applied sciences

MDPI

Article

Fatty Acid Profile, Atherogenic and Thrombogenic Indices, and Meat Quality as the Effect of Feed Additive in African Catfish *Clarias gariepinus* (Burchell, 1822)

Milena Bušová [1,*], Lenka Kouřimská [2], Marek Doležal [3], Vojtech Ilko [3], Diomid Revenco [3], Mahyar Zare [4], Jan Matoušek [4], Ilario Ferrocino [5], Irene Franciosa [5], Petr Smejkal [1,6], Matěj Přeslička [7] and Markéta Prokešová [4]

1 Institute of Hygiene and Epidemiology, First Faculty of Medicine, Charles University and General University Hospital in Prague, 121 08 Prague, Czech Republic; smep@ikem.cz
2 Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, 165 00 Prague, Czech Republic; kourimska@af.czu.cz
3 Department of Food Analysis and Nutrition, Faculty of Food and Biochemical Technology, University of Chemistry and Technology, 166 28 Prague, Czech Republic; marek.dolezal@vscht.cz (M.D.)
4 Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Institute of Aquaculture and Protection of Waters, University of South Bohemia in Ceske Budejovice, 370 05 České Budějovice, Czech Republic; mprokesova@frov.jcu.cz (M.P.)
5 DISAFA—Microbiology and Food Science, University of Turin, 10095 Grugliasco, Italy; ilario.ferrocino@unito.it (I.F.)
6 Department of Hygiene and Epidemiology, Institute for Clinical and Experimental Medicine, 140 21 Prague, Czech Republic
7 ReConsulting ČR, a.s., 360 01 Sadov, Czech Republic; obchod@humaty.cz
* Correspondence: milena.busova@lf1.cuni.cz; Tel.: +420-224-96-8531

check for updates

Citation: Bušová, M.; Kouřimská, L.; Doležal, M.; Ilko, V.; Revenco, D.; Zare, M.; Matoušek, J.; Ferrocino, I.; Franciosa, I.; Smejkal, P.; et al. Fatty Acid Profile, Atherogenic and Thrombogenic Indices, and Meat Quality as the Effect of Feed Additive in African Catfish *Clarias gariepinus* (Burchell, 1822). *Appl. Sci.* **2023**, *13*, 10058. https://doi.org/10.3390/app131810058

Academic Editor: Monica Gallo

Received: 9 August 2023
Revised: 31 August 2023
Accepted: 4 September 2023
Published: 6 September 2023

Abstract: Humic substances (HS) are often used as feesd additives in livestock feeding. The long-term effects of different concentrations of HS additives in aquafeed on growth and production performance, antioxidant status, stress resistance, gut microbiome, overall health condition, final product yield, sensory properties of fresh and cooked meat, and composition of fatty acids of market size in African catfish *(Clarias gariepinus*) originating from aquaculture were compared in this study. *C. gariepinus* were exposed to dietary inclusions of HS (0, 1, and 3, and 6% *w*/*w*) in a long-term experiment (six months in total) until fish reached market size. The growth parameters, condition factor, and selected somatic indices did not differ ($p > 0.05$) between the tested groups (HS0–HS6). Biochemical parameters were not different between the tested HS groups by the end of experiment, and only glucose (GLC) levels significantly increased ($p < 0.05$) with increasing concentrations of HS fed as an additive. The levels of cortisol (COR) and GLC did not differ between the tested groups ($p > 0.05$) after the stress challenge, but the results of GLC levels before and after the stress challenge showed an increasing tendency with increasing levels of HS addition in the diet. The levels of COR were slightly lower in groups HS3 and HS6 than in HS1 and the control group. In the case of protein and fat contents, differences between groups (HS0–HS6) were statistically insignificant. Significant differences were found, however, in water and ash content. In some cases, statistically significant differences were found in fatty acid profiles and in nutritional indices assessing fatty acids between samples. Sensory characteristics of fresh fillets did not differ between the tested groups HS0–HS6. The total content of polyphenols increased depending on the addition of humates. The main gut microbiota of samples analyzed (HS0–HS3 group) comprised the following three genera: *Ralstonia*, *Pseudomonas* and *Cetobacterium*; other genera were present in all samples at a low relative abundance: *Staphylococcus*, *Bradyrhizobium*, *Bacillus*, and *Anaerobacillus*. The relative abundance of *Pseudomonas* decreased while the presence of *Cetobacterium* increased in samples fed with 3% of HS. The results of our study yielded a comprehensive set of experimental results about African catfish fed with HS as additives. Although a significant effect of HS on overall performance of *C. gariepinus* was not proven, a positive effect on antioxidant status was seen as well as a decrease in gut microorganisms that can be present as pathogenic contaminants in aquatic environments.

Keywords: humic substances; health effect; antioxidant status; microbiome; sensory analysis

1. Introduction

High workload, stress, and poor diet leads to numerous human diseases in developed European countries. The highest number of deaths in Europe is due to cardiovascular diseases. Slightly over 1.8 million people have died from diseases of the circulatory system, mainly heart attacks and strokes. These are the main causes responsible for 36% of all deaths in the European Union [1].

One of the causes of these unfavourable conditions may be incorrect nutrition with an excess of dietary fats or an excessive intake of saturated fatty acids (SFA). The recommended intakes of total fat and fatty acids for a healthy population (adults, children, and infants) have been published earlier [2]. A number of studies confirm the fact that the quality and quantity of fatty acids in the diet can influence the cardiovascular risk. The benefits of dietary long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) on human health were studied and reported through clinical, pathological, observational, and case studies over a long period. Regular intake of polyunsaturated fatty acids (PUFA) in the diet is an important factor for the normal growth, development, and prevention of cardiovascular diseases (CVD) and atherosclerosis in humans [3–5]. The American Heart Association reported the specific effect of the n-3 polyunsaturated fatty acid (n-3 PUFA) supplementation on clinical cardiovascular events [3]. The LC n-3 PUFA, eicosapentaenoic acid (EPA, 20:5n3), and docosahexaenoic acid (DHA, 22:6n3) are most closely associated with a lower CVD risk [6]. Some authors deal with the preventive effects of PUFA and the nutritional value of fish meat in the diet. Fish meat contains a significantly lower amount of SFA compared to red meat and is beneficial for nutrition due to it being a rich source of LC n-3 PUFA, especially EPA and DHA. Fat is the third major constituent in fish muscle. The fat content varies among species and also among different organs within the species [7–10]. The fatty acid composition, quality, and fat content of fish meat depends on the fish species, on the age of fish, on the composition of feed, and their origin (wild versus farmed) [11,12]. Fish oil is also the most important natural source of PUFA, which includes EPA and DHA [13,14].

In 2002, the American Heart Association (AHA) published a scientific statement on LC n-3 PUFA in marine and freshwater fish and supplements in relation to CVD; they also reported the beneficial effects of LC n-3 PUFA on the prevention of CVD [3]. This recommendation by AHA for using generous quantities of fish in the diet in order to obtain sufficient quantities of protein without excess fatty acids and lipids was supported by other authors [2,5]. Fish muscle is a good source of n-3 PUFAs that are considered to be the most important in human nutrition due to their therapeutic role in reducing certain cardiovascular disorders [11,15–17].

Sufficient intake of n-3 PUFA in the diet prevents arrhythmias, ventricular tachycardia, and fibrillation, inhibits the synthesis of cytokines and mitogens, has anti-inflammatory, antithrombotic, and hypolipidemic properties, with effects on triacylglycerols (TAG), has very low-density lipoproteins (VLDLs), and can inhibit atherosclerosis [2,18,19]. According to older studies, the preventive effect of regular additions of PUFAs in the human diet is obvious. Over the last several decades, a number of authors have reported the beneficial composition of fish meat. Essential polyunsaturated fatty acids such as α-linolenic acid (18:3 n-3, ALA), EPA and DHA are not synthesized in the human body, but they are synthesized by aquatic organisms. Therefore, humans must receive these essential fatty acids in food. The available resources are marine and freshwater fish [7,20].

Fish consumption varies from country to country depending on regional use, historical context, access to the sea, and affordability. In landlocked countries, freshwater fish are reared under traditional fish farming conditions [21]. African catfish *Clarias gariepinus* (Burchell, 1822) is a promising freshwater fish species suitable for intensive aquaculture due to its fast growth at high stocking densities [22], excellent feed conversion [23], the

ability to breathe atmospheric air due to the accessory supra-branchial respiratory organ, tolerance to extreme environmental and water quality conditions [24], as well as tasty flesh with a high nutritional value. African catfish constitutes a good source of protein and PUFA [25,26].

Humic substances (HS) are often applied in agriculture to improve soil quality [27] and for other purposes, for example, in veterinary practice for antiseptic, antioxidant, and detoxifying properties [28,29]. They are often used as feed additives in livestock feeding. Islam et al. [30] published the beneficial effects of HS as feed additives on growth and feed conversion and other positive health effects on farm animals. With these properties, they have potential to be used in fish farming. Some authors reported beneficial effects in common carp [31,32], rainbow trout [33,34], and African catfish juveniles [35]. However, there is still insufficient information on the general properties, overall performance, and nutritional value of fish muscle from market size African catfish fed a diet supplemented with HS.

The aim of this study was to compare the long-term effects of different concentrations of HS additives in aquafeed on growth, production performance, antioxidant status, stress resistance, the gut microbiome, overall health condition, final product yield, sensory properties of fresh and cooked meat, and the composition of fatty acids in market size African catfish originating from aquaculture.

2. Materials and Methods

2.1. Fish Stock

African catfish were originally imported from an intensive recirculation farm (AGRO Fish Farm Ltd., Handlová, Slovakia) and were transported to the Institute of Aquaculture and Protection of Waters (České Budějovice, Czech Republic). The fish (body weight 28.1 ± 6.2 g) were firstly exposed to HS dietary inclusions (0, 1, and 3, and 6% w/w) in a short-term experiment that lasted for two months; see [35]. Thereafter, the same fish (body weight 274.1 ± 66.1 g) were transferred to a larger recirculation aquaculture system (RAS) and the study continued in a long-term experiment (six months in total) until the fish reached market size (body weight 733.0 ± 202.9 g) which, in Europe, is about 1 kg or more.

2.2. Experimental Design

After the first experiment with African catfish juveniles, published by Prokešová et al. [35], the experiment continued. The fish were moved into larger recirculating aquaculture systems to continue the study until achieving a market size of fish (weight of around 1 kg). At the start of the experiment, 240 fish were tagged (PIT-tags, BTS-ID®) and unique four-digit codes were recorded. Then the fish were placed into each of four rearing tanks (n = 80 fish/group) in four separate RASs. Each RAS consisted of a submerged biofilter tank (volume 2500 L) with bio-elements (BT 10, Ratz), four circular rearing tanks (volume 650 L), and a 900 L sump tank with a drum filter (ECO15, DVS-FilterTechniek, Kerkrade, The Nederland). The tanks were supplied with a water flow of 6 L s^{-1} and covered by a net to prevent fish jumping. Water circulation was maintained using a regulated pump (Jecod DCP 12000, Jebao Co., Ltd.). The water was heated to 24.0 ± 0.1 °C using a 1.5 kW heater (900 EVO Aquatic Heater, Elecro Engineering, Hertfordshire, England). The oxygen concentration of outlet water was maintained at 63.0 ± 2.0% (Secoh JDK400, Secoh, Japan) and a pH of about 7.0 ± 0.04. As for the photoperiod, the natural light mode of the May–September summer season was set. The fish were acclimatized to these experimental conditions for two weeks.

During this period, the fish were continuously fed with the experimental feed containing different HS contents: 0, 1, and 3, and 6% of food weight (HS0, HS1, and HS3 and HS6, respectively). Commercial feed (4.5 mm size of pellets, Aller Bona Float, Aller Aqua, Christiansfeld, Denmark) was spray coated with a commercial liquid feed additive Humafit (Humáty s.r.o., Czech Republic) containing HS from Siberian leonardite mineraloid in the required amounts. Then the HS-enriched pellets were dried in a laboratory dryer (UN 75,

Memmert GmbH, Munchen, Germany) at 40 °C for 24 h. For more details on the proximate composition of the HS commercial product and experimental diets, see [35].

The fish in each tank were fed by hand twice a day (8.00, 15.00). The daily food ratio was adjusted according to the manufacturer's instructions (1 to 2% of biomass). The tanks were cleaned of sediment daily and drained water (~1/3 of each rearing tank volume) was replaced with tap water.

The water temperature, pH, and oxygen level were measured daily using a multimeter (HI-98194, Hanna Instruments, Italy). The levels of NH_4^+, NH_3, NO_2^-, and NO_3^- were checked regularly using an aquaculture multi-parameter photometer with pH meter (HI-83303, Hanna Instruments).

