Applied Microbiology
Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Significant improvement of intestinal microbiota of gibel carp (*Carassius auratus gibelio*) after traditional Chinese medicine feeding

Z.B. Wu[1,2], F.-J. Gatesoupe[3], T.T. Li[4], X.H. Wang[1], Q.Q. Zhang[1,5], D.Y. Feng[6], Y.Q. Feng[1,2], H. Chen[7] and A.H. Li[1,5]

1 State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China
2 University of Chinese Academy of Sciences, Beijing, China
3 NUMEA, INRA, University of Pau and Pays de l'Adour, Saint Pée sur Nivelle, France
4 Department of Applied Biology, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou, China
5 Freshwater Aquaculture Collaborative Innovation Centre of Hubei Province, Huazhong Agricultural University, Wuhan, China
6 National Fisheries Technical Extension Centre, Ministry of Agriculture, Beijing, China
7 Fisheries Technical Extension Centre of Jiangsu Province, Nanjing, China

Keywords
gibel carp, high-throughput sequencing, intestinal microbiota, microbial balance, traditional Chinese medicine.

Correspondence
Hui Chen, Fisheries Technical Extension Centre of Jiangsu Province, Nanjing 210036, China.
E-mail: chenhuijsbf@163.com
and
Aihua Li, State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China.
E-mail: liaihua@ihb.ac.cn

2017/1531: received 23 August 2017, revised 27 November 2017 and accepted 12 December 2017

doi:10.1111/jam.13674

Abstract

Aims: Increasing attention has been attracted to intestinal microbiota, due to interactions with nutrition, metabolism and immune defence of the host. Traditional Chinese medicine (TCM) feed additives have been applied in aquaculture to improve fish health, but the interaction with fish gut microbiota is still poorly understood. This study aimed to explore the effect of adding TCM in feed on the intestinal microbiota of gibel carp (*Carassius auratus gibelio*).
Methods and Results: Bacterial communities of 16 fish intestinal contents and one water sample were characterized by high-throughput sequencing and analysis of the V4–V5 region of the 16S rRNA gene. The results showed that the composition and structure of the bacterial community were significantly altered by the TCM feeding. Some phyla increased markedly (Proteobacteria, Actinobacteria, Acidobacteria, etc.), while Fusobacteria were significantly reduced. Concurrently, the richness and diversity of the taxonomic units increased, and the microbiota composition of TCM-treated fish was more homogeneous among individuals. At the genus level, the addition of TCM tended to reduce the incidence of potential pathogens (*Aeromonas*, *Acinetobacter* and *Shewanella*), while stimulating the emergence of some potential probiotics (*Lactobacillus*, *Lactococcus*, *Bacillus* and *Pseudomonas*).
Conclusions: These data suggested that the feed additive could regulate the fish intestinal microbiota by reinforcing the microbial balance.
Significance and Impact of the Study: This study may provide useful information for further application of TCM for diseases prevention and stress management in aquaculture.

Introduction

The intestinal tract of vertebrates is a complicated ecological niche which is colonized by a large and diverse microbial community (Ley *et al.* 2008). Researches on the intestinal microbiota have revealed that the microbial community plays vital roles in the digestive tract function, such as stimulating the growth and development of the intestinal epithelium, preventing it from pathogen invasion, contributing to the digestion of complex nutrients and synthesizing beneficial secondary metabolites (Verschuere *et al.* 2000; Nayak 2010). On the other side, the gut microbiota harbours opportunistic bacterial pathogens, the overgrowth of which may disrupt

bacterial homeostasis, thus resulting in functional disorder of immune defence mechanisms of the host and even causing serious diseases (Wu *et al.* 2010; Roeselers *et al.* 2011). As intensive aquaculture has been increasingly popular in China, severe outbreaks of epidemic diseases occur frequently. Therefore, further investigation on the intestinal microbiota is essential to secure fish health and rearing performances in aquaculture.

Many endogenous and exogenous factors impact the composition of intestinal microbiota in fish, including species, developmental stage, intestinal structure, diet, nutritional status and living conditions (Ley *et al.* 2008; Nayak 2010; Schwab *et al.* 2011). It is crucial to understand how such factors may influence the gut microbiota, with a view to regulate and control the bacterial community. Previous study showed that the fish intestinal microbiota composition depended on dietary factors to a large extent (Nayak 2010; Tarnecki *et al.* 2017). Moreover, this dependence concerns mainly some specific bacterial groups (Navarrete *et al.* 2013).

Due to high stocking density constraints, fish diseases jeopardize the aquaculture industry, hence causing some abuse of antibiotics in aquaculture. As a consequence, more and more antibiotic-resistant bacteria have been detected in aquaculture products (Smith *et al.* 2002). In view of the serious harmful effects of antibiotics, the European Union has forbidden its use as feed additives in fish farming since January 2006. Other feed additives are environment-friendly, especially some products of the traditional Chinese medicine (TCM), one of the most popular alternative option (Gong *et al.* 2014).

In recent years, the potential application of TCM in aquaculture has been increasingly explored. Studies confirmed that some of TCMs may replace vaccines (Secombes 1994), and antibiotics (Galina *et al.* 2009). Besides, herbal medicines had positive effects on fish growth, disease prevention, appetite stimulation and stress relief (Liu *et al.* 2004; Francis *et al.* 2005; Ardo *et al.* 2008). The principle of TCM lies in maintaining or restoring the balance in the body functions, or enhancing the ability of the immune defences (Choi *et al.* 2014).

The health value of TCMs has been already explored in fish, but these studies concerned mainly nonspecific immune responses and the disease resistance of fishes. For example, *Astragalus* root extract improved the survival rate (SR) of common carp infected with *Aeromonas hydrophila* (Yin *et al.* 2009). Rohu fed *Achyranthus* had much higher superoxide anion and lysozyme production levels, and stronger serum bactericidal activity (Vasudeva Rao *et al.* 2006). A TCM formulation containing *Ocimum basilicum*, *Cinnamomum zelanicum*, *Jugpans regia* and *Mentha piperita* strengthened the innate immunity and disease resistance to *A. hydrophila* challenge in common carp (Abasali and Mohamad 2010). In a word, these results showed that some Chinese traditional herb medicines as feed supplements can enhance immunity of fish.

To our knowledge, the effects of TCM on fish intestinal microbiota have been scarcely investigated so far, and only with culture-dependent methods (Liu *et al.* 2004; Guozhang *et al.* 2008). Considering the promising prospect of TCM applications in aquaculture, it is essential to explore further the interactions of such treatments with fish gut microbiota. Yu-Ping-Feng (YPF, Jade screen) powder, a famous TCM prescription, is known to enhance immunity due to its anti-inflammatory action (Song *et al.* 2013). It may prevent viral infections (Du *et al.* 2015). In the current study, the objective was to analyse the effect of Jade screen powder feed additives on the intestinal microbiota in healthy adults of gibel carp. The microbial community in the culture water was also considered concurrently. To this end, 16S rRNA gene high-throughput sequencing was applied to water sample and to the intestinal contents of gibel carp fed a TCM additive or not fed.

Materials and methods

Chinese herbal compound and TCM fish feed preparation

In the present study, YPF powder was prepared with proportions of 1 : 2 : 2 of its three components, Chinese parsnip root, *Astragalus membranaceus* and *Atractylodes macrocephala* Koidz, respectively. The three compounds were cut and grinded to 80 US Mesh separately before mixing. YPF powder was incorporated at 5% into commercial fish feed powder (10 kg drug was blended into 190 kg feed), and then the mixture was re-pelleted. The same feed without YPF powder was used as control. Both of the feeds were provided by Jiangsu Yancheng YuDa Feed Co., Ltd (China) (crude protein ≥34·0%; crude fat ≥7·5%; available phosphorus ≥0·5%; lysine ≥1·7%; methionine ≥0·5%).

