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ORIGINAL PAPER

Effects of the antibiotic growth promoters flavomycin and florfenicol on the autochthonous intestinal microbiota of hybrid tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂)

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Abstract The 16S rDNA PCR-DGGE and *rpo*B quantitative PCR (RQ-PCR) techniques were used to evaluate the effects of dietary flavomycin and florfenicol on the autochthonous intestinal microbiota of hybrid tilapia. The fish were fed four diets: control, dietary flavomycin, florfenicol and their combination. After 8 weeks of feeding, 6 fish from each cage were randomly chosen for the analysis. The total number of intestinal bacteria was determined by RQ-PCR. The results showed that dietary antibiotics significantly influenced the intestinal microbiota and dramatically reduced the intensity of total intestinal bacterial counts. The intensity of some phylotypes (EU563257, EU563262 and EU563255) were reduced to non-detectable levels by both dietary antibiotics, while supplementation of florfenicol to the diet also reduced the intensity of the phylotypes EU563242 and EU563262, uncultured *Mycobacterium* sp.-like, uncultured *Cyanobacterium*-like and uncultured *Cyanobacterium* (EU563246). Dietary flavomycin only reduced the OTU intensity of one phylotype, identified as a member of the phylum *Fusobacteria*. The antibiotic combination only reduced the phylotypes EU563242 and EU563262. Based on our results, we conclude that the reduced effect of florfenicol on intestinal microbiota was stronger than that of flavomycin, and when flavomycin and florfenicol were added in combination, the effect of florfenicol overshadowed that of flavomycin.

Keywords Hybrid tilapia · Dietary flavomycin · Florfenicol · RQ-PCR · DGGE · Intestinal bacteria

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Introduction

Aquaculture animals are colonized by trillions of microorganisms that have a symbiotic relationship with their host and are distributed in gill, body surface and gastrointestinal (GI) tract (Frenkiel and Mouëza 1995; Armstrong et al. 2001; Izvekova et al. 2007). The majority of these microbes inhabits the GI tract and plays an important role in nutritional, physiological and pathological events (Denev et al. 2009; Merrifield et al. 2010; Nayak 2010). During the last decade, several studies have showed that the composition of fish intestinal microbiota is highly variable and is affected by the developmental stages, diet and environmental conditions (González et al. 1999; Ringø and Birkbeck 1999; Spanggaard et al. 2000; Huber et al. 2004).

In China, antibiotics as growth promoters are widely used in aquafeeds, especially flavomycin and florfenicol. Flavomycin is a glycolipid antibiotic produced by *Streptomyces* species and inhibits peptidoglycan polymerases through impairment of the transglycolase activities of penicillin-binding proteins (Butaye et al. 2003). Flavomycin is known to change the equilibrium of gut microbiota and is active primarily against Gram-positive bacteria but also to

some extent against certain Gram-negative bacteria, such as *Pasteurella* and *Brucella* (Huber and Nesemann 1968). Bacteria species such as *Clostridium perfringens* and many other clostridia species, several species of *Enterococcus*, including *E. gallinarum*, *E. casseliflavus*, *E. faecium*, *E. mundtii* and *E. hirae*, are reported to be of natural resistance to flavomycin (Devriese 1980; Dutta and Devriese 1980, 1982; Butaye et al. 1998, 2000a, b, 2001). Another important antibiotic is florfenicol—a broad-spectrum bacteriostatic antibiotic binding the 50S ribosomal subunit of susceptible pathogens (Plumb 2004). This antibiotic is reported to be effective against important fish pathogens such as *Yersinia ruckeri*, *Flavobacterium psychrophilum* and *Aeromonas salmonicida* (Fukui et al. 1987; Samuelsen et al. 1998; Bruun et al. 2000), and *Pasteurella multocida*, *Mannheimia haemolytica*, *Actinobacillus pleuropneumoniae* and *Streptococcus suis* in vitro (Priebe and Schwarz 2003). However, the influence of antibiotics on intestinal microbiota has been determined by traditional culture-based method (Samuelsen et al. 1998; Bruun et al. 2000; Butaye et al. 2001). A large percentage of the intestinal microbiota cannot be cultured, resulting in limited understanding of the impact of antibiotics on the autochthonous intestinal microbiota. Consequently, the objective of the present study was to obtain better knowledge and understanding of the intestinal bacterial community of hybrid tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂) reared in cages and how supplementation of dietary florfenicol and flavomycin, either singly or in combination, impacts the autochthonous intestinal microbiota.