2.3. Sampling and Analysis

2.3.1. Growth and Production Parameters, Somatic Indices, Fillet Yield, and Overall Mortality

Every 28 days, all fish were starved of food for one day. On the following day, they were one-by-one anesthetized in clove oil (0.03 mL·L^{-1} water) and individual PIT-tag codes, body weight (BW, g), total and standard body lengths (TL and SL, mm) were recorded. Simultaneously, the number of survivors per tank was counted to determine overall mortality. At the end of the experiment, gonads, liver, spleen, visceral fat, and digestive tract (n = 24 fish/group) were eviscerated and weighed to the nearest 0.001 g (Adventurer Pro AV264C, Ohaus, NJ, USA). These fish were then processed for fillet yield, when the individual body weight (BW/10, g), weight of eviscerated body (POT, % of BW), head (PH, % of BW), fillets with skin (PFSK, % of BW), and fillets without skin (PFBK, % of BW) were weighed to the nearest 0.01 g. Their yield was calculated as % of the individual fish BW (g). Afterwards, the viscero- (VSI, %), gonado- (GSI, %), hepato- (HSI, %), splenic- (SSI, %), visceral-fat- (VFSI, %), and gastrointestinal- (GaSI) somatic indices were calculated as a percentage of the organ at the BW of the individual fish (100 × W_{org}/BW). Besides weight gain (WG, g), the condition factor K, and survival rate (SR, %) were calculated as follows:

$$\text{Weight gain (WG, g)} = W_f - W_i$$

$$\text{Condition factor (K)} = (W_f/TL^3) \times 100$$

$$\text{Survival rate (SR, \%)} = (N_f \times 100/N_i)$$

where N_i = initial number of fish per tank, N_f = final number of fish per tank, W_f = final mean body weight (g), W_i = initial mean body weight (g).

2.3.2. Biochemical Analysis

At the end of the experiment, blood was sampled from the anaesthetized fish (n = 10 fish/group). The blood (about 3 mL) was collected from the caudal vessel using a heparinized syringe (50 IU μL^{-1}). Fresh whole blood samples (2 mL), with EDTA (ethylenediaminetetraacetic acid) added as an anticoagulant for blood plasma preparation, were used for biochemical assays in the same way as described by Prokešová et al. [35]. The plasma samples were analyzed for the following biochemical parameters—total protein (TP, g·L^{-1}), alanine aminotransferase (ALT, μkat·L^{-1}), aspartate aminotransferase (AST, μkat·L^{-1}), lactate dehydrogenase (LDH, μkat·L^{-1}). cholesterol (CHOL, mmol·L^{-1}), triglyceride (TAG, mmol·L^{-1}), cortisol (COR, nmol·L^{-1}), and glucose (GLC, mmol·L^{-1}) levels. For the glucose analysis, a part of the whole blood sample was pipetted separately into plastic tubes (Vacuette) with sodium fluoride (NaF), as an inhibitor of glycolysis. All analyses were performed in a third-party lab (Stafila Laboratory, Czech Republic).

2.3.3. Glutathione Antioxidant Status (GSH/GSSG Ratio)

Immediately after blood collection, 100 µL of each whole blood sample was pipetted into plastic microtubes (Eppendorf, Hamburg, Germany) and used for oxidized (GSSG) glutathione analysis, while 50 µL of each whole blood sample was used for reduced (GSH) glutathione analysis in the same way as described by Prokešová et al. [35]. The results are presented as the glutathione ratio (GSH/GSSG).

2.3.4. Stress Challenge, Cortisol, and Glucose Analysis

After the above blood sampling, all remaining non-sampled fish were subjected to a stress challenge for 40 min, i.e., the water level in the rearing tanks was markedly decreased (the water level reached the dorsal fins of the fish). Blood (about 3 mL) was sampled from the caudal vessel (heparinized syringe 50 IU µL) of the fish (n = 10 fish/group) immediately after the stress challenge. Thereafter, the water level was increased to its original level. Each whole blood sample was collected into plastic tubes (Vacuette) with EDTA and separately for a glucose analysis with sodium fluoride (NaF). After separation (centrifuge Mikro 200R, Hettich, Tuttlingen, Germany, at 4 °C, 15 min at 3000 RCF), all plasma samples were frozen and transported to a third-party lab (Stafila laboratory, Czech Republic) for GLU and COR analysis.

2.3.5. Gut Microbiome Analysis

The samples of *C. gariepinus* midgut content (n = 9 fish/group) were squeezed into Eppendorf tubes under sterile conditions and stored frozen at −80 °C. The samples were analyzed by means of a metataxonomic approach in order to highlight any differences in microbiota composition. Total DNA was extracted from midgut samples of *C. gariepinus* and used for 16S rRNA gene analysis (V3-V4 regions) with amplification performed by primers and procedures previously described [36]. Illumina metagenomic guidelines were followed for the PCR products and library pool. The Illumina MiSeq platform with V2 chemistry was used to obtain 250 bp paired-end reads. The raw files generated (*.fastq*) were imported in QIIME 2, 2022.11version software [37]. The *Cutapter* function was used to remove primer sequences and DADA2 algorithms [38] to denoise the reads by using the q2-dada2 plugin in QIIME 2. The taxonomy classification was performed by means of the QIIME2 feature-classifier against the *SILVA* database, excluding the ASVs with less than five read counts in at least two samples to increase the confidence of the sequence reads.

2.3.6. Meat Main Composition

Total water, protein, lipid, and ash content were evaluated in the right fillet of the fish at the end of the experiment. The analyses were performed by a laboratory of the Department of Food Analysis and Nutrition, Faculty of Food and Biochemical Technology, University of Chemistry and Technology in Prague, Czech Republic. The fillets (~200 g) were homogenized using a flesh-suitable mixer and kept in the freezer at −55 °C until the analysis.

Dry Matter, Ash, Protein, and Total Lipid Analysis

The dry matter content was determined gravimetrically according to [39]. The homogenized sample, approximately 1.5 g, was ground with pre-dried sand and dried at 105 °C to constant weight in an oven. The mineral content (ash) was determined after ashing the sample in a muffle furnace at 450 °C. The Kjeldahl method was used to determine nitrogen by the KT200 Kjeltec system (FOSS, Denmark). The protein content in the fillets was estimated by multiplying the nitrogen content by a nitrogen-to-protein conversion factor of 6.25 [40]. The total lipid content was determined gravimetrically by solvent extraction in chloroform–methanol (2:1, v/v) according to the method of Folch et al. [41]. Dry matter, ash, protein, and total lipid analyses were carried out in triplicate.

Fatty Acid Profile

The composition of fatty acids was determined from the aliquot of total lipids, which were extracted from fillets with chloroform–methanol (2:1, v/v) according to the method of Folch et al. [41]. Derivatization of fatty acids was based on the base-catalyzed reaction according to the IUPAC method 2.301 [42]. Fatty acid methyl esters (FAMEs) were then extracted into hexane. FAMEs were analyzed by gas–liquid chromatography using a SP-2560 fused silica capillary column (100 m × 0.25 mm i.d., 20 µm film thickness) (Supelco, USA) in an Agilent 6890 gas chromatograph (Agilent Technologies, USA) equipped with a flame ionization detector (FID) under the conditions described by Pohořelá et al. [43]. Fatty acid quantification was carried out by the internal normalization method and the results were expressed as relative percentages of all identified fatty acids. The FAMEs' analyses were carried out in duplicate.

Nutritional Indices Assessing Fatty Acids

Nutritional indices described by Chen and Liu [44] as suitable for assessing fish samples were calculated from fatty acid profiles of the samples by using formulas summarized in the paper by these authors. They were polyunsaturated fatty acid/saturated fatty acid ratio (PUFA/SFA), index of atherogenicity (IA), index of thrombogenicity (IT), hypocholesterolemic/hypercholesterolemic ratio (HH), unsaturation index (UI), health-promoting index (HPI), and the sum of eicosapentaenoic acid and docosahexaenoic acid (EPA + DHA).

2.3.7. Sensory Analysis of Fillet and Cooked Meat

The sensory analysis was performed by a sensory panel of 10 employees (five men and five women of different ages from 28–70 years) with experience in sensory assessment of fish products at the Institute of Aquaculture and Protection of Waters. The sensory analysis was performed according to ISO 8589 [45]. At the end of the experiment, the fresh fillets were cut into samples (2 × 2 cm squares), and three pieces were placed into a glass jar with a number code (unique for each group) and cooked in an electric oven at 150 °C for 15 min. Afterwards, the sensory evaluators assessed the samples in separate chambers where the glass jars with freshly cooked fish samples (sample/group, n = 4 samples at one time), a fork, a glass of water, a fresh roll, an evaluation form, a pencil, and a paper towel were prepared. Each evaluator assessed the sensory properties (odour, colour, consistency, flavour, and aftertaste) of all samples in the duplicates (the first and second round). The scale for assessment was set up from 1 (the worst result) to 5 points (the best result). There was also a place for verbal notes in the evaluators' forms.

2.3.8. Bacterial, Fungal and Yeast Content in Rearing Environment

For the total count of bacteria, yeasts, and moulds, semi-quantitative Cult-Dip-combi tests (Merck, Germany) were used. The water samples (50 mL/RAS) for each experimental group (HS0–HS6) were collected into sterile plastic tubes for the determination of total bacteria, fungi, and yeasts. The test strip with agar on one side of the strip (TTC agar for determination of bacteria/mL) and potato-dextrose agar on the other side of the strip (for the count of yeast/mL and density of fungi) was fully submerged into sampled water according to the producer's instructions. After 10 s, the strip was returned to the sterile tube and incubated in a dark environment at 28 °C in a Falc ICT 52 laboratory incubator. After 24 h, the total bacterial count (Colony Forming Units, CFU) in 1 mL of water was evaluated by comparing the colony density with a model density chart. After the next 3 days, the numbers of yeast and fungi were evaluated in the same way with the model density chart. Each sample was tested in two repetitions. All manipulations described above were performed carefully and quickly under sterile conditions.

2.3.9. Statistical Evaluation

Data are expressed as means ± standard deviations (SD). The PIT-tagged fish were considered the experimental units for the assessment of BW, TL, SL, K, WG, and somatic

indices. For biochemical parameters, antioxidants, and meat quality parameters, each sampled fish was considered as an experimental unit. In the case of sensory analysis, each evaluator's opinion on the fresh fillets and cooked meat was assessed as an experimental unit. The statistical analysis was performed using STATISTICA 12.0 (StatSoft, Ltd., R version 4.2.3 software Czech Republic). The effects of HS feed additive on the selected parameters were analyzed by one-way ANOVA. Prior to the ANOVA analysis, all data were tested for normal distribution and homogeneity of variance. If these conditions were not met, the Kruskal–Wallis analysis was used. The probability value used for all tests was $p = 0.05$. The Tukey's HSD test assessed the significant differences between groups. In the case of the midgut microbiota composition, the alpha diversity indices were calculated by the diversity script of QIIME2 and a non-parametric Kruskal–Wallis test in R environment was performed to find any differences between the ASV frequency and alpha diversity parameters. The ASV table was used to produce the principal component analysis (PCA) by using the made4 package in R environment (www.r-project.org, accessed on 20 March 2023). The function *dudi.pca* of R based on the ASV table was used for the PCA. Any significant differences in the overall microbial community were detected by ANOSIM statistical test by using the ASV table.

3. Results

3.1. Growth and Production Parameters, Somatic Indices, and FINAL Product

During the experiment, the fish doubled their initial BW. However, the growth parameters (BW, TL, SL, and WG), condition factor K, and selected somatic indices (VSI, GSI, HSI, SSI, VFSI, and GaSI) did not differ ($p > 0.05$) between the tested groups (HS0–HS6) after long-term HS dietary exposure (see Table 1). The survival rate reached 74.7–90.7% of the initially stocked fish in the HS0–HS6 groups.

Table 1. Fish individual body weight (BW), total body length (TL and SL), weight gain (WG), condition factor (K), and somatic indices (VSI, GSI, HSI, SSI, VFSI, and GaSI) of *C. gariepinus* after a long-term exposure to experimental HS diets (HS0–HS6).