Fish feeding protocol

Six cages (4 m × 4 m × 2·5 m) were set up in a fish pond of 10 ha, in a commercial fish farm located in Yancheng City, Jiangsu Province, China. This region is the major production area for gibel carp. Three cages were assigned to each of the experimental groups (G1, G2, G3) and control groups (G4, G5, G6). About 150 healthy juveniles of gibel carp with body weight of *c.* 180 g were reared in each cage. The initial average body weights were 180·24 ± 3·42 g for all cages. The fish were evaluated by

a careful examination of physical appearance and behaviour (e.g. feed behaviour and activity of daily living) and showing no sign of infection. They were fed the commercial diet without YPF at a daily rate of 2% body weight for 15 days before the start of the experiment. The same feeding rate of 2% was applied during the experiment. The experimental group was fed the YPF diet, while the control group was fed the control diet throughout the experiment. The feeding experiment lasted 3 months. No fish disease outbreak occurred during the experiment.

Growth measurements

At the end of the feeding experiment, 10 healthy gibel carp were randomly caught from each cage. Weight gains (WG) and SR were calculated according to the following formulae (Li *et al.* 2015b):

$$\mathrm{WG}(\%) = 100 \times (\mathrm{FBW\text{-}IBW})/\mathrm{IBW}$$

$$\mathrm{SR}(\%) = 100 \times \text{survival number/total number}$$

FBW is the final body weight and IBW the initial body weight of the fish.

Intestinal content collection

Three gibel carp fish were randomly caught from each cage. The sampled fish were transported to the laboratory within 2 h for collecting intestine contents. In the same time, 250-ml water samples were picked up from four locations in the pond at 60-cm depth around the cages with sterile glass bottles. These water samples were pooled together. The fish were euthanized with lethal MS-222 before dissection. The skin was washed with 70% ethanol, and the abdomen was opened immediately with sterile scissors to expose the body cavity. The intestinal content of each fish was squeezed out and separately harvested under aseptic conditions. The intestinal contents randomly from eight individuals of each group were labelled E1–E8 for the experiment groups and C1–C8 for the control groups. One litre of water sample was centrifuged at 12 800 ***g*** (Thermo Scientific, Waltham, MA, USA) for 20 min at 4°C, and the sediment was collected and labelled S. All the 17 samples (E1–E8, C1–C8 plus S) were kept frozen at −80°C until DNA extraction.

Extraction of bacterial DNA

Samples (intestinal content and water sediment) were prepared for genomic DNA extraction using QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions with slight modifications. For each sample, DNA was extracted in duplicates, and the extracts from the same sample were pooled together to minimize the bias in sampling and extraction (Wu *et al.* 2010). DNA concentration and quality were checked using a NanoDrop Spectrophotometer. Extracted DNA was diluted to 10 ng μl^{-1} and stored at −80°C for downstream research.

PCR amplification and MiSeq sequencing

Universal primer 515F (5′-GTGCCAGCMGCCGCGGTA A-3′) and 909R (5′- CCCCGYCAATTCMTTTRAGT-3′) with 12 nt unique barcode at 5′-end of 515F were used to amplify the V4-V5 hypervariable region of 16S rRNA gene. The PCR reaction mix (25 μl) consisted of 1 μl DNA template (*c.* 10 ng), 0·25 U of EX Taq DNA polymerase, 2 μl 10 x Ex Taq buffer (Mg^{2+}), 1·6 μl dNTP mix (TaKaRa Biotechnology Co. Ltd, Dalian, China), 1 μl BSA (10 mg ml^{-1}), forward and reverse primers (1 μmol l^{-1} each), and sterile water added up to 25 μl. Amplification condition consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 50 s with a final extension at 72°C for 10 min. Replicate PCR reactions were carried out for each sample, and their products were pooled and subjected to 1%-agarose gel electrophoresis. The band with a correct size was excised and purified using SanPrep DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China). All PCR products were quantified with NanoDrop, and pooled together for each sample with equal molar amounts. The sequencing samples were prepared using TruSeq DNA kit according to the manufacturer's instruction. The purified DNA library was diluted, denatured, re-diluted, mixed with PhiX (equal to 30% of final DNA amount) as described in the Illumina library preparation protocols, and then applied to an Illumina Miseq system for sequencing at the Environmental Genome Platform of Chengdu Institute of Biology.

Bioinformatics and statistical analyses

The sequence data were processed using QIIME Pipeline–ver. 1.7.0 (http://qiime.org/). All sequence reads were trimmed and assigned to each sample based on their barcodes. The sequences with high quality (length >300 bp, without ambiguous base 'N', and average base quality score >30) were used for downstream analysis. Sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold. The aligned 16S rRNA gene sequences were used for chimera check using the Uchime algorithm (Edgar *et al.* 2011). All the samples were randomly resampled to 10 210 reads to eliminate the effect

of the total number of different sequences on the diversity index. Alpha-diversity was estimated with phylogenetic distance whole tree, chao1 richness, observed species and Shannon's diversity indices and beta-diversity was analysed with PERMANOVA and principal coordinate analysis (PCoA) based on weighted UniFrac metric, for which the rarefaction curves were generated from the observed species. Taxonomy was assigned using the Ribosomal Database Project classifier (Wang *et al.* 2007). Due to the non-normal distribution of the samples, the statistical evaluation was performed with nonparametric tests (SPSS, ver. 19.0., Chicago, IL, USA). In the present study, variations between two independent groups were evaluated by the Mann–Whitney test (MW). The data were displayed as mean value ± standard deviation (mean ± SD) and the significance level of the difference was set at 0·05 or 0·01.

Results

Fish growth and survival

Fish growth and survival are showed in Table S1. Weight gain (WG, %) in the TCM group (111·13 ± 0·48) was slightly higher than that in the control group (111·01 ± 1·70), but without significant difference (MW, $P > 0·05$). Similarly, no significant difference was observed between the mean SR of the two dietary groups (MW, $P > 0·05$). In addition, no significant difference of WG or SR was noted within each group.

Selection of the valid DNA sequences

In this study, 447 131 valid reads (ranging from 14 493 to 32 856 per sample) and 6452 OTUs (ranging from 605 to 2340 per sample) were retrieved in total from the 17 samples after high-throughput sequencing. Among these sequences, 349 reads and seven OTUs corresponded to eukaryotes, and they were removed from the following analysis.

The OTU table was randomly resampled to avoid differences based on high-throughput sequencing, leaving 4618 OTUs for further analysis. These randomly subsampled sequences were clustered into OTUs at the 97% identity threshold, resulting in 318–1338 OTUs per sample. These OTUs were distributed among 43 phyla and unclassified clades. The rarefaction curves tended to approach the saturation plateau (Fig. S1). Good's coverage estimations disclosed that between 93·5 and 98·3% of the bacterial phylotypes present were detected in the microbial community profile of each sample (95·81 ± 1·14%; Table 1).

Richness and diversity analysis

Several alpha-diversity indices were calculated to compare the richness and diversity between the two dietary groups. The number of OTUs covered 45·99–69·47% of the richness estimated by the Chao1 index (Table 1). Statistical analysis revealed that the richness of the microbial community was significantly higher in the TCM group, compared to the control group, as evaluated with the indices of Shannon and Simpson (MW, $P < 0·05$). The diversity indices of Shannon and Simpson were also found significantly higher in the TCM group, compared to the control group (MW; $P < 0·01$, $P < 0·05$, respectively). The Faith' phylogenetic diversity of the microbial community was significantly higher in the TCM group, compared to the control group (PD_whole_tree; MW; $P < 0·01$).

Overall dissimilarity between the bacterial communities of the three groups

Multivariate statistical analyses were performed to compare the integral structure of microbial communities. The global similarity of the bacterial communities between samples was compared by PERMANOVA and PCoA based on weighted UniFrac metric. Nonparametric MANOVA (PERMANOVA) revealed significant differences in the structure

Table 1 Observed diversity richness (OTUs), estimated OTU richness (Chao1), diversity index (Shannon and Simpson), and estimated sample coverage calculated based on a cutoff of 97% similarity of 16S rRNA sequences in the three groups. Group E represents the TCM group (E1–E8); Group C represents the control group (C1–C8); Group S represents the water sample (S)

Indices	Group E	Group C	Group S	MW (Group E and C); *P*-value
OTUs	1033·38 ± 200·31	711·25 ± 228·64	763	0·0135*
Chao1	1752·41 ± 286·69	1344·43 ± 403·08	1098·37	0·0499*
PD_whole_tree	56·96 ± 9·55	39·06 ± 10·18	42·86	0·0019**
Shannon index	6·88 ± 0·94	5·17 ± 1·04	6·02	0·0070**
Simpson index	0·95 ± 0·04	0·86 ± 0·08	0·92	0·0115*
Good's coverage	0·95 ± 0·01	0·96 ± 0·01	0·97	0·0398*

*Means significant difference ($P < 0·05$).

