Effects of phytonutrient-supplemented diets on the intestinal microbiota of Cyprinus carpio

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Abstract

In the aquaculture sector, a strategy for the more efficient use of resources and proper disease control is needed to overcome the challenges of meat production worldwide. Modulation of the gastrointestinal tract microbiota is a promising approach for promoting animal health and preventing infection. This feeding experiment was conducted to discover the phytonutrient-induced changes in the gastrointestinal tract microbiota of common carp (Cyprinus carpio). Acclimatized animals aged 7 months (30 weeks) were divided randomly into five experimental groups to investigate the effects of the applied feed additives. The dietary supplements were manufactured from anthocyanin-containing processing wastes from the food industry, specifically the production of Hungarian sour cherry extract, synbiotics from fermented corn, and fermentable oligosaccharides from Hungarian sweet red pepper seeds and carotenoids from Hungarian sweet red pepper pulps, applied at a dose of 1%. The gut contents of the animals were collected at four time points throughout the 6-week study period. To track the compositional and diversity changes in the microbiota of the carp intestinal tract, V3-V4 16S rRNA gene-based metagenomic sequencing was performed. The growth performance of common carp juveniles was not significantly affected by supplementation of the basal diet with plant extracts. Phytonutrients improve the community diversity, increase the Clostridium and Lactobacillus abundances and decrease the abundances of potentially pathogenic and spoilage bacteria, such as Shewanella, Pseudomonas, Acinetobacter and Aeromonas. The phyla Proteobacteria, Tenericutes and Chlamydiae were positively correlated with the body weight, whereas Spirochaetes and Firmicutes exhibited negatively correlations with the body weight. We hypothesize that the application of phytonutrients in aquaculture settings might be a reasonable green approach for easing the usage of antibiotics.
Introduction

As the human population is expanding, fish have become an nutrition source of increasing importance [1]. The production of total edible aquatic animal food exhibits a greater annual increase than the total terrestrial meat production [2]. The enormously developing aquaculture sector has become the primary source of fish protein and is expected to further expand in order to address the growing needs of the world’s population [3].

The intensification of production has been linked to depressed immunity and decreased resilience to pathogens. Fish diseases have become more significant and lead to serious economic losses [4, 5]. In addition, the lack of proper infection control and limited treatment options negatively affect animal development.

Common carp (Cyprinus carpio) is one of the most important freshwater fish species in aquaculture, with a global production of more than 4 million tonnes, which accounts for 7.7% of total finfish production [6]. Additionally, common carp can be produced in both extensive polyculture fish pond systems and intensive monocultural recirculation aquacultures [7–9], which explains its high economic significance [10–13]. Common carp can consume a wide range of different natural foods and is a flexible and opportunistic feeder that can switch from its preferred to alternative diets according to food availability.

The gastrointestinal tract (GIT) microbiota is a diverse population of microorganisms that play various important roles in the biology of multicellular hosts. The genetics, age, physiological status, pathology, metabolic activity, water chemistry, temperature, locations, and trophic level of a specific organism can notably influence the intestinal microbiota of fish [12, 14]. Due to advances in high-throughput sequencing techniques, metagenomes are receiving increased attention, but few studies have investigated fish-associated microbiomes.

One of the key roles of the GIT microbiota is affecting host immunity and nutrition via multiple mechanisms. GIT microorganisms complement digestive processes by providing enzymes that are not encoded by the host’s genome and play important roles in the breakdown of polysaccharides and the synthesis of vitamins [15]. The GIT microbiome also plays a crucial role in colonization prevention and in defence against pathogenic microorganisms by competing for nutrients and adhesion sites and producing antimicrobial substances [15, 16]. Preserving the balance of the GIT microbiota is crucial for maintenance of the intestinal health of fish [17].

Microbial dysbiosis can be triggered by numerous factors leading to either the depletion of beneficial bacteria or the expansion of potential pathogens. By referring to the opening lines of Leo Tolstoy, “all happy families are all alike; each unhappy family is unhappy in its own way”, the Anna Karenina principle can be used to draw a parallel between microbiome-associated diseases and healthy or sick microbiomes: “healthy” microbiomes are alike, and each disease-associated microbiome is “sick” in its own way [18]. One of the key factors in effective disease control is the timely diagnosis of microbial dysbiosis to mitigate devastating outcomes. Increasing our knowledge of the structure of the healthy GIT community, which is known as the symbiome, can aid the recognition of significant shifts in the abundance of certain microorganisms that indicate dysbiosis in aquaculture systems.

Antibiotics and other chemotherapeutics are still indispensable for the control of infections [19], but their wide and excessive use can cause irreversible damage [20–23]. Furthermore, the fish metabolism does not inactivate drugs, and as a result, the pollution of our ecosystems with antibiotic residues has become a major global concern [23]. In particular, many aquatic ecosystems have been destroyed or severely degraded [23].

Bacterial fermentation involves anaerobic carbohydrate metabolism, which converts indigestible dietary carbohydrates into short-chain fatty acids (SCFAs) that can modulate immune
responses, disease resistance and energy homoeostasis [24]. In aquaculture, SCFAs and their salts have been used as growth promoters and immune stimulators [25, 26]. The fermentation of SCFAs is related to specific bacteria, but dietary intake has a significant influence on the formation of SCFAs [27].

The application of natural, bioactive components in aquacultures is a fast-growing area. Herbal medicines have recently become the focus of the meat industry by supplying consumers with clean-labelled products free of artificial ingredients or synthetic chemicals.

The use of bacteria for probiotic purposes is a new approach for improving fish health and nutrition. Furthermore, dietary probiotics are known to stimulate nutrient digestion and release vitamins and amino acids to the host. Prebiotics are capable of selectively promoting the growth of beneficial microbiota in the gut of aquatic animals, such as common carp [28–30]. A previous study showed that fermentable oligosaccharides exert positive effects on the bacterial microbiome of aquatic animals [31].

Aquaculturists are interested in natural, bioactive components that might reduce the risk of infections. A growing body of evidence related to improving the gut and overall health of animals indicates that pro- and prebiotics have been widely and successfully used as feed additives to prevent and control infectious diseases in fish [5, 22]. However, further research is needed to determine their optimal dosage and long-term effects [5].

Sour cherry (Prunus cerasus) and sweet red pepper (Capsicum annuum), which are natively grown plants in Hungary, are famous worldwide for being rich in antioxidants, vitamins and flavonoids and thus exerting several beneficial health effects [32–35].

In this feeding experiment, we investigated the effects of a phytonutrient-enriched diet on the microbiota of the Cyprinus carpio GIT. To this end, waste materials from the food industry that are rich in natural bioactive compounds, specifically the waste from the processing of sour cherry (rich in anthocyanins) and sweet red pepper pulp (rich in carotenoids) and seed (rich in fermentable-oligosaccharides), were recycled. We believe that this green and cost-effective approach has exciting potential and might lead to improvements in intensive aquaculture farming.

Materials and methods

Experimental protocol

The study was approved and performed in accordance with the guidelines of local ethics committee at the University of Debrecen (University of Debrecen Committee of Animal Welfare) under the registration number DEMAB/15/2019. A schematic view of the feeding trial is presented in Fig 1. Common carp juveniles aged 7 months (30 weeks) were used in this pilot study. Fish with an initial body weight of 123.45±0.37 g were obtained through artificial propagation and reared in a water recirculation system prior to the experiment. A total of 165 fish were equally distributed into five treatments groups 1 week before initiation of the feeding test for acclimation (acclimated animals; AA). After 7 days of acclimatization, the AA animals were further randomly sorted into five treatment groups, and the fish belonging to one of three replicates of each treatment group, which consisted of 11 individuals, was placed in each tank. The fish belonging to the negative control group received a basal diet (BD), whereas the fish in the treatment groups received one of the following supplements: BD+1% anthocyanins (ANTH) provided by sour cherry extract, BD+1% synbiotics (SYN) provided by fermented corn, BD+1% fermentable oligosaccharides (fOS) provided by sweet red pepper seed extract, and BD+1% carotenoids (CAR) provided by sweet red pepper pulp extract. The treatments were set up in a completely randomized design. The experiment lasted for 42 days.
The experiment was performed in a water recirculation system provided with mechanical and aerated biofilters and UV lamps. The water volume of the circular plastic tanks was 350 L. During the experiment, the oxygen saturation level was maintained at 85±0.9% by aeration stones, and the temperature was controlled at 23.51±0.5°C. The photoperiod consisted of 12 hours of light and 12 hours of darkness. The water temperature, pH, total dissolved solids (TDS, HANNA HI98130), dissolved oxygen (DO, HACH HQ30d), and NO₂⁻, NO₃⁻ and NH₄⁺

Fig 1. Overview of the study protocol showing the feeding and sampling strategies. AA stands for acclimatized animals aged 7 months (30 weeks). The fish were divided randomly into five experimental groups and fed either the commercial basal diet (BD, negative control) with no dietary supplement or the following dietary treatments as supplemented feed: BD+1% anthocyanins (ANTH) provided by Hungarian sour cherry extract, BD+1% synbiotics (SYN) provided by fermented corn, BD+1% fermentable oligosaccharides (fOS) provided by Hungarian sweet red pepper seed extract, and BD+1% carotenoids (CAR) provided by Hungarian sweet red pepper pulp extract. The fish intestines were collected on the 1st, 14th, 28th and 42nd days of the experimental period. The letter and number codes (D6-H7) refer to the code used during the 16S metagenomic sequencing library preparation.