Parameters	Initial	HS0	HS1	HS3	HS6	F^{test}	n^{fish}	p
Period (d)	**Day 0**	**Day 84**	**Day 84**	**Day 84**	**Day 84**	**Day 84**	**Day 84**	**Day 84**
BW (g)	368.94 ± 87.38	746.37 ± 212.38	727.81 ± 180.46	732.18 ± 224.08	713.50 ± 181.42	0.51 ^{KW}	All	0.92
TL (mm)	354.68 ± 25.53	444.90 ± 38.42	443.56 ± 35.01	442.32 ± 42.27	440.79 ± 33.47	0.09 ^{1WA}	All	0.97
SL (mm)	317.98 ± 23.45	396.82 ± 36.08	396.73 ± 32.83	395.97 ± 38.99	396.79 ± 30.51	0.01 ^{1WA}	All	0.99
WG 0–84 d (g)	-	375.73 ± 164.02	348.93 ± 142.77	374.08 ± 178.96	315.67 ± 119.02	1.87 ^{KW}	All	0.60
K	0.81 ± 0.08	0.83 ± 0.07	0.82 ± 0.08	0.82 ± 0.07	0.82 ± 0.08	0.92 ^{KW}	All	0.82
VSI (%)	-	7.67 ± 2.08	9.58 ± 4.09	7.81 ± 2.21	8.13 ± 2.85	6.39 ^{KW}	96	0.09
GSI (%)	-	2.09 ± 2.40	2.55 ± 2.67	2.28 ± 2.83	2.85 ± 3.28	1.18 ^{KW}	96	0.76
HSI (%)	-	0.93 ± 0.54	1.03 ± 0.46	0.88 ± 0.31	0.98 ± 0.46	1.11 ^{KW}	96	0.77
SSI (%)	-	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	3.25 ^{1WA}	96	0.35
VFSI (%)	-	0.87 ± 0.43	0.76 ± 0.44	0.95 ± 0.66	0.61 ± 0.39	6.75 ^{KW}	96	0.08
GaSI (%)	-	1.46 ± 0.32	1.46 ± 0.33	1.45 ± 0.31	1.52 ± 0.34	0.46 ^{KW}	96	0.93

Note: The values are presented as means ± SD. The number of considered experimental units (n). One-way ANOVA (^{1WA}) or Kruskal–Wallis (^{KW}) test results are power of test (F) and level of significance ($p = 0.05$). No significant differences (Tukey HSD test, $p > 0.05$) were observed between the groups.

At the end of the experiment, the fish were processed and the yields of eviscerated body, head, fillets with skin and fillets without skin were calculated as % of fish BW. While the weight of the head and fillets with skin differed ($p < 0.05$) between the tested groups (HS0–HS6) by the end of the long-term HS dietary exposure, the yield of eviscerated body and fillets without skin did not significantly differ ($p > 0.05$) between the tested groups (see Figure 1). The yield of the eviscerated body was 90.4–92.3%, head 22.9–25.2%, fillets with skin 49.0–51.7%, and fillets without skin reached 41.5–42.7% of fish BW.

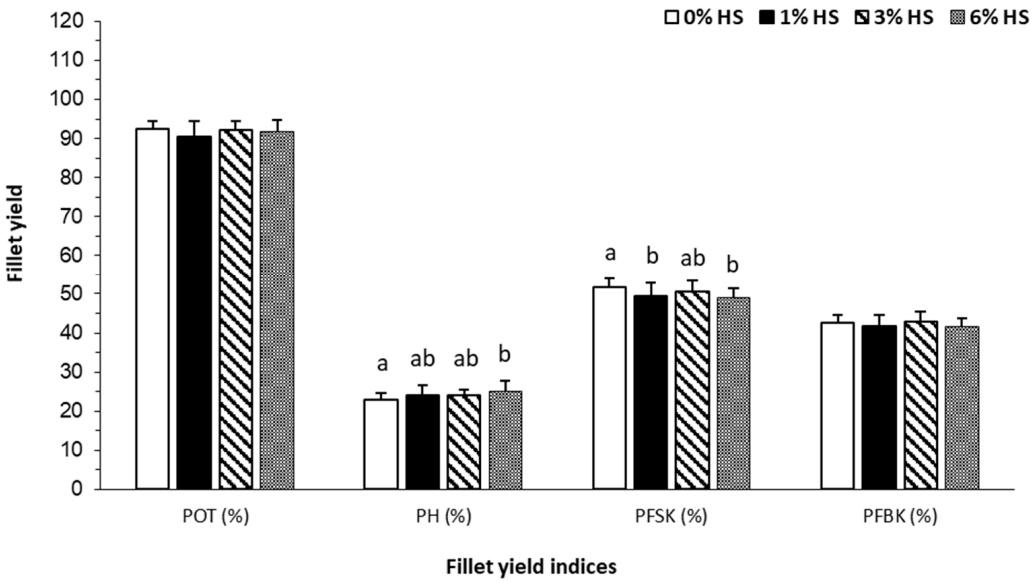


Figure 1. Individual body weight of fish (BW/10, mean ± g), eviscerated body (POT, % of BW), head (PH, % of BW), fillets with skin (PFSK, % of BW), and fillets without skin (PFBK, % of BW) of *C. gariepinus* exposed to experimental diets (HS0–HS6) for a long-term exposure. The values are presented as means ± SD. The number of considered experimental units (n = 96). No superscripts (a,b) above the slopes indicate no significant differences (Tukey HSD test, $p > 0.05$) between the groups within one parameter.

3.2. Biochemical Analysis

Biochemical parameters (TP, ALT, AST, LDH, CHOL, TAG, and COR) were not significantly different ($p > 0.05$) between the tested HS groups by the end of the experiment (see Table 2).

Table 2. Biochemical parameters (total proteins, TP; alanine aminotransferase, ALT; aspartate aminotransferase, AST; cholesterol, CHOL; triglycerides, TAG; lactate dehydrogenase, LDH; glucose, GLC, cortisol, COR and glutathione (reduced glutathione, GSH; oxidized glutathione, GSSG; glutathione ratio, GSH/GSSG) of *C. gariepinus* after long-term exposure to four experimental HS diets (HS0–HS6).

Parameters	**HS0**	**HS1**	**HS3**	**HS6**	***F***	***n***	***p***
Period (d)	**Day 84**	**Day 84**	**Day 84**	**Day 84**	**Day 84**	**Day 84**	**Day 84**
TP ($g \cdot L^{-1}$)	40.79 ± 6.25	40.25 ± 3.74	38.11 ± 4.33	36.79 ± 3.90	4.68 ^{KW}	40	1.97
ALT ($\mu ka \cdot L^{-1}$)	0.19 ± 0.11	0.16 ± 0.13	0.16 ± 0.12	0.15 ± 0.05	1.78 ^{KW}	40	0.62
AST ($\mu kat \cdot L^{-1}$)	1.18 ± 0.39	1.19 ± 0.98	0.94 ± 0.23	1.30 ± 0.68	2.82 ^{KW}	40	0.42
CHOL ($mmol \cdot L^{-1}$)	3.19 ± 0.66	3.10 ± 0.42	3.37 ± 0.46	2.88 ± 0.36	4.99 ^{KW}	40	0.17
TAG ($mmol \cdot L^{-1}$)	1.29 ± 0.32	1.26 ± 0.28	2.32 ± 2.53	1.31 ± 0.36	0.16 ^{KW}	40	0.98
LDH ($\mu kat \cdot L^{-1}$)	2.51 ± 1.28	2.65 ± 2.17	2.23 ± 1.09	3.44 ± 2.70	0.94 ^{KW}	40	0.82
* GLC ($mmol \cdot L^{-1}$)	3.22 ± 0.66 ^{a}	3.29 ± 0.88 ^{ab}	4.26 ± 0.97 ^{ab}	4.55 ± 1.53 ^{b}	9.45 ^{KW}	40	0.02
* COR ($nmol \cdot L^{-1}$)	113.80 ± 124.13	191.30 ± 122.02	208.50 ± 202.99	142.80 ± 118.16	5.63 ^{KW}	40	0.13
GSH (µM)	223.08 ± 45.97	213.02 ± 53.58	247.36 ± 54.51	210.19 ± 17.55	1.38 ^{1WA}	40	0.26
GSSG (µM)	40.35 ± 6.77	41.82 ± 77.84	45.33 ± 14.00	36.05 ± 14.29	0.77 ^{1WA}	40	0.52
GSH/GSSG (µM)	3.63 ± 1.31	3.69 ± 2.11	3.96 ± 2.38	4.55 ± 2.23	0.41 ^{1WA}	40	0.75
** GLC ($mmol \cdot L^{-1}$)	4.97 ± 1.27 ^{a}	5.50 ± 2.13 ^{ab}	5.67 ± 1.55 ^{ab}	7.24 ± 1.58 ^{b}	3.45 ^{1WA}	40	0.03
** COR ($nmol \cdot L^{-1}$)	177.90 ± 113.99	181.10 ± 156.08	112.70 ± 58.64	104.00 ± 84.03	4.76 ^{KW}	40	0.19

Note: * before the stress challenge, ** after the stress challenge. The values are presented as means ± SD. The number of considered experimental units (n). One-way ANOVA (^{1WA}) or Kruskal–Wallis (^{KW}) test results with power of test (F), and level of significance (P). Different superscripts (a,b) indicate significant differences (Tukey HSD test, $p < 0.05$) within one row.

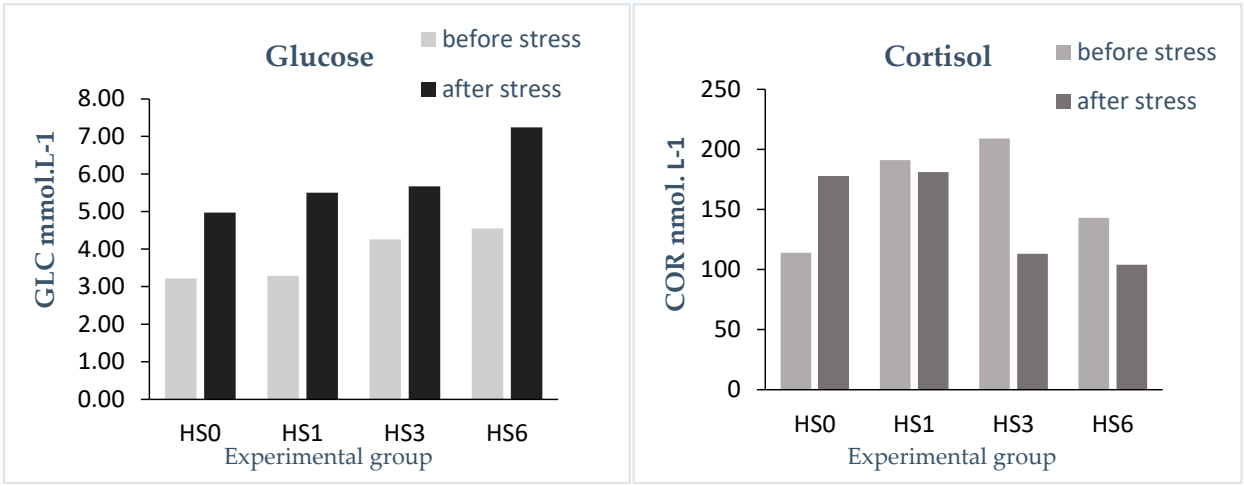
Only the GLC levels significantly increased ($p < 0.05$) with increasing concentration of HS feed additives. The same increase in GLC levels was observed even after the 40 min stress challenge (see Table 2). The results of the GLC and COR levels after the stress challenge in the experimental groups (HS0–HS6) are described below (see Section 2.3.4. Stress Challenge).

3.3. Glutathione Antioxidant Status (GSH/GSSG Ratio)

At the end of the experiment, the levels of reduced glutathione GSH, oxidized glutathione GSSG and the GSH/GSSG ratio were not significantly different ($p > 0.05$) between the tested groups. There was an obvious moderate increase in GSH levels in the HS3 group in comparison with the control HS0 group. Similarly, the GSH/GSSG ratio had a moderately higher value in the HS3 and HS6 groups compared with the control (see Table 2).

3.4. Stress Challenge

After the stress challenge (i.e., lowering levels of water in rearing tanks HS0–HS6 for 40 min), the blood plasma samples were analyzed for GLC and COR levels. These results were compared with the COR and GLC levels in the same fish before the stress challenge (see Table 2). The levels of COR and GLC did not differ between the tested groups ($p > 0.05$) after the stress challenge. In comparison, the results of the GLC levels before and after the stress challenge had an increasing tendency with increasing levels of HS addition to the diet (Figure 2a). On the other hand, the levels of COR were slightly lower in groups HS3 and HS6 than in HS1 and the control group (Figure 2b).



(**a**) (**b**)

Figure 2. Concentration of (**a**) glucose (mmol·L^{-1}) in blood before and after the stress challenge, (**b**) cortisol (nmol·L^{-1}) in blood before and after the stress challenge.

