

Do stocking densities affect the gut microbiota of gibel carp *Carassius auratus gibelio* cultured in ponds?

Z. Zhou^{1*}, S. He¹, Y. Liu¹, P. Shi¹, B. Yao¹ and E. Ringø²

¹Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, P. R. China

²Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, 9037 Tromsø, Norway

Abstract

The aim of the present study was to evaluate the intestinal microbial communities of gibel carp (*Carassius auratus gibelio*) cultivated in two beach ponds at different stocking densities. The two ponds were both ~3.33 hm² in acreage and ~1.5 m in depth. The stocking densities included one intensive with 2 fish m⁻³ while the other treated as semi-intensive with 1 fish m⁻³. The gut microbiota (both allochthonous and autochthonous) were sampled after 135 days of feeding. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene segments was used to evaluate the bacterial community. Actinobacteria, Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria and some unclassified bacteria taxa were identified in gut samples and feed. Similar bacterial communities ($C_s=0.83$) were observed with respect to the autochthonous and allochthonous gut microbiota of gibel carp cultured in the intensive culture pond. In contrast to these results, some difference ($C_s=0.61$) was observed in the gut microbiota of fish reared in the semi-intensive culture pond. Our results indicated that the difference in the bacterial communities between allochthonous bacteria and gut associated bacteria of gibel carp was not constant and was modulated by the stocking density.

Keywords: Beach pond; *Carassius auratus gibelio*; Gut microbiota; Stocking density

Introduction

In order to expand aquaculture production, the increase in stocking density is of high importance because the cultured area in China has decreased from 7,281,252 ha to 6,549,932 ha from 2004 to 2008 [1]. However, it has been reported that higher stocking density could affect the production and nutritional status, health and stress indicators of the fish [2,3]. There are present of many bacteria that have adhered to the mucosal surface of the intestine which may contribute to indigenous microbiota called autochthonous microbiota [4]. These microbiota play an important role in the health and disease control of fish, which is also affected by the developmental stages, diet and environmental conditions [4-6]. The composition of the Atlantic salmon gut microbiota was sensitive to diets with cellulose or non-starch polysaccharides from soybean meal [7]. The intestinal bacterial flora of hybrid tilapia cultured in earthen ponds varied with seasonal change, the highest total viable counts of bacteria were appeared in autumn, *Pseudomonas* spp. were found only in winter, other bacteria *Photobacterium damsela*, *Pasteurella* spp., *Cellulomonas* sp. and *Bacillus* sp. were present in some seasons of the year [8]. Dietary supplementation with Cu²⁺-exchanged montmorillonite significantly reduced ($P < 0.05$) the total intestinal aerobic bacterial counts and affected the composition of intestinal microflora with a tendency of *Aeromonas*, *Vibrio*, *Pseudomonas*, *Flavobacterium*, *Acinetobacteria*, *Alcaligenes*, *Enterobacteriaceae* decreasing as compared with control [9].

One of the traditional cultured fish species in China, gibel carp (*Carassius auratus gibelio*) is well adapted to varying culture conditions and have therefore received much attention [7]. In an early study, He et al. [8] showed that higher stocking density impaired growth and feed conversion of gibel carp cultured in cages, which might be due to low utilization efficiency of nitrogen and energy [9]. However, to our knowledge, no information is available about modulation of the intestinal microbiota of fish reared at different stocking densities. Such effect might be related to disease control [10-12]. The relationship between high stocking density and increased disease incidence has

previously been suggested [13]. Bullock et al. [14] put forward the hypothesis that reduction in stocking density could improve disease resistance. The main purpose of the present study was to evaluate the intestinal microbial communities of gibel carp cultivated in ponds at two different stocking densities. In order to obtain reliable information about the gut microbiota both allochthonous and autochthonous microbiota were investigated.

Materials and Methods

Sample preparation

Two saline-alkali beach ponds, along the East Sea of China, located in Yancheng City, Jiangsu Province, China were used. Each pond was ~3.33 hm² in acreage, ~1.5 m in depth, pH 8.5 and 2‰ salinity. Both ponds were mono-cultured with gibel carp fed similar commercial diet (crude protein level, 33%; Jiangsu Nanshan Feed Co., Ltd, China) under identical situations except for the stocking density. Initial body weight (~50 g), feeding frequency (4 meals per day) and feeding time (08:00, 11:00, 15:00, and 18:00) were similar in both ponds. The fish were continuously fed for 45 minutes until almost all individuals ceased feeding. Aeration was carried out for 3 hours from 12:00 to 15:00. In one pond the stocking density was ~2 fish m³ (HD) while the stocking density was ~1 fish m³ (LD) in the 2nd pond. These values represent intensive - and semi - intensive culture ponds in China [15].