**Means extremely significant difference ($P < 0·01$).

(PERMANOVA, $F = 4{\cdot}66$, $P = 0{\cdot}009$) of gut microbiota between the two dietary groups. PCoA showed that bacterial communities in the TCM group were separated from those of the control group (except sample E5, E6 and E8; Fig. 1). The water community was separated far from the intestinal microbiota, showing distinct differences between intestinal microbiota and water bacterial community. Overall, the two principal coordinates obtained from PCoA explained 65·49% of the variations between all the samples. The PCoA plot visualized the PERMANOVA result, confirming that intestinal microbiota changed after TCM feeding. Further hierarchical cluster analysis based on weighted UniFrac metric also disclosed overall dissimilarity of intestinal microbiota profiles between the two dietary groups, as well as the water community profile (Fig. S2).

The measures of dispersion (including the coefficient of variation (CV), SD and range) of these five richness and diversity within the TCM group were lower than those in the control group (Table S2), suggesting that the bacterial community of the TCM group was more uniform than that of the control group.

Differences in bacterial composition among the three groups

The composition of the 17 bacterial communities was compared at the phylum level (Fig. 2). A total of 43 different phyla or unclassified clades were identified from the eight samples in the TCM group, while only 36 phyla or unclassified clades were present in the control group.

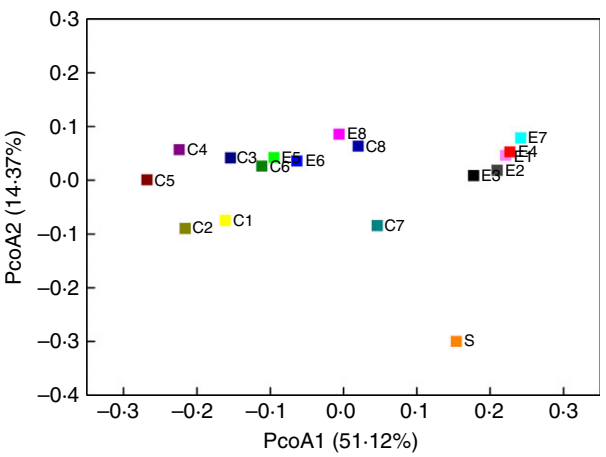

Figure 1 Principal coordinate analysis on the basis of weighted UniFrac metric of all the 17 samples. The percentages indicate the relative contribution of the principal components. Squares E1–E8 stand for the bacterial communities from the TCM group; squares C1–C8 stand for the bacterial communities from the control group; squares S stand for the water bacterial community. [Colour figure can be viewed at wileyonlinelibrary.com]

Crenarchaeota, Elusimicrobia, Gno2, Lentisphaerae, Synergistetes, Thermotogae and WWE1 were present in the TCM group, but not in the control group. Some variability was observed among samples from the same dietary group. In addition, the water community (S) had the simplest bacterial diversity, which consisted of only 18 phyla or unclassified clades. There were significant differences between relative abundances in the two dietary groups at the phylum level. The primary phyla, Proteobacteria, Actinobacteria and Verrucomicrobia in the TCM group appeared distinctly more abundant than in the control group (MW, $P < 0{\cdot}01$), while a significant reduction in *Fusobacteria* was observed in the TCM group compared with the control group (MW, $P < 0{\cdot}01$) (Table 2). Similarly to Crenarchaeota, Elusimicrobia was found only in the TCM group. In addition, four rare phyla and clades (below 1·00% abundance) were significantly more abundant in the TCM group than in the control group: Acidobacteria and KSB3 (MW, $P < 0{\cdot}05$), and TM7 and WS5 (MW, $P < 0{\cdot}01$) (Table 2).

Hierarchically clustered heatmap analysis based on the bacterial community profiles at the family level (average abundance ≥0·20%) disclosed visible differences of 31 main families among the bacterial profiles of the three groups. The fish intestinal microbiota of the two dietary groups showed distinguishing bacterial communities at the family level (Fig. 3). The water bacterial profile was distant from all intestinal profiles, consistent with hierarchical cluster analysis based on weighted UniFrac metric (Fig. S2).

At the genus level, the sequences from the 17 samples represented 226 genera in total. A majority of 181 of these genera were present in the TCM group, compared to only 136 in the control group, and 81 in the water sample. The intestinal samples of the two dietary groups shared in common 112 genera, while 69 and 24 genera were found only in the groups fed the TCM and groups not fed the TCM, respectively. The relative abundance of each genus specific to one dietary group was less than 0·05% of the total reads per group. In comparison to the fish gut microbiota, we observed 21 unique genera in the water community, which was dominated by *Aquirestis* (1·14% of the total reads in the water sample), *Mycoplana* (0·22%) and *Polynucleobacter* (0·14%). More than 79% of all the sequences detected in the 17 samples corresponded to 13 genera and 19 other taxa, unclassified at the genus level, each one with an average abundance higher than 0·5% of the total reads per sample (Fig. 4). In the TCM group (group E), the intestinal microbiota was dominated by *Cetobacterium*, unclassified Chromatiales, followed by *Dechloromonas*, *Desulfobulbus* and unclassified Vibrionaceae. The most abundant genera in the control group (group C) were *Cetobacterium*, *Planktothrix*,

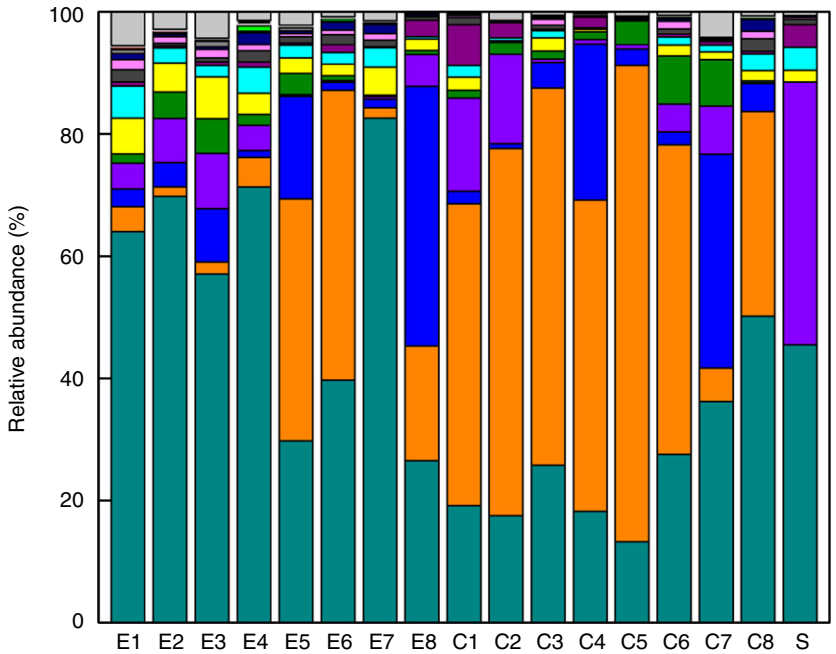

Figure 2 Bacterial composition of the different communities (% of relative abundance of different bacterial phyla within each community). Lanes E1–E8 stand for the bacterial communities from the TCM group; lanes C1–C8 stand for the bacterial communities from the control group; lanes S stand for the water bacterial community (□ others; ■ KSB3; ■ TM7; ■ Chlorobi; ■ WS5; ■ Acidobacteria; ■ Chloroflexi; ■ Planctomycetes; ■ Bacteroidetes; ■ Verrucomicrobia; ■ Actinobacteria; ■ Tenericutes; ■ Cyanobacteria; ■ Firmicutes; ■ Fusobacteria; ■ Proteobacteria). [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Significant different bacteria between group E and group C at the phylum level. Group E represents the TCM group (E1–E8); Group C represents the control group (C1–C8)

