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# THE MULTI-ENZYMES AND PROBIOTICS MIXTURE IMPROVES THE GROWTH PERFORMANCE, DIGESTIBILITY, INTESTINAL HEALTH, AND IMMUNE RESPONSE OF SIBERIAN STURGEON (*ACIPENSER BAERII*)

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**Abstract**

**The inclusion of exogenous digestive enzymes and probiotics is well established in the aquafeed industry. The mixture of multi-enzymes and probiotics improves the feed utilization and wellbeing of aquatic animals compared to the individual supplementation. Herein, we evaluated the exogenous multi-enzyme mixture (beta-glucanase, cellulase, alpha-amylase, protease, xylanase, and phytase) at 250 mg/kg and multi-species probiotic (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum*, and *Pediococcus acidilactici*; $1 \times 10^{10}$ CFU/g for each bacterial strain) at 2 g/kg on the performances of Siberian sturgeon. The final weight, weight gain, SGR, and PER were markedly enhanced while the FCR was reduced in fish fed multi-enzyme and probiotics premix ($P<0.05$). Multi-enzymes and probiotics mixture significantly increased the total body protein content ($P>0.05$). Multi-enzymes and probiotics mixture also improved the digestibility of crude protein, dry matter, and crude lipids nutrients ($P<0.05$). The count of goblet cells, microvilli diameter, microvilli length, outer muscle wall diameter, and enterocyte total absorptive surface were markedly increased ($P<0.05$) by dietary multi-enzymes and probiotics mixture. The WBCs and neutrophils showed marked improvements ($P<0.05$). The levels of glucose, triglycerides, blood urea nitrogen, and total bilirubin were markedly higher in fish fed the control than fish fed the multi-enzymes and probiotics mixture ($P<0.05$). Significantly, Siberian sturgeon-fed dietary multi-enzymes and probiotics had improved lysozyme activity, total immunoglobulin, and total protein in the skin mucus and serum samples ($P<0.05$). Further, the serum complement C3 and C4 was higher in fish-delivered multi-enzymes and probiotics mixture than in control ($P<0.05$). In conclusion, dietary probiotics synergistically enhanced the activity of multi-enzymes and resulted in increased feed utilization, nutrient digestibility, and health status of Siberian sturgeon.**

**Key words: aquaculture, exogenous enzymes, probiotics, digestibility, growth promoter, nutrient digestibility**

Aquaculture activity is significantly contributing to food safety for the increased population (FAO, 2020). The sustainability of aquaculture requires suitable alternatives for the traditional resources involved in aquatic animals farming (Galappaththi et al., 2020). Aquafeed, water quality, seed production, and infection control are the primary requirements for a successful aquaculture industry (Tachibana et al., 2020; Dawood et al., 2021). The high cost of fish meal and low availability result in lower-cost plant ingredients in the aquafeed formulation (Randazzo et al., 2021). Aquatic animals are monogastric species that cannot digest plant ingredients thoroughly (Dawood and Koshio, 2020). Incorporating growth-promoting agents (Dawood et al., 2020), exogenous digestive enzymes (Maas et al., 2021 a), and probiotics (Assan et al., 2022) may help improve the digestibility and absorption of nutrients. Plant ingredients contain high amounts of fibers and antinutritional factors (ANFs) that cannot be digested efficiently in the gastrointestinal tract (GIT) (Tidwell et al., 2021). Consequently, exogenous digestive enzymes are applied to enhance the digestibility of plant ingredients in aquafeed (Velázquez-De Lucio et al., 2021). Phytase, cellulase, amylase, xylanase, and hemicellulase can catalyze the carbohydrates and improve their digestibility in the GIT (Adeola and Cowieson, 2011). Exogenous digestive enzymes increase the digestibility of plant ingredients that contain high amounts of non-starch polysaccharides (NSP) to volatile fatty acids (VFA) that can be easily absorbed through the GIT (Williams et al., 2001; Abdel-Latif et al., 2020). Indeed, the inclusion of exogenous digestive enzymes improved the utilization of plant-based ingredients in several fish species (Huang et al., 2020; Luo et al., 2020; Monier, 2020). Concurrently, feed digestibility, intestinal health, GIT microbiota, physiological function, and growth performances did not deteriorate.

Probiotics, on the other hand, have been proven as functional feed additives (El-Saadony et al., 2021). Mark-

edly, probiotic supplementation caused high feed digestibility through the secretion of digestive enzymes in the GIT (Assan et al., 2022). Lactic acid bacteria (LAB) are the most functional bacterial species associated with feed utilization, intestinal digestion, metabolic and physiological regulation, and the entire body's immunity (Melo-Bolívar et al., 2021). The feed utilization and digestion capacity of probiotics have been investigated in many studies related to aquatic animals (Wuertz et al., 2021). Various bacterial species (e.g., *Bacillus*, *Lactobacillus*, and *Pediococcus*) were included in aquafeed (Adel et al., 2021).

