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ORIGINAL ARTICLE

Characterization of the gut microbiota of three commercially valuable warmwater fish species

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Abstract

Aims: Due to the strong influence of the gut microbiota on fish health, dominant bacterial species in the gut are strong candidates for probiotics. This study aimed to characterize the gut microbiota of channel catfish *Ictalurus punctatus*, largemouth bass *Micropterus salmoides* and bluegill *Lepomis macrochirus* to provide a baseline for future probiotic studies.
Methods and Results: The gut microbiota of five pooled individuals from each fish species was identified using 16S rRNA pyrosequencing. Microbiota differed significantly between fish species in terms of bacterial species evenness. However, all gut communities analysed were dominated by the phylum Fusobacteria, specifically the species *Cetobacterium somerae*. Relatively high abundances of the human pathogens *Plesiomonas shigelloides* and *Fusobacterium mortiferum*, as well as members of the genus *Aeromonas*, suggest these species are normal inhabitants of the gut.
Conclusions: The overwhelming dominance of the genus *Cetobacterium* in all species warrants further investigation into its role in the fish gut microbiota.
Significance and Impact of the Study: This study provides the first characterization of the gut microbiota of three economically significant fishes and establishes a baseline for future probiotic trials.

Introduction

Aquaculture is the fastest growing animal food-producing sector and is set to overtake capture fisheries as a source of food fish (Subasighe 2005). Currently, one of the main factors limiting expansion and profitability of aquaculture is lack of disease control (FDA 2012). In the USA, treatment options against bacterial diseases are limited to four US Food and Drug Administration (FDA)-approved drugs, three of which are antibiotics (FDA 2011). Growing concern over the presence of antibiotic compounds in foods and an increase in antibiotic resistant microbes has led to an interest in alternatives to antibiotics such as probiotics for treatment and prevention of diseases (Balcazar *et al.* 2006; Ringo *et al.* 2010). Probiotics are live microbial feed supplements which beneficially affect the host by improving its intestinal balance. The goal of administering probiotics is to manipulate the gut microbiota to improve the fitness of the host, mainly through the exclusion of opportunistic pathogens (Balcazar *et al.* 2006). However, the gut microbiota strongly influences fish health in other ways such as assisting in the development of the gut epithelium, providing essential nutrients and stimulating the innate immune system (Nayak 2010). Thus, alteration of gut bacterial communities with probiotics may prevent disease through a variety of mechanisms.

In choosing potential probiotics, dominant strains from fish species of interest are often good candidates (Verschuere *et al.* 2000). Nevertheless, most probiotics used in aquaculture include lactic acid bacteria and members of the genus *Bacillus* isolated from mammals or terrestrial environments (Verschuere *et al.* 2000). Although there are several examples where exogenous bacteria proved beneficial for fish, many studies using lactic acid bacteria or *Bacillus* were inconclusive or showed no beneficial effect on the host (Oliva-Teles 2012; Ran *et al.* 2012). Recent studies (Roeselers *et al.* 2011; Sullam *et al.*

2012; Larsen *et al.* 2013) have shown that 'host species' and not the environment is the primary driving force shaping microbial communities in fishes. Hence, the microbial communities of each aquaculture species should be fully characterized to identify significant changes produced by the administration of probiotics, and to provide targets for the development of new probiotics.

In the USA, the top aquaculture species is channel catfish *Ictalurus punctatus* (Rafinesque 1818) with an estimate value of over $390 million annually (FAO Fisheries and Aquaculture Department, Statistics and Information Service 2011). In addition, aquaculture not only provides fish for the food market but also individuals for stocking of recreational fishing ponds. When it comes to recreational fishing, angler surveys indicate the top targeted species in the USA include largemouth bass *Micropterus salmoides* (Lacepède 1802), and panfish such as bluegill *Lepomis macrochirus* Rafinesque 1819 (Leonard 2005). Despite their economic significance, the normal gut microbiota of *I. punctatus*, *M. salmoides* and *L. macrochirus* has not been characterized, and thus, dominant community members that are potential targets for the development of probiotics aimed at these fish species have not been determined.

