Dispersal patterns of endogenous bacteria among grass carp (*Ctenopharyngodon idellus*) guts

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Abstract

The formation and regulation of vertebrate endogenous intestinal microbiota has been widely studied as the microbiota plays a crucial role in the host nutrition, development, and health. Despite the importance of microbiota for host health, it is still unclear whether the endogenous intestinal microorganisms are genetically distinct or whether they are genetically related with each other in different host individuals. In the present study, the dispersal situation of the endogenous intestinal bacteria in grass carp was investigated by constructing bacterial 16S rRNA gene clone libraries. The results indicate that the bacteria harbored in the grass carp gut could be separated into the following two groups: a- the private operational taxonomic units (OTUs), which include *Cetobacterium somerae*, *Aeromonas jandaei*, *Citrobacter freundii*, *Achromobacter xylosoxidans* and *Bacteroides* species; b- the shared OTUs, which include *Vibrio cholerae*, *Plesiomonas shigelloides* and *Pasteurella* speices. The results obtained in this investigation provide valuable information for assessing the mechanism of spread of the endogenous intestinal bacteria, especially the pathogenic ones. However, the mechanisms involved in different modes of bacterial dispersal in the grass carp gut still require further research.

Keywords: Dispersal barrier, Intestinal bacteria, Clone library, Grass carp

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Introduction

The intestinal tract in vertebrates is colonized by a vast number of microorganisms which have a significant impact on the host biology including regulation of host nutrients (Stevens and Hume, 1998; Hooper *et al.*, 2002; Booijink *et al.*, 2010), protection against epithelial cell injury (Xu *et al.*, 2003; Mazmanian *et al.*, 2005) and maturation of the immune system and intestinal angiogenesis (Hooper *et al.*, 2001; Stappenbeck *et al.*, 2002). The significance of the endogenous intestinal microbiota is such that some researchers have proposed that it be considered as an essential "organ" (Hooper *et al.*, 2002; Eckburg *et al.*, 2005). It is widely accepted that endogenous intestinal microbiota have colonized vertebrate intestinal tract postpartum and have reached a relatively stable state (Favier *et al.*, 2002; Hooper *et al.*, 2002). It has further been shown that early environments of the host can significantly affect the endogenous intestinal microbiota (Thompson *et al.*, 2008; Thompson and Holmes, 2009). However, it is still unclear whether the endogenous intestinal microbiota is completely genetically distinct or related to each other among different host individuals, despite the importance of this for assessing the impact of antibiotics on the endogenous intestinal microbiota, antibiotic resistant bacteria diffusivity and re-formation of the endogenous intestinal microbiota after it has been disturbed by antibiotic treatment.

There are various ways to analyze the connectivity of microorganisms including biochemical, genetic and epidemiological methods (Pusch *et al.*, 1998; Berchtold *et al.*, 1999; Hohl *et al.*, 2013). In this study a genetic approach was used to analyze the connectivity of endogenous intestinal microbiota among different grass carp individuals by constructing four clone libraries of bacterial 16S rRNA genes.

Materials and methods

Sampling and pre-processing

Four fish were caught from an aquaculture base located in the middle reach of the Yangtse River in China (28°49´N, 112°22´E). The fish were frozen and transported to a laboratory and measurements of the body length and weight were taken. They were then dissected under sterile conditions to remove the intestinal tract. The midguts were subsequently cut out and transferred to 2.0 ml sterile centrifuge tubes for storage at -20ºC.

DNA extraction

The endogenous intestinal microbial DNA was extracted as previously described (Ni *et al.*, 2012).

16S rDNA clone library construction and sequencing analysis

The universal bacterial primers 9bfm and 1512uR were used to amplify the near-full-length bacterial 16S rDNA fragments by PCR as previously described (Mühling *et al.*, 2008). PCR products were purified using a DNA purification kit (DBI® Shanghai Xinghan BioScience Co., Ltd). The purified DNA was incorporated into pMD 18-T plasmid

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vector (Takara, Japan) and introduced into *E. coli* DH5α cells. The cells were then cultured and the plasmid isolated for sequencing of the inserted fragment. DNA sequencing was conducted by Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd., China.