Phylum	Group E	Group C	MW; *P*-value
Crenarchaeota	0·01 ± 0·01%	0	0·010*
Acidobacteria	0·92 ± 0·63%	0·39 ± 0·61%	0·018*
Actinobacteria	3·96 ± 1·86%	1·26 ± 0·84%	0·003**
Elusimicrobia	0·01 ± 0·02%	0	0·027*
Fusobacteria	15·00 ± 18·62%	48·72 ± 21·59%	0·005**
KSB3	0·21 ± 0·19%	0·05 ± 0·05%	0·010*
Proteobacteria	55·09 ± 20·77%	25·98 ± 12·15%	0·006**
TM7	0·24 ± 0·11%	0·07 ± 0·07%	0·003**
Verrucomicrobia	2·68 ± 1·53%	1·14 ± 0·87%	0·027*
WS5	0·36 ± 0·29%	0·06 ± 0·06%	0·004**

*Means significant difference ($P < 0·05$).
**Means extremely significant difference ($P < 0·01$).

unclassified Vibrionaceae, *Anoxybacillus* and unclassified Chromatiales (Table S3). The water community was overwhelmed by *Planktothrix* (40·66%) in Cyanobacteria, followed by two clades of Proteobacteria: unclassified Gammaproteobacteria (14·19%) and unclassified Comamonadaceae (4·89%). Significant differences were also found at the genus level between the two dietary groups. Among the 69 most abundant bacterial clades at the genus level (average abundance >0·1%), 31 genera or other taxa were significantly higher in the TCM group than in the control group, while *Cetobacterium* and unclassified Aeromonadaceae were significantly reduced in the TCM group (Table S3). These 33 genera, with significant difference of abundance between dietary groups, accounted for 53·94% of the total bacteria in the TCM group and 60·53% of the total bacteria in the control group, respectively. These results indicated that the relative abundance of most of the genera in the intestinal microbiota altered after the TCM feeding.

An investigation of the 10 most abundant OTUs was used to highlight the main differences among the three groups. The 10 most abundant OTUs in the TCM group (samples E1–E8) were *Cetobacterium* sp. OTU65, *Dechloromonas* sp. OTU585, Vibrionaceae OTU64, Chromatiales OTU118, *Ruminantium* sp. OTU9064, *Desulfobulbus* sp. OTU476, *Anoxybacillus* sp. OTU10, Sinobacteraceae OTU61, Burkholderiales sp. OTU54 and *Planktothrix* sp. OTU84 (Table S4). The intestinal microbiota in the control group (samples C1–C8) was dominated by the genus *Cetobacterium*, which included OTU65, OTU95, OTU11, OTU39, and OTU98, *Anoxybacillus* sp. OTU10, *Planktothrix* sp. OTU84, Vibrionaceae sp. OTU64, *Dechloromonas* sp. OTU585 and Chromatiales OTU456 (Table S4). The water community was dominated by the genus

	E1	E2	E3	E4	E5	E6	E7	E8	C1	C2	C3	C4	C5	C6	C7	C8	S
At425_EubF1	0·57	0·28	0·47	0·42	0·26	0·16	0·60	0·06	0·09	0·04	0·21	0·03	0·00	0·06	0·07	0·33	0·00
Microbacteriaceae	0·51	0·19	0·82	0·16	0·24	0·06	0·18	0·21	0·18	0·04	0·07	0·04	0·04	0·11	0·40	0·06	0·03
Bacteroidaceae	0·02	0·00	0·01	0·00	0·08	0·24	0·02	0·82	4·45	1·17	0·01	1·00	0·04	0·18	0·00	0·09	0·00
Phormidiaceae	3·83	3·20	7·28	3·62	0·19	0·19	0·37	4·28	14·28	13·06	0·37	0·61	0·62	3·99	7·38	0·09	40·95
Microcystaceae	0·12	3·53	1·31	0·30	0·01	0·10	0·00	0·75	0·69	1·41	0·06	0·09	0·06	0·42	0·29	0·01	0·88
Peptostreptococcaceae	0·43	0·77	1·27	0·23	0·30	0·38	0·30	38·98	0·92	0·16	1·24	21·70	0·09	1·06	0·89	3·19	0·00
Bacillaceae	0·74	1·34	4·69	0·23	14·87	0·01	0·32	0·03	0·51	0·09	0·50	0·14	2·23	0·10	32·72	0·09	0·01
Clostridiaceae	0·81	0·77	1·38	0·33	0·84	0·28	0·31	0·99	0·31	0·20	1·94	1·09	0·15	0·34	0·43	0·22	0·01
Erysipelotrichaceae	0·04	0·14	0·30	0·04	0·09	0·44	0·04	0·23	0·14	0·16	0·22	2·22	0·14	0·29	0·07	0·86	0·00
Fusobacteriaceae	4·05	1·48	1·95	4·80	39·64	47·44	1·68	18·80	49·41	59·59	61·73	50·91	77·94	50·63	4·94	33·47	0·00
Pirellulaceae	1·48	0·32	0·38	1·55	0·81	1·28	0·83	0·31	0·97	0·08	0·41	0·03	0·03	0·70	0·10	1·79	0·48
Desulfobulbaceae	13·23	8·79	14·09	4·71	6·04	3·38	18·50	0·31	2·11	0·52	6·00	0·41	0·63	2·61	0·62	4·43	0·00
Vibrionaceae	0·16	27·56	6·35	0·24	0·40	0·08	0·01	0·20	0·21	3·59	0·08	4·52	7·58	0·28	17·96	0·12	0·00
Rhodocyclaceae	7·92	4·96	5·95	6·90	3·32	2·61	8·87	1·17	1·43	1·33	5·15	2·24	1·12	3·88	2·31	3·29	1·95
Syntrophaceae	5·79	2·34	5·82	3·50	4·71	1·87	7·09	0·64	0·62	0·23	2·33	0·50	0·28	1·04	0·46	2·32	0·03
Sinobacteraceae	2·78	1·08	1·82	4·93	1·59	3·13	5·74	3·41	0·60	0·38	1·14	0·55	0·12	2·20	0·09	4·51	0·27
Desulfobacteraceae	3·32	1·68	2·53	4·93	1·24	3·19	3·66	0·69	0·39	0·53	0·87	0·71	0·05	1·63	0·19	3·59	0·03
Chromatiaceae	2·02	1·36	2·29	0·99	0·46	0·95	2·99	0·15	0·35	0·14	0·38	0·20	0·06	0·89	0·35	0·64	0·05
Comamonadaceae	1·17	0·35	0·96	1·38	0·53	0·53	0·46	0·43	0·11	0·17	0·23	0·36	0·03	0·51	0·18	0·53	6·11
Aeromonadaceae	0·31	1·28	0·34	0·12	0·22	0·43	0·01	0·22	0·73	2·27	0·43	2·40	0·40	0·44	2·28	0·19	0·01
Xanthomonadaceae	0·51	0·35	0·63	0·69	1·09	0·17	0·50	3·54	0·24	0·33	0·28	0·04	0·24	0·23	1·55	0·13	1·34
Crenotrichaceae	0·85	0·55	0·66	1·71	0·27	0·68	1·08	0·13	0·16	0·10	0·25	0·12	0·05	0·80	0·13	1·47	0·00
Methylococcaceae	0·68	0·58	0·24	1·32	0·08	0·58	1·44	1·93	0·09	0·17	0·23	0·04	0·04	0·41	0·19	0·71	0·07
Alcaligenaceae	0·78	0·25	0·45	1·05	0·22	0·71	0·63	0·91	0·19	0·11	0·26	0·07	0·01	0·40	0·17	1·12	0·57
Acetobacteraceae	0·44	0·61	0·30	0·66	0·13	0·38	0·45	2·33	0·97	0·44	0·05	0·09	0·03	0·18	0·15	0·23	0·45
Syntrophorhabdaceae	0·58	0·27	0·26	1·78	0·07	0·70	0·24	0·19	0·07	0·00	0·13	0·13	0·00	0·26	0·01	0·72	0·02
Enterobacteriaceae	0·34	0·47	0·66	0·30	0·96	0·07	0·23	0·08	0·35	0·06	0·42	0·07	0·25	0·19	0·83	0·07	0·02
Rhodobacteraceae	0·81	0·39	0·82	0·44	0·05	0·12	0·32	0·21	0·09	0·02	0·12	0·05	0·05	0·07	0·12	0·07	1·57
Oxalobacteraceae	0·37	0·52	0·79	0·28	0·30	0·04	0·51	0·08	0·09	0·04	0·26	0·04	0·08	0·10	0·62	0·14	0·25
Syntrophobacteraceae	0·39	0·15	0·33	0·69	0·02	0·59	0·48	0·09	0·06	0·07	0·10	0·09	0·00	0·22	0·05	0·46	0·05
[Chthoniobacteraceae]	3·20	1·57	0·65	2·49	1·10	1·13	1·52	0·18	1·63	0·31	0·70	0·10	0·17	0·98	0·99	1·39	2·08