The purpose of this study was to compare the bacterial diversity associated with the gut of three commercially valuable warmwater fish species *I. punctatus*, *M. salmoides* and *L. macrochirus* using pyrosequencing to provide a baseline for future probiotic studies. We collected sympatric individuals from an experimental recreational fishing pond to minimize the effect of the local environment on the gut microbiota (Sakata *et al.* 1980; MacFarlane *et al.* 1986; Bacanu and Oprea 2013).

Methods

Sample collection

Sampling for all fish species occurred at Auburn University's E. W. Shell Research Station pond S8 in Auburn, Alabama (32°40′18·7″N, 85°30′36·00″W) in February 2012. Pond S8 is an experimental recreational fishing pond with limited access that was stocked in 1991 with *Micropterus salmoides* and *Lepomis macrochirus* reared at a state hatchery (Dr. DeVries, Auburn University, personal communication). Yearling *L. macrochirus* were stocked at 2500 fish ha^{-1} in spring, and age-0 *M. salmoides* were stocked in fall at 250 fish ha^{-1}. *Ictalurus punctatus* individuals reached pond S8 as escapees from nearby aquaculture ponds and have maintained a constant population since the late-1990s. Poststocking, fish were allowed to exist naturally without artificial feeding. Five individuals each of *I. punctatus*, *L. macrochirus* and *M. salmoides* were captured as follows. Catfish jugs were baited, set in the evening and allowed to fish overnight for approximately 15 h to collect *I. punctatus*. *Micropterus salmoides* and *L. macrochirus* were caught on baited hooks and spinning reels. Fish were kept alive in separate aerated coolers filled with lake water until processing (approximately 3 h). Total lengths of sampled fish are given in Table S1.

DNA extraction

Upon arrival at the laboratory, fish were immediately euthanized with an overdose of tricaine methanesulfonate (300 mg l^{-1}). The lower one-third of the intestine was aseptically removed, and the contents squeezed into a sterile tube. All five individuals of each species were pooled to form a single sample. This sample was homogenized for 2 min with a hand-held homogenizer. To account for intrinsic variability associated with DNA extraction and downstream nucleic acid analysis, each fish sample was divided into three subsamples of 25 mg. These replicates were immediately subjected to DNA extraction with the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) following manufacturer's instructions, including pretreatment for lysis of Gram-positive bacteria. DNA was quantified using a NanoDrop ND-1000 (Thermo Scientific, Rochester, NY).

Bacterial community composition determined by sequencing

Samples were subjected to Roche titanium 454 sequencing of the 16S rRNA using individual barcodes and primer 27F (5′-AGRGTTTGATCMTGGCTCAG-3′). Resulting sequences were processed using an exclusive analysis pipeline (MR DNA, Shallowater, TX). Barcodes and primers were removed from the sequences. Sequences with <Q25 (base call error rate <0·3%), <200 base pairs in length, ambiguous base calls and stretches of identical bases longer than 6 base pairs were removed. Denoising was performed, followed by removal of chimeras and singleton sequences. Cut-off for operational taxonomic units (OTUs) was defined at a <3% sequence difference in agreement with the current accepted prokaryote species concept (Rossello-Mora and Amann 2001). OTUs were taxonomically identified using BLASTN against the Greengenes database (DeSantis *et al.* 2006). As species richness and evenness can only be compared between samples when sample sizes are equal (Hurlbert 1971), resulting sequences were randomly selected so as to standardize to the sample with the least number of obtained sequences ($n = 2109$). Rarefaction curves, Good's coverage,

abundance-based coverage estimation (ACE), Chao1, Shannon evenness and shared OTUs based on defined OTUs were generated using Mothur ver. 1.30.0 (Schloss *et al.* 2009). Rarefaction curves were standardized to the sample yielding the least number of total sequences. A one-way ANOVA was performed on all diversity indexes, followed by a Tukey's *post hoc* test when significant ($P < 0{\cdot}05$). A genera abundance table was loaded into PRIMER v6 (Clarke and Gorley 2006) and similarity percentages (SIMPER) analysis was performed to determine the genera responsible for differences between fish species. Cut-off for low contributions was set at the default 90%.