Data analysis

All near-full-length sequences excluding the vector sequences and the PCR primer-binding sites were tested for possible chimeric artifacts using the Chimera check with Bellerophon (version 3) program (DeSantis *et al.*, 2006). Putative chimeras were excluded from further analysis. Sequences from both the intestinal clone libraries and the public databases were aligned using the Clustal X 1.83 program. The resulting alignment was inspected and manually adjusted using the alignment editor in the Bioedit software package. A Phylip distance matrix was generated and used as the input file for the mothur software to determine operational taxonomical units (OTUs) with >97% and >99% similarity (Schloss *et al.*, 2009). The coverages of the clone libraries were calculated according to Good's method (1953).

To estimate whether there is a dispersal limitation of a specific OTUs, we defined the term of 'private OTUs' as the OTUs just appear in a single sample. However, as it is impossible to obtain a complete enumeration of all of the intestinal microorganisms (Eckburg *et al.*, 2005), the 'private OTUs' does not certainly mean the OTUs with dispersal limitation and it is necessary to assess the reliability and validity of the microbiota data, especially when the number of sequences obtained is insufficient. To compensate for this, we introduced a probability, p_m, for estimating whether there is a dispersal limitation of 'private OTUs' in microbiota. The p_m was calculated using the formula $p_m = n^{-(m-1)}$, where n and m are the number of samples and the number of sequences within the private OTU, respectively. In the present study, n equals 4, and on the basis of $p_m<0.05$, m was much more than 3 sequences then the private OTU could be considered to be dispersal limitation. For calculating the proportion of OTUs with dispersal limitation, P_{AL}, all of OTUs that included less than 4 sequences were excluded. P_{AL} was calculated using the formula $P_{AL}=L_{OTU}\ T_{OTU}^{-1}$, where L_{OTU} was the number of OTUs with dispersal limitation, and T_{OTU} is the total number of OTUs that excluded those including less than 4 sequences.

Results

A total of 438 (103, 103, 107 and 125 from Sample 1, Sample 2, Sample 3 and Sample 4, respectively) high quality near-full-length 16S rDNA sequences excluding 27 putative chimeras were obtained (GenBank accession numbers JN032762-JN033199). The length of the sequences ranged from 1381 to 1482 base pairs (bp). It was realized that the different strains belonging to a single species were divided into different OTUs when the OUTs were defined as a group of sequences with > 99% similarity. For instance, the sequences of *Vibrio cholerae*, *Clostridium gasigenes*,

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Citrobacter freundii, and *Stenotrophomonas maltophilia* were divided into different OTUs (see Table 1 in Supporting Information). Therefore, the threshold was set at the more plausible 97%, even though the sequences of *V. cholerae* were still divided into different OTUs (Table 1).

Table 1: Phylogenetic affiliation of 16S rDNA gene phylotypes isolated from the gut contents of grass carps.

