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Gut content microbiota of introduced bigheaded carps (*Hypophthalmichthys* spp.) inhabiting the largest shallow lake in Central Europe

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ABSTRACT

Studying the microbiota in the alimentary tract of bigheaded carps (*Hypophthalmichthys* spp.) gained special interest recently, as these types of investigations on non-native fish species may lead to a better understanding of their ecological role and feeding habits in an invaded habitat. For microbiological examinations, bigheaded carp gut contents and water column samples from Lake Balaton (Hungary) were collected from spring to autumn in 2013. Denaturing Gradient Gel Electrophoresis (DGGE) and pyrosequencing of the 16S rRNA gene were performed to reveal the composition. According to the DGGE patterns, bacterial communities of water samples separated clearly from that of the intestines. Moreover, the bacterial communities in the foreguts and hindguts were also strikingly dissimilar. Based on pyrosequencing, both foregut and hindgut samples were predominated by the fermentative genus *Cetobacterium* (Fusobacteria). The presence of some phytoplankton taxa and the high relative abundance of cellulose-degrading bacteria in the guts suggest that intestinal microbes may have an important role in digesting algae and making them utilizable for bigheaded carps that lack cellulase enzyme. In turn, the complete absence of typical heterotrophic freshwater bacteria in all studied sections of the intestines indicated that bacterioplankton probably has a negligible role in the nutrition of bigheaded carps.

KEY WORDS

bigheaded carp, *Hypophthalmichthys* spp., intestinal microbiota, Lake Balaton, Fusobacteria

1. Introduction

During the past three decades, an increasing attention was paid to studying the versatile role of microbes in the nutrition of fishes (Rahmatullah and Beveridge, 1993; Nayak, 2010; Ray et al., 2012; Ghanbari et al., 2015). Microorganisms may contribute to fish nutrition either directly as part of the ingested and digested food (Rahmatullah and Beveridge, 1993; Matěna et al., 1995; Kamjunke and Mehner, 2001) or indirectly by colonizing their digestive tracts and participating in digestion processes, influencing absorption and synthesis of important nutrients (Ray et al., 2012; Li et al., 2013; Clements et al., 2014). So far, we have evidence of direct usage of microbes as food only in a limited number of fish species. In addition, the importance of this food resource often seems to be marginal (Rahmatullah and Beveridge, 1993; Matěna et al., 1995; Kamjunke and Mehner, 2001). However, all fish species have specific microbe communities thriving in their digestive tracts (Ghanbari et al., 2015). Composition and functioning of these intestinal microbes change along sections of the digestive tract and vary among fish species and across habitats (Sullam et al., 2012; Li et al., 2014; Ni et al., 2014; Li et al., 2015).

Deliberate consumption of microbes by fish requires specific filtering apparatus which is possessed only by some specialized planktivorous species. Silver carp (*Hypophthalmichthys molitrix*), bighead carp (*H. nobilis*) and their hybrids (collectively referred as bigheaded carps) are among the most widely known and distributed fishes with such capabilities. They are able to filter and consume even nanoplankton (<10 μm; Cremer and Smitherman, 1980; Xie, 1999; Görgényi et al., 2016) and other very small suspended particles (5−6 μm in diameter) from the water (Xie, 2001). Moreover, it is also known that aquatic bacteria are often attached to the surface of algae (Paerl, 1976; Worm and Søndergaard, 1998) and abioseston (Kirchman and Mitchell, 1982; Pedros-Alio and Brock, 1983). Significant part of the bigheaded carps' diet consists of suspended particulate matter

(Boros et al., 2014), and thus bacteria may be ingested adventitiously. On the other hand, bigheaded carps have been reported to have diverse and unique endogenous microbiota as well. High throughput cultivation-independent approaches revealed that intestinal microbiota in the guts of Asian carps (including bigheaded carps) is strongly affected by internal (e.g. genetic and physiological features of the host) and external factors (e.g. the trophic status of the habitat or the feeding habits of the fish) (Li et al., 2014; Ye et al., 2014; Li et al., 2015). Accordingly, bigheaded carps are ideal study objects to investigate the trophic interaction between bacteria and fish.

In order to control algal blooms (Virág, 1998) and increase fishery yields (Specziár, 2010), bigheaded carps were introduced into Lake Balaton (Hungary) in 1972 and were stocked until 1983. Bigheaded carps are important food-fishes worldwide, but at the same time are among the most problematic invasive species in certain parts of the world, including Hungary (Kolar et al., 2007). These filter-feeding and predominantly planktivorous fishes proved to be successful invaders in Lake Balaton; their stock gradually increased and accumulated as a result of the difficulties in their harvesting (Bíró, 2000). The present bigheaded carp stock in the lake consists mainly of hybrid (bighead carp × silver carp) individuals (Boros et al., 2014; 2015). Surprisingly, these hybrids with various types of gill rakers (intermediate in development between those typical for the parental species) consume food within the same size-spectrum and have very similar diet composition, independently of the rate of hybridization (Battonyai et al., 2015). Despite low productivity and consequently scarce food resources which have advanced as a result of a successful nutrient controlling program in the drainage, bigheaded carps grow intensively in Lake Balaton and their condition factor (the "plumpness" of fish) is relatively high compared to other ecosystems (Boros et al., 2014). However, zooplankters which according to the results of microscopic analyses dominate the diet of bigheaded carps (Vitál et al., 2015) are not too abundant nowadays (G.-Tóth et al.,