Results

Diversity analysis

Pyrosequencing yielded a total of 58 164 bacterial sequences and 311 OTUs. After standardizing for sample size, 18 981 sequences remained with a total of 278 OTUs. Sequence coverage was ≥97% in all cases (Table 1), supported by the rarefaction curves generated by Mothur (Fig. 1). Total expected richness as calculated by ACE and Chao1 did not differ significantly by fish species. However, the Shannon evenness index was significantly higher in *Lepomis macrochirus* and *Ictalurus punctatus* than in *Micropterus salmoides*. Over 38% of all OTUs were shared by all three fish species (Fig. 2). *Micropterus salmoides* shared the least with the other two species and also had the highest number of unique OTUs.

Gut microbiota composition

Eight bacterial phyla were identified from the gut content of all fish species (Fig. 3). From each fish species, the phylum Fusobacteria made up the majority of all sequences (82·6% in *L. macrochirus*, 90·6% in *M. salmoides* and 94·9% in *I. punctatus*). Proteobacteria was the second most common phylum, varying in abundance from 5 to 16%. Within the Proteobacteria, each fish microbiota was composed of mostly Gammaproteobacteria, followed by Betaproteobacteria and Alphaproteobacteria. The less common phyla varied in abundances between fish species, with some unique members of each community. For example, only *M. salmoides* contained Actinobacteria while *L. macrochirus* and *I. punctatus* lacked representatives from the cyanobacteria and from the Spring Alpine Meadow; candidate division, respectively.

The gut microbiota of each fish species was composed of 11 shared genera and two to four unique genera per fish species (Table 2). Of these, most sequences were identified as *Cetobacterium*. Other relatively abundant genera included *Aeromonas* and *Fusobacterium* in *L. macrochirus, Aeromonas* in *I. punctatus* and *Plesiomonas* in *M. salmoides*. The 11 shared genera made up >98% of all identified sequences in each species, suggesting highly similar bacterial composition in the gut of these fishes at the genus level.

SIMPER analysis (Table 3) by bacterial genera indicated the largest difference in gut community composition between *L. macrochirus* and *M. salmoides*. A majority of the differences between all fish species were due to varying abundances of the genus *Cetobacterium*. Within

Table 1 Diversity indexes as calculated by MOTHUR software (ver. 1.30.0). Operational taxonomic units (OTUs) are defined at 97% sequence similarity

			# Predicted OTUs			
Sample ID	Fish species	# Observed OTUs	ACE	Chao1	Shannon evenness	Good's coverage
04	*Lepomis macrochirus*	146	198	218	0·757	0·978
05	*L. macrochirus*	160	250	208	0·727	0·975
06	*L. macrochirus*	141	187	199	0·732	0·979
40	*Micropterus salmoides*	137	177	173	0·696	0·981
41	*M. salmoides*	120	156	164	0·683	0·982
42	*M. salmoides*	131	180	189	0·679	0·978
76	*Ictalurus punctatus*	148	208	218	0·726	0·975
77	*I. punctatus*	116	183	153	0·729	0·982
78	*I. punctatus*	143	192	183	0·714	0·978
Totals	*L. macrochirus*	149	212	208	0·739	0·977
	M. salmoides	129	171	175	0·686*	0·980
	I. punctatus	136	194	185	0·719	0·979

ACE, abundance-based coverage estimation.
Significance among total values for each fish species was determined by a one-way ANOVA followed by Tukey's *post hoc* test.
*Statistically significant (ANOVA: $F_{2,6} = 17{\cdot}52$, $P < 0{\cdot}01$).