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concentrations (HACH DR3900) were checked daily. Any uneaten feed and faeces were removed daily.

Prior to the experiment, the basal diet was supplemented with four different feed additives at 1%. The composition of the experimental diets is provided in S1 Table. The fish were fed manually three times each day (0800, 1200 and 1600), and the feeding rates were equal to 3% of the total biomass.

The wet body weight (BW) of the common carp individuals was measured at the beginning and end of the feeding trial (Table 1). The survival of the fish was 100% during the experiment. The growth performance and feed conversion were estimated by calculating the weight gain (WG, %), specific growth rate (SGR, %) and feed conversion ratio (FCR) of the fish using the following formulas:

- \( \text{WG} (\%) = \frac{(W_f - W_i)}{W_i} \times 100 \), where \( W_f \) is the final wet body weight (g) and \( W_i \) is the initial wet body weight (g);
- \( \text{SGR} (\%) = \frac{(\ln W_f - \ln W_i)}{t} \times 100 \), where \( W_f \) is the final wet body weight (g) and \( W_i \) is the wet initial wet body weight (g), \( t \) is the time (day); and
- \( \text{FCR (g day}^{-1}) = \frac{F}{(W_f - W_i)} \), where \( F \) is the feed intake, \( W_f \) is the final wet body weight (g) and \( W_i \) is the initial wet body weight (g).

Statistical analyses of the growth performance and feed conversion were performed using the SPSS/PC+ software package. The variance homogeneity was tested by Levene’s test, and a \( P \) value higher than 0.05 was considered to indicate homogeneity. The effects of the treatments on the BW, WG, SGR and FCR results were assessed by one-way ANOVA. The significance of the differences was determined using Tukey’s multiple comparison test, and \( P < 0.05 \) was considered to indicate significance.

### Determination of natural feed additives

The ANTH supplement was prepared as described by Nemes et al. [36]. Anthocyanins were extracted from Hungarian sour cherry (*Prunus cerasus*). Cherries were deseeded and homogenized, and a methanol:water:acetic acid solution at a ratio of 25:24:1 was then used to extract the anthocyanins. The sample was mixed with a magnetic stirrer (MSH 300, BioSan, Riga, Latvia) for 1 hour. After filtration, centrifugation was performed at 10,000 RPM for 5 minutes, and a simple fraction was then obtained in preconditioned tubes (Supelclean ENVI-18 SPE.
tubes). For preconditioning, 5 mL of MeOH and 5 mL of H₂O were mixed with 1 mL of the fruit sample. Elution was conducted with methanol containing 20% H₂O, and vaporization was performed at 40˚C. The sample was dried in a vacuum to yield a powder. A VWR-Hitachi ChromasterUltraRs UHPLC system (Hitachi, Tokyo, Japan) with a Phenomenex Kinetex® column (2.6 µm, XB-C18, 100 A, 100 x 4.6 mm) (Phenomenex, Torrance, CA, USA) was used to determine the anthocyanin profile. Two solvents were applied for gradient elution: A, which consisted of MeOH, and B, which consisted of 3% formic acid. Elution was conducted using the following parameters: 0 minutes, 15% solvent A; 0–25 minutes, 30% solvent A; 25–30 minutes, 40% solvent A; and 30–40 minutes, 50% solvent A. UV-VIS detection was performed at 534 nm, the flow rate was maintained at 0.7 mL/minutes at 25˚C, and the injection volume was 10 µL. The UHPLC profiles of the anthocyanins and the identified main compounds, including the relative areas and retention times, are shown in S1 Fig.

The synbiotics in the SYN supplement include probiotics (Bifidobacterium bifidum, B. infantis, B. lactis, B. longum, Lactobacillus acidophilus, L. buchneri, L. casei, L. paracasei, L. plantarum, L. salivarius, and L. lactis), prebiotics (fructo-, xylo-, and mannooligosaccharides and arabinoxylan), vitamins (B group vitamins and vitamins C, D2, D3, E and K2), unsaturated fatty acids (ω-3, ω-6, and ω-9), minerals/trace elements (sodium, potassium, calcium, iodine and phosphorous) and lactose. The GC profile of the oligosaccharides and the identified monomer units with the greatest relative areas and retention times are shown in S2 Fig.

The carotenoids in supplemental CAR were determined as described by Remenyik et al. [33] and Csernus et al. [37]. Carotenoids were extracted from Hungarian sweet red pepper (Capsicum annuum) powder (1–5 g) using a mixture of dichloroethane:acetone:methanol at a ratio of 2:2:1 as the solvent. The mixture was stirred in an ultrasonic water bath for 30 minutes and purified through Munktell-292 filter paper (VWR International, Debrecen, Hungary). For additional purification, a 0.22-µm PTFE syringe filter (TPP Techno Plastic Products AG, Switzerland) was applied. The filtered sample was vaporized at 40˚C and 0.2 bar and then dissolved in an HPLC pigment reagent (isopropanol:acetonitrile:methanol at a ratio of 55:35:10) (Merck, Darmstadt, Germany). HPLC separation was conducted with a Phenomenex Kinetex® column (2.6 µm, XB-C18, 100 A, 100x4.6 mm) (Phenomenex, Torrance, CA, USA) using two elution gradients of elution: A, 11% methanol; and B, isopropanol:acetonitrile:methanol (55:35:10 V/V/V%) mixture. The gradient elutions were performed with the following settings: 0–3 minutes, 100% solvent A; 15–20 minutes, 20% solvent A; 25–45 minutes, 100% solvent B; and 48–50 minutes, 100% solvent A. For sample detection, a diode array detector (DAD) was used with a flow rate setting of 0.6 mL/minutes. The sample was injected in a 10-µL volume, and DAD detection was applied at 460 and 350 nm. The HPLC profile of the carotenoids and identified monomer units with the greatest relative areas and retention times are shown in S3 Fig.

The fermentable oligosaccharides in the fOS supplements was determined as described by Csernus et al. [37] Hungarian sweet red pepper seeds (Capsicum annuum) were used for the extraction of fermentable oligosaccharides with a high arabino-galactose content. An HP 5890 gas chromatograph with an SP-2380 capillary column (30 m x 0.25 mm, 0.2 µm) was used to measure the composition of oligosaccharides. The samples were lyophilized and extracted with trifluoroacetic acid:acetic acid:water at a ratio of 5:75:20 as the solvent. A reduction step was used to convert the oligosaccharides into alditol-acetate with NaBH₄ at alkaline pH. The sugars were then converted to sugar alcohols (alditols) with acetic anhydride in pyridine, which removed interfering isomers and anomers. The nitrogen gas flow rate was 1.2 mL/min. The injector temperature was set to 300˚C with a split ratio of 1:20. A flame ionization detector (FID) was applied for the identification of oligosaccharides. The GC profiles of the oligosaccharides and the identified monomer units, including the greatest relative areas and retention times, are shown in S4 Fig.
Sample collection

During the experimental period, intestinal samples were obtained from the fish at ages of 31 weeks (day 0), 33 weeks (14th day), 35 weeks (28th day), and 42 weeks (42nd day). The average body weight (ABW) was measured at the beginning and end of the feeding period (Table 1). The fish were euthanized with clove oil [38] and disinfected with 96% EtOH, and the whole intestine was placed into a tube. During the gut collection procedure, sampling was also performed from the air and surgical table to monitor the environmental contamination, which can bias sequencing. The bowel was stored and transported on ice in sterile PBS (Thermo Fisher Scientific, MD, USA). Residual tissue was removed from the guts under class II laminar flow hood (Thermo Fisher Scientific, MD, USA), and a 10-g sample was digested in 100 mL of lysis buffer (500 mM NaCl, 50 mM Tris HCl, 50 mM EDTA, 4% SDS, 0.1 mg of Proteinase-K, and 10% Triton X) at 56˚C for 16 hours.