3.5. Meat Main Composition

Total protein, lipid, and mineral contents in meat was evaluated in the right fish fillet at the end of the experiment (Table 3). Statistically significant differences were found only between the water content in HS1 and HS6 and in ash content among all samples. In the case of protein and fat content, the differences between the groups (HS0–HS6) were statistically insignificant ($p > 0.05$).

Table 3. Content of water, protein, fat, and ash (in g/100 g) in tested fish meat samples.

Analyte	HS0	HS1	HS3	HS6
Water	77.50 ± 0.76 ^{ab}	76.71 ± 0.76 ^{b}	77.23 ± 0.98 ^{ab}	77.74 ± 0.53 ^{a}
Protein	17.15 ± 0.59	17.13 ± 0.54	16.73 ± 0.24	16.84 ± 0.49
Fat	4.17 ± 0.71	5.01 ± 0.78	4.91 ± 1.20	4.39 ± 0.59
Ash	1.18 ± 0.02 ^{a}	1.14 ± 0.01 ^{b}	1.12 ± 0.02 ^{c}	1.02 ± 0.02 ^{d}

Note: The values are expressed as means ± standard deviation (SD). The number of independent replicate experiments n = 10. Numbers followed by different lowercase letters in the same row are statistically different ($p \leq 0.05$).

3.6. Fatty Acid Profile, and Nutritional Indices Assessing Fatty Acids

Table 4 shows the relative representation of fatty acids in the fish samples analyzed. The most represented was oleic acid, followed by linoleic and palmitic acid, each of which was more than 10%. Other acids with a content of more than 1% were α-linolenic, stearic, asclepic, DHA, icosatetreanoic, and palmitoleic. The other fatty acids listed in Table 4 had a minor representation (content below 1%). There was a significant presence of nutritionally valuable essential fatty acids with 20 and 22 carbons, including EPA and DHA. Table 5 shows the sums of individual groups of fatty acids according to their number and position of double bonds, together with the nutritional indices calculated from the fatty acid profile of the samples analyzed; see Table 5.

Table 4. Fatty acid profile of the tested samples in relative % of all detected fatty acids.

Fatty Acid Name	Formula	HS0	HS1	HS3	HS6
Saturated fatty acids					
Capric	C10:0	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.02
Lauric	C12:0	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.11 ± 0.05
Myristic	C14:0	0.72 ± 0.11	0.73 ± 0.04	0.73 ± 0.07	0.69 ± 0.07
Pentadecanoic	C15:0	0.28 ± 0.04	0.28 ± 0.03	0.25 ± 0.04	0.28 ± 0.03
Palmitic	C16:0	14.76 ± 0.69	14.78 ± 1.13	15.18 ± 1.31	14.65 ± 0.94
Margaric	C17:0	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.02
Stearic	C18:0	4.75 ± 0.37	4.60 ± 0.37	5.03 ± 0.42	4.81 ± 0.33
Arachic	C20:0	0.34 ± 0.02	0.34 ± 0.02	0.36 ± 0.02	0.34 ± 0.02
Behenic	C22:0	0.20 ± 0.02	0.20 ± 0.01	0.20 ± 0.02	0.20 ± 0.02
Lignoceric	C24:0	0.12 ± 0.03	0.10 ± 0.04	0.22 ± 0.09	0.12 ± 0.04
Unsaturated fatty acids					
Hexadecenoic	C16:1 cis-7	0.34 ± 0.04	0.35 ± 0.03	0.33 ± 0.03	0.35 ± 0.05
Palmitoleic	C16:1 cis-9	1.30 ± 0.13	1.35 ± 0.16	1.36 ± 0.15	1.30 ± 0.19
Hexadecenoic	C16:1 cis-11	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
Heptadecenoic	C17:1 cis-9	0.45 ± 0.19	0.48 ± 0.06	0.39 ± 0.11	0.53 ± 0.12
Octadecenoic	C18:1 trans isomers	0.10 ± 0.03	0.12 ± 0.06	0.12 ± 0.03	0.13 ± 0.08
Oleic	C18:1 cis-9	41.29 ± 1.51 ^{ab}	41.23 ± 1.13 ^{ab}	42.68 ± 1.50 ^{a}	40.78 ± 1.22 ^{b}
Asclepic	C18:1 cis-11	3.02 ± 0.24	2.87 ± 0.15	3.11 ± 0.14	3.02 ± 0.32
Octadecenoic	C18:1 cis isomers	0.12 ± 0.01	0.14 ± 0.03	0.13 ± 0.01	0.13 ± 0.02
Octadecadienoic	C18:2 cis,trans isomers	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.01	0.13 ± 0.02
Linoleic	C18:2 cis, cis-9,12	17.21 ± 0.78 ^{ab}	17.59 ± 0.73 ^{ab}	16.61 ± 0.87 ^{b}	17.72 ± 0.67 ^{a}
Octadecatrienoic	C18:3 cis,trans isomers	0.11 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.11 ± 0.02
γ-Linolenic	C18:3 all cis-6,9,12	0.87 ± 0.13	0.82 ± 0.12	0.78 ± 0.13	0.95 ± 0.19
α-Linolenic	C18:3 all cis-9,12,15	4.12 ± 0.20 ^{ab}	4.30 ± 0.23 ^{a}	3.93 ± 0.36 ^{ab}	3.84 ± 0.31 ^{b}
Stearidonic	C18:4 all cis-6,9,12,15	0.44 ± 0.06	0.43 ± 0.06	0.40 ± 0.06	0.47 ± 0.08
Eicosenoic	C20:1 cis-11	1.60 ± 0.09 ^{b}	1.56 ± 0.05 ^{b}	1.69 ± 0.06 ^{a}	1.58 ± 0.07 ^{b}
Eicosadienoic	C20:2 cis, cis-8,14	0.10 ± 0.02	0.09 ± 0.01	0.10 ± 0.02	0.10 ± 0.02
Eicosadienoic	C20:2 cis, cis-11,14	0.50 ± 0.07	0.50 ± 0.05	0.49 ± 0.05	0.48 ± 0.04
Eicosatrienoic	C20:3 all cis-8,11,14	1.08 ± 0.12	1.02 ± 0.10	0.92 ± 0.23	1.09 ± 0.14

Table 4. *Cont.*

Fatty Acid Name	Formula	HS0	HS1	HS3	HS6
Eicosatrienoic	C20:3 all cis-11,14,17	0.16 ± 0.03	0.17 ± 0.01	0.14 ± 0.03	0.15 ± 0.02
Arachidonic	C20:4 all cis-5,8,11,14	0.58 ± 0.12	0.54 ± 0.09	0.41 ± 0.17	0.64 ± 0.19
Eicosatetraenoic	C20:4 all cis-8,11,14,17	0.22 ± 0.02	0.22 ± 0.02	0.19 ± 0.03	0.21 ± 0.03
Eicosapentaenoic	C20:5 all cis-5,8,11,14,17	0.69 ± 0.16	0.69 ± 0.10	0.51 ± 0.13	0.63 ± 0.11
Erucic	C22:1 cis-13	0.19 ± 0.05	0.17 ± 0.01	0.19 ± 0.02	0.18 ± 0.03
Docosatetraenoic	C22:4 all cis-7,10,13,16	0.08 ± 0.01	0.08 ± 0.01	0.06 ± 0.02	0.09 ± 0.02
Docosapentaenoic	C22:5 all cis-4,7,10,13,16	0.09 ± 0.03	0.09 ± 0.03	0.06 ± 0.02	0.11 ± 0.05
Docosapentaenoic	C22:5 all cis-7,10,13,16,19	0.46 ± 0.08	0.44 ± 0.06	0.34 ± 0.11	0.48 ± 0.12
Docosahexaenoic	C22:6 all cis-4,7,10,13,16,19	2.66 ± 0.70 ^{ab}	2.61 ± 0.41 ^{abc}	1.77 ± 0.66 ^{c}	2.72 ± 0.80 ^{a}

Note: The values are expressed as means ± standard deviations (SD). The number of independent replicate experiments n = 10. Significant differences were calculated for major fatty acids which had their content at least once more than 1%. Numbers followed by different lowercase letters in the same row are statistically different ($p \leq 0.05$).

Table 5. Sums of different groups of fatty acids (in relative % of all detected fatty acids) and their nutritional indices.

Fatty Acid Group	HS0	HS1	HS3	HS6
SFA	21.50 ± 0.95	21.29 ± 1.50	22.24 ± 1.68	21.41 ± 1.20
MUFA cis	48.35 ± 1.38 ^{ab}	48.19 ± 0.99 ^{b}	49.93 ± 1.50 ^{a}	47.91 ± 1.17 ^{b}
PUFA cis	29.24 ± 1.54 ^{a}	29.58 ± 0.99 ^{a}	26.71 ± 2.12 ^{b}	29.66 ± 1.68 ^{a}
UFA cis	77.59 ± 0.95	77.77 ± 1.49	76.64 ± 1.69	77.57 ± 1.13
Trans isomers	0.34 ± 0.04	0.34 ± 0.06	0.37 ± 0.04	0.37 ± 0.09
Omega-3	8.73 ± 0.89 ^{a}	8.86 ± 0.58 ^{a}	7.27 ± 1.13 ^{b}	8.49 ± 1.05 ^{ab}
Omega-6	20.52 ± 0.79 ^{ab}	20.73 ± 0.68 ^{a}	19.43 ± 1.07 ^{b}	21.16 ± 0.89 ^{a}
Omega-3/omega-6	0.425 ± 0.035 ^{a}	0.427 ± 0.028 ^{a}	0.373 ± 0.042 ^{b}	0.401 ± 0.043 ^{ab}
Nutritional index				
PUFA/SFA	1.36 ± 0.11 ^{ab}	1.40 ± 0.14 ^{a}	1.21 ± 0.17 ^{b}	1.39 ± 0.15 ^{ab}
IA	0.229 ± 0.014	0.229 ± 0.020	0.238 ± 0.024	0.226 ± 0.018
IT	0.332 ± 0.026	0.328 ± 0.031	0.371 ± 0.049	0.335 ± 0.035
HH	2.18 ± 0.17	2.20 ± 0.19	2.00 ± 0.24	2.24 ± 0.23
UI	130.0 ± 5.7 ^{a}	130.3 ± 3.6 ^{a}	121.0 ± 7.2 ^{b}	130.6 ± 7.1 ^{a}
HPI	4.37 ± 0.26	4.40 ± 0.38	4.24 ± 0.41	4.44 ± 0.35
EPA + DHA	3.34 ± 0.84 ^{a}	3.29 ± 0.51 ^{a}	2.28 ± 0.79 ^{b}	3.35 ± 0.89 ^{a}

Note: Values are expressed as means ± standard deviations (SD). The number of independent replicate experiments n = 10. Numbers followed by different lowercase letters in the same row are statistically different ($p \leq 0.05$).

In some cases, statistically significant differences were found between the samples. The addition of 3% humic substances resulted in an increased relative content of oleic acid and total MUFA. At the same time, there was a decrease in linoleic, α-linolenic, icosatrienoic, and DHA, which was reflected in a decrease in the sum of PUFA. The nutritional indices of the fish samples were generally very favourable. In some cases, there were statistically significant differences between them, reflecting changes in the composition of the individual fatty acids.

3.7. Gut Microbiota

The principal component analysis did not show a significant difference through samples HS0–HS3 according to the feeding concentrations of HS (Figure 3, $p > 0.05$). The alpha diversity indices showed higher ASV numbers in samples belonging to the 3% HS group (Figure 4). Only HS6 samples were not analyzed for the gut microbiome due to technical problems in the sample shipment to the laboratory.