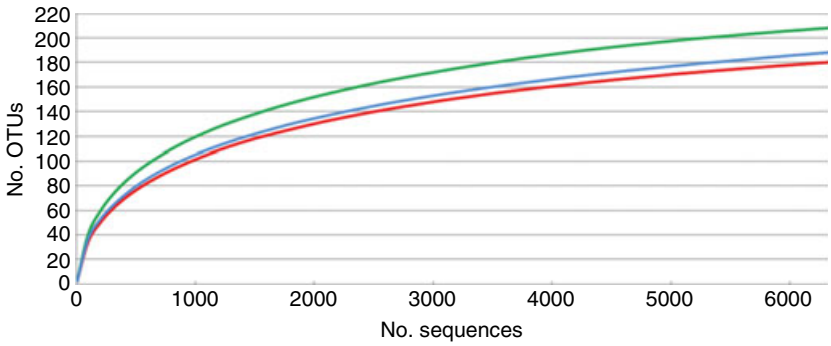

Figure 1 Rarefaction curves by fish species. Sequences were standardized to equal sample sizes for direct comparison. Red, *Lepomis macrochirus*; green, *Micropterus salmoides*; blue, *Ictalurus punctatus*.

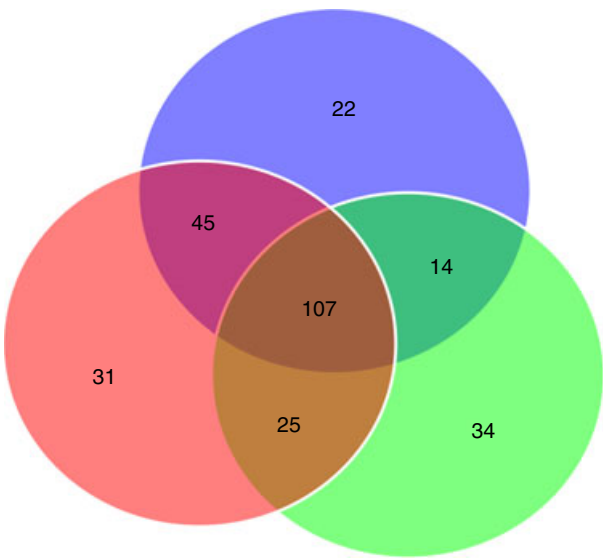

Figure 2 Venn diagram representing shared operational taxonomic units (OTUs) between fish species. Red, *Lepomis macrochirus*; green, *Micropterus salmoides*; blue, *Ictalurus punctatus*.

this genus, all identified sequences shared high 16S sequence similarity with the bacterium *Cetobacterium somerae*. High numbers of *Fusobacterium* in *L. macrochirus* as compared to *I. punctatus* contributed to the dissimilarity in gut microbiota between these two species. *Fusobacterium mortiferum* contributed the majority of sequences from this genus in *L. macrochirus*, while *I. punctatus* had approximately equal abundances of *Fus. mortiferum* and *Fusobacterium gonidiaformans*. Relatively high abundances of *Plesiomonas* spp. in *M. salmoides* contributed to the clearer separation between this species and the other two fish species. All sequences within the genus *Plesiomonas* were closely related to *Plesiomonas shigelloides*. Similarly, relatively high abundances of *Aeromonas* spp. in *L. macrochirus* separated this fish species from the others. Most *Aeromonas* species cannot be differentiated by 16S alone (Janda and Abbott 2010), and thus, species composition of this genus cannot be described in this study.