Using exogenous digestive enzymes and probiotic mixtures is a possible strategy to improve feed digestion and productivity of aquatic animals (Velázquez-De Lucio et al., 2021). In this regard, the incorporation of probiotics (*Bacillus amyloliquefaciens*) and enzymes (xylanase and phytase) enhanced the nutrient digestibility and GIT microbial balance in Nile tilapia (*Oreochromis niloticus*) (Maas et al., 2021 c; Maas et al., 2021 b). Further, *Bacillus pumilus* and exogenous protease regulated the growth performance, feed utilization, and blood haemato-biochemical indices in Nile tilapia (Hassaan et al., 2021). In snakehead (*Channa argus*), the mixture of *B. amyloliquefaciens*, amylase, protease, and papain enhanced feed digestibility, growth performance and regulated the diversity of microorganisms in the GIT (Dai et al., 2019). Herein, we hypothesized that the mixture of exogenous digestive enzymes and probiotics may increase digestibility, feed utilization, growth performance, and the health status of Siberian sturgeon (*Acipenser baerii*), a leading aquaculture candidate.

## Material and methods

### Formulation of the experimental diets

The ingredients and proximate composition of the experimental diets are displayed in Table 1. Two isocaloric, isolipidic, and isonitrogenous diets were formulated to have a diet with a mixture of 250 mg/kg exogenous multi-enzymes and 2 g/kg commercial probiotic mixture (Enz+Pro cocktail diet) and also a diet without any additives (control diet). First, the dietary components were ground and sieved to make a fine powder. The powder was thoroughly mixed with specific amounts of the supplements, and then the oils were added. Afterward, water (200–300 g/kg) and molasses were poured gradually into the mixture to make a stiff paste. After mixing, the dough was pelleted via a meat grinder (3 mm diameter), and the prepared strands were air-dried overnight. During the drying, the pellets were hand-stirred frequently to dry evenly and consequently kept in sealed bags at –20°C until daily feeding.

The commercial exogenous multi-enzyme (Kemzyme® WP dry; beta-glucanase, cellulase, alpha-amylase, protease, xylanase, and phytase) was prepared from Kemin® company (Kemin Industries Herentals, Belgium), and the selected concentration was based on the optimum growth dose for Beluga sturgeon (*Huso huso*) (Ghomi et al., 2012) and the recommendation of the manufacturer. The commercial multi-species probiotic (BIOguil™; *Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici*) was obtained from Zist Yar Varna Company, which is located in Guilan Technology Park, Rasht, Iran. The selected concentration of the commercial probiotic mixture was based on the company's recommendation. LINDO 6.1 software was also used to formulate the diets according to the nutritional requirements of *A. baerii* (Falahatkar, 2018).

### Fish farming system

In the present study, the fish husbandry was carried out in the Dadman International Sturgeon Research Institute (Guilan, Iran). Initially, 150 Siberian sturgeon were selected and maintained for 14 days to adapt to the new conditions. The juveniles were fed with the control diet during the acclimatization period. After that, 120 fish with an average weight of 151.1 ± 2.7 g (mean±SD) were randomly assigned to 6 indoor circular tanks (1 m diameter, 70 cm height, and 350-L). The water was supplied from a mixture of the Sepid-Rud River and well water, and it was at a maximum flow rate of 20 l/min in each tank. The fish containers were continuously aerated using two air stones per tank. The water quality parameters were monitored during the rearing period, and they were 20.3±1.4°C temperature, 6.8±0.3 mg/l dissolved oxygen, and 7.9±0.1 pH. The nitrite and non-ionized ammonia were also measured by photometry and were less than 0.1 and 0.03 mg/l, respectively. This study was performed under the natural photoperiod (average 13.5 h: 10.5 h, light: dark).

Fish were fed the experimental diets by hand three times per day at 7:30, 12:30, and 17:30 up to the satiation level for eight weeks. All the tanks were siphoned to remove wastes and feces every day.

### Zootechnical performance and efficiency

All the fish were bio-assayed at the beginning and end of the trial in each tank. Accordingly, the juveniles were deprived of feed for 24 h, anesthetized with clove powder (flower buds powder, *Syzygium aromaticum*, 150 mg/l), and subsequently, each fish's fork length and weight were measured. The growth and nutritional aspects were calculated using the following mathematical equations:

Weight gain (WG, g) = $W_i$ (g) – $W_f$ (g)

Body weight increase (BWI, %) = 100 × ($W_f$ (g) – $W_i$ (g)/ $W_i$ (g))

Specific growth rate (SGR, %/day) = 100 × [Ln $W_f$ (g) – Ln $W_i$ (g)] / d

K (condition factor, g/cm) = 100 × [$W_f$ (g) /

$W_i$, initial weight; $W_f$, final weight; d, days (=56);

$No_i$, initial number; $No_f$, final number.

fork length ($cm^3$)]

Feed intake (FI, g/fish/day) = total feed intake per fish/d

Feed conversion ratio (FCR) = consumed feed (g) / weight gain (g)

Protein efficiency ratio (PER) = 100 × (weight gain (g) / consumed protein (g))

SR (%) = 100 × $No_f$ of fish / $No_i$ of fish

**Blood and epidermal mucus sampling**

At the end of the rearing period, the blood samples were drawn from the caudal vein of the fish (4 fish from each replicate) by syringes. Afterward, 0.5 ml of the blood was mixed with heparin to prevent blood clots for hematological assays. The remaining blood sample (~ 1.5 ml) was transferred into non-heparinized tubes and centrifuged at 7500 ×*g* for 12 min at 4ºC to collect the supernatants (serum samples).

