Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (Cyprinus carpio L.)

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Abstract
The microbes in the gastrointestinal (GI) tract are of high importance for the health of the host. In this study, Roche 454 pyrosequencing was applied to a pooled set of different 16S rRNA gene amplicons obtained from GI content of common carp (Cyprinus carpio) to make an inventory of the diversity of the microbiota in the GI tract. Compared to other studies, our culture-independent investigation reveals an impressive diversity of the microbial flora of the carp GI tract. The major group of obtained sequences belonged to the phylum Fusobacteria. Bacteroidetes, Planctomycetes and Gammaproteobacteria were other well represented groups of micro-organisms. Verrucomicrobiae, Clostridia and Bacilli (the latter two belonging to the phylum Firmicutes) had fewer representatives among the analyzed sequences. Many of these bacteria might be of high physiological relevance for carp as these groups have been implicated in vitamin production, nitrogen cycling and (cellulose) fermentation.

Keywords: intestinal tract, biodiversity, carp, aquaculture, pyrosequencing, 16S rRNA

Introduction
The intestine is a multifunctional organ system involved in the digestion and absorption of food, electrolyte balance, endocrine regulation of food metabolism and immunity against pathogens (Ringo et al. 2003,). The gastrointestinal (GI) tract is inhabited by many different micro-organisms. As in mammals, this dynamic population of micro-organisms is of key importance for the health of the piscine host (Ringo et al. 2003,; Rawls et al. 2004,). The gut is also a potential route for pathogens to invade and infect their host. The micro-organisms in the GI tract are involved in the protection against these pathogens by the production of inhibitory compounds and competition for nutrients and space. As in mammals, the intestinal microbiota of fish can influence the expression of genes involved in epithelial proliferation, nutrient metabolism and innate immunity (Rawls et al. 2004,). Due to their importance in animal health, the investigation of the intestinal microbiota of fish is highly relevant for aquaculture practice. We investigated the diversity of the microbiota in common carp (Cyprinus carpio), one of the most cultivated freshwater fish species worldwide (FAO, 2011).

The morphology of the GI tract of fishes varies greatly among species. Common carp belong to the family of Cyprinidae, which are herbivorous, stomachless fish. These fish lack pyloric caeca, the finger-like blind sacs in the proximal intestine that increase the absorptive surface of the intestines in many fish (Ringo et al. 2003,; Buddington and Diamond 1987,). The composition of the gut microbiota of common carp has previously been investigated using culture-dependent methods (Sugita et al. 1990,; Namba et al. 2007,; Tsuchiya et al. 2008,). Most bacterial species found in these studies were aerobic and facultative anaerobes. Two studies demonstrated a high abundance of Aeromonas species (Namba et al. 2007,; Sugita et al. 1990,). Other bacteria isolated were Enterobacteriaceae (Sugita et al. 1990,; Namba et al. 2007,), Pseudomonas (Sugita et al. 1990,; Namba et al. 2007,), Bacteroidetes (Sugita et al. 1990,; Tsuchiya et al. 2008,), Plesiomonas (Sugita et al. 1990,; Namba et al. 2007,), Moraxella (Sugita et al. 1990,; Namba et al. 2007,).
DNA extraction, PCR amplification and sequence analysis

Ten fish were euthanized using 0.1% ethyl-m-amino-benzoate methane sulfonate salt (MS-222, MP Biomedicals, Illkirch, France, pH adjusted to 7) followed by decapitation. The body surface of the fish was washed with 70% ethanol and the GI tract was removed aseptically. The whole content of the GI tract was removed by carefully flushing with PBS and DNA was extracted from this material using a cetyltrimethylammoniumbromide (CTAB)-based extraction method (Zhou et al. 1996). Briefly, samples were mixed with CTAB-extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% CTAB, 675 μg per 250 mg sample) and protease K (10 mg/ml) and incubated for 30 min at 37°C. After protease treatment 10% SDS was added, followed by incubation at 65°C for 2 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation and the resulting DNA pellet was resuspended in 1 ml ultrapure water. Before additional purification, DNA was treated with RNase. The DNA thus obtained was purified using Sephadex beads (Amersham Bioscience, USA) according to the manufacturer’s protocol and its integrity was checked on agarose gel. DNA concentrations were estimated spectrophotometrically using NanoDrop® technology (Thermoscientific, USA).