Figure 3 Hierarchically clustered heatmap analysis based on the bacterial community profiles at the family level. Rows represent the 31 predominant bacterial family (average abundance ≥0·20%), columns represent the 17 samples, and the values in the heatmap represent the relative percentage (%) of each bacterial family. The values are depicted by colour intensity with the legend indicated at the lower corner of the figure. [Colour figure can be viewed at wileyonlinelibrary.com]

Planktothrix, which included OTU84, OTU578, OTU1663, and OTU1672, followed by Gammaproteobacteria OTU227, Gammaproteobacteria OTU546, Betaproteobacteria OTU2388, Myxococcales OTU2394, Comamonadaceae OTU10699 and Acidimicrobiales OTU329 (Table S4).

Core bacterial community

The distribution of OTUs was analysed to provide a deep insight into core intestinal microbiota. The eight libraries (E1–E8) in the TCM group have 149 OTUs in common, which contained nearly all of the most abundant OTUs in the eight libraries (Table S5). The 149 shared OTUs consisted of 50·38–81·20% of the reads in each library (E1–E8, 67·34% in average), corresponding to 4·34% of the total OTUs in this group. Among these shared community distributed among 13 phyla, 96, 8 and 6 OTUs belonged to Proteobacteria, Fusobacteria and Firmicutes, respectively (Table S5). For the control group, 70 shared OTUs were related to nine phyla, which contained 42·68–91·85% of the reads in the each library (C1–C8, 73·51% in average), corresponding to 2·63% of the total OTUs in this group (Table S6). Among these shared community, 35, 15 and 4 OTUs belonged to Proteobacteria, Fusobacteria and Firmicutes, respectively (Table S6). The water community, which comprised 763 OTUs in total, was dominated by Proteobacteria and Cyanobacteria (Fig. 2). Four hundred and eight and 139 OTUs belonged to these two phyla, respectively.

Discussion

Gut microbiota has drawn increasing attention for its essential role in maintaining the host's health (Ley *et al.* 2008; Tarnecki *et al.* 2017). With the implementation of green cultivation, TCM is more and more widely applied in aquaculture (Francis *et al.* 2005; Galina *et al.* 2009;

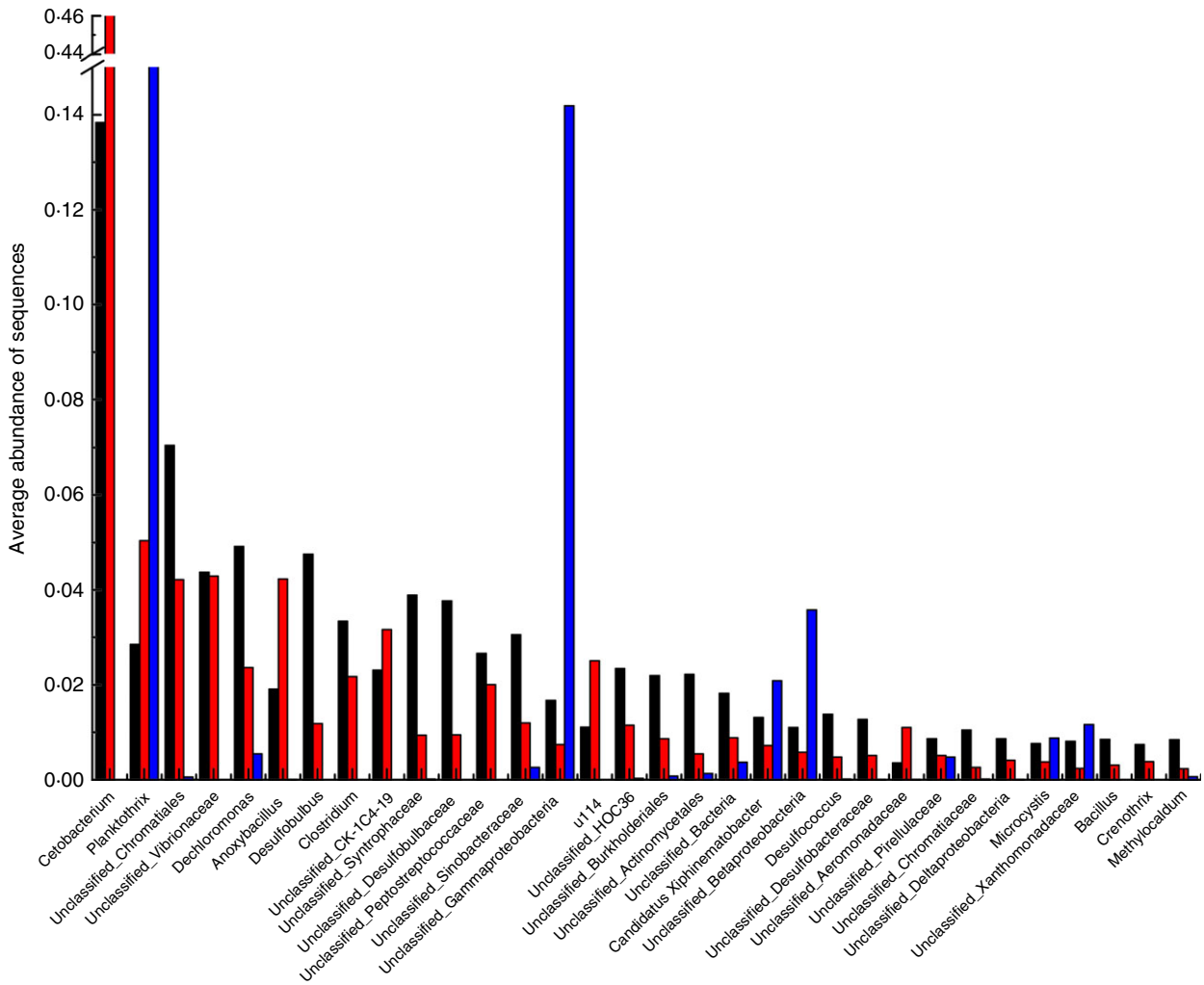

Figure 4 Relative abundance of the predominant genera in the bacterial communities (average abundance, >0·5%) of the three different groups. Group E (■) stands for the TCM group, group C (■) stands for the control group, and group S (■) stands for the water sample. [Colour figure can be viewed at wileyonlinelibrary.com]

Choi *et al.* 2014). Until now, however, studies regarding effects of TCM feed additives on intestinal microbiota have been relatively few, especially about gibel carp, a very important species for aquaculture in China. The present study compared the gut microbiota structure in gibel carp fed TCM feed additive or not fed TCM additive, with a complementary regard to the bacterial community sampled in the surrounding water.