Skin mucus collection was performed at the end of the feeding experiment according to Subramanian et al. (2007) method with slight modification. Briefly, 12 fish from each treatment (4 fish per tank) were randomly caught, and each fish was individually placed in sealed polyethylene bags (Badook™ double-zip bags) containing 5 ml of 50 mM sodium chloride (NaCl, Merck). The bags were gently rubbed for 1–2 min to allow the fish to secret enough mucus. Finally, the fresh mucus was collected in 15 ml sterile tubes and centrifuged to obtain the supernatants by centrifugation method (500 ×*g*, 10 min, 4°C). The supernatants were labeled and stored in a freezer at –80°C until further testing. After the mucus collection, the fish were transferred to a pre-oxygenated tank to recover.

Table 1. Dietary ingredients and proximate composition (g/kg) of the experimental Siberian sturgeon (*Acipenser baerii*) diets

| Feedstuffs | Experimental diets (g/kg) | |
|---|---|---|
| | Control | Enz+Pro cocktail |
| Herring fishmeal[1] | 338 | 338 |
| Soybean meal[2] | 180 | 180 |
| Wheat flour | 229 | 229 |
| Fish oil | 65 | 65 |
| Soybean oil | 65 | 65 |
| Molasses | 20 | 20 |
| Mineral premix[3] | 20 | 20 |
| Vitamin premix[4] | 20 | 20 |
| Monocalcium phosphate | 5 | 5 |
| Anti-oxidant[5] | 0.2 | 0.2 |
| Anti-myotoxin[6] | 1.8 | 1.8 |
| Filler (CMC)[7] | 50 | 47.75 |
| Lecithin | 1 | 1 |
| DL-methionine | 5 | 5 |
| Kemzyme® WP (multi-enzyme)[8] | 0 | 0.25 |
| BIOguil™ (multi-strain probiotic)[9] | 0 | 2 |
| Total | 1000.00 | 1000.00 |
| Proximate composition (g/kg dry matter) | | |
| Crude protein (CP) | 407.5 | 409.1 |
| Ether extract (EE) | 149.4 | 148.0 |
| Ash | 64.4 | 65.9 |
| Moisture | 79.0 | 75.2 |
| NFE[10] | 299.8 | 301.8 |
| Gross energy (kJ $g^{-1}$)[11] | 20.7 | 20.7 |

[1]Kilka fishmeal (Clupeidae) with 735.0 g/kg CP, 87.7 g/kg EE, 108.1 g/kg ash, and 72.5 g/kg moisture (Pars Aquatic Feed Factory, Mazandaran, Iran).

[2]ShaySoy™ (Shayan Energy and Protein Co., Qazvin, Iran).

[3]The mixture of minerals (unit/kg of the premix): iron (6 g), zinc (1.5 g), selenium (20 mg), cobalt (100 mg), copper (0.6 g), magnesium (34 mg), manganese (5 g), iodine (3 mg), calcium phosphate (5 mg), choline chloride (6 g).

[4]The elements used in the vitamin supplement (unit/kg of the premix): ascorbic acid (C, 80 g), menadione ($K_3$, 2 g), retinol acetate (A, 9000 IU), DL-cholecalciferol ($D_3$, 6000 IU), thiamine hydrochloride ($B_1$, 2.5 g), riboflavin ($B_2$, 30 mg), nicotinic acid ($B_3$, 175 mg), calcium pantothenate ($B_5$, 50 mg), pyridoxine hydrochloride ($B_6$, 3.5 g), biotin ($B_7$, 3 mg), DL-alpha tocopherol acetate (E, 40 g), inositol ($B_8$, 1 g), folic acid ($B_9$, 220 mg), cyanocobalamin ($B_{12}$, 120 mg).

[5]Butylated hydroxytoluene (BHT; Yasho Industries, Gujarat, India).

[6]Takgen Company (Tehran, Iran).

[7]Sodium carboxymethyl cellulose (CMC; Fortune Biotech, Shandong, China).

[8]A stabilized multi-enzyme mixture contains 5,000 IU $g^{-1}$ beta-glucanase, 5,000 IU/g cellulase complex (pentosanase, pectinase, and hemicellulase), 2000 IU/g alpha-amylase, 2,000 IU/g protease, 20,000 IU/g xylanase, and 2,000 IU/g phytase (Kemin® Industries, Inc., Herentals, Belgium).

[9]A commercial multi-probiotic contains *Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici* with $1 \times 10^{10}$ CFU/g for each bacterial strain (Guilan Science and Technology Park, Rasht, Iran).

[10]NFE (nitrogen-free extract, g/kg) were calculated by a mathematical calculation (1000 – [CP + EE + ash + moisture]).

[11]Gross energy was estimated based on 1 g CP being 23.6 Kj, 1 g EE being 39.5 Kj, and 1 g carbohydrate being 17.2 Kj.

**Complete blood count test**

The white blood cells (WBC) and red blood cells (RBC) were counted by loading and counting the blood in the Neubauer hemocytometer chambers under a conventional optical microscope (Olympus CX31, Japan) (Barham et al., 1980). The leukocyte differential count (lymphocytes, monocytes, neutrophils, and eosinophils) was quantified by preparing the blood smears and staining in 5% Giemsa. The prepared slides were studied for the WBC differential count under the microscope (Olympus CX31).

The hemoglobin (Hb) concentration was measured by spectrophotometry using Drabkin's reagent (ParsAzmun, Alborz, Iran) at 540 nm (Thrall et al., 2012). The hematocrit percentage or packed cell volume (PCV) was estimated by capillary tubes and laboratory microcentrifuge apparatus (TAT-Mic, Teifazma, Tehran, Iran). Finally, the hematological indices, including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), were calculated using the formulas described by Campbell (2004).