Retrieval of 16S RNA gene sequences

Obtained DNA (20 ng) was used for amplification in 20 μl reactions using Phuslon Flash enzymes (Finnzymes, Finland). In order to target as many bacterial taxa as possible, the Pla46 F primer was combined with Eubl R, EubII R or EubIII R and for the 616 F primer the same set of reverse primers was used (Table 1). This resulted in 6 different combinations. All reactions were done for individual fish separately. PCR reactions were started by an initial denaturation at 98°C for 1 min followed by 35 amplification cycles (98°C for 6 s, 10 s at annealing temperature, 72°C for 20 s) and a final extension step for 1 min at 72°C. PCR products were examined for size and yield using agarose gel in TAE buffer (20 mM Tris-HCl, 10 mM sodium acetate, 0.5 mM Na₂EDTA, pH 8.0). After successful amplification, obtained products of different reactions were pooled and 9.2 μg PCR product was used for pyrosequencing using the Roche 454 GS

Table 1 Primer specifications

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Pla46 F</td>
<td>Planctomycetales</td>
<td>CGATTAGGCATGCAAGTC</td>
<td>Neef et al. 1998</td>
</tr>
<tr>
<td>616 F</td>
<td>Most bacteria</td>
<td>AGAGTTGATYMTGGCTCAG</td>
<td>Juretscho et al. 1998</td>
</tr>
<tr>
<td>Eubl R</td>
<td>Most bacteria</td>
<td>GCTGCTCACCCTAGGGAGT</td>
<td>Amann et al. 1990</td>
</tr>
<tr>
<td>EubII R</td>
<td>Planctomycetales</td>
<td>GCAAGCCACCCCCGAGGAGT</td>
<td>Daims et al. 1999</td>
</tr>
<tr>
<td>EubIII R</td>
<td>Verrucomicrobiales</td>
<td>GCTGCCACCCCCGAGGAGT</td>
<td>Daims et al. 1999</td>
</tr>
</tbody>
</table>
FLX Titanium sequencer (Roche, Switzerland). A problem with 454 pyrosequencing is ‘blinding’ of the camera due to flashing caused by incorporation of the same nucleotide in many spots, which can occur when many similar DNA templates are sequenced (Kip et al. 2011). This was circumvented by mixing 16S rRNA gene products in a 1:1 ratio with pmoA PCR products (targeting a subunit of the particulate methane monooxygenase) from a non-related experiment (Kip et al. 2011).

**Phylogenetic analysis**
A Megablast search (using default parameters) of all sequenced reads larger than 100 nt against the Silva SSURef database (version 102) was done to extract all 17,892 16S rRNA gene sequences (average length 314 nt). The taxonomic annotations available in the Silva SSURef database were used to classify the sequenced reads. Each read was assigned to the taxonomic clade of its highest scoring Megablast hit, when a sequence was assigned to more than one clade, its vote was divided equally. Furthermore, obtained sequences were processed using the Classifier tool (Wang et al. 2007) of the RDP pyrosequencing pipeline http://pyro.cme.msu.edu/. The confidence threshold used was 50%. The sequence reads are available at the MG-Rast Metagenome analysis server http://metagenomics.anl.gov/ under Project ID 4449604.3 and from the Sequence Read Archive (SRA) at http://www.ebi.ac.uk/ena/data/view/ under accession number ERP000995.