2011) and are also intensively harvested by several native and abundant fish species (e.g. bleak *Alburnus alburnus*, common bream *Abramis brama* and razor fish *Pelecus cultratus*) as well as by juveniles of almost all fishes in Lake Balaton (Specziár & Rezsu, 2009; Specziár, 2010). According to a recent survey, bigheaded carps can harvest almost all algal taxa that are available in the ambient water, but only a portion of the filtered algae are digested and utilized (Görgényi et al., 2016). Moreover, the intensive growth of bigheaded carps in Lake Balaton is especially interesting in the light of the high inorganic matter content (up to 80% in dry mass) of the ingested food (Boros et al., 2014).

Considering the intensive growth and high condition factor of bigheaded carps in the oligo-mesotrophic Lake Balaton, we hypothesized that in addition to phytoplankton and zooplankton, bacterioplankton or intestinal microbes- which remained hidden during the microscopic diet analysis procedure - may also play an important role in their nutrition either by serving directly as food or increasing the efficiency of digestive processes.

The objective of this study was to compare the taxonomic composition of microorganisms in lake water and in gut content samples from the alimentary tract of bigheaded carps of different sex, age and physiological features, in order to shed light on possible role of planktonic microbes in the nutrition of bigheaded carps. We applied a combined genetic approach based on the examination of denaturing gradient gel electrophoresis (DGGE) patterns and pyrosequencing data.

2. Material and methods

2.1.Sample collection and initial sample processing

Samples were collected from Lake Balaton, which is located in the Transdanubian region of Hungary and is the largest shallow lake in Central Europe. It has a surface area of 596 km^2 and an average depth of about 3 m (Szabó et al., 2011). Bigheaded carps and water samples

were collected from the eastern basin of Lake Balaton in April, May, June, September and October 2013.

Fish were captured by the local fishery company (Balaton Fish Management Non-Profit Ltd), using 12 cm knot-to-knot mesh gill nets. Fish were killed immediately after catching by severing the central nervous system and transported into the laboratory within 30 min. This procedure was conducted by the researchers of the Balaton Limnological Institute (Centre for Ecological Research, Hungarian Academy of Sciences). The Institute has a permit for delivery, breeding and the use of animals (permit reg. no.: VE-I-001/01890-3/2013, issued by the Food-security and Animal Health Directorate, Governmental Office of Veszprém County, Hungary). During the survey, a total of 10 bigheaded carps were submitted to detailed examinations (2 fish per sampling date).

Foregut and hindgut samples were removed aseptically as described by Görgényi et al. (2016). Approximately 5 g subsamples of the obtained gut contents were placed into sterile Eppendorf tubes and were stored at -20 °C until laboratory processing, done within 24 h.

Parallel to fish samplings, water column samples were collected around the fishing nets and samples were stored in sterile bottles until processing. Following sample transportation to the laboratory, 1 liter of the lake water was filtered through a 0.45 µm pore diameter polycarbonate filter (Millipore, Billerica, MA, USA), and the filter was stored at -20 °C until the community DNA isolation started within 24

2.2.DNA extraction

The Ultra Clean Soil DNA extraction kit (MO BIO Inc., CA, USA) was applied for DNA extraction from the planktonic mass captured on the filters and from approximately 0.5 g subsample of each gut content sample, according to the manufacturer's instructions.

2.3.Denaturing Gradient Gel Electrophoresis (DGGE)

Amplification of partial 16S rRNA gene sequences for DGGE analysis was performed by two consecutive polymerase chain reaction (PCR) steps for all collected samples. The first PCR was carried out using 27F (Lane, 1991) and 1401R (Nübel et al., 1996) primers, while for the second PCR, 27F-GC and 519R (Turner et al., 1999) primers were applied (which resulted in 16S rRNA gene amplicons containing the V1-V3 variable regions to get high resolution DGGE patterns; Yu and Morrison, 2004; Youssef et al., 2009). The PCR mixtures contained 2 µl of purified genomic DNA, 0.2 mM of each deoxynucleotide, 2 mM $MgCl_2$, 1 U LC *Taq* DNA Polymerase (Fermentas, Vilnius, Lithuania), 1× PCR Buffer (Fermentas, Vilnius, Lithuania) and 0.325 µM of the primers in a final volume of 50 µl. Temperature profile of both PCRs included an initial denaturation at 95 °C for 5 min, followed by 32 cycles (denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 1 min) and a final extension at 72 °C for 10 min. The PCR products were checked by electrophoresis in ethidium bromide stained 1% agarose gel under UV light.