**Serum biochemistry aspects**

The concentrations of total cholesterol (T-Cho), triglyceride (TG), total protein (TP), glucose, total bilirubin, and blood urea nitrogen (BU) were measured according to the cholesterol oxidase, glycerol phosphate oxidase–phenol aminoantipyrine peroxidase (GPO–PAP), glucose oxidase, diazo-sulfanilic acid, and diacetyl monoxime methods using colorimetric assay kits (ParsAzmun, Alborz, Iran) by a Prestige 24i automatic biochemical analyzer (Boeki Group, Tokyo, Japan). In addition, the activities of hepatic enzymes, including aspartate transaminase (AST) and alanine aminotransferase (ALT), were also assayed by the relevant biochemical kits (ParsAzmun, Alborz, Iran), which were previously used for fish (Hedayati et al., 2021).

**Serum and skin mucus immunological parameters**

The lysozyme (LYZ) activity was measured by a turbidimetric method based on the lysis of Gram-positive *Micrococcus lysodeikticus* (Sigma-Aldrich), which was fully explained by Ellis (1990). Total protein (TP) was assessed by photometry using the Lowry (1951) method. Total immunoglobulin (Ig) was estimated according to Siwicki and Anderson (1993) method after the precipitation of immunoglobulins by 12% polyethylene glycol solution (Sigma-Aldrich). The mucus TP content was also determined as the method described in the serum biochemistry section.

**Proximate composition**

Four fish (12 fish in each treatment) were randomly selected and euthanized with an overdose of the clove powder to collect the fish carcass. Then, the gastrointestinal tract of each fish was removed, and the remaining body was subjected to the proximate composition. The crude protein, lipid, ash, and moisture of the faeces, body, and diets were calculated following the standard protocols of AOAC (1995).

In the faeces samples, $Cr_2O_3$ concentration was determined using atomic absorption flame photometry (TAS-990, General Instruments Co. Ltd., Beijing, China) based on AOAC (1995) method by converting chromium oxide to chromic acid.

**Intestine histomorphology**

The hind-gut of four fish in each tank euthanized for the body composition assay was removed and fixed in 10% buffered formalin to evaluate the intestine morphological features. After 24 h, the formalin of the samples was replaced with 70% alcohol and transferred to the laboratory of the Science and Research Branch, Islamic Azad University (Tehran, Iran) for histomorphological studies. The tissue processing stages were followed based on the standard methods (Roberts, 2012). Then, the sections (4–5 μm) were prepared from the paraffin molded tissue samples using a Leica RM2245 microtome (Leica Biosystems, Heidelberger, Germany). The slides were stained with hematoxylin and eosin (H&E) and screened under an inverse microscope (Eclipse TS100, Nikon, Japan). The slides were photographed to evaluate the number of goblet cells (GC), microvilli length (MD), microvilli diameter (ML), inner muscle wall (IMW) diameter, and outer muscle wall (OMW) diameter. Digimizer image analysis software (Digimizer® version 5.4.3, MedCalcSoftware, Ostend, Belgium) was used to measure the above morphological aspects. In addition, the enterocyte total absorptive surface (ETAS) was $= 2\pi \times ML \times \frac{MD}{2}$ (Sakamoto et al., 2000).

**Apparent digestibility coefficients (ADC)**

In the ninth week of the study, chromium oxide ($Cr_2O_3$; Sigma-Aldrich, CAS No1308-38-9) as an indigestible marker was added to the experimental diets at 5 g/kg to determine ADC for crude protein ($ADC_{CP}$), crude lipid ($ADC_{CL}$), and dry matter ($ADC_{DM}$). The remaining fish were fed to apparent satiation with the marked diets for ten days, and the faeces were collected by daily siphoning the bottom of the ponds 30 min after the feeding. The collected faeces were immediately frozen at –80°C for further analysis. The ADC of macronutrients was computed according to the formulas described by Hosseini Shekarabi et al. (2021).

**Statistical analysis**

The Kolmogorov-Smirnov test was used to check the normal distribution of data, and the Levene's test was used to determine the equality of variances. Possible differences between the two treatments were assessed using an independent samples *t*-test. In all statistical tests, a 95% confidence level was considered ($P<0.05$). All the statistical analyses were performed using SPSS ver. 22 software.

## Results

### Growth, feed utilization, digestibility, and carcass chemical traits

The final weight, weight gain, SGR, and PER were markedly enhanced while the FCR was reduced in fish fed multi-enzymes and probiotics premix (P<0.05) (Table 2). No significant differences were seen on the carcass traits except for the total protein, which was significantly increased by multi-enzymes and probiotics mixture (P>0.05) (Table 3). The digestibility of crude protein, dry matter, and crude lipids was also improved by multi-enzymes and probiotics mixture (P<0.05) (Figure 1).

### Intestinal functional topography

The count of goblet cells, microvilli diameter, microvilli length, outer muscle wall diameter, and enterocyte total absorptive surface were markedly increased (P<0.05) by dietary multi-enzymes and probiotics mixture (Table 4). Figure 2 shows increased villi length and width with obvious villi branching with normally arranged absorptive enterocytes, lamina propria submucosa, tunica muscularis, and tunica serosa.

