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Research Article

Effect of mixture of *Artemisia argyi* and *Houttuynia cordata* Thunb extracts on growth performance, antioxidant activity, serum and hepatic lipid levels, and gut bacteria in grass carp (*Ctenopharyngodon idella*)

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Abstract

Artemisia argyi and *Houttuynia cordata* Thunb are common herbs in most regions of China. In order to test their composite effect as additives on fish, the effects of their extracts mixture on growth performance, antioxidant activity, serum and hepatic lipid levels, and gut bacteria in juvenile grass carp were evaluated. 180 fish were divided into 9 tanks with 3 replicate tanks per treatment, and fed with a control diet (A0) or one of two treatment diets containing 500 (A1) or 1000 mg/kg (A2) mixture of extracts, with equal mass of *A. argyi* and *H. cordata*, for 8 weeks. Water flow rate was 8.0 L/min, and water quality parameters were in normal range. At the end of feeding trial, fish weight gain, feed conversion ratio, and survival rate showed no difference among all groups. Significantly higher catalase (CAT) activity and lower malondialdehyde (MDA) content were observed in A1 and A2 groups. Serum triglyceride content was much lower in the A1 group and total cholesterol was lower in experimental groups compared with the control group. Transmission electron microscopy showed that the hepatic lipid droplets were smaller in the experimental groups. High-throughput sequencing identified 10 predominant phyla from gut samples, and Fusobacteria, Bacteroidetes, and Proteobacteria were the most dominant groups. Compared with the control group, in A1 group operational taxonomic unit (OTU) number, species richness (Chao1, and ACE), and α diversity (Shannon index) were higher, and gut bacteria composition was altered in A1 and A2 groups at phylum and genus levels. These results indicated that the extracts mixture of *A. argyi* and *H. cordata* can increase antioxidant activity, lower serum and hepatic lipid levels, and improve gut bacteria composition, which can be used as a green additive for grass carp.

Keywords: Plant extracts; Antioxidant; Lipids; Gut bacteria; Grass carp; Growth; Fatty liver

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Introduction

For many years, China has had the largest aquaculture industry in the world (FAO, 2017). Grass carp (*Ctenopharyngodon idella*) is one of the most important freshwater fish species in China. In 2018, 5.5 million tons of grass carp were farmed, representing 18.6% of total freshwater aquaculture production (CFSY, 2019). Fatty liver usually occurs in grass carp during the most active feeding and growing periods and resulting in reduced growth and feed efficiency, impaired immune response, and decreased nutritional quality. Development of fatty liver is complex and difficult to treat (Du, 2014).

Chinese herbs are shown to reduce fat content and protect liver in mammals (Yin *et al.*, 2008; Guo *et al.*, 2009; Dong *et al.*, 2012; Yan *et al.*, 2015). In fish, a few studies focused on the effects of Chinese herbs on lipometabolism and fatty liver. Some of these herbs or their active components are proven to regulate lipid metabolism in fish. For example, the extract of *Penthorum chinense* Pursh (Miao, 2012), *Sedum sarmentosum* (Wu, 2013), and chlorogenic acid (Yang *et al.*, 2018) could reduce triglyceride (TG) and cholesterol (CHO) level in the blood in grass carp. Similar findings are observed with the extract of *Coptis chinensis* in zebrafish (Chen, 2015). Although use of Chinese herbs as feed additives to prevent fatty liver in fish is feasible, this may not be economically viable owing to the demand for Chinese herbs in human medicine.

A. argyi and *H. cordata* are common plants and abundant natural resources, with a moderate price and wide distribution across most regions of China. Many researches indicated that both *A. argyi* and *H. cordata* have numerous biological functions including antibiotic, antiviral, antioxidant, anti-inflammatory, and immune enhancing activities in animal production (Tan *et al.*, 2014; Xu *et al.*, 2016; Liu *et al.*, 2018; Shingnaisui *et al.*, 2018). These plants can also be used as an antioxidant in feed (Li, 2013). Usually the whole plants of *A. argyi* and *H. cordata* are crushed into powders as feed additives, which produce high effective doses in the above researches. In this study, we examined the effects of extracts of *A. argyi* and *H. cordata* as a dietary supplement on growth performance, antioxidant activity, serum and hepatic lipid levels, and gut bacteria in grass carp.

Materials and methods

Plant extracts

Extracts of *A. argyi* and *H. cordata* were obtained from Shaanxi Sciphar Natural Products Co., Ltd (Shanxi, PR China).

Diets and experimental design

Formulation and chemical composition of the basal (A0) diet is listed in Table 1. Two experimental diets containing 500 mg/kg (A1 group) and 1000 mg/kg (A2 group) plant extract with equal quantities of *A. argyi* and *H. cordata*, were prepared. All ingredients were ground into a fine powder through a 100 mesh, thoroughly mixed with soybean

oil, and formed into a stiff dough by adding an equal volume of water. The dough was then pelleted with a handle noodle machine and air-dried for 24 h at room temperature (24–32°C). The pellets were then smashed into pieces, and proper particles were collected by sieving (40 mesh) and stored at -20°C until use.

Table 1: Diet formulation and chemical composition.