Sample preparation and mechanical cell lyses

The lysate was pipetted into a 50-mL centrifuge tube. The samples were centrifuged at 2,000 x g for 5 minutes, and the upper colloidal phase was discarded. The middle phase was transferred to a new 50-mL centrifuge tube, and PBS was added to the lysate at a ratio of 1:1. The samples were shaken for approximately 3 minutes, the mixture was centrifuged at 500 x g for 5 minutes, and the supernatant was collected in a sterile 50-mL centrifuge tube. This step was repeated three times. We performed mechanical cell lysis: 1,000 μL of sample was added to a PowerBead tube (Qiagen, Hilden, Germany) and lysed using a MagNA Lyser (Roche Applied Sciences; Penzberg, Germany) at 5,000 RPM for 30 seconds. The samples were centrifuged at 16,000 x g for 1 minute, and the supernatant was pipetted into a sterile Eppendorf tube.

DNA extraction

The conventional isolation method was used for total bacterial genomic DNA extraction. Eight hundred microliters of phenol:chloroform:isoamyl alcohol (25:24:1) (Thermo Fisher Scientific, MD, USA) was mixed with 800 μL of lysate, and the mixture vortexed thoroughly for approximately 15 seconds. After sample homogenization, we incubated the samples at room temperature for 3 minutes and centrifuged them for 10 minutes at 16,000 x g and 4˚C. The upper aqueous layer was carefully collected into a new sterile Eppendorf tube. For DNA precipitation, a mixture of 1 μL of glycogen (20 μg/μL), ammonium acetate (0.5x volume, 7.5 M NH₄OAc) and 100% ethanol (2.5x volume) was added to the supernatant. Samples were incubated at -70˚C for 16 hours and then centrifuged them for 30 minutes at 16,000 x g and 4˚C to pellet the DNA. We carefully discarded the supernatant without disturbing the pellet, and 500 μL of 70% EtOH was added to the sample. The mixture was shaken for 20 seconds and centrifuged at 4˚C and 16,000 x g for 5 minutes, and the supernatant was carefully removed. This washing step was repeated twice. We dried the DNA pellet at room temperature and then resuspended it in 15 μL of nuclease-free water. The DNA concentrations were determined using a Qubit® Fluorometric Quantitation dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). The DNA quality and quantity were confirmed using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific), and the DNA integrity (shearing/fragmentation) was measured with a 4200 TapeStation System (G2991AA, Agilent Technologies; Santa Clara, CA, USA). The DNA elutes were stored at ~20˚C.
Library construction and sequencing

Library preparation was performed according to the standard Illumina (San Diego, CA, USA) 16S metagenomic sequencing protocol (15044223 Rev. B). The V3 and V4 hypervariable regions of the bacterial 16S rRNA gene were targeted to generate amplicons of ~460 using the universal primer set 341F (5’ CCTACGGGNGGCWGCAG 3’) and 785R (5’ GACTACHVGGTATCTAATCC 3’) flanked by Illumina overhang adapter sequences (forward overhang: 5’ TCCTCGGAGATGTGTATAAGACAG 3’, reverse overhang: 5’ GTCTCGTGGGCTCGAGATGTGTATAAGACAG 3’) (Sigma Aldrich, MO, USA). After completion of the amplicon PCR with 2x KAPA HiFi HotStart ReadyMix, dual indexing of the samples with adaptor sequences (i7-N7xx-12 items, i5-S5xx-8 items) was performed using the Illumina Nextera XT Index Kit (FC-131-1001/2). PCR clean-up and amplicon size selection were performed using with KAPA Pure Beads (KAPA Biosystems) based on the technical data sheet (KR1245 – v3.16) provided by the manufacturer, which resulted in final ~580-630-bp libraries. For each case, verifications were performed with PCR Agilent D1000 screen tapes (5067–5582) and D1000 reagents (5067–5583). The 16S amplicon libraries for each sample were quantified by qPCR, normalized with respect to the amplicon sizes and pooled into a single library at equal molar quantities. Finally, 5 μL of the 4 nM DNA library pool was prepared for sequencing on the Illumina MiSeq platform. The library pool was denatured with 0.2 M NaOH and diluted to a final concentration of 6 pM. Sequencing was performed with a MiSeq Reagent Kit v3–618 cycle (MS-102-3003) following the manufacturer’s protocols (Illumina, Inc., San Diego, CA, USA). Paired-end sequencing (2×301 nt) was performed on an Illumina MiSeq platform with 5% PhiX spike-in quality control (PhiX Control Kit v3—FC-110-3001).

Preparing sequence reads for downstream analysis

To demultiplex the paired end reads and construct FASTQ files, Illumina BaseSpace software was used. The data were analysed using the Quantitative Insight Into Microbial Ecology pipeline (QIIME2, ver 2019.1) [39]. Cutadapt software integrated in QIIME2 software was used to check for the presence of adapter sequences (CTGTCTCTTATACACATCT) and trim the 3’ end of the reads. Quality trimming, filtering and chimera removal were performed with DADA2 software [40]. The trimming parameters were set as follows: the forward read length was set to 299 bases; for the reverse reads, the length was set to 249 bases. No bases were cropped from the 3’ end of the forward and reverse reads.

Bioinformatic analyses

QIIME2 integrated in MAFFT software was used for multiple sequence alignment [41], and the reads were taxonomically classified using a Naïve Bayesian classifier trained on the Green-genes (ver13_8) [42] reference database by selecting mapping points according to the forward-reverse primer set that was used for amplifying the V3-V4 regions of the 16S rRNA genes of the bacterial community (341F, 806R). Phylogenetic trees were constructed with the FastTree plugin [43]. The QIIME2 pipeline was used for the alpha diversity analyses. For sample normalization, a read depth of 1494 was set. To analyse the alpha diversity, Shannon’s index [44, 45], Faith’s phylogenetic diversity index [46], Simpson evenness [47], and Chao-1 index [48] were calculated using the QIIME2 pipeline. The differences in the alpha diversity were assessed using the Kruskal-Wallis test. To estimate beta diversity differences weighted UniFrac distance [49] was calculated. For visualization of beta diversity distance-based dissimilarity matrices PCoA plots were generated using the Emperor plugin [50]. Beta diversity group significances were calculated with Permutational multivariate analysis of variance (PERMANOVA) pseudo-F statistical test [51]. QIIME2 artifact files were exported from the pipeline and converted to

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TSV files that could be used with different visualization packages. We used the Python (ver 3.6.5) Seaborn package to generate heatmaps, donut plots, and boxplots were constructed with the pandas and matplotlib packages. Correlational analyses were performed with the Corrplot R package.

Results

Growth performance

To investigate the effects of phytonutrients on the growth performance of *Cyprinus carpio*, the average body weight (ABW) was measured (Table 1). At the beginning of the feeding experiment, the ABW was 123.45±49.28 g, whereas at the end of the experiment, the ABW reached 239.51±70.25 g. We estimated that the phytonutrients -based diet had no significant effect (p>0.05) on the ABW of common carp juveniles. The highest ABW was observed in the CAR animals (254.89±76.95 vs. 227.67±78.51 g for the controls). The supplementation of the diet with plant extracts did not have an impact on the specific growth rate (SGR) or feed conversion ratio (FCR) of the fish. However, the highest SGR (1.73±0.10% day$^{-1}$) values and the best FCR results (1.76±0.14 g$^{-1}$) were observed with the CAR treatment (Table 1).

Correlation between average body weight and fish microbiota

We managed to identify taxa showing correlations with body weight. Specifically, we captured notable associations between the GIT microbiota and the body weight of *Cyprinus carpio*. Alterations in the strength and direction of the correlations were measured at five taxonomic levels (Fig 2). At the phylum level, *Proteobacteria* (R = 0.65), *Tenericutes* (R = 0.58) and *Chlamydiae* (R = 0.56) showed the strongest positive correlations, and the phyla *Spirochaetes* (R = -0.47) and *Firmicutes* (R = -0.41) showed the most notable negative correlations with body weight gain. Further important positive correlations were observed for the classes *Gammaproteobacteria* (R = 0.59), the order *Vibrionales* (R = 0.62), the families *Pseudoalteromonadaceae*

Fig 2. Spearman correlation analysis was performed to measure the associations between the ABW and the intestinal taxa of *Cyprinus carpio*. The correlation values range from –1 to +1 and indicate the level of consistency of the positive (≥0; red) and negative (<0; grey) correlations. The correlations were calculated at five taxonomic levels: phylum, class, order, family, and genus.