OTU	Abundance					Closest relative in GenBank (accession no.)	Similarity to the closest relative	Phylogenetic group
	Total	1	2	3	4			
OTU 1	12	10	2	0	0	*Clostridium gasigenes* strain DSM 12272 (NR 024945)	99%	Firmicutes
OTU 2	1	1	0	0	0	*Clostridium disporicum* strain DS1 (NR 026491)	98%	Firmicutes
OTU 3	1	1	0	0	0	*Clostridium subterminale* (NR 041795)	98%	Firmicutes
OTU 4	2	2	0	0	0	Uncultured *Clostridium* sp. clone PW11 (DQ355180)	96%	Firmicutes
OTU 5	10	8	1	1	0	Clostridiaceae bacterium JC13 (JF824807)	93%	Firmicutes
OTU 6	1	1	0	0	0	Erysipelotrichaceae bacterium canine oral taxon 311 (JN713479)	93%	Firmicutes
OTU 7	66	22	1	4	39	*Cetobacterium somerae* C32 (AB353124)	99%	Fusobacteria
OTU 8	34	5	0	0	29	Uncultured *Clostridium* sp. clone Zcy008 (JQ083411)	94%	Firmicutes
OTU 9	1	1	0	0	0	*Leptotrichia* sp. canine oral taxon 294 clone ZW130 (JN713461)	94%	Fusobacteria
OTU 10	3	2	0	0	1	*Shewanella putrefaciens* CN-32 (CP000681)	99%	Proteobacteria
OTU 11	19	6	7	0	6	*Aeromonas jandaei* strain LC205 (FJ940814)	100%	Proteobacteria
OTU 12	1	1	0	0	0	Uncultured *Neisseria* sp. (AM419995)	96%	Proteobacteria
OTU 13	1	1	0	0	0	*Sphingomonas aurantiaca* strain MA306a (AJ429237)	99%	Proteobacteria
OTU 14	10	**10**	0	0	0	Uncultured spirochete clone LiUU-11-182 (AY509522)	86%	Spirochaetes
OTU 15	1	1	0	0	0	Uncultured Verrucomicrobia bacterium clone BF_73 (HM23835)	95%	Verrucomicrobia
OTU 16	3	2	1	0	0	Uncultured planctomycete clone KWK1S.17 (JN656748)	96%	Planctomycetes
OTU 17	41	12	27	0	2	*Paludibacter propionicigenes* WB4 (CP002345)	89%	Bacteroidetes
OTU 18	13	3	1	9	0	*Bacteroides* sp. Tilapia9 (JQ317228)	97%	Bacteroidetes
OTU 19	8	1	7	0	0	*Bacteroides massiliensis* (AB510703)	92%	Bacteroidetes
OTU 20	19	4	14	0	1	*Bacteroides acidofaciens* (AB021157)	87%	Bacteroidetes
OTU 21	3	1	2	0	0	*Bacteroides acidofaciens* (AB021157)	87%	Bacteroidetes
OTU 22	3	3	0	0	0	*Abiotrophia defectiva* strain 99383068 (AY879306)	87%	Firmicutes
OTU 23	6	5	0	0	1	*Exiguobacterium undae* (FN870071)	87%	Firmicutes
OTU 24	2	0	2	0	0	*Merismopedia tenuissima* 0BB46S01 (AJ639891)	99%	Cyanobacteria
OTU 25	2	0	2	0	0	Uncultured Firmicutes bacterium (CU922533)	94%	Firmicutes
OTU 26	1	0	1	0	0	Methylococcaceae bacterium OS501 (AB636299)	95%	Proteobacteria
OTU 27	1	0	1	0	0	Alcaligenaceae bacterium BL-169 (DQ196633)	96%	Proteobacteria
OTU 28	1	0	1	0	0	*Azospirillum* sp. TSA14w (AB542380)	93%	Proteobacteria
OTU 29	3	0	3	0	0	Uncultured *Caulobacter* sp. clone O-B73 (JN886933)	99%	Proteobacteria
OTU 30	1	0	1	0	0	*Brevundimonas vesicularis* (AB680247)	99%	Proteobacteria
OTU 31	1	0	1	0	0	*Methylobacterium aquaticum* (AJ785572)	99%	Proteobacteria
OTU 32	2	0	2	0	0	Uncultured *Nordella* sp. clone PLYFP59 (JN792325)	98%	Proteobacteria

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Table 1 (continued):