Table 2. Growth performance and feed utilization of Siberian sturgeon (*Acipenser baerii*) fed with the experimental diets for 56 days

| Indices | Experimental diets | | Sig. (2-tailed) |
|---|---|---|---|
| | control | Enz+Pro cocktail | |
| $W_i$ (g) | 154.97±2.55 | 153.30±3.03 | 0.507 |
| $W_f$ (g) | 360.07±7.62 | 387.93±9.55* | 0.018 |
| WG (g) | 205.10±8.42 | 234.63±11.22* | 0.024 |
| FCR | 1.93±0.07 | 1.74±0.05* | 0.025 |
| SGR (%/day) | 1.51±0.05 | 1.92±0.07* | 0.038 |
| K (g/cm$^3$) | 0.46±0.01 | 0.50±0.06 | 0.518 |
| FI (g/day) | 7.05±0.36 | 7.28±0.24 | 0.406 |
| PER (%) | 1.28±0.05 | 1.41±0.04* | 0.024 |
| SR (%) | 100 | 100 | – |

Data are expressed as means ± standard deviation (n=12). * Indicates significant differences between the groups at P<0.05.

$W_i$, initial weight; $W_f$, final weight; FCR, feed conversion ratio; SGR, specific growth rate; FI, feed intake; K, condition factor; BWI, body weight increase; PER, protein efficiency ratio; SR, survival rate.

Enz+Pro cocktail: Kemzyme® WP a commercial exogenous multi-enzyme mixture (beta-glucanase, cellulase, alpha-amylase, protease, xylanase, and phytase) at 250 mg/kg + BIOguil™ as a commercial multi-species probiotic (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici*; $1 \times 10^{10}$ CFU/g for each bacterial strain) at 2 g/kg.

Table 3. Body proximate composition (g/kg in dry matter) of Siberian sturgeon (*Acipenser baerii*) fed with the experimental diets for 56 days

| Parameters (%) | Experimental diet | | Sig. (2-tailed) |
|---|---|---|---|
| | control | Enz+Pro cocktail | |
| Moisture | 73.35±0.21 | 73.96±0.33 | 0.020 |
| Crude protein | 12.29±0.18 | 14.50±0.26* | 0.000 |
| Crude fat | 7.52±0.08* | 6.16±0.03 | 0.000 |
| Ash | 6.39±0.13 | 5.24±0.10 | 0.000 |

Data are expressed as means ± standard deviation (n=12). * Indicates significant differences between the groups at P<0.05.

Enz+Pro cocktail: Kemzyme® WP a commercial exogenous multi-enzyme mixture (beta-glucanase, cellulase, alpha-amylase, protease, xylanase, and phytase) at 250 mg/kg + BIOguil™ as a commercial multi-species probiotic (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici*; $1 \times 10^{10}$ CFU/g for each bacterial strain) at 2 g/kg.

Table 4. Intestine histomorphological aspects of Siberian sturgeon (*Acipenser baerii*) fed the experimental diets at 16.5 ±1.0°C for 56 days

| Characteristic | Experimental diets | | Sig. (2-tailed) |
|---|---|---|---|
| | control | Enz+Pro cocktail | |
| Goblet cells (per 100 μm) | 39.13±3.56 | 48.67±1.50* | 0.000 |
| Microvilli diameter (μm) | 246.00±32.48 | 284.29±53.45* | 0.061 |
| Microvilli length (μm) | 842.61±89.73 | 1467.24±135.86* | 0.000 |
| OMW diameter (μm) | 363.12±48.10 | 476.77±60.28* | 0.001 |
| IMW diameter (μm) | 561.53±82.84 | 636.42±110.65 | 0.095 |
| ETAS ($\times 10^3$ μm$^2$) | 649.73±102.72 | 1306.99±261.22* | 0.000 |

Data are expressed as means ± standard deviation (n=12). * Indicates significant differences between the groups at P<0.05.

IMW, inner muscle wall; OMW, outer muscle wall; ETAS, enterocyte total absorptive surface.

Enz+Pro cocktail: Kemzyme® WP a commercial exogenous multi-enzyme mixture (beta-glucanase, cellulase, alpha-amylase, protease, xylanase, and phytase) at 250 mg/kg + BIOguil™ as a commercial multi-species probiotic (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici*; $1 \times 10^{10}$ CFU/g for each bacterial strain) at 2 g/kg.

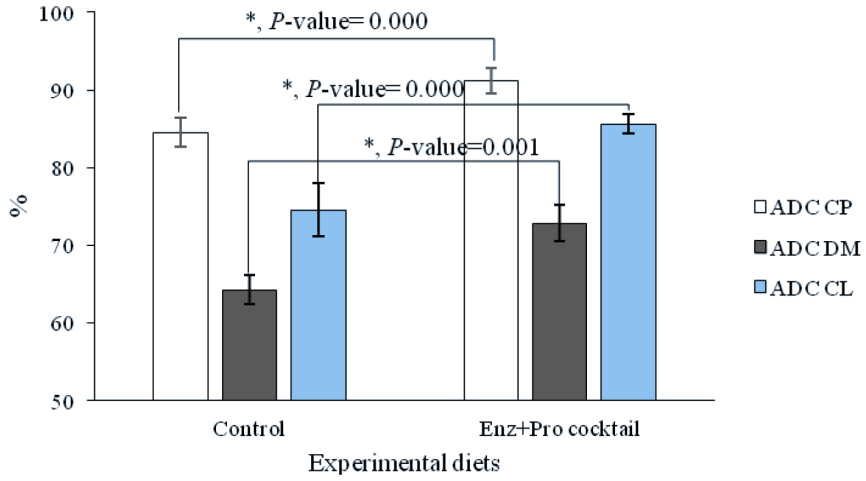


Figure 1. Changes in the apparent digestibility coefficients (ADC) of crude protein ($ADC_{CP}$), crude lipid ($ADC_{CL}$), and dry matter ($ADC_{DM}$) in Siberian sturgeon (*Acipenser baerii*) fed diets supplemented with a mixture of dietary exogenous enzymes and probiotics (Enz+Pro cocktail diet) and basal diet. * Indicates significant differences between the groups at P<0.05

