**Beneficial effects of dietary supplementation of papain in juvenile Sterlet (*Acipenser ruthenus*): growth, intestinal topography, digestive enzymes, antioxidant response, immune response and challenge disease.**

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Abstract

Juvenile sterlet (*Acipenser ruthenus*) were fed commercial feed (group C) and experimental feed supplemented with papain in doses 10 g kg-1 (P1) and 20 g kg-1 (P2) feed for 8 weeks. Growth, digestive enzyme activity, immunity parameters, pathological changes of the liver and intestine, chemical body composition and oxidative response were assayed. Challenge test using *Yersinia ruckeri* was also performed. Final body weight of groups P1 (107.07 ± 7.66 g) and P2 (111.98 ± 1.93 g) was significantly (P < 0.05) higher compared to control group (99.73 ± 2.71 g). The highest intestinal enterocytes was detected in group P2, and the highest supranuclear surface of the intestinal enterocytes was found in groups P1 and P2. The activities of α-amylase, trypsin, lipase and leucine aminopeptidase were significantly (P < 0.05) higher in posterior intestine of fish fed group (P2) than other treatment groups. Ceruloplasmin, total Immunoglobulin (Ig), metabolic activity of splenic macrophages (PMA) and potential killing activity of splenic phagocytes (PKA) were significantly higher in groups P1 and P2 compared with control group. While, the proliferative activity of spleen T and B-lymphocytes were significantly (P < 0.05) higher in group P2 in comparison to the control and group P1. Total antioxidant status (TAS), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were significantly increased in groups P1 and P2 compared with control group. The activities of glutathione reductase was significantly (P < 0.05) decreased with increasing levels of papain supplementation. Furthermore, during challenge test, survival of fish was significantly higher in groups supplemented with papain. Finally, the current study indicated that supplementing feed with doses of 10 g and/or 20 g papain kg-1 for a period of 56-day improved growth efficiency and feed utilization, and also stimulated immunity in aquaculture conditions.

Key words: Sturgeon; fruit enzymes; feed utilization; RAS; immunostimulation

1. Introduction

Intensification of aquaculture production over the past two decades has increased demand for artificial feed (Bowyer et al., 2019; Davies et al., 2019; Hassaan et al., 2019). Fish nutritionist suggested that artificial feed couldn’t meet all requirements of nutrient (Hassaan et al., 2021b; Hassaan et al., 2018; Mohammady et al., 2021; Oluwaseyi, 2016). Feed additives such as veterinary drugs are added as therapeutic or growth agents and stimulant of immunity system (Rico et al., 2013). As known antibiotic treatment can cause drug resistance of bacteria thus there is a trend to using bioactive materials in artificial feeds (Harikrishnan et al., 2010; Hassaan et al., 2018). The criteria for selective feed additive are natural and safety and with high active compound content. Thus, exogenous enzymes play significant roles in the proper development and functioning of the digestive system in larval and juvenile fishes (Kolkovski, 2001; Wiszniewski et al., 2019). Adding exogenous enzymes to fish feed can improve the utilization of feed components thus reducing losses. Exogenous enzymes have been confirmed to improve the nutritional value of feed (Hassaan et al., 2018). Dietary incorporation of enzymes may considerably improve the utilization efficiency of plant-based protein and decrease the load of phosphorus into the aquatic environment (Ai et al., 2007; Liebert and Portz, 2005; Singh et al., 2011).