OTU	Abundance					Closest relative in GenBank (accession no.)	Similarity to the closest relative	Phylogenetic group
	Total	1	2	3	4			
OTU 33	4	0	**4**	0	0	Uncultured *Desulfovibrio* sp. clone AL5IA3 (EU616635)	99%	Proteobacteria
OTU 34	1	0	1	0	0	*Propionibacterium acnes* TypeIA2 P.acn33 (CP003195)	99%	Actinobacteria
OTU 35	1	0	1	0	0	*Actinobacterium* sp. CH9 (FN554394)	96%	Actinobacteria
OTU 36	2	0	2	0	0	Uncultured planctomycete clone KWK1S.17 (JN656748)	96%	Planctomycetes
OTU 37	3	0	3	0	0	Uncultured planctomycete clone IMS3D25 (JN233032)	95%	Planctomycetes
OTU 38	1	0	1	0	0	Uncultured planctomycete clone KWK1S.17 (JN656748)	97%	Planctomycetes
OTU 39	1	0	1	0	0	Uncultured *Pirellula* sp. clone XZELH73 (EU703162)	95%	Planctomycetes
OTU 40	1	0	1	0	0	Uncultured planctomycete clone KWK6S.87 (JN656824)	96%	Planctomycetes
OTU 41	2	0	2	0	0	Uncultured planctomycete (FN668205)	98%	Planctomycetes
OTU 42	2	0	2	0	0	*Isophaera* sp. Schlesner 657 (GQ889437)	89%	Planctomycetes
OTU 43	1	0	1	0	0	Uncultured Planctomycetaceae bacterium YL037 (HM856408)	94%	Planctomycetes
OTU 44	7	0	**7**	0	0	*Bacteroides acidofaciens* (AB021157)	87%	Bacteroidetes
OTU 45	41	0	0	**41**	0	*Vibrio cholerae* strain PIM9 (GQ359963)	99%	Proteobacteria
OTU 46	4	0	0	**4**	0	*Vibrio cholerae* strain PIM9 (GQ359963)	99%	Proteobacteria
OTU 47	34	0	0	17	17	*Citrobacter freundii* (AB54577)	99%	Proteobacteria
OTU 48	9	0	0	**9**	0	*Plesiomonas shigelloides* strain PIC3 (GQ359957)	99%	Proteobacteria
OTU 49	7	0	0	5	2	*Pseudomonas poae* strain LS172 (FJ937922)	99%	Proteobacteria
OTU 50	6	0	0	5	1	*Stenotrophomonas maltophilia* strain 2681 (HQ185399)	99%	Proteobacteria
OTU 51	11	0	0	5	6	*Achromobacter xylosoxidans* strain M66 (HQ676601)	99%	Proteobacteria
OTU 52	9	0	0	7	2	*Alcaligenes faecalis* subsp. faecalis strain AE1.16 (GQ284565)	99%	Proteobacteria
OTU 53	1	0	0	0	1	Uncultured planctomycete clone P-B-An-15 (JN867671)	96%	Planctomycetes
OTU 54	9	0	0	0	**9**	*Pasteurella aerogenes* MCCM 01550 (AF224288)	94%	Planctomycetes
OTU 55	1	0	0	0	1	*Acinetobacter* sp. 5g (EU916711)	99%	Planctomycetes
OTU 56	1	0	0	0	1	*Propionivibrio limicola* strain GolChi1 (NR 025455)	96%	Planctomycetes
OTU 57	1	0	0	0	1	*Halochromatium* sp. MTK6IM088 (FN293083)	93%	Planctomycetes
OTU 58	2	0	0	0	2	*Clostridium sticklandii* str. DSM 519 (FP565809)	98%	Firmicutes
OTU 59	1	0	0	0	1	Uncultured *Clostridium* sp. clone PW11 (DQ355180)	97%	Firmicutes
OTU 60	1	0	0	0	1	*Blastobacter denitrificans* strain IFAM 1005 (NR 041827)	99%	Proteobacteria
OTU 61	1	0	0	0	1	*Desulfovibrio desulfuricans* strain Ser-2 (EU980606)	99%	Proteobacteria
	438	103	103	107	125			

The OUTs are defined as a group of sequences with >97% similarity. Bold type shows the OTUs with dispersal limitation.

At this threshold, a total of 61 OTUs (23, 31, 11, and 21 in Sample 1, Sample 2, Sample 3, and Sample 4, respectively) were detected which belonged to 9 phyla including Bacteroidetes, Proteobacteria, Firmicutes, Planctomycetes, Verrucomicrobia, Lentisphaerae, Fusobacteria, Actinobacteria and Cyanobacteria (Fig. 1). In the 61 OTUs, only 1 OTU (OTU7) was detected from

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all 4 samples and 4 OTUs (OTU5, OTU17, OTU18 and OTU20) were detected from 3 samples. They could be considered as common species and were closest to *Cetobacterium somerae* C32, Clostridiaceae bacterium JC13, *Paludibacter propionicigenes* WB4, *Bacteroides* sp. Tilapia9 and *Bacteroides acidofaciens* in phylogeny, respectively (Table 1). Forty one percent (25) of the OTUs were detected just once and 70.5% (43) of the OTUs were private (Table 1). In addition, the coverage of the clone libraries 1, 2, 3 and 4 were 60.87%, 51.61%, 90.91% and 47.62%, respectively. These findings suggest that there is a considerable amount of rare OTUs in the intestinal bacterial community of grass carp.

The differences of intestinal bacterial compositions among different individuals are widely studied. For instance, Booijink *et al.* (2010) report they detect high temporal and inter-individual variation in the human ileal microbiota. Lankau *et al.* (2012) prove ecological drift and local exposures drive enteric bacterial community differences within species of Galápagos iguanas. In the present study, we also found obvious differences in the intestinal bacterial compositions among different samples. At the phylum level, Firmicutes and Fusobacteria were the dominant microorganisms in Sample 1, while Bacteroidetes were the dominant microorganisms in Sample 2. Proteobacteria were the dominant microorganisms in Sample 3, but Fusobacteria and Proteobacteria were the dominant microorganisms in Sample 4 (Fig. 1).