	Percentage		
Ingredient	**A0**	**A1**	**A2**
Fish meal	2	2	2
Soya bean meal	20	20	20
Rape seed meal	23	23	23
Cotton seed meal	9	9	9
Rice bran	5	5	5
Flour	28	28	28
Soya bean oil	3	3	3
Vitamin mixture[a]	0.5	0.5	0.5
Mineral mixture[b]	0.5	0.5	0.5
Choline	0.5	0.5	0.5
CaH_2PO_4	2	2	2
VC fat	0.5	0.5	0.5
Microcrystalline cellulose	6	5.95	5.9
Plant exacts	0	0.05	0.1
Chemical composition			
Crude protein	26.32	26.34	26.32
Crude lipid	3.71	3.71	3.71
Crude ash	6.71	6.73	6.72
Moisture	11.73	11.75	11.73

Notes: A0, basal diet; A1, basal diet containing 500 mg/kg extract mixture; A2, basal diet containing 1000 mg/kg extract mixture; a, Vitamin mix (per kg mix): vitamin A, *350 000 IU;* vitamin D, *3 450 000 IU;* vitamin E, *20 g;* menadione, *7.5 g;* thiamin, *10 g;* riboflavin, *10 g;* pyridoxamine, *12 g;* cobalamin, *20 mg;* nicotinamide, *40 mg;* folic acid, *3g;* calcium pantothenate, *30 g;* biotin, *100 mg;* ascorbic acid, *60 g;* inositol, *60 g*; b. Mineral mix(per 100 g mix): NaH2PO4, *10 g;* KH2PO4, *21.5 g;* Ca(H2PO4)2· 2H2O, *26.5 g;* CaCO3, *10.5 g;* Ca-lactate, *16.5 g;* MgSO4·7H2O, *10 g;*AlCl3·2H2O, *1.2 g;* ZnSO4·7H2O, *0.511 g;* Fe-citrate, *0.061 g;* MnSO4· 4H2O, *0.143 g;* KI, *0.058 g;* CuCl2, *0.051 g;* CoCl2·6H2O, *0.176 g;* KCl, *2.8 g.*

Experimental fish and feeding trial

Juvenile grass carp were obtained from an aquaculture farm at Jiangxi Institute of Fishery Sciences, Nanchang city. Fish were acclimatized to experimental tanks (60.0 cm×60.0 cm×80.0 cm) in a recirculating aquaculture system for 2 weeks at Jiangxi Academy of Agricultural Science (Nanchang city, China) prior to the feeding trial.

This experiment used 9 tanks with 3 replicate tanks per treatment and 180 fish of uniform size (mean body weight=7.50±0.08 g) were randomly distributed into the tanks (20 fish per tank). Each diet was randomly assigned to the 3 tanks. Fish were hand fed to

apparent satiation twice daily (09:00 and 15:00) for 8 weeks, and the feeding rate was ～3% at the beginning, and then adjusted according to ingestion of fish. Throughout the experiment process, 1/3 of the water volume was renewed with aerated water daily using an air pump to guarantee continuous aeration. Water quality parameters including pH (6.6–7.6), dissolved oxygen (>6.10 mg O/l), temperature (28.0±0.8°C), and nitrate (<0.08 mg N/l) were monitored each day.

At the end of the 8-week feeding trial, fish from each tank were deprived of feed for 24 h, and then weighed to calculate growth rate and feed utilization efficiency. Nine fish were randomly selected from each group and anesthetized with MS-222 (50.0 mg/L), and the blood samples were collected from the caudal vein with disposable syringes. The fish were then dissected and the liver and intestine were sampled. The liver was cut into small pieces with a scalpel and fixed with 2.5% glutaraldehyde. The hindgut (MacDonald *et al.*, 1986; Cahill, 1990) was gently agitated twice in PBS (pH 7.2) to remove the digesta and stored at -80°C for analysis of gut adhesive bacterial communities.

Enzyme activity and lipid peroxidation assays

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) of serum were assayed to understand the influence of plant extract supplemented fish diets on free radical levels and oxidation. The activity of antioxidant enzymes and lipid peroxidation were measured with commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China) following the manufacturer's instructions.

Serum lipid level examination

Triglyceride and total cholesterol content of serum were measured with commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China) following the manufacturer's instructions.

Hepatic lipid

Transmission electron microscopy (TEM) was performed to observe lipid droplets in the liver under different treatments. The methods were described by Ringø *et al.* (2007).

Intestinal microbial analyses

Since inter-individual variation of fish intestinal microbiota is large (Fjellheim *et al.*, 2012), equal weight hindgut pieces from 3 fish were pooled to ameliorate the variation. Total DNA was extracted using the method described in He *et al.* (2009). Illumina MiSeq high-throughput sequencing was employed to determine the predominant adhesive bacterial community in grass carp. The universal 16S rRNA 515F-806R primer pair (V3–V4) was used for PCR reaction. Barcode sequences were attached at the 5′ termini of forward and reverse primers. Standard PCR reaction conditions were employed for reactions with a high-fidelity thermostable DNA polymerase–10 μL

5×Phusion HF buffer, 0.2 μM dNTPs, 0.5 μM each primer, 1.0 U DNA polymerase, and 0.25 μg DNA template (Phusion, China). The V3–V4 region PCR amplification conditions were 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 2 min. After PCR reaction, quality of the amplified PCR products was confirmed by electrophoresis with 50 μL of the PCR reaction mixture in 1% agarose gel (1×TAE buffer) and purified using the Tiangen PCR purification kit (Tiangen, China). An equal quantity (50 ng) of each PCR amplicon was pooled and subsequently sequenced on an Illumina MiSeq high-throughput sequencing platform.