Materials and methods

Experimental diets

Basal diets containing 26.0% protein and 3.0% lipid (Table 1) were formulated according to Li (2001). In the present study, four different diets were used: basal diet (CK), diet supplemented with 20 mg florfenicol kg^{-1}, diet supplemented with 20 mg flavomycin kg^{-1} and a diet supplemented with 10 mg florfenicol kg^{-1} and 10 mg flavomycin kg^{-1}.

Feeding trial

The culture experiment was conducted in a 4,000-m^2 earthen pond at a local aquaculture farm, Jiaxing, Zhejiang, China. Juvenile hybrid tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂) was acclimated in a floating net cage (4.0 m × 2.0 m × 1.5 m) for 2 weeks. Water depth of the pond was approximately 1.5 m. After 2 days of starvation, uniform fish (50.89 ± 0.27 g) were randomly distributed into 12 floating net cages (1.1 m × 1.1 m × 1.1 m). Each dietary group was fed in triplicate cages, and each cage contained 20 fish. The fish were hand-fed 3% of initial body weight three times a day (08:00, 11:30 and 17:30), and the feed ration was adjusted weekly to insure tilapias in each cage consume the diet pellets within 1 h. Each cage was individually aerated, and one tenth of the experimental pond water was exchanged by fully aerated tap water each week. During the feeding period, rearing temperature was 27.0 ± 3.0°C, while dissolved oxygen (DO) >5.0 mg oxygen l^{-1}, pH 7.8, NH_4^+-N < 0.50 mg nitrogen l^{-1} and NO_2-N < 0.05 mg nitrogen l^{-1}. The photoperiod was fixed at a natural condition from 5:00 to 19:00.

Table 1 Ingredients and chemical compositions of the experimental diets (%)

Ingredients	CK	Flavomycin	Florfenicol	Combination of antibiotics
Basal diet[a]	99.4	99.4	99.4	99.4
Florfenicol[b]	0.0	0.0	0.002	0.001
Flavomycin[b]	0.0	0.002	0.0	0.001
Mineral/vitamin mix[c]	0.6	0.6	0.6	0.6
Chemical composition				
Crude protein	26.0	26.0	26.0	26.0
Crude lipid	3.0	3.0	3.0	3.0
Moisture	9.4	9.6	10.2	9.9
Ash	7.4	7.8	7.3	7.6

[a] Basal diet: Cotton seed meal, Shandong, P.R. China (CP 40.0%), 15.0; Rapeseed meal, Henan, P.R. China (CP 38.0%), 23.0; Single cell protein, Zhejiang, P.R. China (CP 73.8%), 1.0; Intestine casing meal, Zhejiang, P.R. China (CP 55.6%), 1.0; Malt sprouts, Zhejiang, P.R. China (CP 26.3%), 6.0; Wheat middings, Zhejiang, P.R. China (CP 16.7%), 13.0; Wheat flour, Shandong, P.R. China (CP 12.7%), 10.0; DDGS, Shandong, P.R. China (CP 26.6%), 6.0; Corn, Shandong, P.R. China (CP 9.5%), 3.0; Bentonite, Zhejiang, P.R. China, 6.0; Rice bran, Jiangsu, P.R. China (CP 14.2%), 12.0; Betaine, Shandong, P.R. China, 0.1; Phospholipid oil, Jiangsu, P.R. China, 1.4; Calcium phosphate, Jiangsu, P.R. China, 1.6; Vc phosphate, Beijing, P.R. China, 0.02; Choline chloride, Shandong, P.R. China, 0.1; Antioxidantr, Shanghai, P.R. China, 0.03; Antimouldr, Shanghai, P.R. China, 0.1

[b] Supplied by Zhejiang Yiwu Huatai Feed Company

[c] See the reference (Zhou et al. 2007)

Sampling of the autochthonous gut microbiota

Six fish from each cage were randomly collected after 8-week feeding for gut bacterial analysis. Sampling of the autochthonous microbiota from the whole intestine was carried out after two days of starvation as previously described (Zhou et al. 2007). Briefly, the digestive tracts were aseptically removed in their entirety, slit open with a sterile scalpel, and the contents and non-adherent bacteria were rinsed three times in phosphate-buffered saline (PBS; 0.1 M, pH 7.2). The surface of each intestine was

homogenized using a glass homogenizer as described elsewhere (LeaMaster et al. 1997) and stored in 2-ml Eppendorf tubes at −20°C until analysis (Zhou et al. 2009a).