Discussion

Studies characterizing the microbiota associated with wild fish are often limited to analysis of one fish species (MacFarlane *et al.* 1986; Ringo and Strom 1994; Ugur *et al.* 2002; Uchii *et al.* 2006; Wilson *et al.* 2008; Wu *et al.* 2010; Bacanu and Oprea 2013), but results from studies that compared several fish species collected from the same area showed a marked specificity between fish species and microbiota (Skrodenyte-Arbaciauskiene *et al.* 2008; Smriga *et al.* 2010; Larsen *et al.* 2013). Our results showed significant differences in bacterial species evenness between the gut microbiota of *Lepomis macrochirus*, *Micropterus salmoides* and *Ictalurus punctatus* that were sharing the same environment and were not artificially fed. These fish species are known to have varying diet preferences and pond S8 offers a balanced ecosystem to satisfy their feeding requirements. Adult *M. salmoides* are primarily piscivorous, consuming fish, including *Lepomis* spp. and crayfish (Hickley *et al.* 1994; Olson and Young 2003; Wheeler and Allen 2003). Although *I. punctatus* are omnivorous (Jearld and Brown 1971; Griswold and Tubb 1977), individuals larger than 300–400 mm are reported to be primarily piscivorous (Jearld and Brown 1971; Michaletz 2006). As all of our *I. punctatus* were larger than 500 mm, they were likely consuming fish as their main diet. Adult *L. macrochirus* are generalists, eating primarily macroinvertebrates and zooplankton, as well as plants and small fish (Turner 1955; Seaburg and Moyle 1964; Harris 1999). Although stomach content was not analysed in this study, our patterns potentially reflect these diet differences.

Previous studies have seen differences in fish gut microbiota due to diet (Heikkinen *et al.* 2006; Silva *et al.* 2011; Mouchet *et al.* 2012; Sullam *et al.* 2012; Di Maiuta *et al.* 2013), and an increased diversity from carnivores to omnivores to herbivores has been demonstrated in

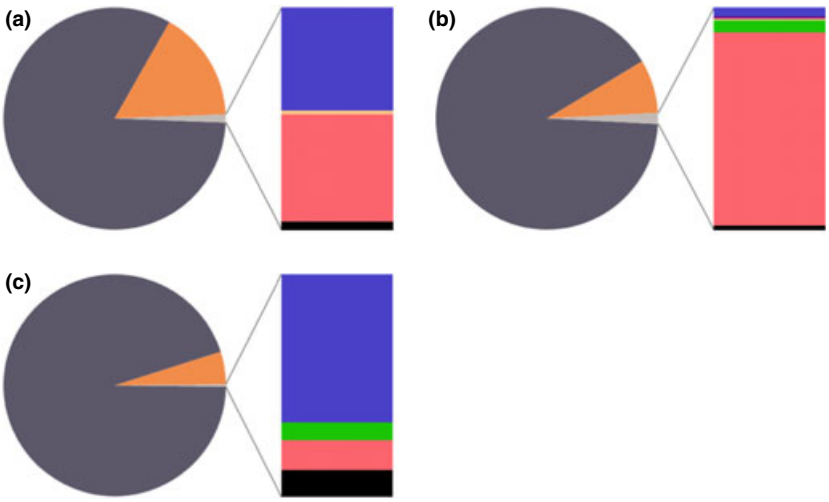

Figure 3 Phylum composition of each fish species as obtained through sequencing. (a) *Lepomis macrochirus*; (b) *Micropterus salmoides*; (c) *Ictalurus punctatus*. Pie: Dark grey, Fusobacteria; orange, Proteobacteria; light grey, all other phyla. Bar: Blue, Bacteroidetes; purple, Actinobacteria; peach, 'Spring Alpine Meadow' (candidate division); green, cyanobacteria; pink, Firmicutes; black, Tenericutes.