Amplicons were extracted from 2% agarose gels, purified using an AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, U.S.), according to manufacturer's instructions, and quantified using the ABI StepOnePlus real-time PCR system (Life Technologies, Foster City, USA). Purified amplicons were pooled in equimolar proportions and paired-end sequenced (2×250) on an Illumina platform, according to standard protocols. Raw data containing adapters or low quality reads would affect subsequent assembly and analysis. Thus, to get high quality clean reads, raw reads were filtered according to the following rules using FASTP (https://github.com/OpenGene/fastp): i) remove reads containing more than 10% of unknown nucleotides (N); ii) remove reads containing less than 60% of bases with quality (*p*-value) ＞20 (Chen *et al.*, 2018). Paired end clean reads were merged as raw tags using FLASH (version 1.2.11), with a minimum overlap of 10 bp and mismatch error rates of 2%. Noisy raw tag sequences were filtered using QIIME pipeline, with specific filtering conditions (Bokulich *et al.*, 2013) to obtain high-quality clean tags. Clean tags were searched against a reference database (http://drive5.com/uchime/uchime_download.html) to perform reference-based chimera checking using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar *et al.*, 2011). All chimeric tags were removed to obtain final effective tags for use in subsequent analyses. Effective tags were clustered into operational taxonomic units (OTUs) of ≥97% similarity using UPARSE pipeline. The tag sequence with highest abundance within each cluster was selected as representative sequence. Representative sequences were used to identify organisms by a naive Bayesian model using RDP classifier (version 2.2), based on SILVA Database (https://www.arb-silva.de/), with confidence threshold values ranging from 0.8 to 1. Abundance statistics of each taxon were visualized using Krona (version 2.6). Alpha diversity and beta diversity indices were calculated in QIIME.

Statistical analysis

Results are expressed as mean±standard deviation. Differences between

treatments were quantified using GraphPad Prism 5 software package (GraphPad Software Inc.). Significant differences were accepted at $p<0.05$.

Results

Growth performance

No difference was observed between groups in terms of growth performance parameters, including weight gain, feed conversion ratio, and survival rates ($p>0.05$; Table 2).

$$\text{Weight gain \%} = \frac{\text{final weight(g)} - \text{initial weight(g)}}{\text{initial weight(g)}} \times 100\%$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{total feed consumption (total feed casting - total food residue) (g)}}{\text{total final weight (g) - total initial weight (g) + total morality weight (g)}} \times 100\%$$

Survival rate % = (# of fish counted/# of stocked fish) × 100.

Table 2: Effects of dietary extract mixture on weight gain (WG), feed conversion ratio (FCR) and survival rates (SR) of grass carp[1].

Treatments	IBW	WG (%)	FCR	SR (%)
A0	151.57±0.87	214.42±14.60	2.15±0.13	100
A1	151.13±0.98	209.50±15.09	2.17±0.18	100
A2	150.80±0.44	208.33±12.88	2.24±0.14	100

[1]A0, basal diet; A1, basal diet containing 500 mg/kg extract mixture; A2, basal diet containing 1000 mg/kg extract mixture.

Enzyme activity and lipid peroxidation

After the feeding trial, no difference in SOD and GSH-PX activities was observed between any of the groups ($p>0.05$; Figure 1A, 1B). CAT activity was significantly higher in the treated groups than in control group ($p<0.05$; Fig. 1C). MDA content was significantly lower in the treated groups than in control group ($p<0.05$; Fig. 1D). No difference was observed between the treated groups ($p>0.05$).

Serum lipid level

Serum lipid level is given in Figure 2. The content of TG and total CHO were significantly lower in the treated groups than those of the control group ($p<0.05$), and no difference was observed between the treated groups ($p>0.05$).

Hepatic lipid

As observed by TEM, droplet size in the control group was larger than those of the treated groups (Fig. 3).

Intestinal microbial analyses

A total of 531,794 valid reads were obtained from 9 samples of hindgut of grass carp fed control or experimental diets. For each sample, the OTU number ranged from 47 to 257, with coverage of 99%. No difference was detected between the control group and group A2

in terms of OTU number and α diversity, including Simpson and Shannon indices, as well as species richness estimates, calculated as Chao1 and ACE (p>0.05). However, group A1showed significantly higher number of OTUs, species richness and α diversity, except the Simpson index, (p<0.05) compared with those of the control group (Table 3).