DGGE analysis

Pooled gut samples from six fish in each cage (~200 mg) were used to avoid erroneous conclusions due to individual variation in gut microbiota as described by Ringø et al. (1995), Spanggaard et al. (2000) and He et al. (2009). The total genomic DNA from the pooled gut samples were extracted using cetyltrimethylammonium bromide (CTAB; Griffiths et al. 2000) and lysozyme methods (Miller et al. 1999) with some modifications. Briefly, the gut samples were mixed with 500 µl 5 mg ml^{-1} lysozyme solution. After incubation at 37°C for 2 h, 50 µl 10 mg ml^{-1} proteinase K (Sigma, St. Louis, MO, USA) was added and mixed gently, followed by incubation at 55°C for 20 min. Then 500 µl CTAB lysis buffer (100 mM Tris–HCl, 100 mM Na-EDTA, 1.5 M NaCl, 1% CTAB, 2% SDS, pH 8.0) was added and incubated at 65°C for 2 h. The DNA was recovered by precipitation with isopropanol and purified as described by Liu et al. (2008). The V3 region of the 16S rRNA gene was amplified with primers 338f (5′-ACTC CTACGGGAGGCAGCAG-3′) with a 40 base GC clamp at the 5′ end and 518r (5′-ATTACCGCGGCTGCTGG-3′). The 50-µl PCR reaction system contained 1× PCR buffer (20 mM Tris–HCl (pH 8.4) and 50 mM KCl), 200 µM dNTP, 500 nM each primer, 1.75 mM $MgCl_2$, 670 ng μl^{-1} bovine serum albumin, 1.25 U Platinum® Taq DNA polymerase (Invitrogen, USA) and 2 µl purified DNA. The PCR conditions were as follows: 5 min of initial denaturation at 94°C, followed by 28 cycles of 30 s of denaturation at 94°C, 30-s annealing at 65°C (decreasing 1°C per cycle until 56°C), and 30-s extension at 72°C and a final extension at 72°C for 10 min. PCR products were examined by 2% agarose gel electrophoresis. DGGE was performed with a D-Code universal mutation system (Bio-Rad, Hercules, CA, USA). PCR products (~800 ng) were loaded onto polyacrylamide gels in 0.5× TAE buffer, with a gradient of 40–60% denaturant. Electrophoresis was performed at 60°C, 65 V for 16 h. After electrophoresis, gels were stained for 20 min in distilled water containing ethidium bromide (0.5 µg ml^{-1}) and visualized under UV light. DGGE bands were excised from the gels, resuspended in 100 µl distilled water and kept at 4°C overnight. The supernatant was used as the template for a second round of PCR under the same conditions. The PCR products were ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) for sequencing (Invitrogen, Shanghai, China). Representative sequences were deposited in the NCBI database under accession numbers EU563242–EU563265.

Total intestinal bacteria analysis

Enumeration of total bacteria was conducted by real-time PCR according to Takahashi et al. (2006) and Silkie and Nelson (2009) with some modifications. Briefly, several cultured bacteria were selected based on the predominant microbiota present in DGGE with an abundance index greater than 5%. In this study, *Clostridium thermocellum* B108 (Gram-positive bacteria) and *Sphingomonas* sp. B222 (Gram-negative bacteria) were chosen as standards. Both bacterial species were cultured overnight in Luria–Bertani (LB) medium, and the total number of bacteria was counted using a hemocytometer. Thereafter, each bacterial strain was mixed equally at 0.5×10^8 cells ml^{-1}, and total genomic DNA was extracted from 1 ml of the combined mixture using CTAB (Griffiths et al. 2000) and lysozyme methods (Miller et al. 1999). To increase the concentration of purified intestinal DNA, the genomic DNA was pre-cooled at −70°C for ~2 h, freeze-dried (CHRIST, Osterode, Germany) overnight and solved in TE buffer to the ideal concentration of 50 ng μl^{-1}.