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(R = 0.61) and Peptostreptococcaceae (R = 0.53) and the genera Lactobacillus (R = 0.59) and Luteolibacter (R = 0.51). In contrast, striking negative correlations were detected between the ABW and the class Brevinematae (R = -0.47), its order Brevinematales (R = -0.47) and its family Brevinemataceae (R = -0.47) and with the order Clostridiales (R = -0.41), the family Clostridiaceae (R = -0.41) and the genera Enterobacter (R = -0.52) and Pseudoxanthomonas (R = -0.56).

Ageing- and diet-driven alterations in the alpha and beta diversities

Microbial diversity is considered a key factor influencing animal productivity through disease tolerance. Diverse microbiota shows increased resilience, and only tendentious shifts can push them toward an unhealthy state. Alpha and beta diversity metrics were calculated to track the notable conversions in the community diversity of the control (BD) and treatment groups (ANTH, SYN, fOS, and CAR) (Fig 3). Specifically, Faith’s PD, Chao-1, Shannon and Simpson diversity indices were used to evaluate the species abundance, richness and evenness of the GIT microbiota. Annotated heatmaps represent the community alpha diversities in relation to the time of sampling and treatments (Fig 3A). In the case of ANTH, SYN, and fOS treated samples the analysis of Faith’s PD showed a steady increase by week 4 (day 28th) of the study period followed by a sharp decline at week 6 (day 42nd) of the experimental period (mean Faith’s PD: 9.3 > 4.9). Chao-1, Shannon’s, and Simpson’s indices did not show a marked decrease after the 4th week (day 28th). Interestingly, the most pronounced decline in the community alpha diversity was observed with the ANTH supplementation during the last 2 weeks of the study period. Moreover, the lowest value of Simpson’s index (day 42nd: 0.85 vs. day 1st: 0.89) was detected during the last quarter of the experimental period. In general, sweet red pepper seed extract (fOS) was able to ameliorate the community diversity according to Faith’s PD and Chao-1 indices, and this treatment group was the only group that did not show a decline in Shanon’s and Simpson’s indices during the last 2 weeks of the study period.

Four beta diversity metrics were investigated; specifically, the weighted and unweighted UniFrac, Bray-Curtis, and Jaccard distances, (Fig 3B) between the different experimental groups were determined. Distance-based dissimilarity matrices showed that nutraceuticals did not exert a significant (p<0.05) influence on the overall community variations between the different treatment groups.

Principal coordinate analysis (PCoA) using weighted UniFrac distances was performed to measure the age dependency of the community taxonomy data, and this analysis yielded two clusters (clusters 1 and 2) representing different spatial ordinations between the fishes at days 14th (aged 33 weeks), 28th (aged 35 weeks) and 42nd (aged 37 weeks) (Fig 3C). The microbiota of 37-week-old Cyprinus carpio showed a distinct clustering pattern compared with the microbiota at earlier time points (aged 33–35 weeks) (Fig 3C). When marking the samples according to diet, no distinct patterns were observed between the treatment groups (Fig 3D). Based on the PCoA plots, we concluded that age induced more pronounced shifts in the microbial community than diet.

Characterization of the Cyprinus carpio gut symbiome

Our interest in understanding phytonutrient-induced taxonomic shifts in the symbiotic microbiota of cultured, healthy Cyprinus carpio has grown. The observed community compositions relative to that of the healthy controls are shown in Fig 4. Differences in normalized abundance data were attained by considering 11 phyla, 16 classes, 25 orders, 36 families and 34 genera. Graphical representations were generated by calculating the log2 differences in taxa abundances between fish fed phytonutrient-supplemented diets (ANTH, SYN, fOS, and CAR)
and those given the basal diet (BD). Taxonomic shifts were obtained when comparing untreated vs. treated healthy *Cyprinus carpio* symbiomes.

Nutraceuticals favoured the growth of *Cyanobacteria*, and anthocyanins (ANTH) induced the growth of the phylum *Actinobacteria*, the family *Nocardiaceae*, and the genera *Sphingomonas* and *Acidovorax*. In contrast, synbiotics (SYN) stimulated the classes *Flavobacteria* and *Betaproteobacteria*, the orders *Burkholderiales* and *Neisseriales*, the families *Weeksellacea*, *Pep- tostreptococcaceae*, *Phyllobacteriaceae* and *Neisseriaceae* and the genera *Chryseobacterium*, *Epulopiscium*, *Pelomonas*, and *Vogesella*. Anthocyanins (ANTH) and synbiotics (SYN) induced marked enrichment of *Firmicutes*, *Clostridia*, *Clostridiales*, *Clostridiaceae* and *Clostridium*. The abundances of the families *Lachnospiraceae* and *Phyllobacteriaceae* and the genus *Shinella* were increased by synbiotics (SYN) and fermentable oligosaccharides (fOS). Fermentable oligosaccharides (fOS) induced the families *Ruminococcaceae* and *Plantomycetaceae*, and

Fig 3. Alpha diversity metrices used in the comparison analysis. a) Annotated heatmaps showing the values of Faith’s PD, Chao-1, Shannon’s and Simpson’s diversity indexes as a function of the time of sample collection (y axis) and the experimental settings (x axis). b) The sample distances were calculated based on quantitative (Bray-Curtis, weighted UniFrac) and qualitative (Jaccard, unweighted UniFrac) dissimilarity-based statistics. c, d) Weighted UniFrac analysis was performed to identify ageing- and treatment-driven differences between the groups. The beta diversity relationships are summarized in two-dimensional scatter plots. The distances between dots are representative of differences in microbiota compositions. BD represents fish that received a basal diet, which served as a negative control with no dietary supplement or the following dietary treatments as supplemented feed: BD+1% anthocyanins (ANTH) provided by Hungarian sour cherry extract, BD+1% synbiotics (SYN) provided by fermented corn, BD+1% fermentable oligosaccharides (fOS) provided by Hungarian sweet red pepper seed extract, and BD+1% carotenoids (CAR) provided by Hungarian sweet red pepper pulp extract.

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the pulp extract (CAR) favoured the growth of the class *Bacteroidia* and order *Bacteroidales* and the families *Bacteroidaceae*, *Rhodospirillaceae*, and the genera *Chryseobacterium*, *Enterococcus*, and *Acidovorax*.

**Diet affects the distribution of beneficial and potentially pathogenic and spoilage bacteria**

The beneficial or detrimental effects of microorganisms on the intestinal microbiota can be strongly influenced by several factors, such as the fish species, prebiotic substrates, composition of the complex microbial populations, pH, living environments, and seasons. Because both biotic and abiotic stressors decrease the abundances of beneficial bacteria, opportunistic...
members of the community might take advantage and become infectious [52], leading to serious seasonal losses in aquaculture.

High-throughput sequencing of the bacterial 16S rRNA genes indicated that a phytonutrient-enriched diet induced significant shifts in the core phyla of the *Cyprinus carpio* GIT (Fig 5). During this feeding experiment, the carp gut was dominated by *Fusobacteria* (54.9 ± 16.6%) and *Proteobacteria* (37.1 ± 16.0%), and significant differences were found between the treatment groups (Fig 5A). The relative frequencies of the two dominant phyla showed the greatest most equalization in the fermented corn-treated (SYN) samples and were most pronounced in the pepper seed (fOS, P/F ratio: 0.61, 35.3% *Proteobacteria*, 58.2% *Fusobacteria*)- and pepper pulp extract-treated samples, (CAR P/F ratio: 0.57, 34.1% *Proteobacteria*, 59.6% *Fusobacteria*).

By modulating the optimal nutrition and energy utilization of the host, the phyla *Bacteroides* and *Firmicutes*, which are associated with SCFA synthesis, can improve the growth performance of aquatic animals [53]. According to data, elevated *Firmicutes* levels can be associated with increased nutrient absorption, whereas *Bacteroidetes* is correlated with enhanced hydrolysis of glycogen, starch and polysaccharides. Under our experimental settings, the highest and lowest F/B ratios were obtained with the ANTH-fed fish (227.7) and the CAR-fed carp (1.6), whereas the control animals (BD) had an F/B ratio of 3.1.