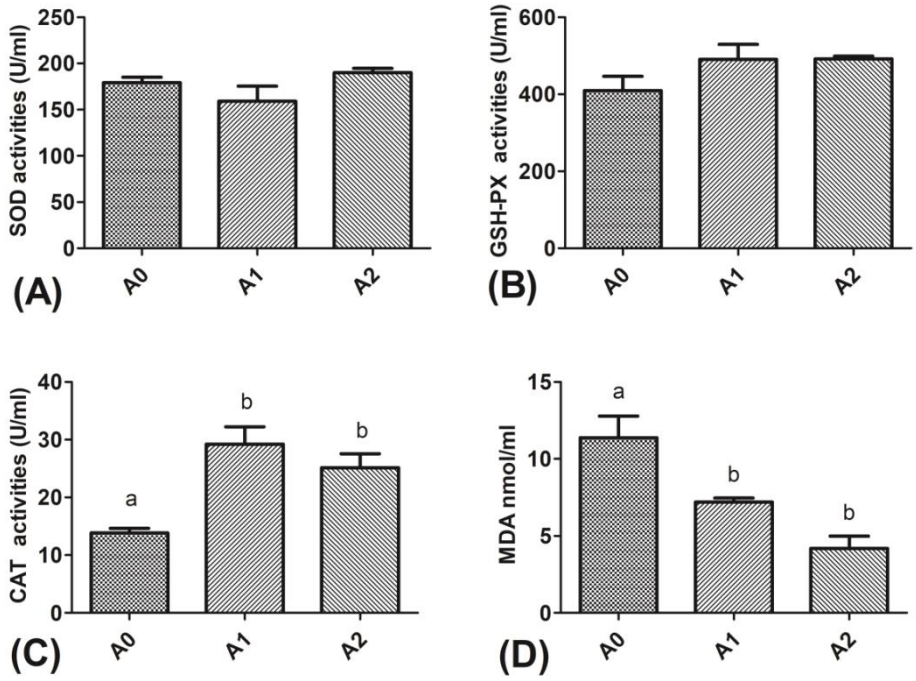


Figure 1: Effect of extract mixture on enzyme activity and lipid peroxidation of grass carp[1]
[1]A0, basal diet; A1, basal diet containing 500 mg/kg extract mixture; A2, basal diet containing 1000 mg/kg extract mixture.
Means sharing a common superscript were not significantly different (p>0.05).

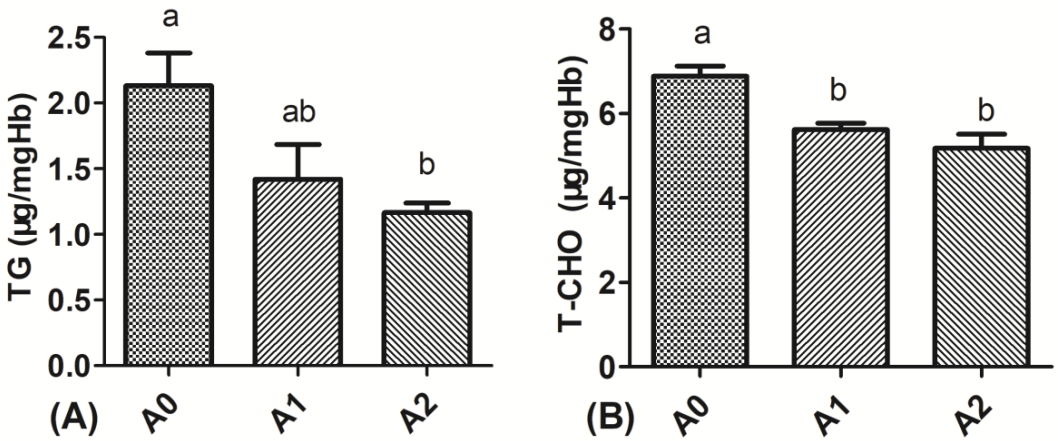


Figure 2: Effect of extract mixture on plasma lipids of grass carp[1].
[1]A0, basal diet; A1, basal diet containing 500 mg/kg extract mixture; A2, basal diet containing 1000 mg/kg extract mixture.
Means sharing a common superscript were not significantly different (p>0.05).

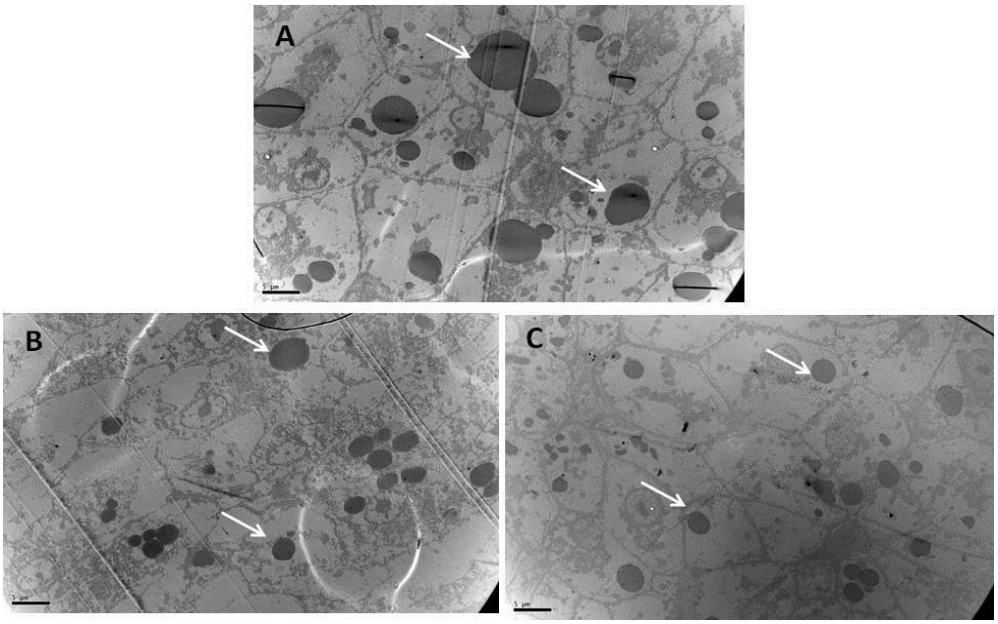


Figure 3: Effect of extract mixture on liver lipids of grass carp[1].
[1]A, basal diet; B, basal diet containing 500 mg/kg extract mixture; C, basal diet containing 1000 mg/kg extract mixture.