Bacterial community profiles were revealed and compared by DGGE using 7% (w/v) polyacrylamide gel containing a 40 to 60% gradient of denaturants (100% is defined as 40% formamide and 7 M urea). The electrophoresis was carried out at 60 °C in 1× Tris-acetate-EDTA (TAE) buffer at 120 V for 14.5 hours using a phorU-2 electrophoresis system (Ingeny International, Goes, Netherlands). The gel was stained with ethidium-bromide, washed in 1× TAE and photographed under UV light. TotalLab (TL 120) version 2006 (Nonlinear Dynamics Inc., Newcastle upon Tyne, UK) software was used to compare the microbial community structures by an UPGMA method on the basis of the detected DGGE patterns.

To identify the dominant members of the communities, discrete DGGE bands were excised, and the DNA was extracted by an overnight incubation in 30 µl DEPC-treated water (G-Biosciences, St. Louis, MO, USA). DNA derived from the excised DGGE bands was

reamplified and sequenced by Sanger method using 27F primer. Identification of the obtained sequences was carried out using the EzTaxon-e tool (Kim et al., 2012).

The obtained sequences were submitted to the GenBank under the accession numbers LN881423-LN881434.

2.4.Pyrosequencing

For pyrosequencing, the V3-V4 region of the 16S rRNA gene was amplified using universal bacterial primers: S-D-Bact-0341-b-S-17 forward (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 reverse (5'-GACTACHVGGGTATCTAATCC-3') (which have the best coverage of bacterial phyla according to a thorough primer evaluation survey; Klindworth et al., 2012), with the proper sequencing barcodes and adapters for three selected samples. PCR amplification was performed in triplicates in 20 µL final volume containing 5× Phusion HF Buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.2 mM dNTPs (Fermentas), 0.4 µg μl^{-1} Bovine Serum Albumin (Fermentas), 0.5 µM of each primer, 0.02 U μl^{-1} Phusion High-Fidelity DNA Polymerase (Thermo Fisher). The following thermal conditions were used: initial denaturation at 98 °C for 5 min, followed by 25 cycles (denaturation at 95 °C for 40 s, annealing at 55 °C for 2 min and extension at 72 °C for 1 min) and a final extension step at 72 °C for 10 min. Triplicate PCR products were pooled resulting one library per sample, and were subsequently purified with the High Pure PCR Cleanup Micro Kit (Roche/454 Life Sciences, Branford, CT, USA). Quality control of the amplicon libraries was carried out using a model 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Emulsion PCR, amplicon library processing and pyrosequencing were performed on a GS Junior sequencing platform according to the Lib-L protocol of the manufacturer (Roche/454 Life Sciences).

Resulting sequence reads were processed using the mothur v1.35 software (Schloss et al., 2009) based on the 454 standard operating procedure (http://www.mothur.org/wiki/454_SOP- downloaded at 04/07/2015; Schloss et al., 2011). To minimize the amplification and pyrosequencing bias, sequences were quality filtered and denoised. Furthermore, the removal of chimeric sequence reads using uChime (Edgar et al., 2011) and singleton sequences according to Kunin et al. (2010) was also carried out. As the first step of taxon identification, sequence alignment was performed with the SINA aligner tool (Pruesse et al., 2012). For alignment and classification, the ARB-SILVA SSU NR reference database – SILVA Release 119 (Quast et al., 2012) was used as reference. Sequences classified within the Archaea domain (15 hits from the 1306-5F sample) were excluded from further analysis.

Operational taxonomic units (OTUs) were assigned at 95% and 97% similarity threshold levels, representing bacterial genera and species according to Tindall et al. (2010). Richness estimators, diversity indices and coverage percentage were calculated from randomly subsampled set of sequences (based on the smallest dataset) in mothur. To visualize the number and correlation of taxa at different taxonomic ranks among samples, CoVennTree (Lott et al., 2015) was used, a tool on the Galaxy platform (Giardine et al., 2005; Blankenberg et al., 2010; Goecks et al., 2010). The resulted output was visualized in Cytoscape 2.8.3 (Shannon et al., 2003).

Chloroplast sequences were also retrieved by Bacteria-specific primers, since plastids represent a distinct lineage within phylum Cyanobacteria (Giovannoni et al., 1988). These sequences were analyzed separately, owing to that plastid 16S rRNA gene references are underrepresented in the ARB-SILVA database. In this case, closest related sequences were retrieved from GenBank using BLAST, and subsequent phylogenetic analysis (including the search for the best-fit model) was performed with the MEGA 6.0 software (Tamura et al., 2013).

Raw sequence reads are available in the NCBI Sequence Read Archive under the following Biosample accession codes: SAMN03291236, SAMN03291239 and SAMN03291240.