Anthocyanins increased the abundance of *Shewanella* (ANTH 1.4±1.3% vs. BD 0.9±0.7%) in comparison to that obtained with the basal diet. Synbiotics increased the abundances of *Bacillus* (SYN 1.3±1.8% vs. BD 0.8±0.8%) and *Shinella* (SYN 0.2±0.5% vs. BD 0.04±0.09%) and decreased that of *Shewanella* (SYN 0.5±0.6% vs. BD 0.9±0.7%). The genus *Acinetobacter*, which contains potential fish pathogen species, was not detected in the synbiotic-fed (SYN) fish, whereas the pepper pulp (CAR 0.1±0.03%) and seed extracts (fOS 0.06±0.08%) decreased the abundance of this genus, and the sour cherry extract (ANTH 0.1±0.2%) significantly increased its abundance relative to that found in fish fed the basal diet (BD 0.3±0.1%).

According to other data, *Cetobacterium* is involved in the fermentation of peptides and carbohydrates [54], including in the production of vitamin B12 [15]. Pulp extracts increased the abundances of *Cetobacterium* (CAR 59.6±14.5% vs. BD 50.2±15.6%) and *Enterococcus* (CAR 0.6±0.6% vs. BD 0.3±0.3%) and decreased the abundances of *Bacillus* (CAR 0.3±0.7% vs. BD 0.8±0.8%), *Pseudomonas* (CAR 0.9±0.5% vs. BD 1.8±0.8%), and *Acinetobacter* (CAR 0.1±0.04% vs. BD 0.3±0.2%). The genus *Shinella*, which can potentially eliminate nitrate contamination from the environment, was markedly increased in the synbiotic-fed fish.

**Comparative metagenomics reveals taxonomy associations in carp symbiomes**

Taxonomic interconnections were revealed between and within the GIT microbiota of the control and phytonutrient-fed fish. We also estimated the extent to which genera are likely to play an indispensable role in maintaining the gastrointestinal health of *Cyprinus carpio*. Relative proportions were correlated using Spearman’s correlation method (Fig 6). The analysis revealed 32 significant correlations between genera, and these included 12 positive (Fig 6A), two negative (Fig 6B), and 18 opposite (Fig 6C) correlations. Furthermore, 31 unique (26 in BD, five in phytonutrients-fed fishes) significant correlations were found between *Cyprinus carpio* GIT genera (Fig 6D). Significant positive associations were found among *Planctomycetes*, *Enterococcus*, and *Luteolibacter*, among *Pseudoxanthomonas*, *Enterobacter*, and *Cetobacterium*, and among *Pseudomonas*, *Acinetobacter* and *Aeromonas*. However, *Enterobacter* and *Cetobacterium* were negatively correlated with *Bacillus* and *Vogesella*, respectively.
Notably, opposite correlations were detected between Enterococcus and Luteolibacter, between Pseudomonas and Plesiomonas and among Clostridium, Aeromonas, Acinetobacter and Plesiomonas.

We also revealed significant correlations between genera that were unique to a specific diet. The basal diet-fed animals showed the highest number of unique taxonomy matches (26 positive correlations). As such, very strong associations between Lactobacillus and Aerococcus and among Proteocatella, Curvibacter and Gluconacetobacter were exclusively obtained in non-treated carp.

**Discussion**

Aquaculture fish production has replaced the use of capture fisheries since the global demands for fish have surpassed those for beef, pork and poultry products [2, 55]. At present, one-fifth of the world’s population has opposed the use of fisheries, but fish stocks are becoming increasingly overexploited [56]. The establishment of a more sustainable aquaculture sector is needed to supply this high-quality protein to rapidly growing world’s population [57].

Controlling the outbreaks of infections associated with high stocking densities of fish is essential. The routine usage of antibiotics in aquacultures releases significant amounts of drug residues and potentially increases resistance in natural environments. Antibiotic-free breeding systems have become more important in animal farming and in easing pollution and resistance spread. [58–60].

Most of the microbes that can cause infections in aquacultures are part of the healthy baseline fish microbiota. Therefore, their complete elimination from fish rearing systems is not achievable. Additionally, by carrying resistance against a wide range of antibiotics, their zoologic importance is about to escalate [61]. Although the mechanisms underlying “colonization
“Resistance” are unclear, it has been suggested that microbiota compete with pathogens for niches by producing and secreting antimicrobial peptides [14].

As a substitute for antibiotics, the use of natural alternatives for farmed fish via dietary supplementation is a key area of research [62, 63]. The results of other studies have shown that probiotic-based supplements might facilitate this process [5]. However, only a few recent studies on the application of phytonutrients as feed additives in aquaculture settings have provided a detailed analysis. The available data propagate the importance of the dietary application of plant-derived bioactive components as potential growth promoters that support intestinal homeostasis by modulating feed utilization. However, further investigations are needed to understand how these natural compounds are beneficial to aquatic animals.

Common carp is one of the most commonly cultivated species in fish rearing systems worldwide [64]. Due to sustainability concerns, fish meat production continues to increase, which encourages aquaculturists to apply the latest technologies with the aim of improving the maximum yield [16].

The intestine of aquatic animals is an ideal environment for the colonization and proliferation of commensal microbes [65]. The GIT microbiome composition shapes host physiology and growth, but these functions in aquatic animal hosts have not yet been fully investigated. Only a limited number of studies have evaluated the association between the fish microbiota and production outcomes in aquaculture settings [66, 67].

This feeding experiment was conducted to decipher the phytonutrient-induced changes in the GIT microbiota of 7-month-old Cyprinus carpio. Next-generation sequencing targeting the 16S rRNA gene was applied to trace even non-cultivable and low-abundance rare taxa.

Our results showed that dietary supplementation did not exert a significant effect on the ABW of carp juveniles, which is in accordance with the results described by Hoseinifar et al. [68].

A high intestinal microbiota diversity is generally considered beneficial for health [69], and we thus investigated the effects of phytonutrients on the diversity of the healthy *Cyprinus carpio* symbiome. In this study, the acclimation of the *Cyprinus carpio* intestinal microbiota did not result in noticeable changes in the alpha diversity values, which implies that the healthy *Cyprinus carpio* microbiota can dynamically change the community heterogeneity to preserve basic biological functions. We noted that acclimation to the new environmental conditions involved a noticeable adjustment in the alpha diversities of the healthy *Cyprinus carpio* GIT microbial communities. At the beginning of the experiment, the average alpha diversity values were lower, but we observed a general improvement throughout the experimental period. During the study, the highest diversity was observed at the 4th week of the feeding period (day 28th) in the animals fed sweet red pepper pulp (CAR) and sour cherry (ANTH) extracts. The Chao1 index was increased by the bioactive components in sweet red pepper seeds (fOS) and pepper pulp (CAR). Shannon’s and Simpson’s indices did not show remarkable differences over the course of the experiment.

The gut microbiota provides the host with important short-chain fatty acids (SCFAs). In fish, carbohydrate fermentation occurs mostly by members of the genus *Bacteroides*, which are...
known producers of SCFAs that play an important role against gut inflammation [15]. Based on our data, the highest abundances of Bacteroidales were found in the fermented corn (SYN)- and pepper pulp-treated (CAR) groups.

In general, due to shorter retention times, herbivorous and omnivorous carp show lower SCFA levels; therefore, pre- and probiotic supplementation might be appropriate [70]. Different fibre ratios might result in different gut microbiota structures. A proportionality is generally observed between the diversity of the intestinal microbiota and SCFA producers [71]. Additionally, stress-stimulated microbiota dysbiosis is considered a relevant factor that negatively affects the proportions of SCFA-producing microorganisms.

Specifically, synbiotics selectively promoted the growth of Bacillus and Lactobacillus. In contrast, anthocyanins and synbiotics decreased the abundances of Cetobacterium, whereas fermentable oligosaccharides enriched this genus. Clostridia is a member of the endogenous flora of the fish intestine and is involved in pathogen exclusion through its antibacterial activity [54]. Clostridiales species are also associated with carbohydrate degradation and are responsible for producing SCFAs in vertebrates [72]. In this study, higher abundances of the genus Clostridium were found in fermented corn-fed fish compared with the controls [54].

Enterococcaceae are lactic acid bacteria (LAB) comprising both pathogenic and commensal microorganisms that are ubiquitous even as gut symbionts. Their competitiveness is also due to their ability to produce bacteriocins recognized for their wide-range effectiveness against pathogenic and spoilage bacteria [73]. Sweet red pepper seed (fOS) and pulp (CAR) extracts also enriched the genus Enterococcus, whereas sour cherry (ANTH), fermented corn (SYN) and sweet red pepper seed (fOS) extracts did not exert remarkable influence on the abundance of LAB.