Table 3: Effects of extract mixture on diversity of gut adhesive bacteria of grass carp[1].

Sample ID	Reads	0.97					
		coverage	OTUs	ace	Chao	Shannon	Simpson
A0-1	61513	0.99	47	72	60	0.96	0.5203
A0-2	71351	0.99	84	165	128	1.02	0.6041
A0-3	50672	0.99	132	205	173	2.27	0.168
A1-1	73385	0.99	216	229	229	2.21	0.2544
A1-2	54413	0.99	257	264	265	2.49	0.1641
A1-3	65505	0.99	210	229	243	1.99	0.2915
A2-1	52613	0.99	167	215	203	1.1	0.6397
A2-2	92100	0.99	104	124	129	1.2	0.5543
A2-3	10242	0.99	98	127	132	1.47	0.3779
A0	61178±10343	0.99	87.67±34.80^{a}	147.33±39.40^{a}	120.33±17.1^{a}	1.42±0.21^{a}	0.43±0.13
A1	64434±9531	0.99	227.67±20.89^{b}	240.67±11.67^{b}	245.67±10.48^{b}	2.23±0.14^{b}	0.42±0.04
A2	82379±26289	0.99	123.00±31.21^{a}	155.33±29.85^{a}	154.67±24.18^{a}	1.26±0.11^{a}	0.52±0.08

[1]A0, basal diet; A1, basal diet containing 500 mg/kg extract mixture; A2, basal diet containing 1000 mg/kg extract mixture. Means sharing a common letter were not significantly different (p>0.05).

Ten predominant microbial phyla were identified from hindgut samples of fish fed the experimental diet, with majority of sequences (>80% of all sequences) belonging to three phyla: Fusobacteria, Bacteroidetes, and Proteobacteria (Fig. 4A). Compared with relative abundances of bacterial phyla in the control group, relative abundances of Proteobacteria and Thermi were significantly higher in group A1 (p<0.05), while relative abundances of Bacteroidetes and Actinobacteria in group A2, and Verrucomicrobia in group A1 were significantly lower (p<0.05). Relative abundances of Proteobacteria, Actinobacteria, and Thermi were higher in group A1 than in A2, while those of Fusobacteria and Tenericutes were higher in group A2 than in A1 (p<0.05, Table 4).

Table 4: Dominate bacteria of gut at phylum level[1].

Phylum	Groups		
	A0/%	A1/%	A2/%
Fusobacteria	64.73±8.51^{ab}	45.41±7.089^{a}	74.70±6.94^{b}
Bacteroidetes	21.32±4.72^{a}	12.43±7.26^{ab}	7.52±2.76^{b}
Proteobacteria	6.45±5.59^{a}	26.27±5.53^{b}	3.89±2.52^{a}
Spirochaetes	1.58±1.07	1.48±0.82	8.27±3.12
Firmicutes	3.02±1.27	2.69±0.99	3.43±0.88
Verrucomicrobia	1.04±0.11^{a}	0.47±0.13^{b}	1.35±0.61^{ab}
Actinobacteria	1.56±0.34^{a}	0.40±0.10^{a}	0.08±0.03^{b}
Tenericutes	0.05±0.02^{ab}	0.02±0.01^{a}	0.09±0.02^{b}
Thermi	0.01±0.01^{a}	0.47±0.17^{b}	0.07±0.05^{a}
Planctomycetes	0.001±0.003	0.04±0.03	0.002±0.001