Serial dilutions of standards at 10^3, 10^4, 10^5 and 10^6 CFU per template reaction were prepared for calibration. The RNA polymerase β-subunit gene (*rpo*B; one copy in bacteria) was amplified using the primers *rpo*B1698f (5′-A ACATCGGTTTGATCAAC-3′) and *rpo*B2041r (5′-CGT TGCATGTTGGTACCCAT-3′; Dahllöf et al. 2000). The reaction mixture (20 µl) was prepared according to the manufacturer's protocol: 7 µl PCR-grade water, 1 µl each primer (5 µmol l^{-1}), 10 µl 2× real-time PCR master mix (SYBR Green; TOYOBO, Shanghai, China) and 1 µl DNA template (50 ng μl^{-1}). The PCR conditions consisted of initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min.

The concentration of each standard (CFU ml^{-1}) was inputted into the LightCycler 2.0 software using the threshold cycle value (C_T) to construct a standard for absolute quantification analysis. The number of bacteria present in unknown samples (12 samples) was calculated based on the standard curve. Each sample was analyzed in at least four replicates.

Statistical analysis

The gel images were analyzed using the public domain NIH Image program to calculate relative abundance (RA, %; Simpson et al. 1999). Cluster analysis was performed based on the unweighted pair group method using the arithmetic mean algorithm (UPGMA) by the program NTSYS. In this study, pairwise similarity coefficient (*C*s) less than 0.60 is regarded as significant difference; while $0.60 \leq Cs < 0.85$ is

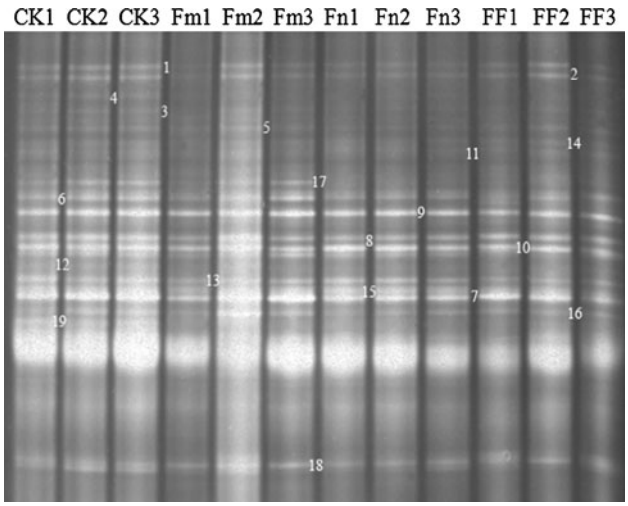

Fig. 1 DGGE profile generated from the V3-16S rDNA fragments of the bacteria from the intestinal wall of hybrid tilapia *O. niloticus* ♀ × *O. aureus* ♂. CK1–3 represent samples taken from the intestine of tilapia fed control diet without antibiotic supplement; Fm1–3 are samples from the intestine of tilapia fed diet supplemented with flavomycin; Fn1–3 are samples from the intestine of tilapia fed diet supplemented with florfenicol; and FF1–3 are samples from the intestine of tilapia fed diet supplemented with flavomycin and florfenicol

marginal difference, and $Cs \geq 0.85$ is very similar according to Sun et al. (2004). The Shannon index of bacterial diversity, H, was calculated as Shannon and Weaver (1963) described.

Results are presented as mean ± SD. Data were subjected to one-way ANOVA to test the effect of dietary treatment. When significant differences were detected ($P < 0.05$), Duncan's multiple range test was used to compare mean values among dietary treatments. All statistical analysis was carried out using the statistic software SPSS version 10.0.