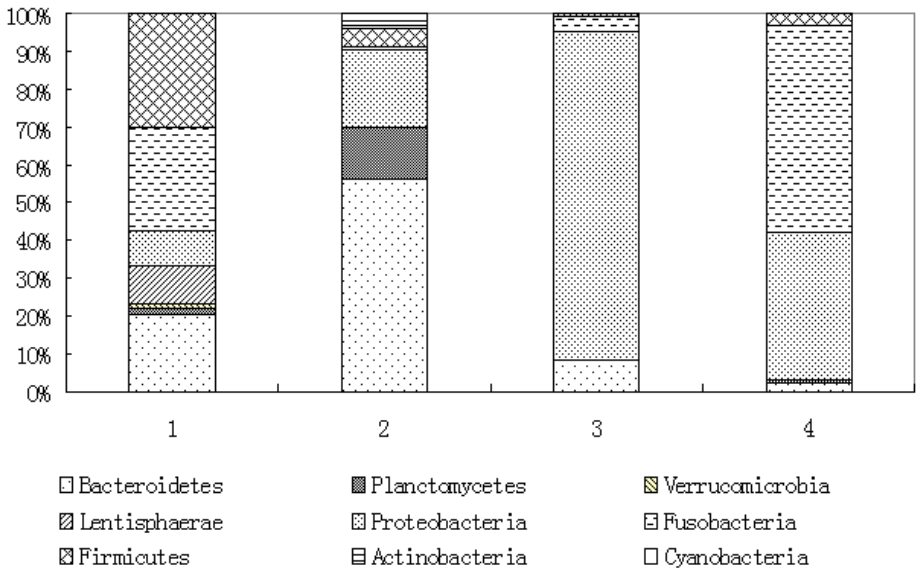

Figure 1: Bacterial phylum composition in the gut contents of grass carps.

In order to screen the OTUs with dispersal limitation, we analyzed the OTUs based on the formula $p_m = 4^{-(m-1)}$. On the basis of p_m<0.05, the private

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OTUs with more than 3 sequences were believed to be dispersal limitation. A total of 7 OTUs with dispersal limitation were detected in the present study, i.e. OTU 14 from Sample 1, OTU 33 and OTU 44 from Sample 2, OTU 45, OTU 46 and OTU 48 from Sample 3, and OTU 54 from Sample 4 (Table 1). Because T_{OTU} was 22, the proportion of private OTUs, P_{AL}, was 31.82%. These OTUs belonged to Spirochaetes, Bacteroidetes, Planctomycetes, and Proteobacteria (Table 1).

Discussion

Vertebrate endogenous intestinal microbiota has attracted extensive investigations in recent years (Zoetendal *et al.*, 2004; Kinross *et al.*, 2011; Shenderov, 2012). It is widely accepted that both environmental factors and physiological status of host affect the structure and function of vertebrate intestinal microbiota (Romero and Navarrete, 2006; Thompson and Holmes, 2009; He *et al.*, 2011; Li *et al.*, 2011; Ni *et al.*, 2012,2014). Booijink *et al.* (2010) reported that there were inter-individual differences in the human ileal microbiota. The present study shows there are significant structure differences among the intestinal microbiota from different grass carp individuals living in the same habitat. Those differences may be due to differences in physiological status of individuals.

We found that the 'shared bacteria' mostly belonged to Fusobacteria, Firmicutes, Bacteroidetes, and a number of Proteobacteria, while the 'private bacteria' mostly belonged to Spirochaetes, Bacteroidetes, Planctomycetes, and a number of Proteobacteria. The results implied that endogenous intestinal bacteria from different phyla would show different responses on the dispersal barrier.

In the present study, *Cetobacterium somerae*, *Aeromonas jandaei*, *Citrobacter freundii*, *Achromobacter xylosoxidans*, and a number of *Bacteroides* speices are the 'shared bacteria' from grass carp guts (Table 1) and are widely distributed in different habitats. For instance, *Bacteroides* and *C. somerae* have been detected in various other host intestinal tracts (Finegold *et al.*, 2003; Xu *et al.*, 2003; Tsuchiya *et al.*, 2008; Brugman *et al.*, 2009; Wu *et al.*, 2010; He *et al.*, 2011; van Kessel *et al.*, 2011) while *A. jandaei* has been isolated from water, leeches, fish, reptiles, and amphibia (Sugita *et al.*, 1994; Sugita *et al.*, 1995; Janda and Abbott, 1998; Siddall *et al.*, 2007). Other researchers have isolated *C. freundii* from tannery effluent and patients (Kumar, *et al.*, 1999; Nakano *et al.*, 2004). *A. xylosoxidans* has been found in infected patients and aqueous environments (Yabuuchi *et al.*, 1974; Igra-Siegman *et al.*, 1980; Reverdy *et al.*, 1984; Spear *et al.*, 1988; de Baets *et al.*, 2007; Wan *et al.*, 2007).