**Results**
The use of next generation sequencing technologies for sequencing of a mixture of 16S rRNA amplicons amplified with primer sets targeting as many phyla as possible will give a much broader taxonomic overview compared to the use 16S rRNA hypervariable regions (Kysela et al. 2011). The use of next generation sequencing technologies for microbial diversity in the GI tract of common carp due to flashing caused by incorporation of the same DNA templates are sequenced (Kip et al. 2011).

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**Discussion**
Almost all Fusobacterial 16S rRNA sequences, 8081 out of 8085, from the carp GI tract belonged to the genus *Cetobacterium*. *Cetobacterium* were not observed in most culture-dependent studies done on the GI tract microbiota of common carp (Sugita et al. 1990; Namba et al. 2007,). Only Tsuchiya et al. (2008) described the isolation and characterization of *Cetobacterium somerae* from the GI tract of five different fresh water fish, including carp. *Cetobacterium* was also shown to be present in the gut of zebrafish (Rawls et al. 2006), a cyprinid species closely related to common carp. Furthermore, *Cetobacterium* isolated from human faeces performed fermentation of peptides and carbohydrates (Finegold et al. 2003). It has also been shown that *Cetobacterium* can produce vitamin B12 (Tsuchiya et al. 2008,). This can well explain why carp do not have a dietary vitamin B12 requirement (Sugita et al. 1991). The combination of a fermentative metabolism together with vitamin production may explain the relevance of *Cetobacterium* sp. in the GI tract of carp.

Another well represented group within the obtained sequences were the *Bacteroidetes* (22% of obtained sequences), a phylum known for a fermentative metabolism and degradation of oligosaccharides derived from plant material (Van der Meulen et al. 2006). The *Bacteroidetes* sequences found could be divided into 4 major groups (Additional file 1): *Marinilabaceae* (or *Cytophaga*, 13%), *Porphyromonadaceae* (39%), *Bacteroidaceae* (15%) and *Bacteroidales_incertae_sedis* (33%). All *Marinilabaceae* sequences belonged to the same group: the *Anaerophaga*. This relatively newly discovered group of bacteria includes strictly anaerobic, chemo-organo-trophic, fermentative bacteria (Denger et al. 2002). These bacteria may play an important role in the fermentation of food in the GI tract of herbivorous carp since anaerobic fermentation is generally an important step in the digestion of plant material.
Figure 1 Phylogenetic diversity of 16S rRNA sequences retrieved from the GI tract content of common carp. Classification the 17,641 reads was performed using the taxonomic annotations available in the Silfva SSURef database. The number of sequences (10log-transformed) belonging to each clade is indicated by the red circles.
Porphyromonadaceae are present in the GI tract of several organisms including human and pigs (Mulder et al. 2009). These bacteria can be pathogens but in this niche they are most probably involved in fermentation. By using labelled glucose, it has been shown that these bacteria are involved in saccharide fermentation (Li et al. 2009). Also the Sphingobacteria present could also be involved in oligosaccharide degradation since Sphingobacterium sp. TN19, an endosymbiont in insects, contains a xylanase encoding gene (Zhou et al. 2009). Xylanases are involved in the breakdown of xylan, a polysaccharide found in plant material. The presence of