We also investigated the effects of natural feed additives on the abundances of potentially pathogenic and spoilage bacteria. Intrinsic characteristics, such as high pH values and high levels of proteins and free amino acids, make fish products highly susceptible to spoilage [74]. Based on their metabolic activities in the Enterobacteriaceae family and ubiquitous Shewanella, Pseudomonas species can have negative effects on fish meat quality and are often responsible for the psychrotrophic spoilage of fish products through the production of hydrolytic enzymes [29]. In this experiment, the presence of Pseudomonas, which can improve the survival of pathogenic bacteria [75], in fish meat was decreased by the fructo-oligosaccharides found in the pepper extracts (fOS, CAR).

The probiotic Shewanella, which is one of the major omega-3-polyunsaturated fatty acid-producing genera, is often used in aquacultures. This genus can also be commonly isolated from the GIT of fish and vertebrates [76]. Nevertheless, the genera Shewanella and Pseudomonas are important psychrotrophic spoilage bacteria that can produce hydrolytic extracellular enzymes and might exert negative effects on fresh meat quality [74]. The highest abundance of Shewanella was detected in the anthocyanin extract-treated samples [14].

The known pathogen Aeromonas is widely distributed in freshwater aquatic environments and is also a known member of the endogenous flora of freshwater fish that participates in the fermentation of organic compounds, cellulose degradation, and antibacterial activity [54]. Intensive practices in aquacultures typically produce weakened populations of to their virulence factors, Aeromonas are often associated with human infections, which leads to significant seasonal financial losses [77]. Aeromonas are often the causative agent of motile Aeromonas septicemia [30]. Due to their virulence factors, Aeromonas are often associated with human infections [78]. Aeromonas have also been identified as important spoilage bacteria that deteriorate the quality of aquatic products [79]. Based on our data, the pepper pulp extracts (CAR) positively stimulated the growth of Aeromonas, whereas anthocyanins (ANTH) and synbiotics
(SYN) decreased the abundance of Aeromonas. Aeromonas was also found to enhance growth in zebrafish [80]; however, our results showed a negative association between the relative frequency of the genus and body weight gain.

There is an indispensable need to investigate the effects of natural and bioactive components in aquacultures. Any successful implementation of an alternative, antibiotic-free meat production systems in animal farming could be beneficial and might be accompanied by high economic significance. Based on their natural sources, phytonutrients carry many advantages over traditional medicines. We demonstrated that phytonutrients positively affect the beneficial bacteria of the carp intestinal microbiota, and thus, their medical and health benefits might be important in preventing or treating infections and diseases in antibiotic-free farming systems. In summary, we believe that phytonutrients rich in minerals, vitamins, and amino acids can provide an efficient strategy for aquaculture that improves the safety of the hosts and the sustainability of fish production.

Conclusions

Increasing studies are investigating how natural feed additives can induce positive changes in the fish GIT microbiota and support pathogen exclusion. It has been envisioned that providing information on the applications of phytonutrients in aquaculture will facilitate the development of future strategies of farmed aquatic animals.

• In this feeding experiment, we applied a culture-independent molecular approach to thoroughly discover the phytonutrient-induced alterations in the GIT microbiome of common carp. The applied phytonutrients were derived from food waste materials, which emphasizes the economic potential of this strategy.

• Farming was performed in a temperate climatic zone, and Cyprinus carpio GIT samples were monitored through a 6-week fish meat production cycle. This approach allows us to decipher the symbiotic microbiome of 33- to 37-week-old healthy carp, and we observed notable correlations between the fish GIT microbiota and the ABW.

• Marked differences were found in the distribution of the two dominant phyla: Fusobacteria and Proteobacteria. We did not find any significant enhancement in animal growth but detected strong positive correlations between the ABW and the relative frequencies of p__Proteobacteria, p__Tenericutes, c__Mollicutes, o__Vibrionales, f__Peptostreptococcaceae, f__Pseudoalteromonadaceae and f__Lactobacillaceae.

• No profound differences in the alpha and beta diversities were detected among the treatment groups, although anthocyanins (ANTH) and the pepper seed (fOS) and pulp (CAR) extracts were found to be promising for improving diversity.

• We did not observe any marked shifts in the abundance of opportunistic pathogen and spoilage genera such as Shewanella, Pseudomonas and Aeromonas. However, we noted that synbiotics (SYN) and fermentable oligosaccharides (fOS) decreased the relative proportions of Aeromonas and Pseudomonas, respectively, but the changes were not significant.

Phytonutrient-based feeding strategies might be a promising alternative for aquaculture systems because they exert a beneficial effect on the bacterial symbionts of the GIT of Cyprinus carpio.

Supporting information

S1 Table. Total amount of the raw materials used in the diets and nutrient contents of the experimental feeds (dry matter %). BD: basal diet (negative control), ANTH: BD+1%
anthocyanins provided by sour cherry extract, SYN: BD+1% synbiotics provided by fermented corn, fOS: BD+1% fermentable oligosaccharides provided by sweet red pepper seed extract, CAR: BD+1% carotenoids provided by sweet red pepper pulp extract. *Vitamin and mineral premix: vitamin A (retinyl acetate), 9000000 IU; vitamin D3 (cholecalciferol), 7200000 IU; vitamin E, 5400 mg kg-1; vitamin K3 (MSB), 9600 mg kg-1; vitamin B1 (thiamin-HCL), 1000 mg kg-1; vitamin B2 (riboflavin), 9600 mg kg-1; vitamin B3 (niacin), 45000 mg kg-1; vitamin B5 (calcium d-pantothenate), 15000 mg kg-1; vitamin B6 (pyridoxine–HCL), 5400 mg kg-1; D-biotin, 100 mg kg-1; folic acid, 1200 mg kg-1; vitamin B12 (cyanocobalamin), 27 mg kg-1; vitamin C, 4000 mg kg-1; and choline chloride, 1500 mg kg-1. **Anchovy fish oil.

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S1 Fig. The UHPLC profiles of the anthocyanins and the identified main compounds, including the relative areas in ANTH. (Y axis: absorbance intensity (mAU); X axis: retention time (min)). The table shows the anthocyanin (ANTH) compounds of sour cherry with their retention areas and retention times.

S2 Fig. The GC profile of the oligosaccharides and the identified monomer units with the greatest relative areas and retention times in SYN. (Y axis: counts; X axis: retention time (min)). The table shows the oligosaccharide monomers comprising the synbiotic (SYN) compounds of fermented corn with their relative retention areas and retention times.

S3 Fig. The HPLC profile of the carotenoids and identified monomer units with the greatest relative areas and retention times in CAR. (Y axis: absorbance intensity (mAU); X axis: retention time (min)). Table identifies carotenoid compounds (CAR) of sweet red pepper pulp extract with relative percentage of areas and retention times.

S4 Fig. The GC profiles of the oligosaccharides and the identified monomer units, including the greatest relative areas and retention times in fOS. (Y axis: counts; X axis: retention time (min)). Table identifies fermentable oligosaccharide monomers (fOS) of Hungarian sweet red pepper seed with relative percentage of areas and retention times.

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References

1. Merino G., Barange M., Blanchard J.L., Harle J., Holmes R., Allen I., et al. Can marine fisheries and aquaculture meet fish demand from a growing human population in a changing climate? Glob. Environ. Change 2012; 22, 795–806. https://doi.org/10.1016/j.gloenvcha.2012.03.003

2. FAO. The State of World Fisheries and Aquaculture 2012. Rome. 209 pp. [last cited: 11.01.2021] In fao.org [internet], available from: http://www.fao.org/3/a-i2727e.pdf last access: 10/12/2020.

3. Thépot V., Campbell A.H., Rimmer M.A., Paul N.A., Meta-analysis of the use of seaweeds and their extracts as immunostimulants for fish: a systematic review. Rev. Aquacult. 2020. https://doi.org/10.1111/raq.12504

4. Li H., Zhou Y., Ling H., Luo L., Qi D., Feng L., The effect of dietary supplementation with Clostridium butyricum on the growth performance, immunity, intestinal microbiota and disease resistance of tilapia (Oreochromis niloticus). PLOS ONE 2019; 14(12): e0223428. https://doi.org/10.1371/journal.pone.0223428 PMID: 31815958

5. Hai N.V., The use of probiotics in aquaculture. J. Appl. Microbiol. 2015; 119, 917–935. https://doi.org/10.1111/jam.12886 PMID: 26119489

6. FAO. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome; 2020. https://doi.org/10.4060/ca9229en

7. FAO Common carp—Natural food and feeding habits. [last cited: 11.01.2021] In: fao.org [internet], available from http://www.fao.org/fishery/affris/species-profiles/common-carp/natural-food-and-feeding-habits/en/.

8. FAO Cultured Aquatic Species Information Programme Cyprinus carpio (Linnaeus, 1758) [last cited: 11.01.2021]. In fao.org [internet], available from http://www.fao.org/fishery/culturedspecies/Cyprinus_carpio/en.