Table 2 Genus identity of sequences, represented by percentage of total sequences. Only genera accounting for at least 0·01% of sequences in at least one fish species are included. Shared genera are those shared among all three fish species. Unique genera are those present in only one or two fish species

Classification				Abundance (%)		
Phylum	Class	Family	Genus	*Lepomis macrochirus*	*Micropterus salmoides*	*Ictalurus punctatus*
Shared genera						
Fusobacteria	Fusobacteria	Fusobacteriaceae	*Cetobacterium*	72·04	89·9	94·02
Fusobacteria	Fusobacteria	Fusobacteriaceae	*Fusobacterium*	10·56	0·66	0·9
Proteobacteria	α-Proteobacteria	Methylobacteriaceae	*Methylobacterium*	0·03	0·02	0·01
Proteobacteria	β-Proteobacteria	Neisseriaceae	*Laribacter*	2·56	0·11	0·03
Proteobacteria	γ-Proteobacteria	Aeromonadaceae	*Aeromonas*	10·24	0·06	3·18
Proteobacteria	γ-Proteobacteria	Enterobacteriaceae	*Plesiomonas*	2·84	7·64	0·39
Proteobacteria	γ-Proteobacteria	Enterobacteriaceae	*Serratia*	0·18	0·01	0·05
Firmicutes	Clostridia	Clostridiaceae	*Clostridium*	0·57	1·24	0·03
Firmicutes	Clostridia	Lachnospiraceae	*Epulopiscium*	0·01	0·08	0·02
Bacteroidetes	Bacteroidia	Porphyromonadaceae	*Parabacteroides*	0·56	0·06	0·15
Tenericutes	Mollicutes	Mycoplasmataceae	*Mycoplasma*	0·04	0·03	0·04
Unique genera						
Actinobacteria	Actinobacteria	Micrococcaceae	*Kocuria*	0	0·01	0
Proteobacteria	α-Proteobacteria	Unspecified	*Pelagibacter*	0·03	0	0
Proteobacteria	β-Proteobacteria	Neisseriaceae	*Deefgea*	0·02	0	<0·01
Proteobacteria	γ-Proteobacteria	Shewanellaceae	*Shewanella*	<0·001	0	0·01
Proteobacteria	γ-Proteobacteria	Xanthomonadaceae	*Xanthomonas*	0·01	0	0
Firmicutes	Bacilli	Staphylococcaceae	*Salinicoccus*	0	0·01	0

mammals (Ley *et al.* 2008; Tsao 2011). We observed an increase in the number of predicted OTUs from carnivory (*M. salmoides* > *I. punctatus*) to herbivory (*L. macrochirus*), but this trend was not statistically significant. Alternatively, species evenness was significantly higher in *L. macrochirus* and *I. punctatus* as compared to *M. salmoides*. McKenney (2011) saw an increased evenness in the gut microbiota of omnivorous primates as opposed to carnivorous ones. However, this statistic is rarely reported in these types of studies thus, the commonality of this pattern is to the best of our knowledge unknown.

Pyrosequencing identified differences between fish species' gut microbiota in terms of bacterial abundances at each taxonomic level. The phylum Fusobacteria was the main component of all three species' gut communities followed by the Proteobacteria. A few studies have shown Fusobacteria as dominant members of the gut microbiota of freshwater fishes (van Kessel *et al.* 2011; Di Maiuta

Table 3 SIMPER analysis between each combination of fish species. Average abundances include all three replicates within each fish species

Fish species	Bacteria genus	Species 1 average abundance	Species 2 average abundance	Contribution to dissimilarity (%)
1. *Lepomis macrochirus*	*Cetobacterium*	3244	8669	76·6
2. *Micropterus salmoides*	*Plesiomonas*	128	737	8·4
	Aeromonas	461	6	5·8
1. *L. macrochirus*	*Cetobacterium*	3244	4927	71·1
2. *Ictalurus punctatus*	*Fusobacterium*	476	47	11·4
	Aeromonas	461	167	8·5
1. *M. salmoides*	*Cetobacterium*	8669	4927	75
2. *I. punctatus*	*Plesiomonas*	737	20	15·5