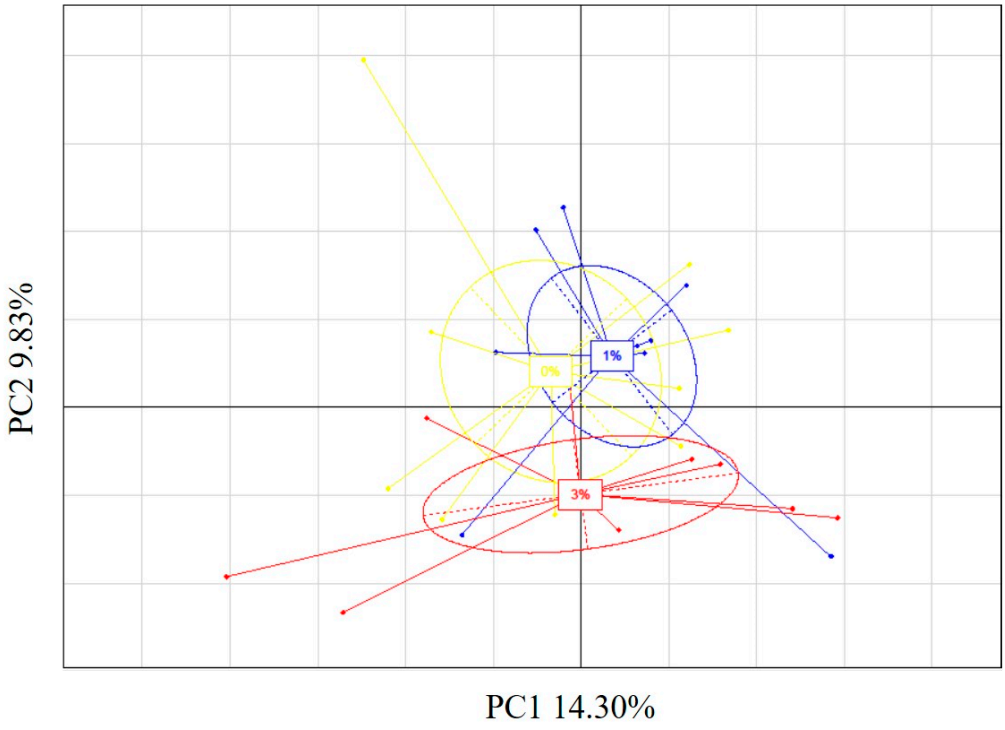


Figure 3. The principal component analysis (PCA) of microbiota in *C. gariepinus* midgut samples (HS0–HS3).

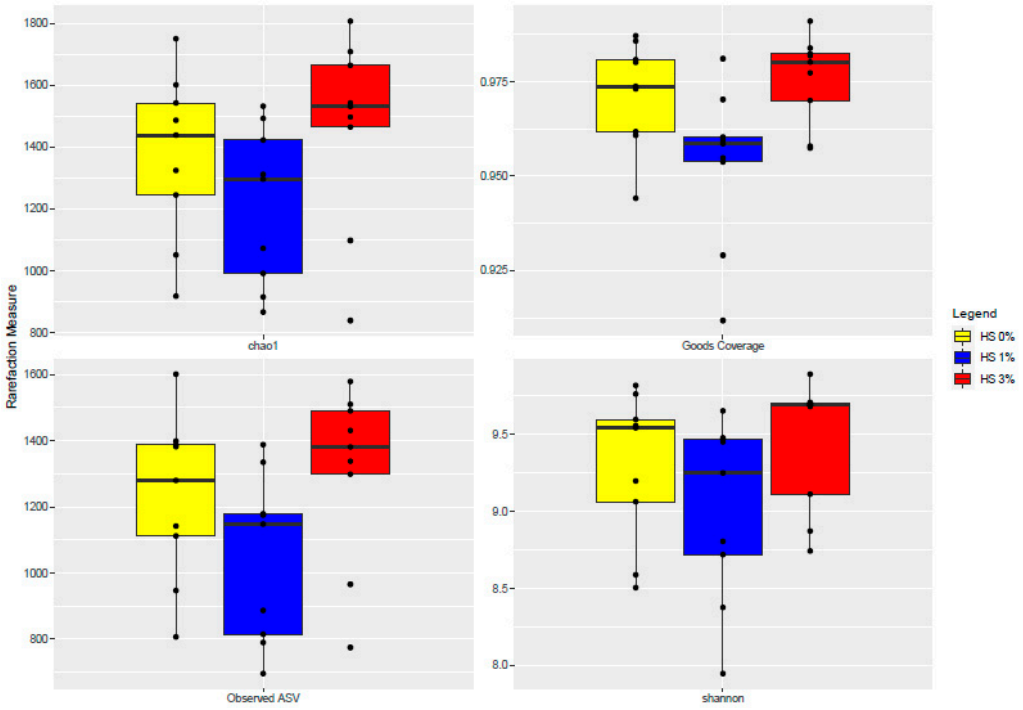


Figure 4. Alpha diversity measure of the microbiota in *C. gariepinus* midgut samples (HS0–HS3).

The main microbiota of the analyzed samples comprised the following three genera: *Ralstonia*, *Pseudomonas*, and *Cetobacterium;* other genera were present in all the samples, but at a low relative abundance: *Staphylococcus*, *Bradyrhizobium*, *Bacillus*, and *Anaerobacillus* (Figure 5).

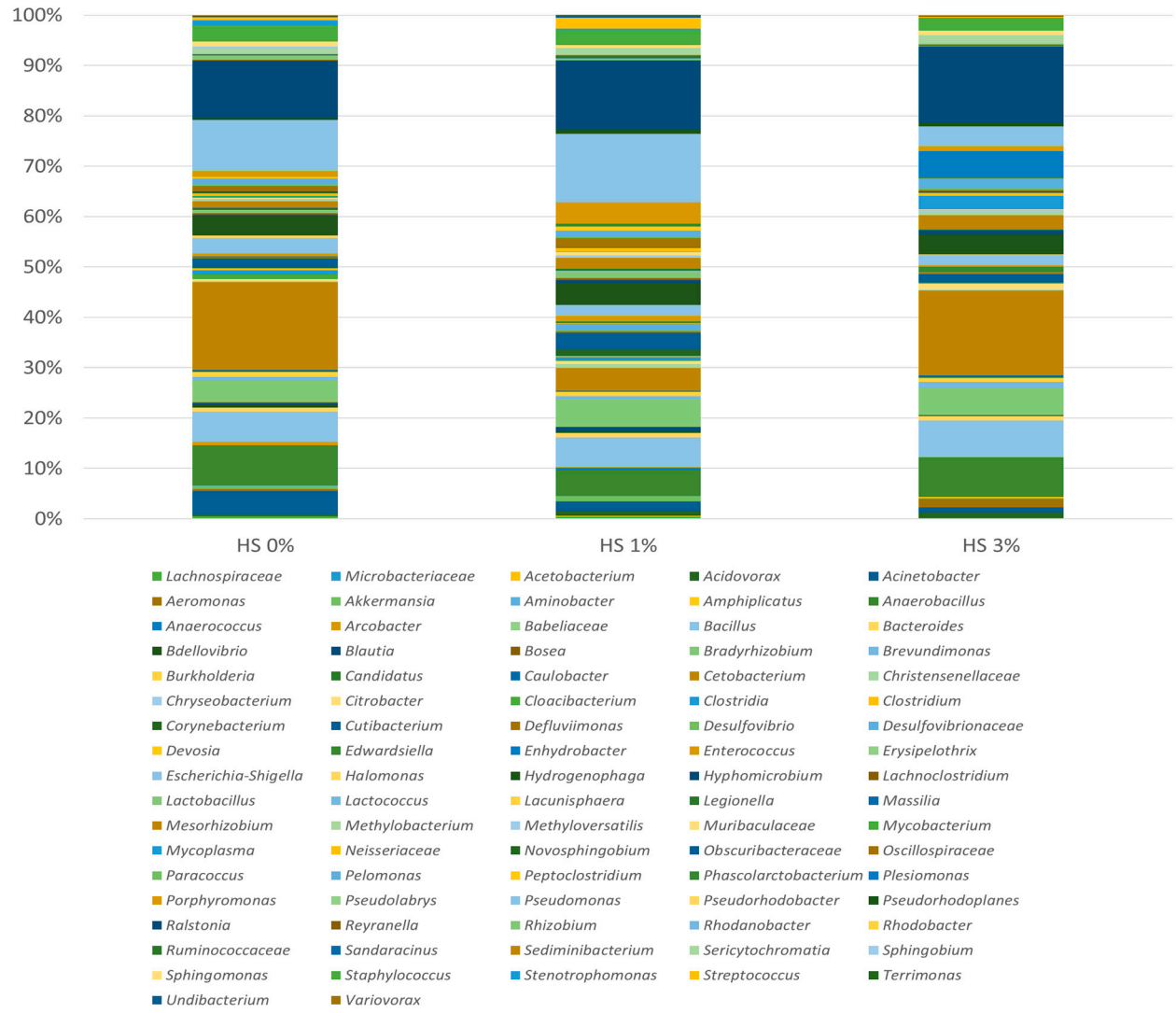


Figure 5. The global composition of the microbiota displays the lowest taxonomic resolution of *C.gariepinus* midgut samples at the genus or family level.

In particular, the relative abundance of *Pseudomonas* decreased while the presence of *Cetobacterium* increased in samples fed with 3% HS (Figure 6, $p < 0.05$). The same samples showed the highest relative abundance of *Cetobacterium* and *Plesiomonas* (Figure 6, $p < 0.05$). *Anaerococcus* genus was more abundant in samples fed with 1% HS, while the relative abundance of *Acinetobacter* genus decreased when the percentage of HS increased (Figure 6, $p < 0.05$).

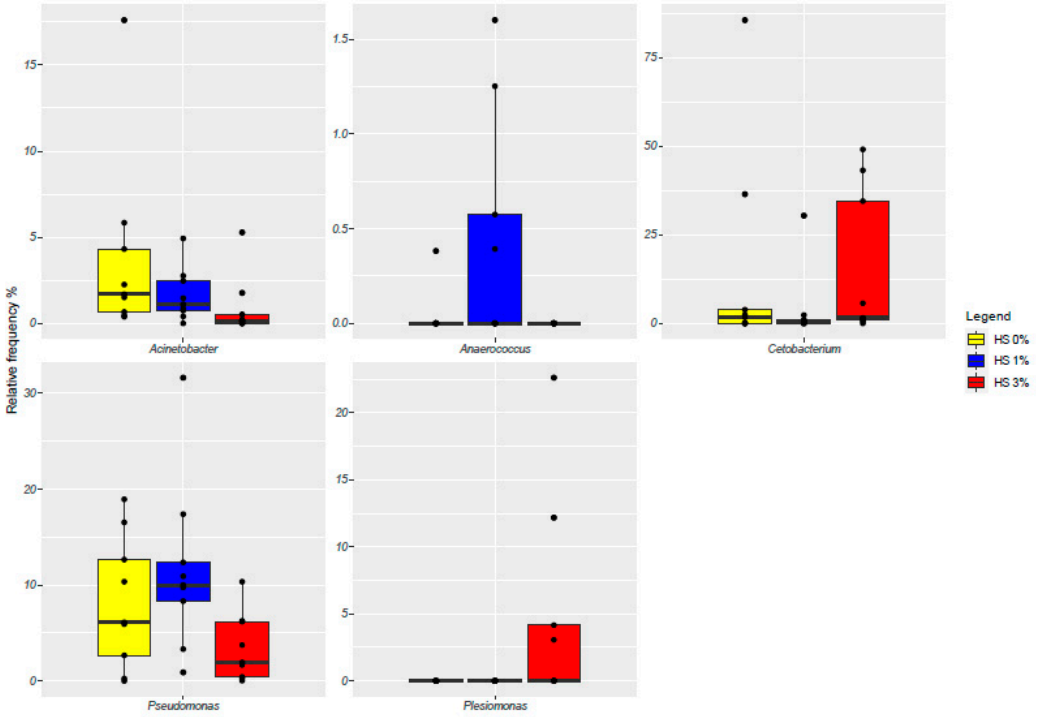


Figure 6. Boxplots of the ASVs at the genus level which were significantly different between the different levels of inclusion in *C. gariepinus* midgut samples (HS0–HS3).

3.8. Sensory Analysis of Fresh Fillets and Cooked Meat

The sensory analysis (odour, colour, texture, overall acceptability) of fresh fillets did not differ ($p > 0.05$) between the tested groups HS0–HS6 (see Figure 7).

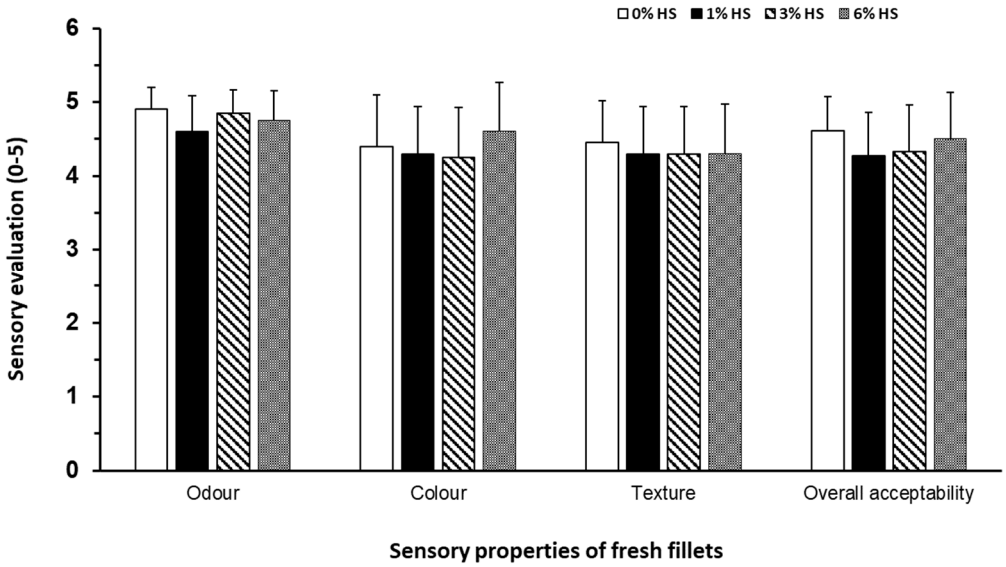


Figure 7. The results of the sensory assessment of odour, colour, texture, and overall acceptability (the sum of the points awarded by evaluators, $n = 10$) of fresh fillets of *C. gariepinus* long-term fed experimental diets (HS0–HS6). The values are presented as means ± SD. No significant differences (Tukey HSD test, $p > 0.05$) between the groups within one parameter.