Previous studies showed that Chinese herbal medicine contributed to promote the growth of aquatic animals. For example, 300 mg kg^{-1} of Perilla seed extract in the diet of gibel carp may significantly improve growth and feed utilization (YaoPing *et al.* 2008). The present study showed that 5% Jade screen (YPF) powder added to the diet of gibel carp for 3 months had no impact on fish growth. In an earlier study, diets supplemented with Jade screen powder at several doses (2·5, 5, 10%) for 2 weeks increased the body WG of tilapia (*Oreochromis niloticus*), and the dose of 10% was the most efficient (Zhang *et al.* 2015). However, Jade screen powder had no effect on the WG of *Acipenser schrencki* (Wang *et al.* 2012). The effect of TCM on fish growth promotion may depend on many factors, such as the composition of the medicine, the dose and duration of treatment, as well as the fish species. In the present study, healthy gibel carp were reared in optimal conditions, and the dietary addition of Jade screen powder had no negative effect neither in terms of growth nor survival. Further experiments would be necessary to test the efficiency of such a treatment in suboptimal conditions.

Many studies showed that the microbial community in the fish intestinal contents may be close to those in

the culture water and sediment, but aquaculture feed has also a significant impact on gut microbiota (Wu *et al.* 2012; Navarrete *et al.* 2013; Tarnecki *et al.* 2017). Gut fullness and feed composition influenced particularly the gut microbiome of grass carp, among other external factors (Ni *et al.* 2014). Some reports suggested that Proteobacteria and Firmicutes are often the most ubiquitous and predominant phyla in the intestinal microbiota of fish, especially Cyprinids (Wu *et al.* 2010; Roeselers *et al.* 2011). In other studies on freshwater fishes including crucian carp, Fusobacteria and Proteobacteria were the two most dominant phyla, while the relative abundance of Firmicutes was relatively low (van Kessel *et al.* 2011; Li *et al.* 2015a). The present data were in agreement with the later trend. Almost all Fusobacteria found in the fish intestinal contents were assigned to the genus *Cetobacterium* (48 917 out of 52 092 reads, i.e. 93·91%). In China, gibel carp (*Carassius auratus gibelio*) were cultivated at high stocking density to utilize fully the ponds and to maximize yield, resulting in an increase of the relative abundance of *Cetobacterium* in gut microbiota by 7–11 times compared to that observed in low stocking density conditions (Zhou *et al.* 2012). In the present study, the high stocking density of gibel carp in net cages may thus explain the overwhelming abundance of *Cetobacterium* in the fish gut. Besides, Firmicutes was the third phylum in terms of abundance, with a relatively stable level around 10% of the intestinal community in gibel carp.

The core gut microbiota received extensive attention and debate among researchers from all over the world (Turnbaugh *et al.* 2009). Previous studies showed that the core gut microbiota in gibel carp may belong to Proteobacteria and Firmicutes (Wu *et al.* 2013). However, a recent study showed that Fusobacteria and Proteobacteria were the two main phyla in the intestinal core microbiota of crucian carp (Li *et al.* 2015a). Further comparisons of gut microbiota from the same species of fish sampled in different living conditions would be essential to delineate the hypothetical 'true' core microbiota of the species. In the current study, Fusobacteria, Proteobacteria and Firmicutes included most of the prevalent bacteria in all intestinal samples, possibly constituting the core gut microbiota of gibel carp.

Proteobacteria were predominant in the culture water, in a similar proportion as in the fish intestinal contents (45·45 *vs* 40·54%, respectively). The second most abundant phylum in the culture water community was Cyanobacteria (43·02%), which were far more abundant than in the intestinal contents of gibel carp (4·71%). Moreover, the fish intestinal contents shared 9·93% bacterial OTUs in common with the culture water, which confirmed that the fish gut microbiota was partly derived from the culture water, but remaining globally distinct, as previously observed (Romero and Navarrete 2006). A much lower richness and diversity was observed in the water samples compared with gut microbiota, suggesting that the surrounding community may be unstable. Many reports emphasized that culture water is an open ecosystem highly susceptible to environmental factors (Wu *et al.* 2013).

Recent studies have revealed that a distinct and stable intestinal microbiota is established in the healthy gut of different fish species, despite its susceptibility to environmental factors (Freese and Schink 2011; Li *et al.* 2015a). However, some variability was generally detected among individuals or replicates of the same fish species (Wu *et al.* 2013; Li *et al.* 2015a). In this study, a clearly higher proportion of shared OTUs were found within the TCM group (4·34%) compared to the control group (2·63%), and the richness and diversity indices in the TCM group had relatively lower CV, SD and ranges. These results showed clearly the higher homogeneity of the intestinal community among fish individuals of the TCM group. It might result from the selection pressure within the gut due to long-term TCM feeding, as suggested by several studies (Bevins and Salzman 2011; Wong and Rawls 2012). A previous study indicated that the immune system exerted a critical role in the establishment of sustainable host–microbe relationship, contributing thus to the remarkable diversity of ecosystems (Peterson *et al.* 2007). Jade screen powder can improve the nonspecific immune response and the expression of related genes in fish, which may influence the intestinal microbiota of fishes (Zhang *et al.* 2015). The present features of fish gut microbiota confirmed the opinion that TCM can balance the gut ecosystem (Jia *et al.* 2008; Li *et al.* 2009a). A diet supplemented with five Chinese herbal medicines modified the gut microbiota composition and increased the quantity of some intestinal bacteria in *Cyprynus carpio* (Liu *et al.* 2004). The OTU richness, and the Shannon and Simpson indices observed in the present experiment showed that the bacterial diversity was also increased in the intestine of gibel carp after TCM feeding. Such diversity may contribute to the homeostasis of gut microbiota. Compared with the control group, Actinobacteria were more abundant in the TCM group (MW; $P < 0{\cdot}05$). Actinobacteria are widely distributed in various ecosystems, including in terrestrial and aquatic environment, where they can degrade refractory biomaterials through decomposition and humus formation (Trzebiatowski *et al.* 1981). Moreover, Actinobacteria can produce abundant secondary metabolites, including various potent antibiotics (Ventura *et al.* 2007), which can inhibit the growth of the intestinal pathogenic bacteria. Therefore, the increase of

Actinobacteria due to the TCM treatment may be beneficial to fish metabolism and immunity. Disturbances in gut microbiota may be caused by the colonization and infection with pathogens (Sekirov and Finlay 2009). The maintenance of balance and homeostasis of the intestinal community plays a key role in the defence against pathogens in coordination with the host immune system (Ringo and Birkbeck 1999; Round and Mazmanian 2009).

The increased bacterial diversity observed after TCM feeding corresponded mainly to the decrease of the most abundant genus, *Cetobacterium*. Since the high abundance of *Cetobacterium* was associated with high stocking density of gibel carp in a previous study (Zhou *et al.* 2012), a working hypothesis for future experiments may be to investigate whether the relative abundance of this genus could be considered as an indicator of the stress due to intensive rearing conditions, and whether TCM feeding could mitigate the effects of such stress in the suboptimal conditions created by very high stocking density. Previous studies showed that such conditions impaired growth and feed conversion of fishes cultured in cages (Kilambi *et al.* 1977), while increasing the disease incidence (Trzebiatowski *et al.* 1981). The high stocking density could affect the gut microbiota composition and damage the intestinal microecological balance in gibel carp (Zhou *et al.* 2012). In the present study, however, the intestinal bacterial balance of gibel carp cultured in high density was consolidated after TCM feeding. The possible benefit of TCM as feed additive in stressful conditions such as very high density breeding remains to be investigated in gibel carp.

The fish digestive tract is a reservoir for many opportunistic pathogens (Wu *et al.* 2010). The genera *Aeromonas* and *Acinetobacter* have been demonstrated to be related with bacterial septicaemia and other bacterial fish diseases (Li and Huang 2003; Wenhao *et al.* 2010). *Aeromonas*, *Acinetobacter* and *Shewanella*, the three genera that contain most of the important opportunistic pathogens for gibel carp, were slightly reduced after TCM feeding (Table 3). On the one hand, this might be due to chromones, the main bioactive constituents of Radix Saposhnikoviae with anti-inflammatory and immunomodulatory effects (Dai *et al.* 2008). On the other hand, it might result from interactions between bacteria after the alteration of the intestinal microbiota due to TCM feeding. Among the other ingredients of TCM, Chinese parsnip root has been already documented for stimulating bacteriostasis (Sun *et al.* 2016). *Atractylodes macrocephala* Koidz might also contribute to the effect, due to its antibacterial and immunostimulant properties (Li *et al.* 2009b). The decreasing trend of potential pathogens may reduce the risk of fish disease outbreak, which is consistent with other studies reporting that TCM could enhance the immunological function of fish and lower the risk of disease outbreak (Vasudeva Rao *et al.* 2006; Yin *et al.* 2009; Choi *et al.* 2014).