Results

DGGE profiles of intestinal microbiota in tilapia

Bacterial DGGE profiles of four different treatments showed significant differences (Fig. 1, Tables 2, 4), and the difference of the interior-group was more significant than that of inter-group. There were 18.67 ± 0.47, 15.00 ± 0.82, 14.33 ± 0.47 and 14.66 ± 0.47 OTUs in CK, flavomycin, florfenicol and antibiotic combination groups, respectively (Table 2). The pairwise similarity coefficients (*Cs*) matrix for the intestinal bacterial community of hybrid tilapia based on the DGGE fingerprints is shown in Table 3. The bacterial community in flavomycin group was similar to that of CK with a *Cs* value of 0.84. The bacterial community of CK was marginally different to that of florfenicol and the combination of flavomycin and florfenicol.

Identification of dominant DGGE bands

A total of 19 representative OTUs were retrieved from the bacterial DGGE profiles (Table 4). *Proteobacteria* (2 OTUs), *Actinobacteria* (2 OTUs), *Cyanobacteria* (3 OTUs), *Fusobacterium* (1 OTU) and *Firmicutes* (1 OTU) were the predominant autochthonous bacteria in hybrid tilapia intestine. The relative abundance (RA) results showed that OTU 3 (uncultured bacterium, EU418508), 7 (uncultured bacterium, EF532770), 8 (uncultured *Cyanobacterium*, EF630240), 10 (uncultured prokaryote, AJ867878), 11 (uncultured bacterium clone, DQ675149), 13 (*Sphingomonas* sp., EU442226) and 18 (uncultured *β-Proteobacterium*, EF697165) were not affected by dietary antibiotics. In contrast to these results, the intensities of OTU 4 (uncultured bacterium, AB206034), 12 (*Streptomyces* sp., EU159565) and 19 (uncultured prokaryote, AJ867878) were reduced to non-detectable levels by dietary flavomycin and florfenicol. One interesting observation was that OTU 17, an uncultured bacterium with 100% similarity to accession no. EF599665, was reduced to non-detectable levels by dietary florfenicol and the antibiotic combination, but no difference in RA was observed between the control group (CK) and fish that received flavomycin. Compared with CK, supplementation of dietary florfenicol decreased the intensities of OTU 1 (uncultured bacterium, AJ504589), 2 (uncultured *Cyanobacterium*, DQ158167), 5 (uncultured *Cyanobacterium,* EU751409) and 14 (uncultured *Mycobacterium* sp., EF438322), but the intensities of OTU 9 (uncultured bacterium, EF669487) and 16 (uncultured bacterium,

Table 2 Effect of different feeding regimes on the intestinal microbiota of hybrid tilapia *O. niloticus* ♀ × *O. aureus* ♂

Feeding	CK	Flavomycin	Florfenicol	Combination of antibiotics	*P* value
Bacterial counts ($\times 10^7$ CFU g^{-1} dry matter)	1.11 ± 0.10^a	0.47 ± 0.05^b	0.2 ± 0.01^c	0.43 ± 0.08^b	<0.001
OTUs	18.67 ± 0.47^a	15.00 ± 0.82^b	14.33 ± 0.47^b	14.66 ± 0.47^b	<0.001
H	2.64 ± 0.02^a	2.45 ± 0.02^b	2.36 ± 0.01^c	2.48 ± 0.02^b	<0.001
E_H	0.73 ± 0.01^a	0.73 ± 0.02^a	0.70 ± 0.01^a	0.79 ± 0.02^b	0.002

Data (mean ± SD) in the same row sharing a common superscript are not significantly different (Duncan's multiple range test, $P > 0.05$)

Table 3 Pairwise similarity coefficients (*Cs*) matrix for the intestinal microbiota of hybrid tilapia *O. niloticus* ♀ × *O. aureus* ♂

	CK	Flavomycin	Florfenicol	Combination of antibiotics
CK	1.00			
Flavomycin	0.84MS	1.00		
Florfenicol	0.79MS	0.95NS	1.00	
Combination of antibiotics	0.79MS	0.95NS	1.00NS	1.00

NS very similar, *MS* marginal difference

AJ548786) were elevated. An interesting finding was that the intensity of OTU 15 on the DGGE, identified as a member of the phylum *Fusobacteria*, was reduced only by dietary flavomycin. The combination of the two antibiotics significantly decreased OTU 1 as a RA value of 1.37 ± 0.12 was observed in the combined antibiotics group compared to the RA value of 2.60 ± 0.24 in CK.