3. Results

3.1. Main parameters of examined fish

The body length, mass, sex and age of the fish under study are presented in Fig. 1. Based on the investigation of external morphological traits, all individuals in this study proved to be hybrids of silver carp and bighead carp. The sex ratio of the studied individuals was 60% male and 40% female. Total length varied between 94 and 119 cm (with an average of 103.3±6.9 cm). Body mass ranged between 11.2 kg and 25.1 kg (with an average of 15.6±4.2 kg), and the fish were 8-13 years old (determined by counting annual rings on dorsal scales).

3.2.Comparison of gut content and lake water bacterial communities by DGGE

In this study, PCR-DGGE method was applied to reveal the bacterial community shifts in gut content samples of bigheaded carps and the planktonic bacterial communities of Lake Balaton. An UPGMA dendrogram (Fig. 1) was constructed on the basis of the DGGE patterns of the almost 500 bp long PCR products, obtained from 10 foregut and 10 hindgut samples as well as from 5 lake water samples. The first branching of the dendrogram separated clearly the lake water samples from the gut content samples. According to the DGGE patterns, the planktonic bacterial communities at different seasons showed higher than 60% similarity. Samples taken from spring and autumn formed two sub-clusters. A clear distinction could also be observed between the bacterial communities of foreguts and hindguts in bigheaded carps. According to the similarities on the dendrogram, higher values (53-90%) were detected among the bacterial community structures of the hindgut samples than among the foregut samples (35-62%). The results show that the differences among the DGGE profiles of the

intestinal bacterial communities of bigheaded carps seem to be independent of the sampling month, morphological characteristics, sex, age and size of the studied fish. The only observable factor reflected in the separation of the bacterial DGGE patterns of the bigheaded carp gut contents was the anatomical difference of their origin (foregut versus hindgut) (Fig. 1).

To reveal the taxonomic position of the dominant members of the studied communities, altogether 39 discrete bands were excised from the gel, re-amplified and submitted to sequence analysis. The phylogenetic distribution of the 12 high density DGGE bands (others were mixed or ambiguous) is presented in Table 1. The partial 16S rRNA gene sequences of these bands showed at least 97% sequence similarities to validly described species. Members of genera *Pelomonas*, *Herbaspirillum* and *Aeromonas* (Proteobacteria) were detected in the foregut whereas *Shewanella* (Proteobacteria) and *Cetobacterium* (Fusobacteria) in the hindgut contents of bigheaded carps. DGGE band sequence closely related to '*Candidatus* Planktophila limnetica' (Actinobacteria) was present only in the lake water samples.

3.3.Comparison of gut content and lake water microbial communities by pyrosequencing

Based on the results of the DGGE analysis, representatives of bigheaded carp foregut and hindgut content samples (0613-5F and 0613-5H) and the corresponding lake water sample (0613-LW) were selected for a deep studying of taxonomic diversity by high-throughput DNA sequencing. From the three analyzed samples, a total of 13,910 quality-filtered partial 16S rRNA gene sequences with an average length of 384 nucleotides were obtained. Richness estimators, diversity and coverage values at two cutoff levels (97% and 95% similarities) are presented in Table 2. According to each species richness estimator, the highest values were found in the lake water sample, followed by the hindgut and foregut samples (e.g. the estimated species number values based on Chao1 were 254, 68 and 33, respectively). The

rarefaction analysis of the gut content and lake water samples resulted in a similar trend (Supplementary Fig. 1). Nevertheless, all three samples exhibited adequate sequence coverage as rarefaction curves are asymptotic.

Following the processing of pyrosequencing data, a total of 332 OTUs (defined at 0.03 distance level) of bacteria were recovered, out of which 315 OTUs (94.9%) were not shared among the samples, indicating that at the species-level the three sample types were rather distinct (Supplementary Fig. 2). It is important to note that 13 OTUs common to the gut content samples (representing genera *Cetobacterium*, *Aeromonas*, *Shewanella*, *Microcystis*, *Vogesella* and *Macellibacteroides*) were highly abundant as they accounted for 75.6% of the gut content sequences. Contrary to this, the number of OTUs common to gut content and lake water samples and also their relative abundance proved to be very low (2.7% of total sequences were shared between gut contents and lake water).

From the three data sets, representatives of altogether 13 phyla, 49 orders and 81 genera described were identified which represented 99.8%, 90.2% and 58.4% of the total sequences, respectively. Comparing the taxonomic depth of the samples achieved by sequencing, the proportion of unclassified sequences increased from the phylum to the genus level, although its extent was different from sample to sample (Table 3). The number of identified bacterial phyla, orders and genera was similar in the gut content samples but was lower than in the lake water sample (Table 3).