9. Trites A.W., Marine Mammal Trophic Levels and Trophic Interactions, in: Encyclopedia of Ocean Sciences. Elsevier, 2019; pp. 589–594. https://doi.org/10.1016/B978-0-12-409548-9.11618-5

10. Gyalog G., Oláh J., Békéfi E., Lukácsik M., Popp J., Constraining Factors in Hungarian Carp Farming: An Econometric Perspective. Sustainability 2017; 9, 1–13. https://doi.org/10.3390/su9112111

11. Neori A., Nobre A.M., Relationship Between Trophic Level and Economics in Aquaculture. Aquac. Econ. Manag. 2012; 16, 40–67. https://doi.org/10.1080/13657305.2012.649046

12. Kashinskaya E.N., Simonov E.P., Kabilov M.R., Izvekova G.I., Andree K.B., Solovyev M.M., Diet and other environmental factors shape the bacterial communities of fish gut in an eutrophic lake. J. Appl. Microbiol. 2018; 125: 1626–1641. https://doi.org/10.1111/jam.14064 PMID: 30091826

13. Vilizzi L., Copp G.H., Global patterns and clines in the growth of common carp Cyprinus carpio. J. Fish Biol. 2017; 91, 3–40. https://doi.org/10.1111/fj.13346 PMID: 28691399

14. Butt R.L., Volkoff H., Gut Microbiota and Energy Homeostasis in Fish. Front. Endocrinol. 2019; 10:9. https://doi.org/10.3389/fendo.2019.00009 PMID: 30733706

15. Ramirez C., Coronado J., Silva A., Romero J., Cetobacterium Is a Major Component of the Microbiome of Giant Amazonian Fish (Arapaima gigas) in Ecuador. Animals 2018; 8, 189. https://doi.org/10.3390/ani8110189 PMID: 30352962

16. Taiwar C., Nagar S., Lal R., Negi R.K., Fish Gut Microbiome: Current Approaches and Future Perspectives. Indian J. Microbiol. 2018; 58, 397–414. https://doi.org/10.1007/s12086-018-0760-y PMID: 30262950

17. Yang S., Du J., Luo J., Zhou Y., Long Y., Xu G., et al. Effects of different diets on the intestinal microbiota and immunity of common carp (Cyprinus carpio). J. Appl. Microbiol. 2019; 127, 1327–1338. https://doi.org/10.1111/jam.14405 PMID: 31379377

18. Ma Z. (Sam), Testing the Anna Karenina Principle in Human Microbiome-Associated Diseases. iScience 2020; 23 (4):101007. https://doi.org/10.1016/j.isci.2020.101007 PMID: 32305861
19. Mesalhy Aly S., Albutti A., Antimicrobials Use in Aquaculture and their Public Health Impact. J. Aquac. Res. Dev. 2014; 5:4. https://doi.org/10.4172/2155-9546.1000247

20. de Bruijn I., Liu Y., Wieger M., Raaijmakers J.M., Exploring fish microbial communities to mitigate emerging diseases in aquaculture. FEMS Microbiol. Ecol. 2018; 94(1). https://doi.org/10.1093/femsec/fiy056 PMID: 29206925

21. Preena P.G., Arathi D., Raj N.S., Arun Kumar T.V., Arun Raja S., Reshma R.N., et al., Diversity of antimicrobial-resistant pathogens from a freshwater ornamental fish farm. Lett. Appl. Microbiol. 2020; 71, 108–116. https://doi.org/10.1111/lam.13231 PMID: 31602688

22. Banerjee G., Ray A.K., The advancement of probiotics research and its application in fish farming industries. Res. Vet. Sci. 2017; 115, 66–77. https://doi.org/10.1016/j.rvsc.2017.01.016 PMID: 29157611

23. Papp N., Szilvássy B., Abranko L., Szabó T., Pfeiffer P., Szabó Z., et al., Main quality attributes and antioxidants in Hungarian sour cherries: identification of genotypes with enhanced functional properties. Int. J. Food Sci. Technol. 2010; 45, 395–402. https://doi.org/10.1111/j.1365-2621.2009.02168.x PMID: 20953446

24. Remenyik J, Ledo H, Dudas L, Veres Zs, Fari M. Antioxidant capacity of some red sweet pepper lines and varieties. Cereal Res Comm. 2008; 36:1759 –1726.

25. Srinivasan K., Biological Activities of Red Pepper (Capsicum annuum) and Its Pungent Principle Capsaicin: A Review. Crit. Rev. Food Sci. Nutr. 2016; 56, 1488–1500. https://doi.org/10.1080/10408398.2013.772090 PMID: 25675368

26. Nemes A., Homoki J.R., Kiss R., Hegedüs C., Kovács D., Peitl B., et al., Effect of Anthocyanin-Rich Tart Cherry Extract on Inflammatory Mediators and Adipokines Involved in Type 2 Diabetes in a High Fat Diet Induced Obesity Mouse Model. Nutrients 2019; 11, 1666. https://doi.org/10.3390/nu11091665 PMID: 31438590

27. Csernus B., Biró S., Babinszky L., Komlösi I., Jávor A., Stündl L., et al. Effect of Carotenoids, Oligosaccharides and Anthocyanins on Growth Performance, Immunological Parameters and Intestinal Morphology in Broiler Chickens Challenged with Escherichia coli Lipopolysaccharide. Anim. Open Access J. MDPI 2020; 10, 347. https://doi.org/10.3390/animals10020347 PMID: 32098265

28. Csernus B., Biró S., Babinszky L., Komlösi I., Jávor A., Stündl L., et al. Effect of Carotenoids, Oligosaccharides and Anthocyanins on Growth Performance, Immunological Parameters and Intestinal Morphology in Broiler Chickens Challenged with Escherichia coli Lipopolysaccharide. Anim. Open Access J. MDPI 2020; 10, 347. https://doi.org/10.3390/animals10020347 PMID: 32098265
38. Fernandes I.M., Bastos Y.F., Barreto D.S., Lourenço L.S., Penha J.M., Fernandes I.M., et al. The efficacy of clove oil as an anaesthetic and in euthanasia procedure for small-sized tropical fishes. Braz. J. Biol. 2017; 77, 444–450. https://doi.org/10.1590/1519-6894.15015 PMID: 27683808

39. Bolyen E., Rideout J.R., Dillon M.R., Bokulich N.A., Abnet C.C., Al-Ghalith G.A., et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 2019; 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9 PMID: 31341288

40. Callahan B.J., McMurdie P.J., Rosen M.J., Han A.W., Johnson A.J.A., Holmes S.P., DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 2016; 13, 581–583. https://doi.org/10.1038/nmeth.3869 PMID: 27214047

41. Katoh K., Standley D.M., MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Mol. Biol. Evol. 2013; 30, 772–780. https://doi.org/10.1093/molbev/mst010 PMID: 23329690

42. DeSantis T.Z., Hugenholtz P., Larsen N., Rojas M., Brodie E.L., Keller K., et al. GreenGenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 2006; 72, 5069–5072. https://doi.org/10.1128/AEM.03006-05 PMID: 16820507

43. Price M.N., Dehal P.S., Arkin A.P., FastTree 2 –Approximately Maximum-Likelihood Trees for Large Alignments. PLOS ONE 2010; 5(3): e9490. https://doi.org/10.1371/journal.pone.0009490 PMID: 20224823

44. Spellerberg I.F., Fedor P.J., A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the ‘Shannon–Wiener’ Index. Global Ecology and Biogeography, 2003; 12: 177–179. https://doi.org/10.1046/j.1466-822X.2003.00015.x

45. Shannon C.E., A Mathematical Theory of Communication. Bell Syst. Tech. J. 1948; 27, 379–423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x PMID: 30854411

46. Faith D.P., Baker A.M., Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. Evol. Bioinforma. Online 2007; 2, 121–128., https://doi.org/10.1177/117693430600200007

47. Simpson E.H., Measurement of Diversity. Nature 1949; 163, 688–688. https://doi.org/10.1038/163688a0

48. Chao A., Nonparametric Estimation of the Number of Classes in a Population. Scand. J. Stat. 1984; 11, 265–270., https://doi.org/10.2307/4615964

49. Lozupone C., Knight R., UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Appl. Environ. Microbiol. 2005; 71, 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005 PMID: 16332807

50. Vázquez-Baeza Y., Pirrung M., Gonzalez A., Knight R., EMPorer: a tool for visualizing high-throughput microbial community data. GigaScience 2013; 2(1):16. https://doi.org/10.1186/2047-217X-2-16 PMID: 24280061