Table 3 Relative abundance (%) of genera detected in the intestinal content of gibel carp, and possibly including probiotics and/or pathogen representatives. Group E represents the TCM group (E1–E8); Group C represents the control group (C1–C8)

Taxa	Group E	Group C
Aeromonas	0·0086 ± 0·0133%	0·0391 ± 0·0388%
Acinetobacter	0·0355 ± 0·0322%	0·0416 ± 0·0813%
Shewanella	0·0820 ± 0·1704%	0·1211 ± 0·1049%
Pseudomonas	0·2067 ± 0·1670%	0·0587 ± 0·0672%
Lactobacillus	0·0024 ± 0·0069%	0
Lactococcus	0·0049 ± 0·0074%	0·0037 ± 0·0073%
Bacillus	0·8538 ± 1·3104%	0·3082 ± 0·5264%

Probiotics have been widely applied in aquaculture (Wu *et al.* 2012). The fish intestinal microbiota might be a vital source of potential probiotics for the fishes (Verschuere *et al.* 2000). The aptitude to successful colonization of the intestine may be considered as prerequisite for dietary probiotics, and the candidate strains should come preferably from fish gut microbiota. Previous studies have indicated that lactic acid bacteria (*Lactobacillus*, *Streptococcus* and *Lactococcus*), *Bacillus*, and *Pseudomonas* include important agents for biological control in aquaculture (Verschuere *et al.* 2000; Raoult 2009). In the present study, the relative abundance of lactic acid bacteria was low, while *Bacillus* and *Pseudomonas* were present in relatively high proportions in the gut of gibel carp (Table 3). Some representatives of the last two genera might be potential probiotics, but some others are known as potential pathogens (Wu *et al.* 2012), and further investigations would be necessary to characterize possible beneficial or detrimental features of the particular taxa detected in gibel carp. In the current study, lactic acid bacteria (*Lactobacillus*, and *Lactococcus*), *Bacillus*, and *Pseudomonas* were detected relatively increased in the gut of fish subjected to the TCM treatment (Table 3). Though the relative abundance of these genera was low, that may suggest that TCM feeding could promote the colonization of potential probiotics.

In conclusion, this study showed for the first time the changes in the intestinal microbiota of gibel carp after feeding the TCM, and the differences between the communities present in fish intestine and in culture water. The TCM treatment reinforced the balance and homogeneous of the intestinal microbiota, but there was no visible effect of the treatment on growth and survival of the fish that were healthy and reared in optimal conditions. The TCM feed additive may be beneficial to

enhance fish immune functions and to improve intestinal health condition, but further studies would be required to investigate the effects in suboptimal conditions, like those caused by environmental stress or very high stocking density.

Acknowledgements

This work was supported by grants from FEBL project (2016FBZ04), Jiangsu Marine and Fisheries Bureau project (D2015-11 and D2017-3), Science and technology basic project of science and technology (2014FY120200) and the Natural Science Foundation of China (no. 30670112 and 31070112).

Availability of data and materials

The sequence dataset was deposited to the NCBI/EBI/DDBJ Sequence Read Archive (accession number DRA005770) and was also available from the corresponding author upon request.

Conflict of Interest

The authors declare that they have no competing interests.

References

Abasali, H. and Mohamad, S. (2010) Immune response of common carp (*Cyprinus carpio*) fed with herbal immunostimulants diets. *J Anim Vet Adv* **9**, 1839–1847.

Ardo, L., Yin, G., Xu, P., Varadi, L., Szigeti, G., Jeney, Z. and Jeney, G. (2008) Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila*. *Aquaculture* **275**, 26–33.

Bevins, C.L. and Salzman, N.H. (2011) The potter's wheel: the host's role in sculpting its microbiota. *Cell Mol Life Sci* **68**, 3675–3685.

Choi, W.M., Mo, W.Y., Wu, S.C., Mak, N.K., Bian, Z.X., Nie, X.P. and Wong, M.H. (2014) Effects of traditional Chinese medicines (TCM) on the immune response of grass carp (*Ctenopharyngodon idellus*). *Aquac Int* **22**, 361–377.

Dai, J., Chen, X., Cheng, W., Liu, X., Fan, X., Shen, Z. and Bi, K. (2008) A sensitive liquid chromatography-mass spectrometry method for simultaneous determination of two active chromones from *Saposhnikovia* root in rat plasma and urine. *J Chromatogr B Analyt Technol Biomed Life Sci* **868**, 13–19.

Du, C.Y.Q., Zheng, K.Y.Z., Bi, C.W.C., Dong, T.T.X., Lin, H.Q. and Tsim, K.W.K. (2015) Yu Ping Feng San, an ancient Chinese herbal decoction, induces gene expression of anti-viral proteins and inhibits neuraminidase activity. *Phytother Res* **29**, 656–661.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194–2200.

Francis, G., Makkar, H.P.S. and Becker, K. (2005) *Quillaja* saponins - a natural growth promoter for fish. *Anim Feed Sci Technol* **121**, 147–157.

Freese, H.M. and Schink, B. (2011) Composition and stability of the microbial community inside the digestive tract of the aquatic crustacean *Daphnia magna*. *Microb Ecol* **62**, 882–894.

Galina, J., Yin, G., Ardo, L. and Jeney, Z. (2009) The use of immunostimulating herbs in fish. An overview of research. *Fish Physiol Biochem* **35**, 669–676.

Gong, J., Yin, F., Hou, Y. and Yin, Y. (2014) Review: Chinese herbs as alternatives to antibiotics in feed for swine and poultry production: potential and challenges in application. *Canadian J Anim Sci* **94**, 223–241.

Guozhang, Z.U., Jinnian, L.I., Chuanliang, Z., Xiaohua, Z., Zhong, W.E.I. and Jianxin, X.U. (2008) The effects of Chinese herb medicine compound on the intestinal microflora and blood visible compositions of Grass carp. *Chinese J Microecol* **20**, 4–6.

Jia, W., Li, H.K., Zhao, L.P. and Nicholson, J.K. (2008) Gut microbiota: a potential new territory for drug targeting. *Nat Rev Drug Discov* **7**, 123–129.

vanKessel, M.A.H.J., Dutilh, B.E., Neveling, K., Kwint, M.P., Veltman, J.A., Flik, G., Jetten, M.S.M., Klaren, P.H.M. *et al.* (2011) Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.). *Amb Express* **1**, 41.

Kilambi, R.V., Adams, J.C., Brown, A.V. and Wickizer, W.A. (1977) Effects of stocking density and cage size on growth, feed conversion, and production of rainbow-trout and channel catfish. *Prog Fish-Cult* **39**, 62–66.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R. and Gordon, J.I. (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol* **6**, 776–788.

Li, X. and Huang, W. (2003) Study on the bacterial septicemia of *Carassius auratus* of penze (♀)* *Cyprinus acutidorsalis* (♂) i–isolation and identification of the pathogen. *Microbiology* **30**, 56–60.

Li, H.K., Zhou, M.M., Zhao, A.H. and Jia, W. (2009a) Traditional Chinese medicine: balancing the gut ecosystem. *Phytother Res* **23**, 1332–1335.

Li, R.L., Sakwiwatkul, K., Li, Y.T. and Hu, S.H. (2009b) Enhancement of the immune responses to vaccination against foot-and-mouth disease in mice by oral administration of an extract made from *Rhizoma Atractylodis Macrocephalae* (RAM). *Vaccine* **27**, 2094–2098.