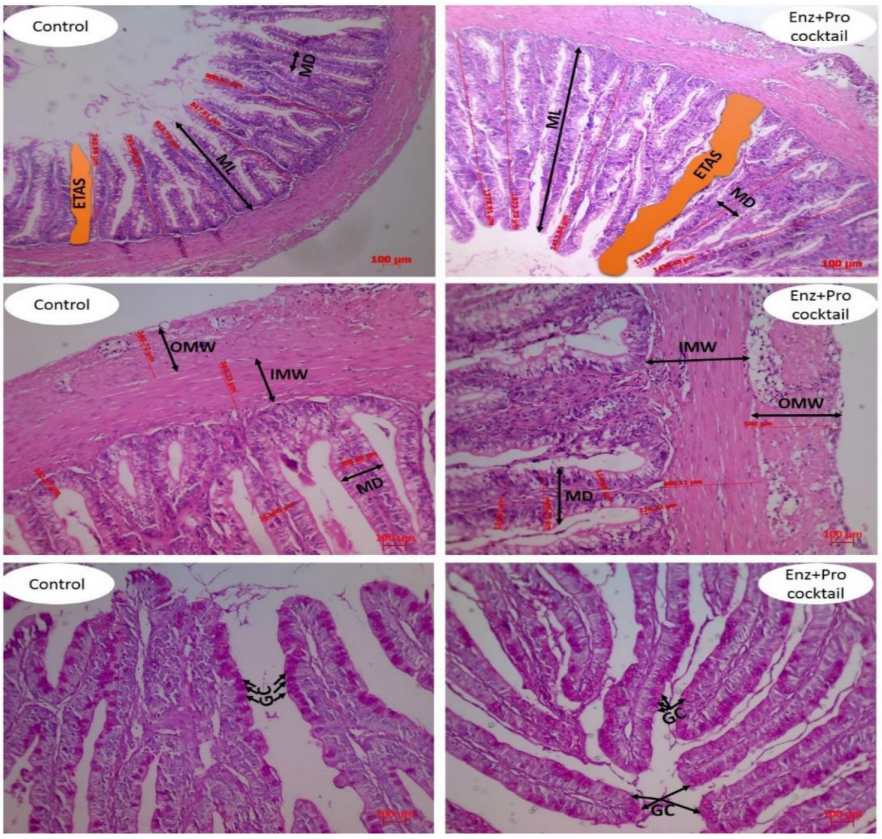


Figure 2. The intestinal functional topography of Siberian sturgeon (*Acipenser baerii*) fed diets supplemented with a mixture of exogenous enzymes and probiotics (Enz+Pro cocktail diet) and basal diet (control). MD, microvilli length; ML, microvilli diameter; ETAS, enterocyte total absorptive surface; IMW, inner muscle wall diameter; OMW, outer muscle wall diameter; GC, goblet cells (H&E; bar = 100 μm)

**Hematological and serum biochemical profiles**

No significant effects (P>0.05) were seen on the hematological profile of Siberian sturgeon fed dietary multi-enzymes and probiotics mixture except for the WBCs and neutrophils, which showed marked improvements (P<0.05) (Table 5). The levels of glucose, triglycerides, blood urea nitrogen, and total bilirubin were markedly higher in fish fed the control than fish fed the multi-enzymes and probiotics mixture (P<0.05) (Table 6).

Table 5. Hematological parameters of Siberian sturgeon (*Acipenser baerii*) fed with the supplemented diets for 56 days

| Parameters | Experimental diets | | Sig. (2-tailed) |
|---|---|---|---|
| | control | Enz+Pro cocktail | |
| WBC ($\times 10^3$ µl$^{-1}$) | 10.08±0.16 | 12.18±0.22* | 0.000 |
| RBC ($\times 10^5$ µl$^{-1}$) | 4.45±0.09 | 4.62±0.13 | 0.074 |
| Hb (g dl$^{-1}$) | 6.30±0.10 | 6.41±0.19 | 0.333 |
| PCV (%) | 24.00±0.82 | 25.50±1.29 | 0.097 |
| MCV (ft) | 540.07±20.07 | 552.17±22.95 | 0.458 |
| MCH (pg) | 141.72±3.57 | 138.64±1.77 | 0.143 |
| MCHC (g dl$^{-1}$) | 26.26±0.73 | 24.92±1.46 | 0.130 |
| Lymphocytes (%) | 76.75±0.96 | 77.50±1.73 | 0.477 |
| Monocytes (%) | 6.50±1.73 | 4.75±0.96 | 0.127 |
| Neutrophils (%) | 15.50±0.58 | 18.25±1.26* | 0.007 |
| Eosinophils (%) | 0.75±0.96 | 0.25±0.50 | 0.390 |

Data are expressed as means ± standard deviation (n=12). * Indicates significant differences between the groups at P<0.05.

WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin, MCHC, mean corpuscular hemoglobin concentration.

Enz+Pro cocktail: Kemzyme® WP a commercial exogenous multi-enzyme mixture (beta-glucanase, cellulase, alpha-amylase, protease, xylanase, and phytase) at 250 mg/kg + BIOguil™ as a commercial multi-species probiotic (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici*; 1 × $10^{10}$ CFU/g for each bacterial strain) at 2 g/kg.

Table 6. Serum biochemical responses of Siberian sturgeon (*Acipenser baerii*) fed with the supplemented diets for 56 days

| Parameters | Experimental diets | | Sig. (2-tailed) |
|---|---|---|---|
| | control | Enz+Pro cocktail | |
| Glucose (mg dl$^{-1}$) | 51.73±3.00* | 44.15±2.21 | 0.000 |
| T-Cho (mg dl$^{-1}$) | 145.23±20.22 | 128.24±17.25 | 0.073 |
| TG (mg dl$^{-1}$) | 980.10±191.04* | 763.21±124.64 | 0.045 |
| BUN (mg dl$^{-1}$) | 2.23±0.28* | 1.89±0.36 | 0.034 |
| Total bilirubin (mg dl$^{-1}$) | 0.06±0.01* | 0.01±0.00 | 0.040 |
| ALT (U l$^{-1}$) | 12.22±2.33 | 11.00±1.22 | 0.183 |
| AST (U l$^{-1}$) | 53.44±8.11 | 56.00±4.99 | 0.410 |

Data are expressed as means ± standard deviation (n=12). * Indicates significant differences between the groups at P<0.05.

BUN, blood urea nitrogen; TG, triglyceride; T-Cho, total cholesterol, AST, aspartate transaminase; ALT, alanine aminotransferase.

Enz+Pro cocktail: Kemzyme® WP a commercial exogenous multi-enzyme mixture (beta-glucanase, cellulase, alpha-amylase, protease, xylanase, and phytase) at 250 mg/kg + BIOguil™ as a commercial multi-species probiotic (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici*; 1 × $10^{10}$ CFU/g for each bacterial strain) at 2 g/kg.

### Humoral and mucosal immune parameters

The blood and skin mucus-related immune responses are shown in Table 7. Significantly, Siberian sturgeon fed dietary multi-enzymes and probiotics had improved lysozyme activity, total immunoglobulin, and total protein in the skin mucus and serum samples (P<0.05). Further, the serum complement C3 and C4 was higher in fish delivered multi-enzymes and probiotics mixture than in control (P<0.05).

Table 7. Serum and skin mucus immunological parameters of Siberian sturgeon (*Acipenser baerii*) fed with the experimental diets for 56 days

| Parameters | Experimental diets | | Sig. (2-tailed) |
|---|---|---|---|
| | control | Enz+Pro cocktail | |
| Mucus LYZ activity (U ml$^{-1}$) | 33.17±1.04 | 47.82±2.76* | 0.001 |
| Mucus total Ig (mg dl$^{-1}$) | 8.48±0.31 | 9.90±0.22* | 0.000 |
| Mucus TP (g dl$^{-1}$) | 0.72±0.02 | 0.89±0.05* | 0.005 |
| Serum LYZ activity (U mL$^{-1}$) | 23.00±0.42 | 30.79±0.27* | 0.000 |
| Serum total Ig (mg dl$^{-1}$) | 47.26±1.02 | 69.63±1.57* | 0.000 |
| Serum TP (g dl$^{-1}$) | 2.06±0.21 | 3.68±0.35* | 0.000 |
| Serum C3 (mg dl$^{-1}$) | 35.56±3.37 | 47.79±5.32* | 0.001 |
| Serum C4 (mg dl$^{-1}$) | 6.34±0.25 | 8.98±0.33* | 0.000 |

Data are expressed as means ± standard deviation (n=12). * Indicates significant differences between the groups at P<0.05.

LYZ, lysozyme; Ig, immunoglobulins; TP, total protein; C3, complement component 3; C4, complement component 4.

Enz+Pro cocktail: Kemzyme® WP a commercial exogenous multi-enzyme mixture (beta-glucanase, cellulase, alpha-amylase, protease, xylanase, and phytase) at 250 mg/kg + BIOguil™ as a commercial multi-species probiotic (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici*; 1 × $10^{10}$ CFU/g for each bacterial strain) at 2 g/kg.

## Discussion

The key factor for healthy and productive aquatic animals is to ensure aquafeed's high digestibility and utilization (Yu et al., 2021). The mixture of multi-enzymes and probiotics improves the digestibility and metabolism of nutrients compared to exogenous enzymes or probiotic additives individually (Maas et al., 2021 b). In addition to the prominent role of probiotics, it can synergistically maximize the activity of digestive enzymes in the GIT (Dawood, 2021), leading to high digestion and absorption capacity. The dual objectives of using exogenous digestive enzymes and probiotics are associated with enriching the microbial balance in the GIT (Dai et al., 2019). Consequently, the abundance of beneficial bacteria increases while pathogenic microorganisms decrease (Maas et al., 2021 c). Herein, we evaluated the role of the multi-enzymes and probiotics mixture on the growth performance, digestion capacity, intestinal health, blood hemato-biochemical indices, and immunity of Siberian sturgeon. The growth performance and protein utilization (PER) were enhanced while the feed conversion ratio (FCR) was decreased in the group of fish delivered by the mixture of multi-enzymes and probiotics. In the same sense, Hassaan et al. (2021) stated that Nile tilapia fed protease and *Bacillus pumilus* had improved growth performance and feed utilization. Further, Maas et al. (2021 b) reported that dietary phytase, xylanase, and *B. amyloliquefaciens* mixture enhanced the growth performance and feed digestibility of Nile tilapia. Dai et al. (2019) also reported that dietary amylase, acid protease, papain, and *B. amyloliquefaciens* improved snakehead's growth performance and feed utilization. Supplementing probiotics and exogenous enzymes improves nutrition-