51. Vázquez-Baeza Y., Gonzalez A., Smarr L., McDonald D., Morton J.T., Navas-Molina J.A., et al., Bringing the Dynamic Microbiome to Life with Animations. Cell Host Microbe 2017; 21, 7–10. https://doi.org/10.1016/j.chom.2016.12.009 PMID: 28081445

52. Boutin S., Bernatchez L., Audet C., Deroïme N., Network Analysis Highlights Complex Interactions between Pathogen, Host and Commensal Microbiota. PLOS ONE 2013; 8(12): e84772. https://doi.org/10.1371/journal.pone.0084772 PMID: 24376845

53. Tran N.T., Li Z., Wang S., Zheng H., Aweya J.J., Wen X., et al. Progress and perspectives of short-chain fatty acids in aquaculture. Rev. Aquac. 2020; 12, 283–298. https://doi.org/10.1111/raq.12317

54. van Kessel M.A., Dutilh B.E., Neveling K., Kwint M.P., Veltman J.A., Flik G., et al. Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (Cyprinus carpio L.). AMB Express 2011; 1, 41. https://doi.org/10.1186/2191-0855-1-41 PMID: 22093413

55. Tacon A.G.J., Metian M., Fish Matters: Importance of Aquatic Foods in Human Nutrition and Global Food Supply. Rev. Fish. Sci. 2013; 21, 22–38. https://doi.org/10.1080/10641262.2012.753405

56. Dittmann K.K., Rasmussen B.B., Castex M., Gram L., Bentzon-Tilia M., The aquaculture microbiome at the centre of business creation. Microb. Biotechnol. 2017; 10(6):1279–1282. https://doi.org/10.1111/1751-7915.12877 PMID: 29064164

57. Carrias A., Ran C., Terhune J.S., Liles M.R., Bacteria and bacteriophages as biological agents for disease control in aquaculture, in: Austin B. (Ed.), Infectious Disease in Aquaculture, Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing, 2012; pp. 353–393. https://doi.org/10.1533/9780857095732.3.353

58. Kraemer S.A., Ramachandran A., Perron G.G., Antibiotic Pollution in the Environment: From Microbial Ecology to Public Policy. Microorganisms 2019; 7(6):180. https://doi.org/10.3390/microorganisms7060180 PMID: 31234491
59. Monteiro S.H., Antibiotic Residues and Resistant Bacteria in Aquaculture. Pharmaceut. Chem. J. 2018; 5, 127–147.

60. Watts J.E.M., Schreier H.J., Lanska L., Hale M.S., The Rising Tide of Antimicrobial Resistance in Aquaculture: Sources, Sinks and Solutions. Mar. Drugs 2017; 15(6):158. https://doi.org/10.3390/md15060158 PMID: 28587172

61. Awan M.B., Maqbool A., Bari A., Krovaček K., Antibiotic susceptibility profile of Aeromonas spp. isolates from food in Abu Dhabi, United Arab Emirates. New Microbiol. 2009; 32, 17–23. PMID: 19382665

62. Barman D., Ren P., Mandal S.C., Kumar V., Immunostimulants for Aquaculture Health Management. J. Mar. Sci. Res. Dev. 2013; 3, 1–11. https://doi.org/10.4172/2155-9910.1000134

63. Akhter N., Wu B., Memon A.M., Mohsin M., Probiotics and prebiotics associated with aquaculture: A review. Fish Shellfish Immunol. 2015; 45, 733–741. https://doi.org/10.1016/j.fsi.2015.05.038 PMID: 26044743

64. Rahman M.M., Role of common carp (Cyprinus carpio) in aquaculture production systems. Front. Life Sci. 2015; 8, 399–410. https://doi.org/10.1080/21553769.2015.1045626

65. Han S., Liu Y., Zhou Z., He S., Cao Y., Shi P., et al. Analysis of bacterial diversity in the intestine of grass carp (Ctenopharyngodon idellus) based on 16S rDNA gene sequences. Aquac. Res. 2010; 42, 47–56. https://doi.org/10.1111/j.1365-2109.2010.02543.x

66. Infante-Villamil S., Huerlimann R., Jerry D.R., Microbiome diversity and dysbiosis in aquaculture. Rev. Aquac. 2020; 12, 539–5131. https://doi.org/10.1111/raq.12513

67. Yilmaz E., Effects of dietary anthocyanin on innate immune parameters, gene expression responses, and ammonia resistance of Nile tilapia (Oreochromis niloticus). Fish Shellfish Immunol. 2019; 93, 694–701. https://doi.org/10.1016/j.fsi.2019.08.033 PMID: 31421240

68. Hoseinifar S.H., Soleimani N., Ringo E., Effects of dietary fructo-oligosaccharide supplementation on the growth performance, haemato-immunological parameters, gut microbiota and stress resistance of common carp (Cyprinus carpio) fry. Br. J. Nutr. 2014; 112, 1296–1302. https://doi.org/10.1017/S0007114514002037 PMID: 25313574

69. Nie L., Zhou Q.-J., Qiao Y., Chen J., Interplay between the gut microbiota and immune responses of ayu (Plecoglossus altivelis) during Vibrio anguillarum infection. Fish Shellfish Immunol. 2017; 68, 479–487. https://doi.org/10.1016/j.fsi.2017.07.054 PMID: 28756287

70. Hao Y.T., Wu S.G., Jakovlić I., Zou H., Li W.X., Wang G.T., Impacts of diet on hindgut microbiota and short-chain fatty acids in grass carp (Ctenopharyngodon idellus). Aquac. Res. 2017; 48, 5595–5605. https://doi.org/10.1111/are.13381

71. Llewellyn M.S., Boutin S., Hoseinifar S.H., Derome N., Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 2014; 5:207. https://doi.org/10.3389/fmicb.2014.00207 PMID: 24917852

72. Eichmiller J.J., Hamilton M.J., Staley C., Sadowsky M.J., Sørensen P.W., Environment shapes the fecal microbiome of invasive carp species. Microbiome 2016; 4(1). https://doi.org/10.1186/s40168-016-0190-1 PMID: 27514729

73. Hanchi H., Mottawea W., Sebei K., Hammami R., The Genus Enteroococcus: Between Probiotic Potential and Safety Concerns—An Update. Front. Microbiol. 2018; 9:1791. https://doi.org/10.3389/fmicb.2018.01791 PMID: 30132008

74. Sternšiš M., Bucar F., Kunert O., Smolč Možina S., Targeting fish spoilers Pseudomonas and Shewanella with oregano and nettle extracts. Int. J. Food Microbiol. 2020; 328 (2020), p. 108664. https://doi.org/10.1016/j.ijfoodmicro.2020.108664 PMID: 32474229

75. Sternšiš M., Purgatorio C., Paparella A., Mraz J., Možina S.S., Combination of rosemary extract and buffered vinegar inhibits Pseudomonas and Shewanella growth in common carp (Cyprinus carpio). J. Sci. Food Agric. 2020; 100, 2305–2312. https://doi.org/10.1002/jsfa.10273 PMID: 31960971

76. Dailey F.E., McGraw J.E., Jensen B.J., Bishop S.S., Løkken J.P., Dorff K.J., et al. The Microbiota of Freshwater Fish and Freshwater Niches Contain Omega-3 Fatty Acid-Producing Shewanella Species. Appl. Environ. Microbiol. 2015; 82, 218–231. https://doi.org/10.1128/AEM.02266-15 PMID: 26497452

77. Curtis A.C., Durborow R.M., Hemstreet W.G., Thune R.L., Hawke J.P., Aeromonas Bacterial Infections N Motile Aeromonad Septicemia SRAC 1998; Publication No. 478.

78. Praveen P.K., Debnath C., Shekhar S., Dalai N., Ganguly S., Incidence of Aeromonas spp. infection in fish and chicken meat and its related public health hazards: A review. Vet. World 2016; 9, 6–11. https://doi.org/10.14202/vetworld.2016.06-11 PMID: 27051177

79. Qian Y.-F., Ye J.-X., Yang S.-P., Lin Z.-Q., Cao W., Xie J., Evaluation of the spoilage potential of Shewanella putrefaciens, Aeromonas hydrophila, and Aeromonas sobria isolated from spoiled Pacific white shrimp (Litopenaeus vannamei) during cold storage. J. Food Saf. 2018; 38, e12550. https://doi.org/10.1111/jfs.12550
80. Rawls J.F., Samuel B.S., Gordon J.I., Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc. Natl. Acad. Sci. U. S. A. 2004; 101, 4596–4601. https://doi.org/10.1073/pnas.0400706101 PMID: 15070763