The sensory analyses (odour, colour, consistency, flavour, after taste, overall acceptability) of cooked meat were assessed by the evaluators as not to be different ($p > 0.05$) between the tested groups HS0–HS6 (see Figure 8). Slightly improved odour, consistency,

flavour, after taste, and overall acceptability seemed to be associated with the HS3 group, but it was insignificant ($p > 0.05$).

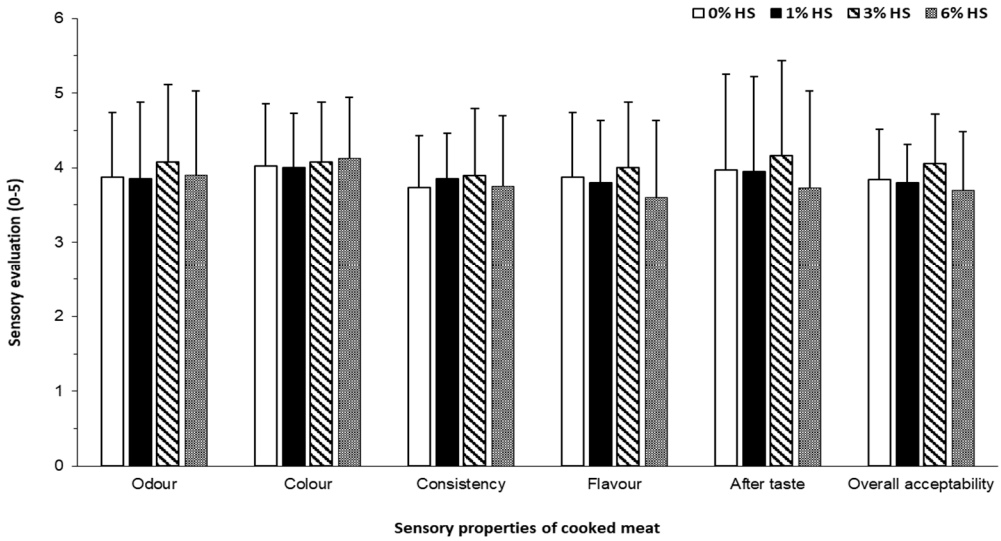


Figure 8. The results of the sensory assessment of odour, colour, consistency, flavour, after taste, and overall acceptability (the sum of the points awarded by evaluators, $n = 10$) of cooked meat of *C. gariepinus* long-term fed experimental diets (HS0–HS6). The values are presented as means ± SDs. The slopes indicate no significant differences (Tukey HSD test, $p > 0.05$) between the groups within one parameter.

3.9. Bacterial, Fungal, and Yeast Content in Rearing Environment

The water samples (50 mL/RAS) from each rearing tank after incubation were evaluated as the total count of bacteria/mL, total count of yeast/mL, and density of fungi (Table 6). The water samples were obtained from one recirculating tank from each group (HS0, HS1, HS3, HS6) of experimental fish. The total content of bacteria indicates a moderate infection in rearing tanks. The occurrence of fungi was not recorded and the number of yeasts was low. Lower numbers of bacteria were present in the tank with the HS1 and HS6 group of experimental fish; see Table 6.

Table 6. Total count of bacteria, fungi, and yeast in rearing water of each tested group (HS0–HS6).

Sample	HS0	HS1	HS3	HS6
Total bacteria count (CFU/mL)	10^6	10^5	10^7	10^5
Fungi (count)	0	0	0	0
Yeast (per mL)	3	0	1	5
Number of fish/tank (*n*)	62	59	62	68

Note: CFU—colony forming units, *n*—a number of fish. Fungi: count (+ slight, ++ moderate, +++ heavy). Yeast count/mL.

3.10. Polyphenols in Fish Diet

In the present study, the data on feed composition were supplemented by analyses of total polyphenols and flavanols in the fish diets (HS0–HS6). The laboratory analyses were performed by the AZL Laboratory of Research Institute of Brewing and Malting in Prague, Czech Republic. The increasing concentrations of HS (HS1–HS6) correspond to higher values of total polyphenols in fish feed, see Table 7.

Table 7. Content of total polyphenols and flavanols in the experimental diets (HS0–HS6).

Experimental Diet	Total Polyphenols (mg·g^{-1})	Flavanols (mg·g^{-1})
HS0	4.7	<1
HS1	4.9	<1
HS3	6.6	3.4
HS6	7.3	2.3

Note: HS0—control pellets without Humafit supplement, HS1—pellets with 1% of Humafit supplement (1 g/100 g), HS3—pellets with 3% of Humafit supplement (3 g/100 g), HS6—pellets with 6% of Humafit supplement (6 g/100 g).

4. Discussion

In the present study, the long-term effects of leonardite mineraloid dietary additive were tested in *C. gariepinus*. In total, fish were fed with four HS inclusions (HS0, HS1, HS3, and HS6) for a six-month period from juveniles (BW 28.1 ± 6.2 g) to adults (BW 733.0 ± 202.9 g). Firstly, the fish were exposed short term to HS dietary additives for a 56-day period (see published manuscript [35]) while the present study observed the prolonged long-term effects of the HS dietary additive on overall performance of intensively reared *C. gariepinus*.

During our study, the experimental fish doubled their initial BW and reached a final size that is considered an average market size for *C. gariepinus* sold in African countries [46]. However, no significant positive/negative effects of leonardite HS dietary additive (that was spray coated on commercial feed pellets) were observed on growth and production performance of *C. gariepinus* in our study. Based on our studies, it seems that the short-term and even long-term leonardite HS dietary exposure had no beneficial/detrimental effects on *C. gariepinus* growth performance from juvenile until market-size stage. Similarly, as in our study, Yilmaz et al. [33]. found no effects of HS dietary additive on fish growth performance, while other authors [18,31,32] described beneficial effects after short-term (maximum 10 weeks) HS dietary exposures.

Furthermore, no positive/negative effects in the somatic indices (VSI, GSI, HSI, SSI, VFSI, and GaSI) were observed after long-term HS dietary exposure. These indices were assessed as indicators of fish health and physical condition that might be negatively affected in relation to stress and body detoxification (e.g., liver and spleen of larger size), but they were at a physiological optimum as they did not differ from the control group.

By the end of this study, most of the sampled fish (93.8%) were sexually mature, which is typical for *C. gariepinus* of this average size (BW 733.0 ± 202.9 g). Overall mortality reached 9.3–25.3% HS0–HS6 groups by the end of our study. This result corresponds to a mortality range of 16.7–55.7% that was reported by Akinwole et al. [47] for juvenile *C. gariepinus* reared under different stocking densities for 42 days.

In the present study, the final product yield of fish (i.e., weight of eviscerated fish, head, and fillets with/without skin) was assessed. The eviscerated fish yield was 90.4–92.3%, the head was 22.9–25.2%, the fillets with skin were 49.0–51.7%, and the fillets without skin reached 41.5–42.7% of the fish BW. These results are in accordance with findings of Kouřil et al. [26] who presented a 40–43% fillet yield without skin after testing different commercial diets. While the yield of eviscerated body and fillets without skin did not differ significantly between the tested groups (HS0–HS6) by the end of the present study, the weight of head and fillets with skin differed—this finding might be a result of the slightly lower size of filleted fish in group HS6, and probably therefore even the head yield was slightly higher in this group compared to the control.

According to results from the main composition of fish meat, the content of ash was continually decreasing with the increasing water content in fish muscles and increasing Humafit concentration in the fish diet from groups HS1 to HS6. This finding corresponds to the assumption that the higher muscle water content corresponds to the lower ash content in muscle samples.

Peters and Bretschneider [48] reported that *C. gariepinus* is even able to survive on dry land for several hours. However, hypoxia, anoxia, or other stress factors can trigger a stress reaction that releases stress hormones to the blood stream [49]. Cortisol is known to increase blood glucose due to glycogenolysis and gluconeogenesis. Cortisol and glucose levels in plasma were used as indicators of stress in our experiment. After the stress challenge, glucose levels of fish exposed to lowering levels of water were significantly higher in all groups (HS1-HS6) in comparison with glucose levels before the stress challenge. Levels of glucose increased by 35%, 40%, 25%, and 33%, respectively, in samples HS0–HS6. These results are in accordance with the assumption that stress increases GLC levels in exposed animals [50]. The lowest increase in GLC levels was found in group HS3 (only by 25%). It is interesting that GLC levels were significantly increased before and after stress only in the HS6 group. The levels of COR were lower and stable in HS3 and HS6 compared to values in the same groups before stress. This finding supported the assumption of a protective effect of Humafit against a stress reaction in fish, as published by Meinelt et al. [51]. Higher cortisol levels in plasma before stress stimulus can be connected with anaesthesia and blood collection. Manipulation of fish during anaesthesia and blood collection can be sources of errors in evaluation of glucose levels [49]. According to these authors, glucose levels are a less precise indicator of stress than cortisol. Nutritional status of fish is a factor that can also affect glucose levels. However, our experimental fish in all groups (H1–H6) were fed with the same feed ad libitum, with only the HS addition being different in each group.

The increase in plasma glucose in fish is not as rapid as cortisol after stress stimuli. Many researchers have documented an increase in glucose in minutes or days after the stress [49]. Velíšek et al. [52] reported a significant increase in glucose in rainbow trout after anaesthesia with clove oil. Some results suggest that elevated glucose values are species dependant [49]. No further information is available on the increase in plasma glucose after a stress stimulus in *C. gariepinus.*

Some authors have studied the proximate composition of fish muscle and fatty acid profile of the African catfish [17,53–55]. These authors reported that the principal fatty acids in the polyunsaturated group were ALA, EPA, and DHA. Marquez-Fernandez et al. [56] studied freshwater fish under similar living conditions to African catfish, from the same order Siluriformes. According to these authors, the content of total lipids in *Pseudoplatystopma magdalenae* was 6.56–7.89% in fish muscles. In comparison with these authors, our results confirm 4.17 $\pm$ 0.71 to 5.01 $\pm$ 0.78% of total lipid content in fish muscles. In the HS1 and HS3 groups, total lipids were higher than in the control group HS0, but this difference was insignificant. With increasing water content in fish muscles (HS0–HS6), a decreasing content of ash was observed.

According to our results, fatty acid profile and nutritional index values were comparable to those for other fish samples given by Chen and Liu [44]. Marques-Fernandez et al. [56] reported a much higher content of MUFAs than PUFAs in fish muscles. In agreement with these authors, fish muscles from HS0–HS6 groups contained more MUFAs and less PUFAs. The nutritional value of fish meat and benefits to human health can be evaluated by the calculation of PUFA/SFA, IA, IT, HH, UI, and HPI indices, and the sum of EPA + DHA. In agreement with Chen and Liu [44], our results showed similar PUFA/SFA values. Kalyoncu et al. [57] reported a higher content of PUFAs than MUFAs or SFAs in *Oncorhynchus mykiss* during seasonal changes. According to these authors, the omega-3/omega-6 ratios ranged from 0.61 to 1.68 in rainbow trout during the year. In our study, the omega-3/omega-6 ratio was from 0.373 $\pm$ 0.042 to 0.427 $\pm$ 0.028 (in HS1–HS6). Significant differences occurred between HS1 and HS3 groups. From the previously published studies, it is generally known that the consumption of food with a high omega-3/omega-6 ratio is favourable for human health [12,18,53]. The fish oils of Pacific fish species studied by Abbas et al. [53] contained a high omega-3/omega-6 ratio, from 6.4 to 18.6. According to these authors, levels of total PUFA and DHA content were much higher in the lean and low-fat fish than in fattier species. Guler et al. [58] reported a lipid content ranging from 1.09 to 4.45% w.w.

during the season, and an omega-3/omega-6 ratio ranging from 0.50 to 1.06 in common carp.