***Corresponding author:** Z. Zhou, Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, No.12 Zhongguancun South Street, Beijing 100081, P. R. China, Tel. +86 10 82106073; Fax. +86 10 82106054; E-mail: zhou_zg@msn.com

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	Auto-HD	Auto-LD	Allo-HD	Allo-LD	Feed
Auto-HD	1.00				
Auto-LD	0.86 ns	1.00			
Allo-HD	0.83 ns	0.81 ns	1.00		
Allo-LD	0.53**	0.61*	0.58**	1.00	
Feed	0.50**	0.47**	0.44**	0.42**	1.00

*Auto-HD: autochthonous gut microbiota of gibel carp cultivated at the high stocking density; Auto-LD: autochthonous gut microbiota of gibel carp cultivated at the low stocking density; Allo-HD: allochthonous gut microbiota of gibel carp cultivated at the high stocking density; Allo-LD: allochthonous gut microbiota of gibel carp cultivated at the low stocking density; Feed: microbiota of the diet.

[†] $C_s < 0.60$ is regarded as significant difference; $0.6 \leq C_s < 0.80$ is marginal difference; and $C_s \geq 0.80$ is similar. ** - significantly different; * - marginally different; ns - similar.

Table 1: Pairwise similarity coefficient (C_s) matrix of bacterial communities in the gastrointestinal (GI) tract of gibel carp (*Carassius auratus gibelio*) cultivated in two saline-alkali ponds of Eastern China at different stocking density and in the feed.

Phylogenetic group	Band no.	Closest relative (obtained from BLAST search)	Identity (%)	Accession no.	Relative abundance (RA, %) [†]				
					Auto-HD	Auto-LD	Allo-HD	Allo-LD	Feed
<i>Actinobacteria</i>	9	Uncultured <i>Actinobacterium</i> (AY795724.2)	95	EU585899	0.5	0.1	2.4	1.4	–
	41	Uncultured <i>Corynebacterium</i> sp. (EU029393.1)	100	EU585931	3.5	4.1	6.7	8.5	–
	11	Uncultured <i>Rubrobacterales</i> bacterium (EF662655)	95	EU585901	3.6	1.6	–	2.2	1.6
<i>Proteobacteria</i>	7	<i>Acinetobacter</i> sp. (EU260356.1)	100	EU585896	–	–	–	–	2.5
	43	Uncultured <i>Acinetobacter</i> sp. (EU704874.1)	99	EU585929	–	–	–	1.2	–
	17	Uncultured <i>Eubacterium</i> sp. (AM422244.1)	98	EU585907	–	–	–	–	2.6
	18	Uncultured <i>Eubacterium</i> sp. (AM422244.1)	98	EU585908	–	–	–	–	1.4
	37	Uncultured <i>Eubacterium</i> sp. (AM422244.1)	99	EU585928	–	–	2.7	3.6	–
	15	Uncultured <i>gamma Proteobacterium</i> (DQ676291.1)	98	EU585905	–	–	–	1.3	–
	16	Uncultured <i>gamma Proteobacterium</i> (DQ676291.1)	98	EU585906	–	–	–	1.7	2.9
<i>Firmicutes</i>	34	Uncultured <i>Proteobacterium</i> (EF664750.1)	98	EU585925	–	–	–	–	11.2
	12	<i>Clostridium bif fermentans</i> (EU887825.1)	98	EU887825	2.6	12.7	7.9	5.1	3.1
	19	<i>Clostridium bif fermentans</i> (EU887825.1)	100	EU585909	–	–	–	10.4	–
	20	<i>Clostridium bif fermentans</i> (EU887825.1)	98	EU585910	0.5	1.4	1.9	9.0	–
	22	<i>Clostridium paraputrificum</i> (AY442815.1)	98	EU585913	–	–	–	1.2	–
	29	<i>Exiguobacterium</i> sp. (EF101987.1)	100	EU585903	–	1.7	–	1.0	–
	40	<i>Lactobacillales</i> bacterium (EU728748.1)	99	EU585930	3.0	5.8	2.8	–	–
	3	<i>Lactobacillus panis</i> (EF412978.1)	100	EU585895	1.2	3.7	–	0.9	–
	42	<i>Lactococcus garvieae</i> (EU727199.1)	99	EU585927	24.6	33.1	16.0	19.4	4.7
	36	<i>Streptococcus infantarius</i> (AJ439568)	100	EU585923	2.0	–	3.0	–	–
<i>Fusobacteria</i>	2	Uncultured <i>Firmicutes</i> bacterium (AB451793.1)	99	EU585891	10.0	6.1	15.4	–	12.8
	6	<i>Anaerobic</i> bacterium G13(AB064328)	96	EU585894	–	–	–	5.6	8.5
	1	<i>Cetobacterium somerae</i> (AB353124.1)	100	EU585890	24.2	2.2	15.9	2.2	4.4
	5	<i>Fusobacteria</i> bacterium (AY579753.1)	97	EU585893	–	2.7	2.6	4.4	0.5
<i>Cyanobacteria</i>	4	Uncultured microorganism (EU181077.1)	99	EU585892	7.6	–	7.9	1.7	10.5
	30	Uncultured bacterium (AB254251.1)	98	EU585922	4.3	8.5	2.5	4.9	4.0
	31	Uncultured bacterium (AB254251.1)	100	EU585924	–	–	1.2	0.8	–
<i>Unclassified_Bacteria</i>	27	<i>Vaccinium corymbosum</i> (DQ445025.1)	94	EU585919	–	–	–	2.5	–
	35	Uncultured <i>Actinobacterium</i> (DQ676371.1)	99	EU585920	–	–	–	–	1.7
	10	Uncultured <i>Cyanobacterium</i> (EU661291.1)	100	EU585900	3.6	6.0	1.8	3.2	2.7
	14	Uncultured <i>Cyanobacterium</i> (EU661291.1)	98	EU585902	2.7	4.8	2.6	3.1	14.4
	U1				1.8	–	–	–	3.3
	U6				–	–	–	–	3.5
	U8				4.4	5.5	5.7	–	–
	U9				–	–	–	1.1	1.9
U11				–	–	–	–	0.8	