Papain is a proteolytic enzyme from the proteinases enzyme family, papain is extracted from different parts of papaya (*Carica papaya*) such as the leaf, unripe fruit and fruit sap (Yogiraj et al., 2014), that belongs to the group of cysteine proteases, which can hydrolyze proteins into short peptides (Mo et al., 2016; Pendzhiev, 2002). Papain is used in the food industry and in medicine (Amri and Mamboya, 2012). This enzyme has anti-inflammatory properties and is a component of many pharmaceuticals (Yogiraj et al., 2014). Amri and Mamboya (2012) noted that papain could break down dietary proteins into amino acids, thus, the fish could digest the feed more easily, in turn, it improved feed efficiency. Also, Muchlisin et al. (2016), noted that papain enzyme (27.5 mg kg-1) increased the feed utilization and growth of Keureling fish (*Tor tambra*). In *Labeo rohita*, however, 10 g kg-1 diet would be enough to increase the feed efficiency (Khati et al., 2015). Furthermore, addition of 15 g papain kg-1 diet significantly increased the weight gain and feed efficiency of *C. gariepinus* (Rachmawati et al., 2019). As a feed supplement, papain increases the digestibility of protein of feed and improves its assimilation, which advantageously affect feed utilization and growth indices (Wong et al., 1996). Based on earlier reports, dietary incorporation level of papain for best performance might be species specific. However, the addition of papain in sterlet (*Acipencer ruthenus*) has not been studied before, and to our knowledge so far, there is a lack of information regarding the effect of papain addition on non-specific immunity parameters and oxidative response for fish cultured. Therefore, the aim of this study was to examine the impacts of papain additive on growth, immunity parameters, enzymatic activity, pathological structure of the liver and intestine, chemical body composition, oxidative response and challenge test using *Y. ruckeri* on sterlet (*Acipenser ruthenus*).

2. Material and methods

2.1. Rearing conditions

The feeding trial was conducted at Stanisław Sakowicz Inland Fisheries Institute (IFI) in Olsztyn, Poland for 56-day. Juvenile sterlet (*Acipenser ruthenus*) with an average initial body weight of 37 ± 3.59 g were selected and acclimated to experimental conditions for two weeks. During the acclimatization period, fish were fed commercial diet (540 g kg-1 crude protein, 22.6 MJ kg-1 gross energy) three times daily. Thirty fish were randomly distributed in each of nine tanks (280 L each tank) connected in recirculating aquaculture system (RAS). The physical and chemical water parameters were measured at the rearing tank outflows during the experiment and were recorded as follows: water temperature 20 ◦C (± 0.2); oxygen content 6.05–7.15 mg O2 L-1 (± 0.59), ammonia nitrogen (TAN = NH4+-N + NH3-N) and nitrate (NO2-N) 0.124–0.167 ± 0.098 mg TAN L-1 and 0.009–0.014 ± 0.004 NO2-N L-1, respectively. The water pH was within the range of 7.4–7.6.

2.2. Design and diets preparing

A commercial feed for sturgeons was used (Nutra T-2.0, Skretting, France, 54 % crud protein and 18 % lipids), whereas this diet was divided into three groups. First one was control without supplementation (control diet), while another group diet 2 (P1) and diet 3 (P2) were supplemented with exogenous enzyme papain (Sigma-Aldrich, USA) 10 g and 20 g kg-1 diet, respectively. Preparation of diets after supplementation with papain was according to Wiszniewski et al. (2019).

2.3. Rearing indices

The fish were weighed (W; ± 0.01 g) and their total length was measured (TL; ± 0.1 cm) at the beginning and end of the experiment. The mean weight of the fish was measured every seven days to set the feed rations. Rearing indices(DGR, SGR, CF, FCR, PER, VSI, HIS) were calculated (Table 1) according to the following formulas described in Jarmołowicz et al. (2012). Growth performance and feed utilization parameters were calculated with the following formulas:

DGR (daily growth rate, g d-1) = (Wf – Wi) × T-1;

SGR (specific growth rate, % d-1) = 100 × [(lnW2 - lnW1) × t-1];

CF (condition coefficient) = (W × 100) × TL-3;

FCR (feed conversion ratio) = TFI × (FB – IB)-1;

PER (protein efficiency ratio) = (FB – IB) × TFP-1.

HSI (hepatosomatic index, %) = 100 × (LW × W-1)

VSI (viscerosomatic index, %) = 100 × (VW × W-1)

where: Wi= initial mean body weight (g), Wf = final mean body weight (g); T = rearing time (d). W – body weight (g); TL = total length (cm); FB = final stock biomass (g); IB = initial stock biomass (g); TFI = total feed intake (g); TFP = total feed protein (g); FBP = final body protein (%); IBP = initial body protein (%); LW = liver weight (g); VW = viscera weight (g).