In all health-promoting indices, the lowest values of HH, HPI, UI, omega-3/omega-6 ratio, sum of EPA + DHA, and PUFA/SFA ratio were observed in the HS3 group. These results correspond with the highest values of pro-thrombotic and atherogenicity indices (IA, IT) in this group. The group HS6 (with 6% of HS additive) did not show the same tendency. This observation can probably be connected with the presence of different microflora colonizing the fish gut in the HS3 group.

In *C. gariepinus*, dominant groups of microorganisms were mainly *Ralstonia, Pseudomonas,* and *Cetobacterium* genera. In fish fed with 3% of HS (from HS3 group), the relative abundance of *Pseudomonas* decreased while the presence of *Cetobacterium* increased. In the same HS3 group, the highest relative abundance of *Cetobacterium* and *Plesiomonas* was observed. *Plesiomonas* was included in the Vibrionaceae family but was recategorized to the family Enterobacteriaceae, consisting only of one species *Plesiomonas shigelloides.* This bacterium is an aquatic organism, present in freshwater and seawater. This is a pathogenic microorganism in humans, connected with foodborne diseases causing gastrointestinal illness and diarrhoea due to the consumption of contaminated water and raw or undercooked fish and shellfish [59]. This microorganism produces a cholera-like enterotoxin. Especially, high mortality occurs among young children and immunocompromised individuals. *Cetobacterium* is an anaerobic bacterium present in aquatic environments and can colonize the intestinal tract of freshwater fish, including goldfish and common carp [60]. *Acinetobacter* genus of bacteria are connected with foodborne diseases. They occur as contaminants of dairy products, undercooked food connected with the consumption of raw fruits and vegetables that are irrigated with contaminated water. They may be risky, especially to immunocompromised individuals and young children. Infections with *Acinetobacter baumannii* are connected with increasing morbidity, mortality, and multiple-drug resistance [61]. Probably due to the high abundance of *Plesiomonas* in the HS3 group, worse values of all nutritional indices were observed in HS3 comparing to the others. The occurrence of a 10 times higher total bacterial count in RAS may contribute to this situation. Probably, due to these factors, fish from RAS in the experimental HS3 group were debilitated. According to our results, the main abundant genus of fish microbiome, *Plesiomonas* and *Acinetobacter,* are considered risky to humans. Their presence in aquatic environments is undesirable. A decreasing relative abundance of *Acinetobacter* genus in the fish microbiome with increasing percentage of HS in the experimental diet is a positive finding reflecting the effectiveness of increasing HS additive in preventing the spread of these pathogenic microorganisms in aquatic environments.

Polyphenolic compounds have many beneficial effects on living organisms. Polyphenols are known as powerful antioxidants. Farm animals (as herbivorous animals) are naturally exposed to substantial amounts of polyphenols in natural feeding [62]. Jahazi et al. [63] reported a positive effect in total antioxidant capacity with increasing levels of polyphenols in fish feed for common carp juveniles fed for 8 weeks in their experiment. Wang et al. [64] reported positive effects of dietary polyphenol supplementation on gut microbiota in animals by increasing beneficial and decreasing harmful microorganisms. In another experiment, the authors reported modulatory effects of tannins allowing the recovery of normobiotic conditions after inflammatory insult in zebrafish [65]. Gong et al. [66] reported a similar significant effect in zebrafish (*Danio rerio*) on the intestinal microbiota after exposure to fermented polyphenols. In our present study, we investigated the content of polyphenols in fish fed with Humafit as an additive in experiments with *C. gariepinus*. According to our results, a higher concentration of HS additive in fish feed reflected a higher value of total polyphenols. The positive effect, observed as a decreasing abundance of the genus *Acinetobacter,* can be connected with an increasing value of total polyphenols in an experimental fish diet. According to our next results regarding the GSH/GSSG ratio expressing the antioxidant status of fish, the highest GSH/GSSG ratio was observed in the HS6 group. This result supports the assumption about the positive effect of HS

supplementation on the antioxidant status of fish. Probably, the higher total polyphenols in the fish diet represents an increasing GSH/GSSG ratio in the long-term experiment in *C.gariepinus.*

After fish processing, the sensory analysis of fresh and cooked fish meat was assessed by 10 experienced evaluators. They evaluated that the HS dietary additive had no significant positive/negative effects on the visual appearance of fresh fillets (odour, colour, texture, overall acceptability) and even the sensory qualities of cooked meat (odour, colour, consistency, flavour, after taste, overall acceptability). Nevertheless, some authors published significant effects of various feed additives or alternative components, e.g., fish oil substitutes published by Turchini et al. [67] on selected organoleptic properties of fish meat.

5. Conclusions

Fish differ in their content of unsaturated fatty acids and are a valuable source of PUFAs and MUFAs in human nutrition due to their protective effects on human health in the prevention of cardiovascular disease. The results of our study yielded a comprehensive set of experimental results about African catfish *C. gariepinus* fed with humic substances as additives. Moreover, it yielded missing information about the composition of fatty acids and nutritional indices, composition of the microbiome, stress resistance, and sensory properties of fish muscles of *C. gariepinus*. Although a significant effect of HS additive on the overall performance of *C. gariepinus* was not proven, the positive effect on antioxidant status was seen, as well as the suppression of gut microorganisms that can be present as pathogenic contaminants in the aquatic environment. Higher concentrations of HS in the diet also slightly improved the defence of *C. gariepinus* against stress. For future research, we recommend repeating the stress test with the same and other stress factors. It would also be good to extend the blood collection time and use a more detailed dilution of HS additives in the range between 3% and 6% in order to obtain more information about the effect of HS additives on the protection of fish against stress and their effect on the composition of the gut microbiota.

Author Contributions: Conceptualization, M.B. and M.P. (Markéta Prokešová); investigation, methodology, M.B. and M.P. (Markéta Prokešová); sample collection and analysis, M.P. (Markéta Prokešová), M.B., M.D., V.I., D.R., M.Z., J.M., I.F. (Ilario Ferrocino), I.F. (Irene Franciosa) and M.P. (Matěj Přeslička); validation, M.P. (Markéta Prokešová), M.B., L.K., I.F. (Ilario Ferrocino), I.F. (Irene Franciosa) and M.D.; statistics, M.P. (Markéta Prokešová), L.K., I.F. (Ilario Ferrocino) and I.F. (Irene Franciosa); writing—original draft preparation, M.B., M.P. (Markéta Prokešová), L.K. and P.S.; visualization, M.P. (Markéta Prokešová), L.K., I.F. (Ilario Ferrocino) and I.F. (Irene Franciosa). All authors have read and agreed to the published version of the manuscript.

Funding: The study was financially supported by the Ministry of Agriculture of the Czech Republic—NAZV project QK1810296 and the project COOPERATIO/PROGRES Q25/1.LF Charles University in Prague. This work was also supported by the METROFOOD-CZ research infrastructure project (MEYS Grant No: LM2023064) including access to its facilities.

Institutional Review Board Statement: The experimental procedure conformed to the Directive No. 2010/63/EU of the European Union and was authorized by the Ministry of Health of the Czech Republic regarding the protection of animals used for scientific purposes.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. EUROSTAT. Causes and Occurrence of Deaths in the EU. 2019. Available online: www.ec.europa.eu/eurostat/web/products-eurostat-news/-/DDN-20190716-1 (accessed on 10 June 2021).
2. Aranceta, J.; Pérez-Rodrigo, C. Recommended dietary reference intakes, goals and dietary guidelines for fat and fatty acids: A systematic review. *Br. J. Nutr.* **2012**, *107*, S8–S22. [CrossRef]

3. Rimm, E.B.; Appel, L.J.; Chiuve, S.E.; Djoussé, L.; Engler, M.B.; Kris-Etherton, P.M.; Mozaffarian, D.; Siscovick, D.S.; Lichtenstein, A.H. American Heart Association Nutrition Committee of the Council on Lifestyle and Cardiometabolic Health. Seafood Long-Chain n-3 Polyunsaturated Fatty Acids and Cardiovascular Disease: A Science Advisory, From the American Heart Association. *Circulation* **2018**, *138*, e35–e47. [CrossRef]
4. Shijo, Y.; Chizuko, M.; Nakamura, E.; Nakano, R.; Shima, M.; Mae, A.; Okabe, Y.; Park, S.; Kameyama, N.; Hirai, S. Japan diet intake changes serum phospholipid faatty acid compositions in middle-aged men: A pilot study. *J. Atheroscler. Thromb.* **2018**, *26*, 3–13. [CrossRef] [PubMed]
5. Breslow, J.L. N-3 fatty acids and cardiovascular disease. *Am. J. Clin. Nutr.* **2006**, *83*, 1477–1482. [CrossRef] [PubMed]
6. Nestel, P.; Clifton, P.; Colquhoun, D.; Noakes, M.; Mori, T.A.; Sullivan, D.; Thomas, B. Indications for omega-3 long chain polyunsaturated fatty acid in the prevention and treatment of cardiovascular disease. *Heart Lung Circ.* **2015**, *24*, 769–779. [CrossRef] [PubMed]
7. Jabeen, F.; Chaudhry, A. Chemical compositions and fatty acid profiles of three freshwater fish species. *Food Chem.* **2011**, *125*, 991–996. [CrossRef]
8. Nestel, P.J.N. Fish oil and cardiovascular disease: Lipids and arterial function. *Am. J. Clin. Nut.* **2001**, *71*, 228–231. [CrossRef]
9. FAO. *The State of World Fisheries and Aquaculture 2008*; Fisheries and Aquaculture Department of the Food and Agriculture Organization (FAO) of the United Nations: Rome, Italy, 2009.
10. Sidhu, K.S. Health benefits and potential risks related to consumption of fish or fish oil. *Regul. Toxicol. Pharmacol.* **2003**, *38*, 336–344. [CrossRef]
11. Ljubojević, D.; Ćirković, M.; Novakov, N.; Jovanović, R.; Janković, S.; Đorđević, V.; Mašić, Z. Productivity and meat nutrient in fish: The diet effect. *Kafkas Univ. Vet. Fak. Derg.* **2013**, *19*, 43–49. [CrossRef]
12. Etherton, K.P.M.; Harris, W.S.; Appel, L.J. Fish consumption, fish oil, omega-3 fatty acids and cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, 20–31.
13. Raffenbeul, W. Fish for a healthy heart. *Eur. J. Fat Sci. Technol.* **2001**, *103*, 315–317. [CrossRef]
14. Saoud, I.P.; Batal, M.; Ghanawi, J.; Lebbos, N. Seasonal evaluation of nutritional benefits of two fish species in the Eastern Mediterranean Sea. *Int. J. Food Sci. Technol.* **2008**, *43*, 538–542. [CrossRef]
15. Lands, W.E.M. *Fish and Human Health*; Academic Press: Cambridge, MA, USA, 1986; p. 170.
16. Stickney, R.R.; Hardy, R.W. Lipid requirements of some warm water species. *Aquaculture* **1989**, *79*, 145–156. [CrossRef]
17. Osibona, A.O.; Kusemiju, K.; Akande, G.R. Proximate composition and fatty acids profile of the African Catfish, *Clarias gariepinus*. *Acta Satech.* **2009**, *3*, 85–89.
18. Gao, Y.; He, J.; He, Z.; Li, Z.; Zhao, B.; Mu, Y.; Lee, J.-Y.; Chu, Z. Effects of fulvic acid on growth performance and intestinal health of juvenile loach Paramisgurnus dabryanus (Sauvage). *Fish Shellfish Immunol.* **2017**, *62*, 47–56. [CrossRef]
19. Raatz, S.K.; Silverstein, J.T.; Jahns, L.; Picklo, M.J. Issues of fish consumption for cardiovascular disease risk reduction. *Nutrients* **2013**, *5*, 1081–1097. [CrossRef]
20. Sushchik, N.N.; Gladyshev, M.I.; Kalachova, G.S. Seasonal dynamic of fatty acid content of a common food fish from the Yenisei River, Siberian grayling, *Thymallus arcticus*. *Food Chem.* **2007**, *104*, 1353–1358. [CrossRef]
21. Bušová, M.; Kouřimská, L.; Tuček, M. Fatty acids profile, atherogenic and thrombogenic indices in freshwater fish common carp (*Cyprinus carpio*) and rainbow trout (Oncorhynchus mykiss) from market chain. *Cent. Eur. J. Public Health* **2020**, *28*, 313–319. [CrossRef] [PubMed]
22. Van de Nieuwegiessen, P.G.; Olwo, J.; Khong, S.; Verreth, J.A.J.; Schrama, J.W. Effects of age and stocking density on the welfare of African catfish, Clarias gariepinus Burchell. *Aquaculture* **2009**, *288*, 69–75. [CrossRef]
23. Mota, V.C.; Limbu, P.; Martins, C.I.M.; Eding, E.H.; Verreth, J.A.J. The effect of nearly closed RAS on the feed intake and growth of Nile tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*) and European eel (*Anguilla anguilla*). *Aquac. Eng.* **2015**, *68*, 1–5. [CrossRef]
24. Ajiboye, A.O.; Awogbade, A.A.; Babalola, O.A. Effects of water exchange on water quality parameters, nutrient utilization and growth of African catfish (*Clarias gariepinus*). *Int. J. Livest. Prod.* **2015**, *6*, 57–60.
25. Appelbaum, S.; Kamler, E. Survival, growth, metabolism and behaviour of *Clarias gariepinus* (Burchell 1822) early stages under different light conditions. *Aquacult. Eng.* **2000**, *22*, 269–287. [CrossRef]
26. Kouřil, J.; Drozd, B.; Prokešová, M.; Stejskal, V. *Intensive Rearing of African Sharptooth Catfish (Clarias gariepinus), Methodological Edition*; FFPW USB: Vodnany, Czech Republic, 2013; Volume 138, p. 60. ISBN 978-80-87437-79-7. (In Czech)
27. Ampong, K.; Thilakaranthna, M.S.; Gorim, L.Y. Understanding the Role of Humic Acids on Crop Performance and Soil Health. *Front. Agron.* **2022**, *4*, 848621. [CrossRef]
28. EMEA. Committee for Veterinary Medicinal Products. Humic Acids and their Sodium Salts. Summary Reports. EMEA/MRL/554/99-Final. 1999. Available online: https://www.ema.europa.eu/en/documents/mrl-report/humic-acids-their-sodium-salts-summary-report-committee-veterinary-medicinal-products_en.pdf (accessed on 20 October 2022).
29. Peña-Méndez, E.M.; Havel, J.; Patočka, J. Humic substances—Compounds of still unknown structure: Applications in agriculture, industry, environment, and biomedicine: A review. *J. Appl. Biomed.* **2005**, *3*, 13–24. [CrossRef]
30. Islam, K.M.S.; Schuhmacher, A.; Gropp, J.M. Humic Acid Substances in Animal Agriculture. *Pak. J. Nutr.* **2005**, *4*, 126–134.
31. Sharaf, M.M.; Tag, H.M. Growth performance, gill, liver and kidney histomorphology of common carp (*Cyprinus carpio*) fingerlings fed humic acid supplemented diets. *Egypt. J. Exp. Biol.* **2011**, *7*, 285–294.