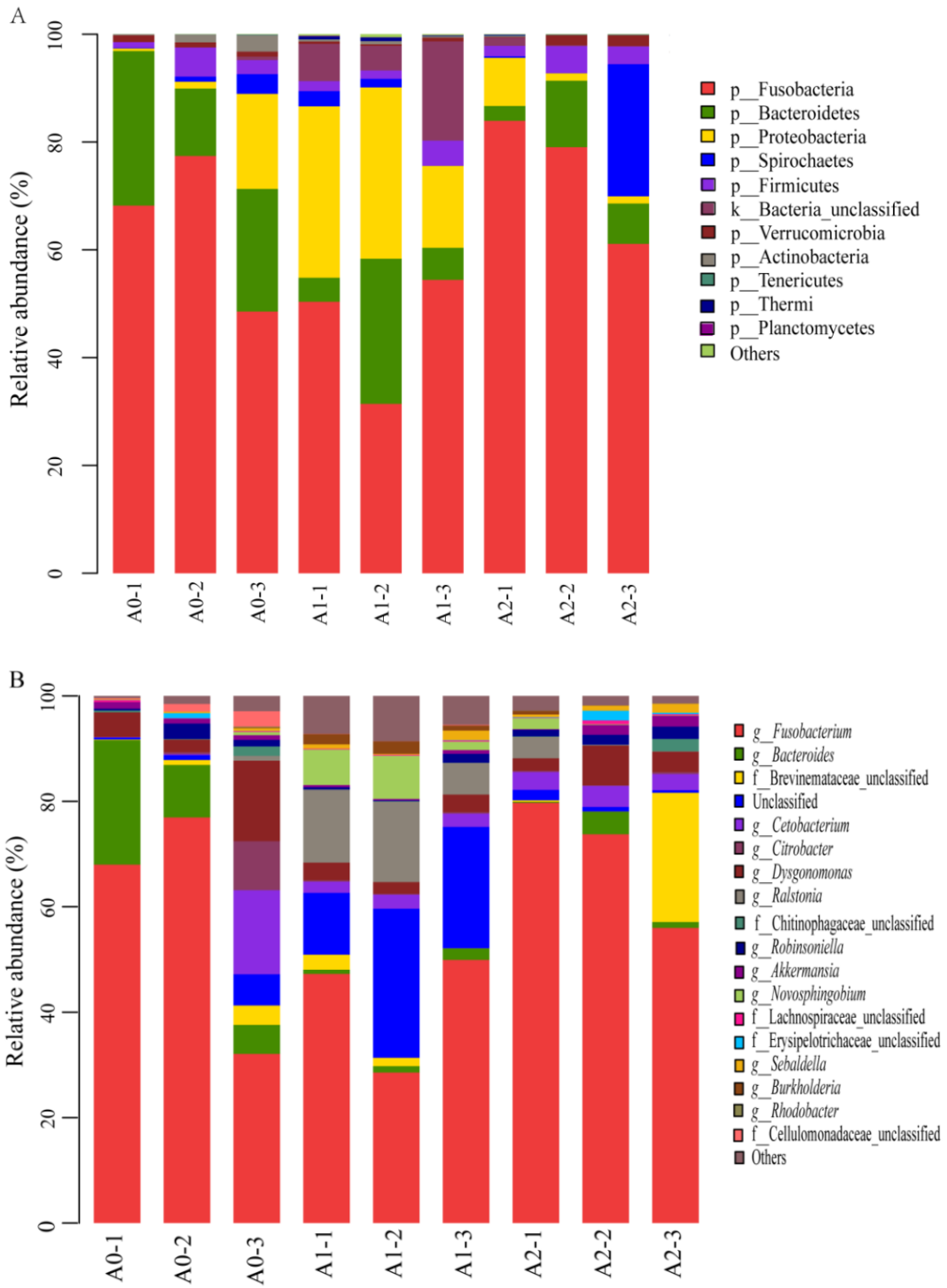
[1]A0, basal diet; A1, basal diet containing 500 mg/kg extract mixture; A2, basal diet containing 1000 mg/kg extract mixture. Means sharing a common letter were not significantly different (p>0.05).

Composition of intestinal bacteria at genus level is presented in Figure 4B. *Fusobacterium* was the dominant gut bacteria in grass carp, followed by *Bacteroides*, *Dysgonomonas*, *Cetobacterium*, *Ralstonia*, *Citrobacter*, *Robinsoniella*, *Akkermansia*, *Novosphingobium*, *Sebaldella*, *Burkholderia*, and *Rhodobacter* (Table 5). Relative abundance of *Fusobacterium* was higher in group A2 than in group A1 (p<0.05). Relative abundances of *Ralstonia* and *Burkholderia* in group A1 were higher than those of the control and A2 groups (p<0.05). Results of principal co-ordinates analysis and heatmap showed that samples of different treatments clustered separately, indicating difference in intestine bacteria among groups (Fig. 4C and 4D).

Table 5: Dominate bacteria of gut at genus level[1].

Genus	Groups		
	A0/%	A1/%	A2/%
Fusobacterium	59.01±23.74^{ab}	41.92±11.64^{a}	69.82±12.35^{b}
Bacteroides	13.03±9.43	1.41±0.71	1.87±2.14
Dysgonomonas	7.43±6.90	2.97±0.63	4.60±2.65
Cetobacterium	5.40±9.13	2.45±0.28	3.49±0.47
Ralstonia	0.32±0.51^{a}	11.72±5.00^{b}	1.43±2.29^{a}
Citrobacter	3.25±5.23	0.22±0.07	0.27±0.13
Robinsoniella	1.50±1.32	0.77±0.83	1.83±0.54
Akkermansia	1.04±0.20	0.46±0.23	1.35±1.06
Novosphingobium	0.22±0.34	5.52±3.47	0.77±1.14
Sebaldella	0.32±0.20	0.94±0.82	1.05±0.59
Burkholderia	0.05±0.08^{a}	1.75±0.71^{b}	0.24±0.40^{a}
Rhodobacter	0.06±0.09	0.002±0.002	0.02±0.01

[1]A0, basal diet; A1, basal diet containing 500 mg/kg extract mixture; A2, basal diet containing 1000 mg/kg extract mixture. Means sharing a common letter were not significantly different (p>0.05).


A
Relative abundance (%)
0
20
40
60
80
100
A0-1
A0-2
A0-3
A1-1
A1-2
A1-3
A2-1
A2-2
A2-3
p__Fusobacteria
p__Bacteroidetes
p__Proteobacteria
p__Spirochaetes
p__Firmicutes
k__Bacteria_unclassified
p__Verrucomicrobia
p__Actinobacteria
p__Tenericutes
p__Thermi
p__Planctomycetes
Others
B
Relative abundance (%)
0
20
40
60
80
100
A0-1
A0-2
A0-3
A1-1
A1-2
A1-3
A2-1
A2-2
A2-3
g__Fusobacterium
g__Bacteroides
f__Brevinemataceae_unclassified
Unclassified
g__Cetobacterium
g__Citrobacter
g__Dysgonomonas
g__Ralstonia
f__Chitinophagaceae_unclassified
g__Robinsoniella
g__Akkermansia
g__Novosphingobium
f__Lachnospiraceae_unclassified
f__Erysipelotrichaceae_unclassified
g__Sebaldella
g__Burkholderia
g__Rhodobacter
f__Cellulomonadaceae_unclassified
Others