Li, T.T., Long, M., Gatesoupe, F.J., Zhang, Q.Q., Li, A.H. and Gong, X.N. (2015a) Comparative analysis of the intestinal bacterial communities in different species of carp by pyrosequencing. *Microb Ecol* **69**, 25–36.

Li, Z.M., Xu, L., Liu, W.S., Liu, Y.C., Ringo, E., Du, Z.Y. and Zhou, Z.G. (2015b) Protein replacement in practical diets altered gut allochthonous bacteria of cultured cyprinid species with different food habits. *Aquac Int* **23**, 913–928.

Liu, H., Zhang, Y., Yang, Y., Lu, T. and Ye, J. (2004) Effects of five Chinese herb medicines as additive in feed on the growth and intestinal microflora in common carp (*Cyprinus carpio*). *J Dalian Fisheries College* **19**, 16–20.

Navarrete, P., Fuentes, P., De la Fuente, L., Barros, L., Magne, F., Opazo, R., Ibacache, C., Espejo, R. *et al.* (2013) Short-term effects of dietary soybean meal and lactic acid bacteria on the intestinal morphology and microbiota of Atlantic salmon (*Salmo salar*). *Aquac Nutr* **19**, 827–836.

Nayak, S.K. (2010) Role of gastrointestinal microbiota in fish. *Aquac Res* **41**, 1553–1573.

Ni, J.J., Yan, Q.Y., Yu, Y.H. and Zhang, T.L. (2014) Factors influencing the grass carp gut microbiome and its effect on metabolism. *FEMS Microbiol Ecol* **87**, 704–714.

Peterson, D.A., McNulty, N.P., Guruge, J.L. and Gordon, J.I. (2007) IgA response to symbiotic bacteria as a mediator of gut homeostasis. *Cell Host Microbe* **2**, 328–339.

Raoult, D. (2009) Probiotics and obesity: a link? *Nat Rev Microbiol* **7**, 616.

Ringo, E. and Birkbeck, T.H. (1999) Intestinal microflora of fish larvae and fry. *Aquac Res* **30**, 73–93.

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K. and Rawls, J.F. (2011) Evidence for a core gut microbiota in the zebrafish. *ISME J* **5**, 1595–1608.

Romero, J. and Navarrete, P. (2006) 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). *Microb Ecol* **51**, 422–430.

Round, J.L. and Mazmanian, S.K. (2009) The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* **9**, 313–323.

Schwab, C., Cristescu, B., Northrup, J.M., Stenhouse, G.B. and Ganzle, M. (2011) Diet and environment shape fecal bacterial microbiota composition and enteric pathogen load of grizzly bears. *PLoS ONE* **6**, e27905.

Secombes, C.J. (1994) Enhancement of fish phagocyte activity. *Fish Shellfish Immunol* **4**, 421–436.

Sekirov, I. and Finlay, B.B. (2009) The role of the intestinal microbiota in enteric infection. *J Physiol-London* **587**, 4159–4167.

Smith, D.L., Harris, A.D., Johnson, J.A., Silbergeld, E.K. and Morris, J.G. (2002) Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proc Natl Acad Sci U S A* **99**, 6434–6439.

Song, J., Li, J., Zheng, S.R., Jin, Y. and Huang, Y. (2013) Anti-inflammatory and immunoregulatory effects of Yupingfeng (cZ parts per thousand a +/- e Z) pound powder on chronic bronchitis rats. *Chin J Integr Med* **19**, 353–359.

Sun, H., Ni, X., Song, X., Wen, B., Zhou, Y., Zou, F., Yang, M., Peng, Z. *et al.* (2016) Fermented Yupingfeng polysaccharides enhance immunity by improving the foregut microflora and intestinal barrier in weaning rex rabbits. *Appl Microbiol Biotechnol* **100**, 8105–8120.

Tarnecki, A.M., Burgos, F.A., Ray, C.L. and Arias, C.R. (2017) Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. *J Appl Microbiol* **123**, 2–17.

Trzebiatowski, R., Filipiak, J. and Jakubowski, R. (1981) Effect of stock density on growth and survival of rainbow-trout (*Salmo-Gairdneri* Rich). *Aquaculture* **22**, 289–295.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J. *et al.* (2009) A core gut microbiome in obese and lean twins. *Nature* **457**, 480–U487.

Vasudeva Rao, Y., Das, B.K., Jyotyrmayee, P. and Chakrabarti, R. (2006) Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol* **20**, 263–273.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F. and van Sinderen, D. (2007) Genomics of actinobacteria: tracing the evolutionary history of an ancient phylura. *Microbiol Mol Biol Rev* **71**, 495–548.

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev* **64**, 655–671.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**, 5261–5267.

Wang, D., Li, S., Lu, T. and Liu, H. (2012) Effect of Jade screen power on the blood biochemical indicators and non-specific immunity of *Acipenser schrencki*. *J Northwest A & F Univ Nat Sci Edn* **40**, 39–46.

Wenhao, L., Hui, C., Yong, Z., Yuanjie, Y., Xida, W. and Chungui, H. (2010) Identification and drug sensitive test of the pathogen in *Acinetobacter* disease from hybrid crucian carp (*Carassius auratus gibelio* ♀ × *Cyprinus carpio* ♂). *Fish Sci* **29**, 156–161.

Wong, S.D. and Rawls, J.F. (2012) Intestinal microbiota composition in fishes is influenced by host ecology and environment. *Mol Ecol* **21**, 3100–3102.

Wu, S.G., Gao, T.H., Zheng, Y.Z., Wang, W.W., Cheng, Y.Y. and Wang, G.T. (2010) Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*). *Aquaculture* **303**, 1–7.

Wu, S.G., Wang, G.T., Angert, E.R., Wang, W.W., Li, W.X. and Zou, H. (2012) Composition, diversity, and origin of

the bacterial community in grass carp intestine. *PLoS ONE* **7**, e30440.

Wu, S.G., Tian, J.Y., Gatesoupe, F.J., Li, W.X., Zou, H., Yang, B.J. and Wang, G.T. (2013) Intestinal microbiota of gibel carp (*Carassius auratus gibelio*) and its origin as revealed by 454 pyrosequencing. *World J Microbiol Biotechnol* **29**, 1585–1595.

YaoPing, L.U., YongXu, C., XuGan, W.U., ZhiYong, Y.U. and MuHua, W. (2008) Effects of extractives of perilla seed on survival, growth and body biochemical composition of *Carassius auratus gibelio*. *Acta Hydrobiol Sin* **32**, 258–266.

Yin, G., Ardo, L., Thompson, K.D., Adams, A., Jeney, Z. and Jeney, G. (2009) Chinese herbs (*Astragalus radix* and *Ganoderma lucidum*) enhance immune response of carp, *Cyprinus carpio*, and protection against *Aeromonas hydrophila*. *Fish Shellfish Immunol* **26**, 140–145.

Zhang, Q., Liu, T., Li, T., Xu, J., Long, M., Wang, X., Gong, X. and Li, A. (2015) The effects of jade screen power on the non-specific immune response and the expression of the related genes in fish. *Acta Hydrobiol Sin* **39**, 1076–1084.

Zhou, Z., He, S., Liu, Y., Shi, P., Yao, B. and Ringø, E. (2012) Do stocking densities affect the gut microbiota of gibel carp (*Carassius auratus gibelio*) cultured in ponds? *J Aquacult Res Dev* **S1**, 003.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Rarefaction analysis of the different samples. Rarefaction curves of OTUs clustered at 97% sequence identity of all the 17 samples.

Figure S2 Cluster analysis based on weighted UniFrac metric for bacterial communities from all the 17 samples.

Table S1 Fish growth and survival in each cage.

Table S2 Comparison of measures of dispersion between the TCM group (Group E) and the control group (Group C).

Table S3 Average relative abundance of significantly different bacterial populations at the genus level in the two groups of intestinal content.

Table S4 The 10 highest average abundant OTUs in the three groups.

Table S5 The distribution of shared reads and OTUs of core bacteria in TCM group.

Table S6 The distribution of shared reads and OTUs of core bacteria in the control group.