al availability and intestinal health and helps maintain a more balanced microbiome diversity (Maas et al., 2021 c). The results also showed improved digestibility of proteins, fibers, and lipids which can explain the improvements in the PER and FCR in fish treated with multi-enzymes and probiotics mixture. The enhancement in the growth performance could be related to the improved digestion capacity in the intestines of Siberian sturgeon by dietary multi-enzymes and probiotics mixture. Increased digestibility of proteins, lipids, and fibers allows the absorption of these nutrients in a simple form (amino acids, volatile fatty acids, vitamins, and minerals), required for the main metabolic and physiological functions (Dawood, 2021).

The intestinal histological features (goblet cells, villi length, and width) were markedly improved by dietary multi-enzymes and probiotics mixture under the current trial conditions (Nikiforov-Nikishin et al., 2021). Similarly, Hassaan et al. (2021) illuminated that Nile tilapia treated with protease and *B. pumilus* mixture had improved intestinal histological features. Probiotics can protect the intestinal mucosal layer from pathogenic microorganisms' toxic secretions, leading to improved local intestinal health (Sagada et al., 2021). Consequently, the enhancement of the intestinal histological features is correlated with increased absorption surface, leading to high digestibility and feed utilization (Yin et al., 2021).

The detection of carcass nutrients is also correlated with the influence of multi-enzymes and probiotics mixture on the accumulation of digested nutrients in the entire body. The results showed increased protein content in the body of Siberian sturgeon, which can be related to the increased digestibility of proteins leading to high accumulation in fish bodies. Also, the increased protein content is associated with the improved PER in Siberian sturgeon fed dietary multi-enzymes and probiotics mixture. Moreover, decreasing the body fat content in the treated fish may indicate fat storage as energy to increase growth rate.

The monitored hematological indices revealed no significant differences between fish fed the control or multi-enzymes and probiotics mixture except for the white blood cells count (WBCs) and neutrophils. Markedly increased WBCs and neutrophils were seen in Siberian sturgeon fed multi-enzymes and probiotics mixture. Similarly, Nile tilapia fed protease and *B. pumilus* had increased WBCs (Hassaan et al., 2021). Increased WBCs and neutrophils indicate improved immunity to counteract the infection with bacterial pathogens. The inclusion of probiotics caused increased immunity and WBCs in several fish species (Hassaan et al., 2014; Akbari et al., 2021). In addition, dietary multi-enzymes and probiotics mixture decreased the glucose, triglycerides, and total bilirubin traits. The reduction of these blood biochemical traits indicates the absence of stress, kidney and liver failure. Probiotics reduce lipid peroxidation and oxidative stress, which may explain the absence of stress and hepato-renal failure (Adawi et al., 2001; Kong et al., 2021).

The immune response of Siberian sturgeon is detected in the serum and skin mucus samples. The skin mucus is the first layer that combats biotic and abiotic stressors and correlates with entire body immunity (Firmino et al., 2021; Mori et al., 2021). Functional feed additives are involved in the activation of local intestinal immunity (Dawood, 2021). Probiotics compete with the harmful microorganisms in the GIT and lower their negative impact on the local intestinal immunity (Ushakova et al., 2021). The detection of total protein, immunoglobulin (Ig), lysozyme activity, and complement (C3 and C4) in Siberian sturgeon fed dietary multi-enzymes and probiotics mixture revealed activated serum and skin mucus immunity. Similarly, Nile tilapia fed dietary protease and *B. pumilus* mixture had increased lysozyme activity, Ig, and total proteins (Hassaan et al., 2021). Further, snakehead fed dietary *B. amyloliquefaciens*, amylase, acid protease, and papain had increased lysozyme activity and total protein (Dai et al., 2019). The activation of skin mucus immunity is also observed in several fish fed dietary probiotics (Dawood et al., 2017). Probiotics can boost immunity by increasing the release of enzymes and proteins in the bloodstream (Wuertz et al., 2021). In addition, exogenous enzymes increase macrophage and monocyte activation, resulting in increased immune-related cytokines (Mohammad and Mehran, 2010). The enhancement in the total proteins, Ig, lysozyme, and complement (C3 and C4) indicate the increased ability of Siberian sturgeon to resist bacterial infection and pathogenic invaders.

## Conclusion

In conclusion, dietary probiotics synergistically enhanced the activity of multi-enzymes and resulted in increased feed utilization and nutrient digestibility in Siberian sturgeon. Besides, the intestinal histological features were improved, leading to increased growth performance. Consequently, fish had regulated hemato-biochemical indices and activated serum and skin mucus immune responses. This work is a basis for future research to elucidate the probiotic properties of the multi-strain probiotic as a potential probiotic in aquaculture nutrition.

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