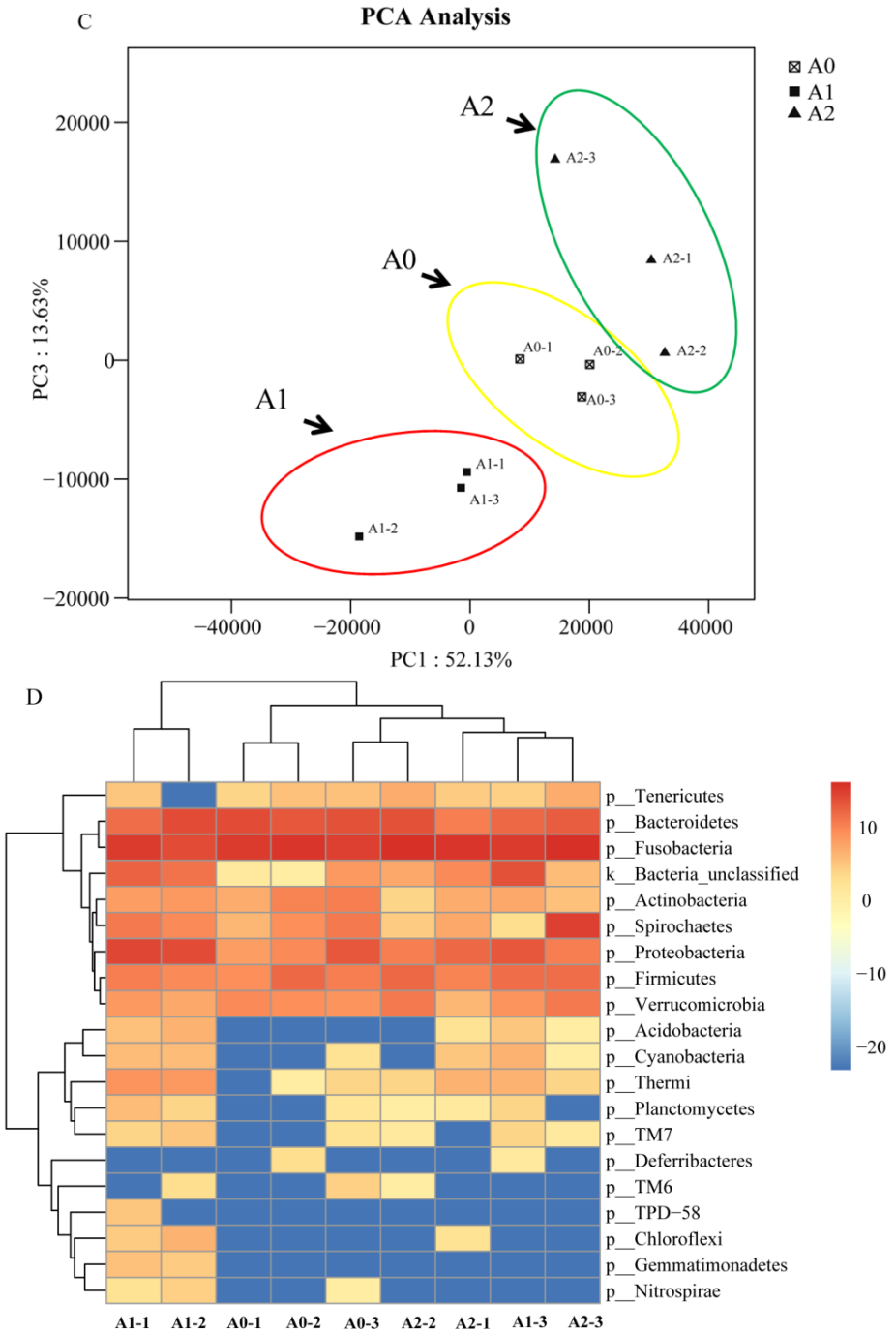


Figure 4: Effect of extract mixture on gut adhesive bacteria of grass carp. A. bacteria composition at phylum level; B. Bacteria composition at genus level; C. PcoA analysis; D. Heatmap of bacteial distribution among different treatments (base on top 20 at phylum level).
[1]A0, basal diet; A1, basal diet containing 500 mg/kg extract mixture; A2, basal diet containing 1000 mg/kg extract mixture.

Discussion

The influence of dietary supplementation with *A. argyi* or *H. cordata* and their extracts on growth performance are evaluated with several domestic and aquatic animals with varied results. In regard to the application of *A. argyi* in animal production, Chen *et al.* (2016) and Liu *et al.* (2018) summarized the positive

effects of powder or extract on growth performance in aquatic and terrestrial domesticated animals. Yan *et al.* (2011) and Cho *et al.* (2012) reported that pigs fed *H. cordata* extract at 1 g/kg had increased average daily weight gain and average daily feed intake. However, in a subsequent study on pigs, no such effect on growth performance was observed (Yan *et al.*, 2012). Tan *et al.* (2014) found that average weight and weight gain rate of grass carp increased significantly when fed 2% *H. cordata* powder. When hybrid catfish (*Clarias macrocephalus*×*C. gariepinus*) were fed *H. cordata* extract solution at 50 mL/kg, 70 mL/kg and 100 ml/kg feed, significant increase was observed in average daily growth, specific growth rate, feed efficiency, and protein efficiency ratio, as well as decrease in feed conversion rate (Panase *et al.*, 2018). In study of Wigraiboon *et al.* (2016), hybrid red tilapia fed with essential oil of *H. cordata* at 1.5 g/kg showed higher growth performance and lower feed conversion ratio than the control group. In the current study, growth performance of grass carp was not affected by the experimental diet supplemented with an extract mixture of *A. argyi* and *H. cordata* at 0.5 and 1.0 g/kg feed. These unexpected results might be related to the dosage, molecular weight, duration of feeding, environmental temperature, route of administration, and species. Thus, perhaps the amounts used here were not sufficient to enhance growth performance in grass carp.