Total intestinal bacterial counts

The total intestinal bacterial counts analyzed by RQ-PCR varied from 0.20 ± 0.01 to 1.11 ± 0.10 CFU $\times 10^7$ g^{-1} as shown in Table 2. Supplementation of dietary florfenicol and flavomycin significantly reduced ($P < 0.05$) the intestinal bacteria. Shannon diversity indexes (H) of the antibiotics treatments were significantly lower ($P < 0.05$) than that of CK. The value of H for florfenicol group was higher than that of flavomycin and antibiotic combination ($P < 0.05$), but the Shannon equitability indexes (E_H) were not affected by dietary antibiotics compared to CK ($P > 0.05$).

Discussion

In the present study, the autochthonous intestinal microbiota was significantly modulated by flavomycin. This result is consistent with previous reports that flavomycin reduced the incidence of the animal pathogens *Salmonella* and *Clostridium* in pre-slaughter broilers (Bolder et al. 1999) and modulated the ruminal gut microbiota (Edwards et al. 2005). In the study of broiler chicks, Gunal et al. (2006) demonstrated that the counts of total bacteria and Gram-negative bacteria were significantly decreased by flavomycin after 21 and 42 days of feeding. Zhou et al. (2009b) observed that dietary flavomycin affected the autochthonous intestinal bacterial community in tilapia. In the present study, several intestinal phylotypes such as *Streptomyces* sp.-like bacterium, uncultured bacterium (EU563262), uncultured prokaryote-like bacterium and *Fusobacteria* bacterium (EU563264) were reduced by flavomycin. *Fusobacterium* species are Gram-negative bacteria (Garcia et al. 1992) and have been reported in bovine rumen, pig, poultry and fish (GU301238; Tan et al. 1996; Anderson et al. 2000; Edwards et al. 2005). *F. necrophorum* is generally regarded as an opportunistic pathogen (Brazier et al. 2002) but has been reported to be inhibited by flavomycin in a study of sheep (Edwards et al. 2005). More recently, Jeong et al. (2009) showed that *Fusobacterium* spp. in human gut were susceptible to flavomycin. Previous studies reported that *Fusobacterium* spp. have a very high rate of deamination by converting excessive dietary amino acids to ammonia (Russell et al. 1991; Attwood et al. 1998), and suppression of *Fusobacterium* by flavomycin has been reported to have a favorable effect on nitrogen metabolism (Edwards et al. 2005).

The present study showed that supplementation of dietary florfenicol reduced a number of autochthonous intestinal bacteria in tilapia compared to flavomycin. For example, the intensity of *Streptomyces* sp.-like bacterium, uncultured bacteria (EU563262 and EU563265) and uncultured prokaryote-like bacterium were reduced to non-detectable levels by dietary florfenicol. On the other hand, some gut bacteria including uncultured *Mycobacterium* sp.-like bacterium, uncultured *Cyanobacterium*-like bacterium, uncultured *Cyanobacterium* (EU563246) and uncultured bacterium (EU563242) were partly decreased. *Mycobacteria* are obligate aerobic, acid-fast, Gram-positive, non-spore forming, non-motile and prevalent in soil and water (Frerichs 1993). In two recent studies (He et al. 2009; Zhou et al. 2009a) using DGGE, *Mycobacterium* sp.-like bacteria were detected in the tilapia intestine. In the present study, florfenicol affected the RA value of *Mycobacterium* sp.-like bacteria from 1.40 ± 0.16 to 0.93 ± 0.21; however, dietary flavomycin had no effect.

Three species of Cyanobacteria were detected in the tilapia intestine (Table 4). Cyanobacteria possess the capability to store abundant nutrients, and some species can convert gaseous nitrogen to ammonia via nitrogen fixation (Stewart 1967). Cyanobacteria are well known for their ability to produce a large number of diverse secondary metabolites (Vining 1992), which cause mortality, initiate or promote tumors or deteriorate the health of several cultivated species (nile tilapia, catfish, white shrimp and rainbow trout; Smith et al. 2008). Previous investigations have reported Cyanobacteria in the intestine of filter-feeding fish such as Atlantic menhaden (*Brevoortia tyrannus*), silver carp (*Hypophthalmichtyhs molitrix*) or tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂; Friedland et al. 2005; Kolmakov et al. 2006; He et al. 2009). In the present study, two species of Cyanobacteria were reduced by florfenicol.