Sequences belonging to phyla Bacteroidetes (17.2%), Proteobacteria (15.0%), Actinobacteria (12.6%), Cyanobacteria (2.6%) and Firmicutes (2.1%) were present in all samples (Fig. 2). Strikingly, a high proportion of the total sequences (45.2%) were of the phylum Fusobacteria which was found only in the gut content samples (Fig. 2). Sequences affiliated with the phylum Lentisphaerae (2.0%) were recovered exclusively from the hindgut sample. Other sequences were obtained mainly from the water of Lake Balaton, and were

related to phyla Verrucomicrobia (2.1%), Chloroflexi (0.5%), Chlorobi (0.2%), Planctomycetes (0.2%), Nitrospirae (0.1%), Elusimicrobia (0.1%) and candidate divisions (0.1%). In general, the three sample types differed markedly at the phylum level; phyla Actinobacteria (41.5%) and Proteobacteria (27.4%) were the highest in relative abundance in lake water sample, while phylum Fusobacteria was the most abundant in the foregut and hindgut (with a relative abundance of 84.7% and 45.6%, respectively).

Comparison of the 16S rRNA gene datasets at genus level resulted in remarkable differences among the studied samples (Fig. 3) because only the genus *Microcystis* (Cyanobacteria) was common to all three samples, and accounted for 0.7% of total sequences. The genera (orders and phyla) common to both gut content samples (according to their decreasing abundance) were *Cetobacterium* (Fusobacteriales, Fusobacteria), *Aeromonas* (Aeromonadales, Gammaproteobacteria) and *Shewanella* (Alteromonadales, Gammaproteobacteria), *Macellibacteroides* (Bacteroidales, Bacteroidetes), *Caryophanon* (Bacillales, Firmicutes) and *Vogesella* (Neisseriales, Betaproteobacteria) (Fig. 3). However, the last three genera were represented less than 1% of the sequences. In addition, sequences related to genera *Clostridium* and *Holdemania* (Firmicutes) were present more than 1% in the hindgut sample.

In the foregut and hindgut samples 84.4% and 40.7% of sequences were closely related to genus of *Cetobacterium*, 10.7% and 1.1% to *Aeromonas* and 0.9% and 4.4% to *Shewanella*, respectively. On the other hand, not only higher number of genera was found in the lake water (Table 3), but as suggested also by the previously mentioned OTU-based comparison (Supplementary Fig. 2), genera identified from the water sample were substantially different from those detected in the gut content samples (Fig. 3).

Plastid sequences (177 sequences, representing 1.3% of total sequences) belonged to lineages Bacillariophyta, Dictyochophyceae, Euglenozoa, Chrysophyceae, Cryptophyta

(including Dinophyceae, due to the endosymbiotic origin of *Dinophysis* plastid from a cryptophyte or as a sign of kleptoplasty; Hackett et al., 2003; Nishitani et al., 2010), Haptophyceae, Chlorophyta and Streptophyta (Fig. 4). Bacillariophyta sequences were present in all three sample types (Chloroplast_OTU_2 on Fig. 4 also belonged to this lineage), while Dictyochophyceae and Cryptophyta (including Dinophyta) sequences were found only in the lake water sample (Chloroplast_OTU_3 and OTU_1 on Fig. 4, respectively). Most plastid lineages were retrieved from the lake water sample which contained 74.0% of total chloroplast sequences, while in the foregut and hindgut samples, only minority of them were found (5.7% and 20.3%, respectively).

4. Discussion

In this study, the 16S rRNA gene-based PCR-DGGE method was applied to compare bacterial community structures in lake water and bigheaded carp gut content samples, while pyrosequencing was used to reveal the diversity and taxonomic composition of the gut content microbiota of bigheaded carps and of the bacterioplankton in Lake Balaton. Knowledge on the importance of bacteria either as direct food resources or as intestinal decomposers of ingested food (algae, detritus, etc.) is important because it helps us more accurately predict where bigheaded carps will find adequate living conditions and be potentially invasive. In addition, deeper understanding on the role of microbes in the nutrition of bigheaded carps may contribute to more effective fish production in aquaculture operations.

The particular intestinal bacterial communities found in bigheaded carps and the dissimilar microbial compositions characteristic to different sections of the digestive tract were in accordance with the findings of other studies (Li et al., 2014; Ye et al., 2014). The remarkable change in composition between gut sections can be attributed to the digestion process that

takes place between the foregut and hindgut and difference in the chemical environment of the two gut sections (Sullam et al., 2012). Thus, we assume that digestion processes caused distinct clustering of hindgut and foregut bacterial communities, independently of sampling date, sex and morphological features of the studied fish (Fig. 1). However, it has to be mentioned that bacterial composition of one foregut and one hindgut sample may not reflect the actual variability among the individuals.