<table>
<thead>
<tr>
<th>Table 2 Niche and possible function of the bacterial classes present within the 16S rRNA amplicons obtained from the GI tract of common carp.</th>
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<tbody>
<tr>
<td><strong>Class/Subclass</strong></td>
</tr>
<tr>
<td>Bacilli</td>
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<tr>
<td>Bacteroides</td>
</tr>
<tr>
<td>Cetobacterium</td>
</tr>
<tr>
<td>Clostridia</td>
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<tr>
<td>Enterobacteriales</td>
</tr>
<tr>
<td>Gemmata</td>
</tr>
<tr>
<td>Isosphaera</td>
</tr>
<tr>
<td>Marinilabiaceae</td>
</tr>
<tr>
<td>Pirellula</td>
</tr>
<tr>
<td>Planctomyces</td>
</tr>
<tr>
<td>Porphyromonadaceae</td>
</tr>
<tr>
<td>Schlesneria</td>
</tr>
<tr>
<td>Sphingobacteria</td>
</tr>
<tr>
<td>Verrucomicrobiaceae</td>
</tr>
<tr>
<td>Vibrio</td>
</tr>
<tr>
<td>Zavarzinella</td>
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</table>
fermenting microorganisms is not suprising, since it has been shown that the GI microbiota of carp is able to ferment different oligosacharides (Kihara and Sakata 2002). The obtained Planctomycete sequences (13% of classified sequences) could be divided into 9 groups (Additional file 1); Gemmata, Pirellula, Schlesneria and Zavavarzinella were the most abundantly found groups. Gemmata and Pirellula are aerobic chemo-heterotrophs, Schlesneria are chemo-organotrophic facultative aerobes and Zavavarzinella are aerobic heterotrophs. The presence of Planctomycetes has been shown before in gut microbiota of fish and other organisms (Ley et al. 2008; Rawls et al. 2006). The exact function of these bacteria in the GI tract is not clear, possibly these bacteria live from products of the metabolism of other bacteria. However, the relatively high abundance of Planctomycetes in close association with other organisms such as kelp, marine sponges and prawn (Bengtsson and Ovreas 2010; Pimentel-Elardo 2003; Fuerst et al. 1997; Lahav et al. 2009) suggests a more important role. Possibly, these bacteria are involved in the metabolism of complex compounds. In a recent study, in which the close association of Planctomycetes with the brown seaweed kelp (Laminaria hyperborea) was investigated, it was hypothesized that these bacteria are degraders of sulfated polysacharides produced by kelp (Bengtsson and Ovreas 2010). The organisms found in the biofilm at the plant’s surface were mainly members of the lineage Pirellulaceae (which includes Pirellula, Rhodopirellula and Blastopirellula). The genome sequence of Rhodopirellula baltica SH1 revealed many genes involved in the breakdown of sulfated polysaccharides (Glockner et al. 2003). Possibly, the heterotrophic Planctomycetes found in carp gut confer a similar ability of polysaccharide breakdown to the host. Furthermore, a separate lineage within the Planctomyces, the anammox bacteria, were present in the carp gut (Figure 1). These anaerobic bacteria, described before in fish gut (Lahav et al. 2009), are involved in nitrogen cycling. Together with the Nitrosomonas and Nitrospira species (also present within the obtained sequences, Figure 1), ammonium can be converted into dinitrogen gas. The removal of nitrogenous compounds from aquaculture systems is one of the most important challenges in aquaculture. The presence of nitrogen cycling bacteria in fishes could offer new in situ solutions for the removal of nitrogen from aquaculture systems.

The Gammaproteobacteria sequences found could be classified as bacteria that are known members of the GI microbiota of many organisms including fish (Wu et al. 2010; Lee et al. 2009). Most Gammaproteobacteria (Additional file 1) found in carp belonged to the Aeromonas group. Members of the genus Aeromonas are mainly distributed in freshwater and sewage, often in association with aquatic animals (Cahill 1990; Sugita et al. 1995). They can cause a diverse spectrum of diseases in both warm- and cold-blooded animals but they also appear to be aquatic environments including in fish intestines (Sugita et al. 1995). Other abundantly present members among the Gammaproteobacterial sequences were the genera Enterobacterium and Vibrio. Enterobacterium spp. are widespread in GI tracts of various organisms (Wu et al. 2010), whereas Vibrio sp. are commonly found in aqueous environments, aquaculture systems and in association with eukaryotes (Wu et al. 2010; Thompson et al. 2004). This phylum also contains Plesiomonas and Acinetobacter species that have been found in carp before (Sugita et al. 1991; Cahill 1990). Furthermore, the presence of high number Proteobacteria has also been shown for zebrafish, which is closely related to carp (Rawls et al. 2006). Also in other fish belonging to the Cyprinidae members of the Gammaproteobacteria (Enterobacter and Citrobacter species) were found (Ray et al. 2010). Enterobacter and Citrobacter species isolated from the GI tract of Indian carp (Cyprinidae) were shown to produce amylase, cellulase and protease (Ray et al. 2010), which indicates that these bacteria can be actively involved in the digestion of food in carp guts.