Auto-HD: autochthonous gut microbiota of gibel carp cultivated at the high stocking density; Auto-LD: autochthonous gut microbiota of gibel carp cultivated at the low stocking density; Allo-HD: allochthonous gut microbiota of gibel carp cultivated at the high stocking density; Allo-LD: allochthonous gut microbiota of gibel carp cultivated at the low stocking density; Feed: microbiota of the diet

[†]Relative abundance (RA, %) was represented by the percentage of a specific band density to the total band density

Table 2: Representative of autochthonous and allochthonous bacteria or clones and their relative abundance (RA, %) in DGGE fingerprint of gibel carp cultivated in high – and low density ponds, Eastern China.

The experiment lasted for 135 days. At the end of the feeding period, twelve fish with an average weight of ~400 g (near the harvest size) were randomly collected from each pond and killed by a sharp blow on the head. All fish were individually sealed in sterile plastic bags,

stored on ice at –4°C, and transported to the Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China within 6 h. Six individual fish full of digesta were selected from each pond. Digesta

was gently squeezed out under sterile conditions as described elsewhere [16]. The autochthonous (adherent) and allochthonous (digesta) microbiota in the whole intestine of 6 pooled samples with identical weight were investigated. Analyses of pooled samples are a normal procedure used when investigating the gut microbiota by DGGE analysis of the 16S rRNA gene [16] to avoid inter-individual variations in the gut microbiota [16].

DNA extraction, PCR amplification, and denaturing gradient gel electrophoresis (DGGE) analysis

From each pond, genomic DNA from the bacterial communities of feed and the allochthonous and autochthonous fish gut microbiota were extracted as described by previous study [16,17]. Amplification of the 16S V3 region and DGGE analysis were carried as described previously [17].

Gels were stained with ethidium bromide (5 µg mL⁻¹) for 20 min, washed with deionized water and photographed with UV transillumination. Computer-assisted comparison of DGGE patterns was performed with BIO-ID++ gel analysis software (Vilber-Lourmat, Torcy, France). Relative abundance (RA, %) was represented by the percentage of a specific band density to the total band density. The Shannon diversity index $H = -\sum p_i \ln(p_i)$ and Shannon equitability index $E_H = H/\ln(S)$ (where p_i is the proportion of the i th band and S is the total number of visual bands) were calculated using spreadsheet software (V0.1, Microsoft Inc., Gardena, CA, USA) [18].

Cluster analysis was based on the unweighted pair group method using the arithmetic mean algorithm (UPGMA) as previously described by Liu et al. [17]. Similarity coefficients (C_s) < 0.60 is regarded as significant difference, $0.60 \leq C_s < 0.80$ as marginal difference, and $C_s \geq 0.80$ as similar.

