimulants on fish. Ardó László et al. (2008) reported that feeding Nile tilapia with Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) singly or in combination for 28 days significantly increased phagocytic and respiratory burst activity of blood phagocytes, and both herbs reduced the mortality following *Aeromonas hydrophila* infection [14]. Won et al. (2010) also found that fish fed a diet supplemented with 3% Siberian ginseng, *Eleutherococcus senticosus*, residuum extract exhibited improved non-specific immunity and resistance to *Edwardsiella tarda* and *Vibrio anguillarum* infection [38]. However, the mechanism of immune stimulation of dietary TCM, especially for the antitoxic decoction, general antiphlogistic decoction, and Herbae Artemisiae Capillariae decoction were unclear, but it may be attributed to one or more of their components, e.g., flavanones, phenolic acids, and glycosides [39]. The role of antioxidants in protecting cellular components from oxidative

Table 5
Effects of dietary TCM on the composition of the culturable gut microflora of *Oncorhyrchus masou*.

Bacteria species	Control			Group A			Group B			Group C		
	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut
Chr	40.0	42.9	30.2	11.9	13.8	13.2	8.9	12.6	11.2	12.9	10.9	23.1
Pan	26.7	–	27.6	26.4	27.7	36.3	29.7	26.3	28.0	14.1	12.9	12.8
Ent	20.0	–	28.4	27.4	15.1	23.1	13.8	16.8	13.6	15.3	14.9	24.8
Ral	13.3	–	–	–	–	–	10.4	22.1	9.6	16.5	17.8	
Klu	–	21.4	–	5.0	–	–	–	–	–	–	–	10.3
Bac	–	35.7	13.8	11.4	16.4	16.5	11.9	11.6	20.8	29.4	20.8	11.1
Pse	–	–	–	6.5	14.5	–	9.7	10.5	8.8	–	12.9	9.4
Psy	–	–	–	–	–	–	–	–	8.0	–	–	–
Mac	–	–	–	6.0	–	–	8.2	–	–	–	–	–
Mic	–	–	–	5.5	–	–	7.4	–	–	–	–	–
Art	–	–	–	–	12.6	11.0	–	–	–	–	–	–
Bur	–	–	–	–	–	–	–	–	–	11.8	9.9	8.5
Genera number	4	3	4	8	6	5	8	6	7	6	7	7

Note: *Chryseobacterium* sp. (Chr); *Pantoea* sp. (Pan); *Enterobacter* sp. (Ent); *Kluyvera* sp. (Klu); *Bacillus* sp. (Bac); *Pseudomonas* sp. (Pse); *Ralstonia* sp. (Ral); *Psychrobacter* sp. (Psy); *Microbacterium* sp. (Mic); *Macrococcus* sp. (Mac); *Arthrobacter* sp. (Art); *Burkholderia* sp. (Bur).

stress has been well demonstrated [40]. The obtained results herein showed that the activities of liver SOD and GSH-px in the treatment groups increased significantly ($P < 0.05$). This study showed that dietary antitoxic decoction, general antiphlogistic decoction, and Herbae Artemisiae Capillariae decoction might be helpful in enhancing the body's antioxidant defense system to some extent, so that the reactive oxygen produced by cell metabolism could be at a low level for maintaining the normal physiological function of the body.

4.3. Effects of dietary TCM on quantity of intestinal microflora

It has been reported that the number of intestinal bacteria in freshwater fish was 10^5–10^8 CFU g^{-1} [41]. Yoshimizu et al. found that it was 10^4–10^7 CFU g^{-1} in Salmonidae [30]. In comparison, the number (8.2×10^4–2.4×10^6 CFU g^{-1}) of culturable gut bacteria in *Oncorhynchus masou* was lower as the culture-based method used in this study only presented a partial picture of the microbial diversity of the intestine. The composition of culturable gut microflora was changed when fish were fed TCM. Wu et al. (2018) reported a higher quantity of intestinal microflora in gibel carp (*Carassius auratus gibelio)* fed with dietary TCM [42]. Similarly, the present study indicated significantly ($P < 0.05$) higher quantity of culturable gut microflora in all treatment groups compared with the control group, especially in group A (antitoxic decoction) and group B (general antiphlogistic decoction). In addition, the highest bacterial population was found in the foregut. However, Fu et al. (2017) found that the bacterial population was the highest in the hindgut [43], which maybe owing to the quantity of intestinal microflora being closely related to the contents of the intestine, the physical and chemical environment in the intestinal tract. Thus, further work is needed to explore the relationship of the quantity of bacteria in the foregut, midgut, and hindgut.