32. Abdel-Wahab, A.M.; El-Refaee, A.M.E.; Ammar, A.A. Effects of humic acid as feed additive in improvement of nonspecific immune response and disease resistance in common carp (*Cyprinus carpio*). *Egypt. J. Aquacult.* **2012**, *2*, 83–90.
33. Yilmaz, S.; Ergun, S.; Çelik, E.S.; Yigit, M. Effects of dietary humic acid on growth performance, haemato-immunological and physiological responses and resistance of Rainbow trout, *Oncorhynchus mykiss* to Yersinia ruckeri. *Aquac. Res.* **2018**, *49*, 3338–3349. [CrossRef]
34. Lieke, T.; Steinberg, C.E.W.; Pan, B.; Perminova, I.V.; Meinelt, T.; Knopf, K.; Kloas, W. Phenol-rich fulvic acid as a water additive enhances growth, reduces stress, and stimulates the immune system of fish in aquaculture. *Sci. Rep.* **2021**, *11*, 174. [CrossRef]
35. Prokešová, M.; Bušová, M.; Zare, M.; Tran Quang, H.; Kučerová, E.; Ivanova, A.P.; Gebauer, T.; Stejskal, V. Effect of humic substances as feed additive on the growth performance, antioxidant status, and health condition of African catfish (*Clarias gariepinus*, Burchell 1822). *Animals* **2021**, *11*, 2266. [CrossRef]
36. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2013**, *41*, e1. [CrossRef]
37. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]
38. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.; Holmes, S.P. DADA2, High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
39. *ČSN ISO 712*; Cereals and Cereal Products—Determination of Moisture Content—Routine Reference Method. (In Czech). Available online: https://www.technicke-normy-csn.cz/csn-iso-712-461014-206263.html (accessed on 23 June 2022).
40. EU. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011. *Off. J. Eur. Union* **2011**, *304*, 18–63.
41. Folch, J.; Lees, M.; Stanley, G.H.S. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [CrossRef]
42. IUPAC; Commission on Oils, Fats, and Derivatives. Methods 2.301—Preparation of the Fatty Acid Methyl Esters. In *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th ed.; Paquot, C., Hautfenne, A., Eds.; Blackwell Sci. Publications: Oxford, UK, 1987; Eurostat, 2019.
43. Pohořelá, B.; Gramblička, T.; Doležal, M.; Dvořáková, D.; Pulkrabová, J.; Kouřimská, L.; Ilko, V.; Pánek, J. Nutritional Quality and Assessment of Contaminants in Farmed Atlantic Salmon (*Salmo salar* L.) of Different Origins. *J. Food Qual.* **2022**, *2022*, 9318889. [CrossRef]
44. Chen, J.; Liu, H. Nutritional Indices for Assessing Fatty Acids: A Mini-Review. *Int. J. Mol. Sci.* **2020**, *21*, 5695. [CrossRef] [PubMed]
45. *ISO 8589*; Sensory Analysis-General Guidance for the Design of Test Rooms. 2007. Available online: http://www.iso.org/iso/catalogue_detail.htm?csnumber536385 (accessed on 23 June 2022).
46. FAO. *Clarias gariepinus. Cultured Aquatic Species Information Programme*; Text by Pouomogne, V; Fisheries and Aquaculture Division: Rome, Italy, 2022.
47. Akinwole, A.O.; Bankole, A.F.; Dauda, A.B.; Saliu, O.E. Growth and survival of *Clarias gariepinus* (Burchell 1822) fingerlings cultured at different stocking densities in Igboora, Oyo State, Nigeria. *J. Agric. Biodivers. Res.* **2014**, *3*, 58–60.
48. Peters, R.C.; Bretschneider, F. Electroreceptive microampullae in the African mudfish *Clarias lazera* (Cuv. And Val., 1840). In *Sensory Physiology of Aquatic Lower Vertebrates*; Szabó, T., Czéh, G., Eds.; Pergamon Press: Oxford, UK, 1981; pp. 13–27. [CrossRef]
49. Martínez-Porchas, M.; Martines-Córdova, L.R.; Ramos-Enriques, R. Cortisol and Glucose: Reliable indicators of fish stress? *PAN-Am. J. Aquat. Sci.* **2009**, *4*, 158–178.
50. Sopinka, N.M.; Donaldson, M.R.; O'Connor, C.M.; Suski, C.D.; Cooke, S.J. Stress Indicators in Fish. In *Fish Physiology*; Tort, L., Farrell, A.P., Brauner, C.J., Eds.; Academic Press: Cambridge, MA, USA, 2016; Volume 35, pp. 405–462. [CrossRef]
51. Meinelt, T.; Schreckenbach, K.; Pietrock, M.; Heidrich, S.; Steinberg, C.E. Humic substances. Part 1, Dissolved humic substances (HS) in aquaculture and ornamental fish breeding. *Environ. Sci. Pollut. Res. Int.* **2008**, *15*, 17–22. [CrossRef]
52. Velíšek, J.; Svobodová, Z.; Piačková, V. Effects of clove oil anaesthesia on rainbow trout (*Oncorhynchus mykiss*). *Acta Vet. Brno* **2005**, *74*, 139–146. [CrossRef]
53. Abbas, K.A.; Mohamed, A.; Jamilah, B. Fatty acids in fish and beef and their nutritional values: A review. *J. Food Agric. Environ.* **2009**, *7*, 37–42.
54. Abaho, I.; Bwanika, G.; Walekhwa, P.; Kwetegyeka, J.; Arinaitwe, A. Fatty acid profiles and growth of African catfish (Clarkias gariepinus, Burchell, 1822) larvae fed on freshwater rotifer (*Brachionus calyciflorus*) and Artemia as live starter feeds. *Int. J. Fish. Aquat. Stud.* **2015**, *4*, 189–196.
55. Ng, W.-K.; Lim, P.-K.; Boey, P.-L. Dietary lipid and palm oil source affects growth, fatty acid composition and muscle α-tocopherol concentration of African catfish, *Clarias gariepinus*. *Aquaculture* **2003**, *215*, 229–243. [CrossRef]
56. Márquez-Fernández, P.M.; Márquez, E.R.; Ruiz-Villadiego, O.S.; Márquez-Fernandez, D.M. Nutritional value of fatty acids of the Neotropical freshwater fishes *Prochilodus magdalenae*, *Pseudoplatystoma magdaleniatum* and *Ageneiosus pardalis*. *Grasas Y Aceites* **2020**, *71*, e342. [CrossRef]
57. Kalyoncu, L.; Yaman, Y.; Aktumsek, A. Determination of the seasonal changes on total fatty acid composition of rainbow trout, *Oncorhynchus mykiss* in Ivriz Dam Lake, Turkey. *Afr. J. Biotechnol.* **2010**, *9*, 4783–4787.

58. Guler, G.O.; Kiztanir, B.; Aktumsek, A.; Citil, O.B.; Ozparlak, H. Determination of the seasonal changes on total fatty acid composition and ω3/ω6 ratios of carp (*Cyprinus carpio* L.) muscle lipids in Beysehir Lake (Turkey). *Food Chem.* **2008**, *108*, 689–694. [CrossRef]
59. Levin, R.E. Bacteria: Plesiomonas shigelloides. In *Encyclopedia of Food Safety*; Yasmine, M., Ed.; Academic Press: Cambridge, MA, USA, 2014; pp. 480–485. ISBN 9780123786135. [CrossRef]
60. Tsuchiya, C.; Sakata, T.; Sugita, H. Novel ecological niche of Cetobacterium somerae, an anaerobic bacterium in the intestinal tracts of freshwater fish. *Lett. Appl. Microbiol.* **2008**, *46*, 43–48. Erratum in: *Lett. Appl. Microbiol.* **2008**, *46*, 282. [CrossRef]
61. Amorim, A.M.; Nascimento, J.D. Acinetobacter: An underrated foodborne pathogen? *J. Infect. Dev. Ctries.* **2017**, *11*, 111–114. [CrossRef]
62. Hashem, N.M.; Gonzalez-Bulnes, A.; Simal-Gandara, J. Polyphenols in Farm Animals: Source of Reproductive Gain or Waste? *Antioxidants* **2020**, *9*, 1023. [CrossRef]
63. Jahazi, M.A.; Hoseinifar, S.H.; Jafari, V.; Hajimoradloo, A.; Doan, H.V.; Paolucci, M. Dietary supplementation of polyphenols positively affects the innate immune response, oxidative status, and growth performance of common carp, *Cyprinus carpio* L. *Aquaculture* **2020**, *517*, 734709. [CrossRef]
64. Wang, X.; Qi, Y.; Zheng, H. Dietary Polyphenol, Gut Microbiota, and Health Benefits. *Antioxidants* **2022**, *11*, 1212. [CrossRef] [PubMed]
65. Orso, G.; Solovyev, M.M.; Facchiano, S.; Tyrikova, E.; Sateriale, D.; Kashinskaya, E.; Pagliarulo, C.; Hoseinifar, H.S.; Simonov, E.; Varricchio, E.; et al. Chestnut Shell Tannins: Effects on Intestinal Inflammation and Dysbiosis in Zebrafish. *Animals* **2021**, *11*, 1538. [CrossRef]
66. Gong, X.; Jiang, S.; Tian, H.; Xiang, D.; Zhang, J. Polyphenols in the Fermentation Liquid of *Dendrobium candidum* Relieve Intestinal Inflammation in Zebrafish Through the Intestinal Microbiome-Mediated Immune Response. *Front. Immunol.* **2020**, *17*, 1542. [CrossRef] [PubMed]
67. Turchini, G.M.; Hermon, K.; Moretti, V.M.; Caprino, F.; Busetto, M.L.; Bellagamba, F.; Rankin, T.; Francis, D.S. Seven fish oil substitutes over a rainbow trout grow-out cycle: II) Effects on final eating quality and a tentative estimation of feed-related production costs. *Aquac. Nutr.* **2013**, *19*, 95–109. [CrossRef]