et al. 2013) but not at the abundances observed in this study. The Fusobacteria are anaerobic, Gram-negative bacilli that produce butyrate (Bennett and Eley 1993), a short-chain fatty acid that is often the end product of the fermentation of carbohydrates including those found in mucins (Titus and Ahearn 1988; von Engelhardt *et al.* 1998). In mammals, butyrate provides many benefits to the host, including providing a majority of the energy supply to gastrointestinal cells (von Engelhardt *et al.* 1998; Collinder *et al.* 2003), enhancing mucus production, acting as an anti-carcinogen and anti-inflammatory, as well as playing a role in satiation (McBain *et al.* 1997; von Engelhardt *et al.* 1998; Andoh *et al.* 1999; Hamer *et al.* 2007). This fatty acid has been found in the gut of herbivorous and omnivorous fishes (Clements *et al.* 1994; Clements and Choat 1995), but is not expected to be present in carnivorous species because of their low carbohydrate diets (Schrama *et al.* 2005). Nuez-Ortin *et al.* (2012) demonstrated the ability of butyric acid to inhibit potential freshwater fish pathogens, and sodium butyrate is currently sold as a food additive to promote fish health and growth. However, trials using blends of sodium butyrate and other additives have not proven beneficial (Owen *et al.* 2006; Gao *et al.* 2011). Due to the large proportion of Fusobacteria in all three of these species, future investigations should determine their role in the fish gut microbiota.

Sequences closely related to the bacterium *Cetobacterium somerae* constituted over 70% of sequences from each fish species. This was a surprising result as it has been demonstrated that bacterial communities whose relative species abundances are near equal are more resilient to environmental stress than those that rely on dominant species for certain functions (Wittebolle *et al.* 2009). *Cetobacterium somerae*, formerly classified as *Bacteroides* type A (Tsuchiya *et al.* 2008), are poorly known, microaerotolerant, Gram-negative rods with fermentative metabolism that were originally described from children with late-set autism (Finegold *et al.* 2003). Since the original description, *C. somerae* has been found in a variety of freshwater herbivorous fish species (Tsuchiya *et al.* 2008; Silva *et al.* 2011; Di Maiuta *et al.* 2013). In this environment, the bacterium produces high amounts of vitamin B12 (Sugita *et al.* 1991). *Cetobacterium somerae* is also capable of inhibiting the growth of other bacterial strains (Sugita *et al.* 1996). The presence of this bacterium in a number of other freshwater fishes as well as its high abundance in this study warrants further studies into its function in the fish gut.

Interestingly, human pathogens including *Fusobacterium mortiferum, Plesiomonas shigelloides* (Bourgault *et al.* 1997; Novotny *et al.* 2004) and *Aeromonas* sp. were the second most commonly identified genera in *L. macrochirus*, *M. salmoides* and *I. punctatus*, respectively. This is the second known study to isolate *Fus. mortiferum* from the gut of a fish (Silva *et al.* 2005) and its role has yet to be examined in fish; however, it has also been isolated from wounds caused by catfish spines (Hargreaves and Lucey 1990). On the other hand, *Ple. shigelloides* seems to be a normal component of the gut of other fishes (Vandamme and Vandepitte 1980; Esteve and Garay 1991; Sugita *et al.* 1996; Silva *et al.* 2005; Donkeng *et al.* 2011). The genus *Aeromonas* not only includes opportunistic human pathogens but also fish pathogens such as *A. hydrophila* (Cipriano *et al.* 1984).

In summary, this study provides the first characterization of the gut microbiota of the economically significant *I. punctatus*, *M. salmoides* and *L. macrochirus*. These bacterial communities were isolated from wild individuals from the same lake. The microbiota composition, despite sharing a high percentage of the same bacterial genera, differed in evenness between fish species. Despite their differences, all three fish species harboured by vast majority the species *C. somerae,* sparking interest in its role in the fish gut. Studies are currently underway to isolate and further characterize this bacterium.

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Conflict of interest

The authors have no conflict of interest to declare.

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Supporting Information

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

Table S1 Total length (mm) of individual fish sampled for each fish species.