4.4. Effects of dietary TCM on composition and dominant bacteria of intestinal microflora

As the conventional culture-based method used in this study only present a partial picture of the microbial diversity of the intestine, which is possible to identify constituents which represent only 1% of the total population in the intestines [44], we recommend or plan on using current methods (16S rDNA sequencing using next generation sequencing or meta genomic sequencing) in future studies evaluating the effect of dietary TCM on the gut microbiota. However, the characterization and identification of the gut microbiota designated with its functional role, conventional methods should be used in combination with molecular methods like 16S rRNA sequence analysis as suggested in some studies [45–48]. As several culturable bacterial species were retrieved in the present study that have rarely, or never, previously reported as part of the intestinal microbiota in salmon, some general information is therefore presented in the following discussion.

In general, normal intestinal flora maintain the ecological balance in fish and have mainly three kinds of functions: nutrition, immune regulation, and antagonism against pathogens [49]. The difference in the normal intestinal flora and the dominant bacteria in freshwater fish are because of the fish species [49], age [50], physical condition [51], perched water environment [52,53], and whether the diets [54] are ingested properly and the category of the diets [55]. According to a previous investigation, the following bacterial species have been isolated from the intestines of Salmonidae fish using the method of the culture medium [56], include *Aeromonas* sp., *Achromobacter* sp., *Acinetobacter* sp., *Bacillus* sp., *Bacteroides* sp., *Carnobacterium* sp., *Clostridium* sp., *Corynebacterium* sp., *Enterobacter* sp., *Escherichia* sp., *Flavobacterium* sp., *Fusobacterium* sp., *Micrococcus* sp., *Moraxella* sp., *Pseudomonas* sp., *Streptococcus* sp., and *Vibrio* sp. In this study, *Pseudomonas* sp., *Enterobacter* sp., *Bacillus* sp., and *Micrococcus* sp. were isolated from the intestinal tract of *Oncorhynchus masou*; other species were also isolated, such as *Psychrobacter* sp., which have not been reported before. Besides, there were also some reported species which were not isolated, e.g., *Acinetobacter* sp. etc. The intestinal bacteria of rainbow trout (*Oncorhynchus mykiss*) vary with environmental and culture conditions [57]. The reasons for the differences observed might be the choice of the culture medium, the ratio of the bacteria, fish species, and their different culture conditions [58,59].

Studies have shown that aerobic or facultative aerobic bacteria predominated in the intestinal tract of salmonids [25]. Meanwhile, the intestinal microbiota of freshwater fish species is dominated by *Aeromonas* sp., *Pseudomonas* sp. and *Bacteroides* sp. type A, with *Plesiomonas* sp., *Enterobacteriaceae* sp., *Micrococcus* sp., *Acinetobacte* sp., *Clostridium* sp., *Bacteroides* sp. type B and *Fusarium* sp. as the less abundant groups [60]. In this study, the ratio of Enterobacteriaceae was 44% (control), 56% (group A), 42% (group B), and 36% (group C) in the intestinal bacteria among the treatments. Additionally, *Aeromonas* sp. was not detected and this may be owing to the different culture media or fish species. Peng et al. reported that the ratio of Gammaproteobacteria was the highest in intestinal bacteria [32]. In this study, the ratio was 44%, 62%, 55%, and 44% in the control, group A, group B, and group C, respectively, which was higher than that of the other intestinal bacteria.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.071.

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