Table 4 Representatives of OTUs or clones isolated from the intestine of hybrid tilapia under the experimental feeding regimes and their relative abundance

Phylogenetic group	Band	Accession no.	Relative abundance (RA, %)				*P* value	Closest relative (obtained from BLAST search)	Identity (%)	Isolated from
			CK	Flavomycin	Florfenicol	Combination of antibiotics				
Proteobacteria	13	EU563263	2.77 ± 0.65	3.07 ± 0.34	4.00 ± 0.43	3.73 ± 0.82	0.213	*Sphingomonas* sp. (EU442226)	100	Deep terrestrial subsurface (Brown, unpublished data, NCBI)
	18	EU563260	10.13 ± 1.06	7.90 ± 0.29	10.43 ± 1.34	9.27 ± 0.42	0.082	Uncultured *β-proteobacterium* (EF697165)	99	Human gastrointestinal resection specimen (Frank et al. 2007)
Actinobacteria	11	EU563256	1.70 ± 0.16	2.03 ± 0.38	2.07 ± 0.17	1.87 ± 0.25	0.492	Uncultured *actinobacterium* (DQ675149)	99	Limnology of Stratified Lakes (Allgaier and Grossart, unpublished data, NCBI)
	12	EU563257	1.20 ± 0.24[a]	–[b]	–[b]	–[b]	<0.001	*Streptomyces* sp. 926 (EU159565)	96	Acidic soil in Yunnan, China (Xu et al. unpublished data, NCBI)
	14	EU563258	1.40 ± 0.16[a]	1.57 ± 0.21[a]	0.93 ± 0.21[b]	1.53 ± 0.21[a]	0.04	Uncultured *Mycobacterium* sp. (EF438322)	98	Coal tar contaminated sediment (DeBruyn et al. 2007)
Cyanobacteria	2	EU563243	3.13 ± 0.29[a]	3.73 ± 0.49[a]	1.87 ± 0.25[b]	3.40 ± 0.45[a]	<0.001	Uncultured *cyanobacterium* (DQ158167)	98	Freshwater lake, Germany (Corredor et al. unpublished data, NCBI)
	5	EU563246	0.93 ± 0.05[a]	1.43 ± 0.17[a]	0.57 ± 0.05[b]	1.13 ± 0.26[a]	0.004	Uncultured *cyanobacterium* (EU751409)	100	Sandstone formations (Kurtz et al. unpublished data, NCBI)
	8	EU563251	10.23 ± 0.42	9.13 ± 1.27	11.77 ± 1.20	10.10 ± 1.53	0.243	Uncultured *cyanobacterium* (EF630240)	97	Sea water (Mohamed et al. 2008)
Fusobacterium	15	EU563264	3.50 ± 0.75[a]	1.97 ± 0.39[b]	2.50 ± 0.22[ab]	3.37 ± 0.46[a]	0.042	*Fusobacteria* bacterium (DQ837051)	100	Human feces (Finegold et al. 2003)
Firmicutes	6	EU563248	8.80 ± 0.94[b]	11.63 ± 1.05[a]	6.43 ± 0.61[c]	9.30 ± 0.51[b]	0.002	Uncultured *Clostridium* sp. (DQ168144)	100	Everglades wetlands (Uz and Ogram 2006)
Unclassified bacteria	1	EU563242	2.60 ± 0.24[a]	2.50 ± 0.16[a]	1.60 ± 0.14[b]	1.37 ± 0.12[b]	<0.001	Uncultured bacterium (AJ504589)	99	Activated sludge (Brown and Turner, unpublished data, NCBI)
	3	EU563245	1.00 ± 0.16	1.20 ± 0.29	1.23 ± 0.17	1.13 ± 0.21	0.717	Uncultured bacterium (EU418508)	100	Intestinal microflora of *Ctenopharyngodon idellus* (Huang et al. unpublished data, NCBI)
	4	EU563262	0.27 ± 0.05[a]	–[b]	–[b]	–[b]	<0.001	Uncultured bacterium (AB206034)	94	Activated sludge (Osaka et al. 2006)