Regarding the role of bacteria as important food resource for bigheaded carps, previously it was thought that cyanobacteria are important in the nutrition of these fish, especially silver carp (Beveridge et al., 1993). Thus, silver carp had been stocked to several lakes throughout the world to improve the poor water quality and control cyanobacterial blooms in eutrophic/hypertrophic water bodies (Xie and Liu, 2001; Radke and Kahl, 2002). The role of cyanobacteria in the nutrition of bigheaded carps was also studied formerly in Lake Balaton and its watershed (Herodek et al., 1989; Vörös et al., 1997) and in other lakes (e.g. Domaizon and Devaux, 1999), and size-selective filtration and taxon-specific digestion of planktonic algae (including cyanobacteria and eukaryotic algae) were revealed (Vörös et al., 1997; Boros et al., 2012; Görgényi et al., 2016). However, it should be noted that phytoplankton has characteristic seasonal dynamics with changing biomass and composition in the temperate climate zone, therefore its importance as a food resource for fish may also differ substantially across seasons and ecosystems. In general, eukaryotic algae dominate the phytoplankton in Lake Balaton, and cyanobacteria proliferate substantially only in August and September (Istvánovics et al., 2005). Since pyrosequencing analysis was performed on samples taken in June, identification of plastid sequences added relevant information to our study. Although the number of plastid 16S rRNA gene copies could significantly differ among taxa (Shi et al., 2011), the present pyrosequencing revealed similar algal communities as previous microscopic analyses (Vörös et al., 1997; Görgényi et al., 2016). Algae-derived 16S rRNA

gene sequences from gut content samples were related mainly to diatoms (Bacillariophyta), while two other algal OTUs, found in high sequence number and related to Dictyochophyceae and Cryptophyta (including Dinophyta) were detected only in the lake water sample (Fig. 4). This could be explained by the effective filtering and digestion of cryptophytes and dinoflagellates by bigheaded carps, while diatoms may survive physical and chemical digestion processes in the alimentary tract (Vörös et al., 1997; Görgényi et al., 2016). Although alterations may exist in lower taxonomic ranks in the digestibility of algae, no common OTUs were detected by pyrosequencing from the lake water and intestine samples. It should be also noted that pyrosequencing analysis primarily was not focused on algae; therefore, algae-derived sequences were captured adventitiously which may introduce some bias in their proportion in the samples.

The relative abundance of cyanobacteria was very low both in the foregut and hindgut contents, and *Microcystis* was the most abundant cyanobacterial genus present in the intestines of fish (Fig. 2). This finding is in accordance with the recent oligotrophication trends of Lake Balaton and the depressed abundance of cyanobacteria which exhibited their last bloom in 1994 (Istvánovics et al., 2007). Nevertheless, algal blooms caused mainly by *Microcystis* seem to play important role in the invasion potential of bigheaded carps, as it was proved by Anderson et al. (2015) in the case of Lake Erie. Some colony forming cyanobacteria (e.g. *Microcystis* and *Aphanizomenon*) are easily filterable from the water by bigheaded carps which have some natural resistance against the toxins produced by cyanobacteria (He et al. 2012). However, so far there is no knowledge of toxic cyanobacterial bloom formations in Lake Balaton. It is also interesting to note that contradictory results have been published regarding the abundance of cyanobacteria in the intestine of bigheaded carps. Cyanobacteria were predominant in the gut microbiota of fish inhabiting different rivers of the USA (Ye et al., 2014), while their abundance was very low in the intestinal microbiota of

both silver carp and bighead carp living in the shallow Wuhu Lake, China (Li et al., 2014). The potential dissimilarities in the planktonic composition of these ecosystems may explain the various rates of cyanobacteria consumption.

A fraction of the ingested cyanobacteria and phytoplankton (e.g. *Microcystis sp.*, diatoms, desmids, volvocalean and chlorococcalean green algae) may remain viable after the passage through the alimentary tract of bigheaded carps, as they are protected either by mucilaginous envelope or cellulose-based cell-wall (Gavel et al., 2004; Görgényi et al., 2016). Although bigheaded carps possess pharyngeal teeth adapted to the mastication of plankton, they lack cellulase enzyme in their gut fluids to break down the cell walls of algae (Kolar et al., 2007). Still, we can deduce that the energy and nutrients derived either from the bacterial decomposition of cellulose molecules or from the digestion of bacteria that used cellulose for growth may be important in the nutrition of bigheaded carps in Lake Balaton. Members of the genus *Aeromonas* were present in the foregut samples with a mean relative abundance of about 11% (based on pyrosequencing data), and several species from this genus produce cellulase enzyme (Jiang et al., 2011; Muñoz et al., 2014). Moreover, the genera *Holdemania* (Mishra et al., 2013) and *Macellibacteroides* (Jabari et al., 2012) were present in the hindguts (with 4% and 1% relative abundance, respectively), and these taxa are able to decompose cellulose- and hemicellulose-derived sugars (e.g. cellobiose or xylose), thus their direct or indirect role in the nutrition of bigheaded carps also can be important. Such type of symbiosis between vertebrates and bacteria has been described in ruminants that are able to meet their energy and nutrient requirements by harboring cellulose-degrading bacteria in their alimentary tract (Hofmann, 1989).