Sequencing of the 16S rDNA V3

The DNA fragments were excised and amplified using the primers without the GC clamp for sequencing [17]. After removing the unreliable sequences at the 3' and 5' ends, all sequences were subjected to similarity searches with the BLAST program [19]. Representative sequences were deposited in the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) database under accession numbers EU585890–EU585896, EU585899–EU585903, EU585905–EU585910, EU585912–EU585913, EU585919–EU585920, EU585922–EU585925, EU585927–EU585931, and EU887825, respectively.

Results

Analysis of the bacterial communities

The 16S rDNA V3 DGGE fingerprints were used to profile the microbial communities of feed and in the gastrointestinal tract of gibel carp cultivated at high density (HD) and low density (LD) (Tables 1–3 and Figure 1). Similar bacterial communities ($C_s=0.83$; Table 1) were observed with respect to the autochthonous (Auto) and allochthonous (Allo) gut microbiota of gibel carp cultured in the HD pond (Auto-HD vs. Allo-HD). In contrast to these results, marginal difference ($C_s=0.61$; Table 1) was observed in the gut microbiota of gibel carp reared in the LD pond (Auto-LD vs. Allo-LD), and the allochthonous gut microbiota showed more bands (25 vs. 17), higher Shannon diversity index (3.026 vs. 2.437), and higher Shannon equitability index (0.824 vs. 0.673) (Table 3). The autochthonous gut communities of gibel carp in both ponds (Auto-HD vs. Auto-LD) were similar ($C_s=0.86$), but significant difference ($C_s=0.58$) existed between the allochthonous gut

microbial communities (Allo-HD vs. Allo-LD). The allochthonous gut microbiota in HD pond (Allo-HD) showed lower visual bands (17 vs. 25) and Shannon diversity index (2.673 vs. 3.026) than those indexes in LD pond (Allo-LD). The gut microbiota was significantly different ($C_s<0.60$) compared to the community of the feed.

Identification of bacterial species

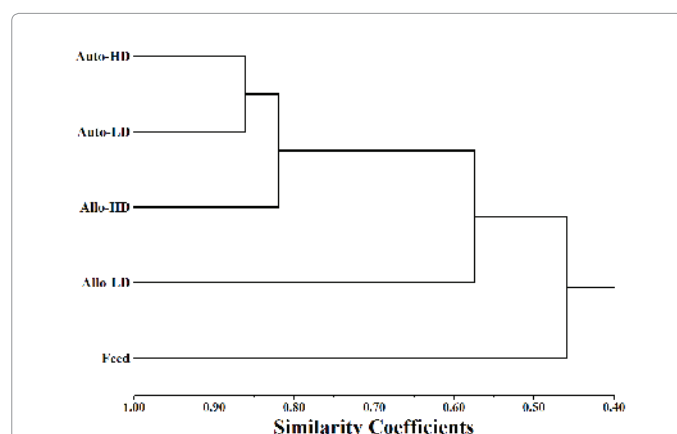
Actinobacteria, Proteobacteria, Firmicutes, Fusobacteria, Cyanobacteria, and some unclassified_bacteria taxa were detected in all samples investigated. However, 4 bands (band 6, 9, 11, and 27) showed identities less than 97% to known sequences and might represent new bacterial species (Table 2). Nine bands (band 1, 9, 10, 12, 14, 20, 30, 41 and 42) were identified in all the intestinal samples (Table 2). Band 29 and 36 were unique for fish cultivated in LD and HD ponds, respectively. Band 31 and 37 were only detected as allochthonous in fish cultivated in both ponds. Four DGGE bands (1, 2, 4 and 42) are found at higher abundance in both the allochthonous and autochthonous populations in the HD pond, while seven bands (2, 5, 12, 20, 30, 41 and 42) are seen at higher levels in the LD pond. Seven bands (band 7, 17, 18, 34, 35, U6 and U11) were only detected in the feed not in the fish.

Parameters	Auto-HD	Auto-LD	Allo-HD	Allo-LD	Feed
Numbers of visual bands	17	17	17	25	21
Shannon diversity index (H)	2.393	2.437	2.673	3.026	2.929
Shannon equitability index (E_H)	0.644	0.673	0.852	0.824	0.891

Auto-HD: intestinal wall of gibel carp at the high stocking density; Auto-LD: intestinal wall of gibel carp at the low stocking density; Allo-HD: intestinal content of gibel carp at the high stocking density; Allo-LD: intestinal content of gibel carp at the low stocking density; Feed: diet.

*The Shannon diversity index $H = -\sum p_i \ln(p_i)$ and Shannon equitability index $E_H = H/\ln(S)$ (where p_i is the proportion of the i th band and S is the total number of visual bands).