Studies have shown that many unilateral and compound Chinese herbs have significant antioxidative effects. Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and glutathione peroxidase (GSH-PX) are principal parameters of oxidative stress (Chlubek and Poland, 2003). In broilers, chick dietary supplementation with *A. argyi* aqueous extract at 500, 1,000 and 2,000 mg/kg increased SOD, CAT, and GSH-PX activities significantly and decrease MDA level in small intestine significantly (Zhao *et al.*, 2016). Niu *et al.* (2019) observed that chick dietary supplementation with fermented *Ginkgo biloba* leaves at concentrations of 1.5, 2.5, 3.5, 4.5, and 5.5 g/kg could increase antioxidant activities including CAT and GSH-PX, and decrease MDA levels to different degrees in pancreas and small intestine. Effects of *A. argyi* and *H. cordata* against peroxidation were less concerned in aquatic animals. Tan *et al.* (2014) found that in grass carp, SOD activity in brain, liver, and kidney increased significantly when fed with *H. cordata* powder (1, 2, and 3%). In the current study, we observed that in the experimental groups CAT activity was significantly higher and MDA level significantly lower than those in control group. This indicates that dietary supplementation with extract mixture of *A. argyi* and *H. cordata* could improve antioxidant capacity of grass carp, as in the previously mentioned studies. However, the mechanism behind this should be further investigated and confirmed using more species.

A few studies showed that Chinese herbs can regulate lipid metabolism in fish. Triglyceride (TG) and cholesterol (CHO) levels can be reduced through dietary supplementation with *P. chinense* (Miao, 2012) and *S. sarmentosum* extract (Wu, 2013) in the blood of grass carp, and *Coptis chinensis* extract in zebrafish (Chen, 2015). In a recent study, grass carp supplemented with chlorogenic acid (an active component of *Lonicera* and *Eucommia*) had lower fat levels in serum and hepatopancreas, and smaller hepatic lipid droplets (Yang *et al.*, 2018). In the present study, effects of mixture of *A. argyi* and *H. cordata* extract were evaluated. The results of TG and CHO levels in serum and the TEM images of liver lipid droplets indicated that the extract mixture had the potential to be used as an additive to prevent fatty liver in grass carp.

It is well known that gut bacteria play an important role in host metabolism and the immune system (Saika *et al.*, 2019), but effects of Chinese herbs and their extracts on gut bacteria of farmed animals are less well understood. Namkung *et al.* (2004) reported that dietary supplementation with herbal extract (0.75% inclusion; containing cinnamon, thyme, and oregano extract) could reduce fecal coliform counts in pigs on day 14 post-weaning. Calvo *et al.* (2006) tested a compound formed by natural extracts from Rutaceae plants, cinnamon oil, and organic acids on intestinal microorganisms of piglets, resulting in lower bacterial counts at intestinal segments (duodenum, jejunum, ileum, and colon). In addition, Castillo *et al.* (2006) showed that dietary supplementation with 0.03% plant extract mixture did not affect total microbial counts but showed an increase in lactobacilli to enterobacteria ratio in the cecum of piglets. Broiler chickens fed with a blend of the three extracts (*Thymus vulgaris*, *Echinacea purpurea*, and *Allium sativum*) had lower colony forming units of *Escherichia coli* in digesta of ileo-cecum, and higher lactic acid bacteria counts (Rahimi *et al.*, 2011). In aquatic animals, use of botanical essential oils attracted increased interest as gut bacteria promoters for the gastrointestinal tract. In rainbow trout (*O. mykiss*), Navarrete *et al.* (2010) observed that no significant change was detected in the TGGE (temperature gradient gel electrophoresis) profiles of *Thymus vulgaris* essential oil-treated fish compared with those of the controls. Further, Giannenas *et al.* (2012) found that fish fed carvacrol or thymol had lower anaerobe counts but higher lactobacillus enumeration by conventional microbiological techniques using selective agar media. Sutili *et al.* (2016) evaluated intestinal microbial communities of drum (*Sciaenops ocellatus*) when supplemented with *Ocimum americanum* oil by denaturing gradient gel electrophoresis (DGGE) and no effect was observed. In a trial focusing on hybrid tilapia (*O. niloticus* ♀×O. *aureus* ♂), Ran *et al.* (2016) observed that carvacrol and thymol could increase the diversity and improve the composition of gut bacteria. Our

study found that dietary supplementation with the extract mixture at 500 mg/kg increased the OTU number and α diversity in the gut of grass carp, with the bacterial composition influenced at phylum and genus levels. The results of the entire study suggest that Chinese herb extracts can promote the status of animal guts, potentially due to antimicrobial activity (Cowan, 1999).

In conclusion, the extracts mixture of *A. argyi* and *H. cordata* can increase antioxidant activity, lower serum and hepatic lipid levels, and improve gut bacteria composition, which can be used as a green additive for grass carp and dosage of 500 mg/kg is recommended.

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