Table 4 continued

Phylogenetic group	Band	Accession no.	Relative abundance (RA, %)				*P* value	Closest relative (obtained from BLAST search)	Identity (%)	Isolated from
			CK	Flavomycin	Florfenicol	Combination of antibiotics				
	7	EU563250	14.73 ± 0.90	14.10 ± 1.92	12.13 ± 1.65	10.53 ± 0.99	0.067	Uncultured bacterium (EF532770)	97	Swine building bioaerosols (Nehme et al. 2008)
	9	EU563252	15.73 ± 0.34^{b}	23.03 ± 1.51^{a}	24.13 ± 1.86^{a}	22.03 ± 0.56^{ab}	0.001	Uncultured bacterium (EF669487)	99	Intestinal microflora of Ctenopharyngodon idellus (Huang et al. unpublished data, NCBI)
	10	EU563255	16.93 ± 0.25	12.30 ± 0.75	14.70 ± 0.45	11.60 ± 0.54	<0.001	Uncultured prokaryote (AJ867878)	98	Lake water (Yuhana, unpublished data, NCBI)
	16	EU563259	2.63 ± 0.21^{b}	2.13 ± 0.24^{b}	5.33 ± 0.56^{a}	3.73 ± 0.39^{b}	<0.001	Uncultured bacterium (AJ548786)	98	Signy Island: Sombre Lake (Pearce et al. 2003)
	17	EU563265	1.30 ± 0.22^{a}	1.47 ± 0.31^{a}	–^{b}	–^{b}	<0.001	Uncultured bacterium (EF599665)	100	Hypersaline water (Dillon et al. unpublished data, NCBI)
	19	EU563255	3.23 ± 0.29^{a}	–^{b}	–^{b}	–^{b}	<0.001	Uncultured prokaryote (AJ867878)	98	Nival Lakes water (Yuhana, unpublished data, NCBI)

Data (mean ± SD) in the same row sharing a common superscript are not significantly different (Duncan's multiple range test, $P > 0.05$)

Based on the results of *C*s matrix and dendrogram (data not shown), the bacterial community in antibiotic combination group was very similar to that of florfenicol group. The effect of antibiotic combination on intestinal bacteria was highly similar to that observed for the florfenicol group, as the intensities of some phylotypes (EU563242, EU563255, EU563257, EU563262, and EU563265) were reduced to non-detectable levels in both the florfenicol and antibiotic combination groups. Finally, the total number of bacteria in the antibiotic combination group was intervenient between both dietary antibiotic groups. Thus, we concluded that the effect of antibiotic combination on intestinal bacteria was not reinforced, but the effect of florfenicol overshadowed that of flavomycin. In the present study, tilapia were fed 20 mg kg^{-1} of florfenicol or flavomycin, but in combination, the dosage of each was 10 mg kg^{-1}. Previous studies showed that the effects of antibiotic on pathogens are heavily dependent on dosage (Akinbowale et al. 2007; Lai et al. 2009). Florfenicol at a daily dose of 10 mg kg^{-1} was effective in decreasing mortalities induced by *Edwardsiella ictaluri* in channel catfish (*Ictalurus punctatus*; Gaunt 2004), and florfenicol showed high antimicrobial activities (MIC90 $\leq$ 1 mg ml^{-1}) in vitro against *Actinobacillus pleuropnemoniae*, *Pasteurella ultocida* and *Mannheimia haemolytica* at 0.12–4.00 mg ml^{-1} (Shin et al. 2005). Future work should aim to enhance our understanding of the effect of dietary florfenicol on gut microbiota in tilapia. In our study, it is interesting to find that some low-abundance bacteria in fish intestine were more easily affected by antibiotics administration (Table 4). Whether this part of bacteria is disadvantaged groups or contributed to the PCR bias still need further confirmation.

In conclusion, dietary florfenicol and flavomycin supplemented alone or in combination could affect the autochthonous intestinal microbiota. The intensity of some bacteria was reduced to non-detectable levels by dietary antibiotics, and the total bacterial diversity was also decreased following the administration of antibiotics. When antibiotics combined together, the effect of florfenicol overshadowed that of flavomycin.

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