Another abundant phylum within our amplicon sequences were the Verrucomicrobiae (including subdivision 3 and 4 (Optitiae)). Verrucomicrobiae species are most commonly found in aquatic environments but are also known members of the gut microbiota in different organisms including seacucumbers (Echinodermata), termites and humans (Wagner and Horn 2006). These bacteria seem to be well adapted to live with eukaryotes, since the genome of some verrucomicrobial species contain a protein secretion system which mediates interactions between eukaryotic and bacterial cells (Wagner and Horn 2006). Verrucomicrobiae usually have an aerobic or obligate anaerobic fermentative metabolism (Schlesner et al. 2006) and could also play a role in the metabolism of plant beta glycans in carp GI tract. Indeed, Pedosphaera parvula Ellin514 (Verrucomicrobia subdivision 3) contains a cellulase in its genome (Kant et al. 2011). Ruminants and postgastric fermenters depend on bacteria containing this gene for the fermentation of plant material in which cellulose is converted to β-glucose. Various fish species do have a cellulase activity in their guts (Saha and Ray 1998; Saha et al. 2006; Ray et al. 2010) which decreases after antibiotic treatments (Saha and Ray 1998), indicating that the GI microbiota is responsible for this activity.

Clostridia and Bacilli, both present in the microbiota of the sampled fish (Figure 1), are members of the phylum Firmicutes. Representative genera of this phylum, including Clostridium, Bacillus, Streptococcus and Staphylococcus spp., have been shown in the microbiota of
fish before (Navarrete et al. 2009; Rawls et al. 2006; Ray et al. 2010; Sugita et al. 1990). Gut isolates belonging to the Firmicutes fermented various carbon sources (Ray et al. 2010), again implicating a role in the utilization of plant materials.

To our knowledge, this is the first detailed analysis of the microbiota of common carp by high throughput sequencing. Our culture independent investigation of the microbial flora of the GI tract gives a more reliable and more complete characterization of the diversity of compared to other studies. Furthermore, great similarities between the microbiota in carp and zebrafish (a closely related fish species) were shown (Roeselers et al. 2011). The GI microbiota is important for the health of the animal and therefore this study could be relevant for aquaculture. Furthermore, the presence of different nitrogen cycling bacteria in the GI tract of fish could offer new possibilities in the removal of nitrogen compounds in aquaculture. The microbiota of the GI tract plays an important role in the digestion and chemical processing of the food as exemplified by the large number of bacteria involved in vitamin production and fermentation of saccharides and beta–glycans (cellulose, hemicellulose) (Table 2). The presence of many different types of bacteria in the herbivorous carp could be predicted since it has been shown that eukaryotes with an herbivorous diet have a higher microbial diversity (Ley et al. 2008,). However, the carp in our study were fed commercially available food with high protein and low plant content. According to their GI microbiota, these fish are very well able to adapt to a more herbivorous diet and this is probably also the case for other cultured fish. Therefore it could be possible to lower the amount of fish meal, one of the major components of fish food, in the food for these fish. Furthermore, it shows that the gut microbes are probably important in the protection of the host against pathogens which should be taken into consideration in aquaculture where a lot of antibiotics are used (Cabello 2006,). It is known that antibiotics have a negative effect on the microbial community in the gut of human (Dethlefsen et al. 2008) and this is possibly also the case for fish. The routinely use of antibiotics may be harmful for the animal. A better knowledge about the microbiota in fish guts is important; it can lead to a better health of cultured fish and therefore to a more efficient fish culture.

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Competing interests
The authors declare that they have no competing interests.

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