Based on the results obtained by molecular biological methods (DGGE and pyrosequencing), community structure and composition of bacteria in the alimentary tract of bigheaded carps differed greatly from that of the lake water (Figs. 1, 2, 3). Actinobacteria

dominated the planktonic communities but were in negligible proportions in the foregut and hindgut. In addition, the proportion of Proteobacteria was also higher in the lake water compared to the foregut and hindgut samples. The well-known freshwater planktonic bacteria (e.g. *Synechococcus*, *Limnohabitans*, '*Candidatus* Limnoluna', '*Candidatus* Planktophila', other actinobacterial genera, members of Flavobacteria and the LD12 group of the SAR11 clade; Hahn, 2009; Jezbera et al., 2009; Felföldi et al., 2011; Newton et al., 2011; Kasalický et al., 2013; Pernthaler, 2013) were abundant in the water of Lake Balaton, but were nearly absent in the intestine samples. One possible explanation for this phenomenon is that members of the bacterioplankton are not or just partially filtered out from the water, since their cells are usually smaller than 10 µm (a filtration-size threshold proposed for bigheaded carps by Vörös et al., 1997). However, some studies reported that particles even smaller than 10 µm are found in the diet of bigheaded carps (e.g., Görgényi et al., 2016), thus we cannot rule out the chance of bacterioplankton consumption by bigheaded carps. In addition, bacteria attached to larger particles or those forming aggregates could also be filtered effectively from the lake water, but compared to the indigenous bacterial community of the foregut, relative abundance of freshwater bacteria was extremely low (in most cases below the detection limit of our pyrosequencing approach). Therefore, our findings do not confirm that heterotrophic bacterioplankton serve as important food resource and that significant bacterioplankton consumption can explain the intensive growth of bigheaded carps.

The gut contents of bigheaded carps were predominated by obligate anaerobic bacteria belonging to phylum Fusobacteria, and Bacteroidetes, Firmicutes and Lentisphaerae were also abundant in the hindgut. In contrast, Fusobacteria were detected only in a minor proportion in the gut contents of bigheaded carps collected in China and in the United States (Li et al., 2014; Ye et al., 2014). In the alimentary tract of bigheaded carps living in Lake Balaton, obligate anaerobic (e.g. *Cetobacterium*, *Clostridium* and *Holdemania*) and facultative

anaerobic (e.g. *Aeromonas* and *Shewanella*) bacteria were frequently detected. These bacteria were found in the intestines of different other fish species too (Sugita et al., 1991; Sugita et al., 1995; Tsuchiya et al., 2008; Li et al., 2014; Ye et al., 2014; Pekala et al., 2015). Moreover, unique taxa (e.g. *Macellibacteroides*) were also found and identified in the gut contents of bigheaded carps inhabiting Lake Balaton (Fig. 3). Due to the strong enzyme (e.g. saccharolytic and proteolytic) activities connected to the fermentative metabolism, the aforementioned anaerobic bacteria (Rainey et al., 2009) may participate in the effective decomposition of consumed organic debris, phytoplankton or zooplankton, which are components of the food of bigheaded carps. Intensive cellulolytic activity of bacteria (e.g. members of the genus *Aeromonas*) isolated from the intestinal tract of grass carp (*Ctenopharyngodon idellus*) was proved by Li et al. (2009, 2016).

However, the question what factors facilitate bigheaded carps in attaining high growth rate under low planktonic productivity (Istvánovics et al., 2007) and high competition for zooplanktonic food resources by other fish species (Specziár & Rezsu, 2009) in Lake Balaton is still open as well as the role of bacteria in its nutrition remains mostly undiscovered.

5. Conclusion

In summary, gut microbiota of bigheaded carps living in the oligo-mesotrophic Lake Balaton is predominated by the strictly anaerobic Fusobacteria, but several other obligate anaerobic and facultative anaerobic bacteria are found in their alimentary tract. The almost complete absence of typical freshwater bacteria in all studied segments of the intestines suggested that heterotrophic bacterioplankton has a negligible role in the nutrition of bigheaded carps, while the high relative abundance of cellulose- and organic matter-degrading bacteria indicated that gut microbiota may contribute significantly to the decomposition of recalcitrant food components, thereby facilitating food utilization. Bigheaded carps in Lake Balaton harbor unique assemblage of bacteria in their intestines that differs in composition

from the gut microbiota of bigheaded carps inhabiting other ecosystems, and this may provide explanation for the intensive growth and relatively high condition factor of bigheaded carps that live in a nutrient-poor (oligo-mesotrophic) ecosystem, such as Lake Balaton.

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Conflict of interest

The authors declare that there is no conflict of interest. All authors have contributed equally to the manuscript and agreed to publication of the work.

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Table 1 Identified sequences retrieved from excised bands (for details see Fig. 1) of bigheaded carp gut content and lake water samples.