Table 3: Numbers of visual bands, Shannon diversity index* and Shannon equitability index of the bacterial communities in the GI tract of gibel carp cultivated in two saline-alkali ponds of Eastern China at different stocking density.



Auto-HD: intestinal wall of gibel carp at the high stocking density; Auto-LD: intestinal wall of gibel carp at the low stocking density; Allo-HD: intestinal content of gibel carp at the high stocking density; Allo-LD: intestinal content of gibel carp at the low stocking density; Feed: diet

Figure 1: The dendrogram of the 16S rDNA V3 PCR-DGGE fingerprints of the bacterial communities in the diet and the GI tract of gibel carp cultivated in two saline-alkali ponds of Eastern China at different stocking density.

Among bacterial species of digesta and those associated to the gut wall of gibel carp in the HD pond (Table 2), six of them: band 1, 2, 4, 5, 27 and 42 were also detected in the feed.

Discussion

In the present study, Actinobacteria, Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria, and some unclassified bacteria were detected in the intestine of gibel carp.

Marginal differences ($C_s=0.61$) existed between the autochthonous and the allochthonous gut microbiota of semi-intensively cultured fish. These results are consistent with the results of *Lutjanus sebae* ($C_s<0.80$; [20]). However, in a poly-cultured pond of grass carp (*Ctenopharyngodon idella*), bluntnose black bream (*Megalobrama amblycephala*) and gibel carp, a C_s value of 0.82 was reported between the autochthonous and allochthonous gut microbiota in gibel carp [21]. High similarity ($C_s=0.83$) was observed between the autochthonous and the allochthonous gut microbiota of fish cultivated in the HD pond, which indicate that the bacterial community between the allochthonous and autochthonous gut microbiota was not constant and was modulated by stocking density. Crowded conditions could impair the non-specific immunity of cultured fish [3], and the authors speculated that this could reduce adherence of the gut microbiota to the gastrointestinal (GI) wall, and enhance pathogen infection. Based on this hypothesis and the results of the present study we recommend that challenge studies be conducted to evaluate the relationship between stocking density and disease susceptibility.

Of the allochthonous and autochthonous gut bacteria detected in the HD pond, one band 1 showed high similarity to the anaerobic bacterium *Cetobacterium somerae* previously reported in the intestinal tract of freshwater fish [16]. Band 5 displayed 97 % identity to a cyclic nitramine - degrading psychrophilic bacterium (Fusobacteria) isolated from marine sediment [22]. In the present study, one band detected in the GI tract of LD fish showed high similarity to a Lactobacillales bacterium previously reported in a porcine bacterial culture collection (Hojberg and Jensen, unpublished data, national Center for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/>). One of the bands detected in the present study showed 99 % identity to *Lactococcus garvieae* isolated and identified from rainbow trout (*Oncorhynchus mykiss*) cultured in Iran (Akhlaghi et al. NCBI, unpublished data). In a study of degrading of turfgrass (Kudo et al. NCBI, unpublished data) information of an uncultured Firmicutes bacterium (AB451793.1) was described. Our DGGE analysis showed that one band showed high identity to the uncultured Firmicutes bacterium.

The results from DGGE analysis displayed an autochthonous *Lactobacillus panis* in the GI tract of fish reared in the HD pond, showed high similarity to a bacterium first reported in the GI tract of chicken [23]. Among the allochthonous bacteria identified in gibel carp cultured in the HD pond, band 37 was most closely related to uncultured Eubacterium sp. (similarity = 99 %) previously isolated from chironomid larvae of *Chironomus plumosus* [24]. *Clostridium paraputrificum* first characterized from the forestomach of an eastern grey kangaroo (*Macropus giganteus*) [25] showed high similarity to band 22 detected in gibel carp reared in the LD pond. The anaerobic (band 6) and uncultured bacteria (band 4) detected in the present study showed high similarity to an anaerobic bacterium isolated from fish intestine (Sugita and Tsuchiya, NCBI, unpublished data), and the uncultured microorganism detected in aquatic bird feces [26],

respectively. The higher abundance microbiota in LD and HD were 7 and 4, respectively, which was also modulated by stocking density. In the future, we will pay much attention to probiotics on intestinal microbiota at these stress conditions because of it contribute to intestinal microbial balance.

Based on the results obtained in the present study some general conclusions can be drawn: Actinobacteria, Proteobacteria, Firmicutes, Fusobacteria, Cyanobacteria, and some unclassified bacteria were identified in the gut of gibel carp. However, reduced intestinal bacterial diversity and the total number of higher abundance microbiota were noticed when the fish were cultured in the HD pond. Whether this finding has any effect on the efficacy of probiotics and prebiotics merits further investigations.

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