These bacteria have been shown to play a part in the host's metabolic pathways. *C. somerae* is involved in the reduction of oxazolone-induced intestinal enterocolitis (Brugman *et al.*, 2009). *C. somerae* can produce acetic acid as the major end product of metabolism of peptides and carbohydrates (Finegold *et al.*, 2003). It can also produce vitamin

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B12 (Tsuchiya *et al*., 2008). *Bacteroides* species are known to break down a wide variety of indigestible dietary plant polysaccharides and to participate in responding to environmental cues (Xu *et al*., 2003).

They are also associated with a number of host diseases. *A. jandaei* is universally recognized to cause speticemia (Joseph *et al*., 1991; Singh and Sanyal, 1997; Janda and Abbott, 1998) whereas *C. freundii* is associated with neonatal bacterial meningitis and transmissible murine colonic hyperplasia (Schauer and Falkow, 1993; Badger *et al*., 1999). *Bacteroides* species can specifically stimulate proliferation of T cell of inflammatory bowel disease patients and show cross reactivity with *Bifidobacterium* and enterobacteria (Duchmann *et al*., 1999). *A. xylosoxidans* has been described as the etiologic agent for a variety of human infections, such as pneumonia, peritonitis, meningitis, and pharyngitis (Igra-Siegman *et al*., 1980; Reverdy *et al*., 1984; Gómez-Cerezo *et al*., 2003; de Baets *et al*., 2007). However, Yan *et al*. (2004) reported that cyclo (L-leucyl-L-prolyl) produced by *A. xylosoxidans* can inhibit aflatoxin production by *Aspergillus parasiticus*. Due to potent carcinogenic and toxic properties of aflatoxins, the complex interaction between *A. xylosoxidans* and *A. parasiticus* and the influence of the interaction on the host require further research.

In the present study, *Vibrio cholerae*, *Plesiomonas shigelloides*, and *Pasteurella* spp. were the major private bacteria in the grass carp guts (Table 1). They are also found in other host and water environments. For instance, *V. cholerae* is found in the intestinal tract and mucus of yellow catfish, the surfaces of live copepods maintained in natural aqueous environments (Huq *et al*., 1983; Heidelberg *et al*., 2000; Wu *et al*., 2010). *P. shigelloides* is a dominant species in the intestinal tract of yellow catfish and is also present in humans, animals and aquatic environments (Foster *et al*., 2000; Gonzalez-Rey *et al*., 2003; González-Rey *et al*., 2004; Wu *et al*., 2010). Considering the potential ability of these organisms to widely disperse, the mechanisms causing the bacterial dispersal limitation in the grass carp gut are still not fully understood. Notably, just like the ‘shared bacteria’, most of the ‘private bacteria’ are also associated with host diseases. For instance, *V. cholerae* is divided into harmless aquatic strains and pathogenic strains, with the pathogenic strains causing cholera (Karaolis *et al*., 1998; Dziejman *et al*., 2002; Zhu and Mekalanos, 2003). *P. shigelloides* is increasingly regarded as an emerging and significant enteric pathogen (Sanyal *et al*., 1980; González-Rey *et al*., 2004). It is also reported that *P. shigelloides* can cause diarrhoea in animals (Foster *et al*., 2000). In addition, a large number of *Pasteurella* species such as *P. multocida* and *P. haemolytica* are reported to be the causative agents of pneumonia and several veterinary diseases (Ackermann and Brogden, 2000; Fuller *et al*., 2000).

Since a large number of multiple antibiotic resistance genes are harbored in the healthy human microbiome (Sommer *et al*., 2009), it is therefore

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reasonable to suggest that the intestinal microbiome of grass carp may also constitute a mobilizable reservoir of antibiotic resistance genes. The vast number of bacteria present in the grass carp gut which are associated with human and animal diseases, raise the potential risk of pathogenic bacteria acquiring antibiotic resistance genes with severe consequences for humans and aquatic animals.

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