DGGE band (Acc.No.)	Phylum/Class	Nearest described relative by EzTaxon	Similarity (bp/bp)
0413-LW (LN881423)	Actinobacteria	*Candidatus* Planktophila limnetica (FJ428831)	97% (402/416)
0413-12F (LN881425)	Betaproteobacteria	*Pelomonas puraquae* (AM501439)	99% (428/430)
0513-4F (LN881426)	Betaproteobacteria	*Herbaspirillum huttiense subsp. putei* (ANJR01000027)	100% (468/468)
0413-9F (LN881424)	Betaproteobacteria	*Herbaspirillum huttiense subsp. putei* (ANJR01000027)	100% (470/470)
0913-13F (LN881429)	Betaproteobacteria	*Herbaspirillum huttiense subsp. putei* (ANJR01000027)	99% (444/445)
1013-5F (LN881430)	Betaproteobacteria	*Herbaspirillum huttiense subsp. putei* (ANJR01000027)	99% (443/445)
0613-9F (LN881428)	Gammaproteobacteria	*Aeromonas veronii* (CDDK01000015)	100% (471/471)
0613-5F (LN881427)	Gammaproteobacteria	*Aeromonas sobria* (X74683)	99% (472/475)
0613-5H (LN881432)	Gammaproteobacteria	*Shewanella hafniensis* (AB205566)	98% (462/468)
0413-12H (LN881431)	Fusobacteria	*Cetobacterium somerae* (AJ438155)	98% (440/447)
1013-5H (LN881433)	Fusobacteria	*Cetobacterium somerae* (AJ438155)	99% (415/418)
1013-11H (LN881434)	Fusobacteria	*Cetobacterium somerae* (AJ438155)	99% (439/443)

Table 2 Pyrosequencing read numbers, richness estimators, diversity and coverage values for bacterial communities from bigheaded carp gut content and lake water samples collected in June 2013. Species are defined at 97%, genera at 95% similarity level according to Tindall et al., (2010). ACE stands for abundance-based coverage estimator.

	Sample identifier	Total high-quality sequence	Coverage (%)	OTUs	Chao1	ACE	Shannon
Species	0613-LW	4222	99.98	254	254	254	3.8
	0613-5F	4886	99.85	31	33	37	0.64
	0613-5H	4802	99.78	66	68	72	1.75
Genera	0613-LW	4222	99.98	204	204	204	3.61
	0613-5F	4886	99.87	26	28	30	0.62
	0613-5H	4802	99.83	51	53	56	1.55

Table 3 Number of different taxa and percentage distribution of bacterial 16S rRNA gene sequences which could be affiliated to previously described taxa from bigheaded carp gut content and lake water samples collected in June 2013.

	Numbers of identified			Percentages of sequences identified in		
Sample identifier	**phyla**	**orders**	**genera**	**phyla**	**orders**	**genera**
0613-LW	10	38	59	99.9	72.6	22.6
0613-5F	8	14	16	100.0	99.4	99.2
0613-5H	8	17	21	99.6	98.7	53.6

Figure captions

Fig. 1 An UPGMA dendrogram constructed on the basis of the DGGE patterns of the bacterial communities of foregut and hindgut contents of bigheaded carps and water samples from Lake Balaton collected in April, May, June, September and October 2013. [Coding: sample identifiers start with sampling date (month and year), after specimen ID number (only in the case of gut content samples), sample type is shown (F – foregut, H – hindgut, LW - lake water samples). Color-coding: red - foregut; yellow - hindgut; blue - lake water. Arrowheads indicate the excised and unambiguously sequenced bands. Phenotypic characteristics: S- sex (M - male, F - female), L- total body length (cm), M - total body mass (kg), A - age (year)]. Samples analyzed also by pyrosequencing are marked with an asterisk.

Fig. 2 Distribution of bacterial phyla based on the 16S rRNA gene pyrosequencing data among the bigheaded carp foregut (0613-5F) and hindgut (0613-5H) content and lake water (0613-LW) samples collected in June 2013.

Fig. 3 CoVennTree showing the ratio and distribution of 16S rRNA gene pyrosequencing data at different taxonomic levels among the bigheaded carp foregut and hindgut contents and lake water samples collected in June 2013 (only the most abundant 50 OTUs are shown as defined at 97% sequence similarity level). OTUs indexing number corresponds to their relative abundance in the dataset in decreasing order. Taxonomic assignments were made when the bootstrap values were higher than 80 based on the ARB-SILVA SSU NR reference database. Numbers in brackets assigned to a parent node are the VDS values of CoVennTree representing similarity among children. For color-coding, see Fig. 1. Unc., unclassified. OTUs detected only in the gut content samples are highlighted in red. Genera detected in all three sample types appear in blue. Root contains all high quality data obtained by pyrosequencing.

Fig. 4 Phylogenetic tree of chloroplast genotypes from the 16S rRNA gene pyrosequencing data of bigheaded carp foregut and hindgut contents and lake water samples collected in June 2013. Tree was constructed using the Maximum Likelihood method with the Kimura 2-parameter nucleotide substitution model and is based on 401 nucleotide positions. GenBank accession numbers are given in parentheses. Sequences differing only in one position are represented with a single sequence, with number of sequences is given in square brackets. For color-coding, see Fig. 1.