2.4. Body proximate composition

The proximate compositions of whole body fish were determined at the end of the experiment in five specimens, from each of the three replicates of the feeding treatments according to the methods were described in Wiszniewski et al. (2019) (Table 2).

2.5. Endogenous enzymes activity analysis and oxidative response

The intestine and liver samples were collected from five fish from each experimental group, and then stored at −80 °C until analysis. All enzyme analyses performed in triplicate were measured with an Infinite 200 Pro device (Tecan Austria, Grödig, Austria). The digestive enzyme activities assay of α-amylase (EC 3.2.1.1), lipase (EC 3.1.1.3), trypsin (EC 3.4.21.4), leucine aminopeptidase (EC 3.4.11.1), to evaluate protein levels samples of sturgeon were first homogenised in buffers and then centrifuged at 4 °C for 15 min. at 15000 × g. The specific enzyme activity was analysed according to the procedures described for amylase (Foo and Bais, 1998), lipase (Winkler and Stuckmann, 1979), trypsin (Erlanger et al., 1961), leucine aminopeptidase (Nagel et al., 1964). Enzyme activities were expressed as the number of micromoles of the reaction product per 1 minute calculated for 1 g of protein (IU g-1 protein) (Table 3).

The activities of oxidative stress enzymes and Total Antioxidative Status (TAS) were performed in the liver. Enzymatic analyses were performed using commercially available kits for: glutathione reductase (BioAssay Systems, no cat. ECGR-100), superoxide dismutase (BioAssay Systems, no cat. ESOD-100), glutathione peroxidase (Randox, no cat. RANSEL RS505) and the total antioxidant status (Randox, no cat. TAS kit NX2332) and according to kit manufacturer instructions and methods described in Palińska-Żarska et al. (2021) and Kapusta et al. (2018) (Table 4). Analyses were performed in triplicates 25 °C. Enzyme activities were expressed as the number of micromoles of reaction product released per 1min calculated for 1mg of protein (U/mg protein). TAS results were expressed as mmol mmol Trolox equivalents per 1 mg of protein (mmol/mg).  (Table 4).

2.6. Histological analysis

On the last day of trial the livers and middle segments of gastrointestinal tracts were removed from seven specimens from each dietary treatment and subjected to histological analysis (Zawistowski, 1986). The histological samples were performed and analyzed with the methods were described by Wiszniewski et al.(2019) (Table 5).

2.7. Immunological indices

At the end of the experiment, for non specific humoral parameters blood was drawn from the caudal vein of 10 individuals from each experimental variant. For cellural immunity, immunocompetent cells were isolated from the spleens that had been removed from ten specimens from each experimental variant. Non specific humoral and cellular immunity analysis were performed and analyzed regarding to the method described by Wiszniewski et al. (2019) (Table 6)*.*

2.8. Challenge test

At the end of the experiment, a challenge test was performed based on the method described by Siwicki et al. (1994). Briefly, 45 fish from three groups were each given a single intraperitoneal injection of *Yersinia ruckeri* (0.2 mL; 1 × 107). *Y. ruckeri* serotype O1, biotype 2 isolated from fatal cases in fish farm of rainbow trout from Poland was used. Before experimental infection, *Y. ruckeri* was cultured on nutrient agar supplemented with 5% of horse blood, tryptone soya agar (TSA, Oxoid) at 25 °C 1 °C for 24 h. Any abnormal behavior, clinical signs and daily mortality in the challenged fish were monitored twice a day for 7 days. Dead fish were removed. For each treatment, three replicates and control groups were used. During the challenge period, fish were continually fed their respective diets (Figure 1).

2.9. Statistical analysis

All data were tested for distribution of normality as well as variances homogeneity using Bartlett's tests. Data was subjected to one-way ANOVA to show the effect of papain enzyme levels addation. Duncan’s multiple range test as a *post-hoc* test using SAS ANOVA procedure (SAS, version 6.03, Soft Inc., Tusla, OK, USA, SAS, 1993). The differences at P < 0.05 were significant. The values are presented as means ± standard deviation (SD).

1. Results

3.1. Growth performance and nutrient utilization

Supplementing the feed with different papain levels significantly (P < 0.05) influenced in the performance indices and feed utilization coefficients. Significant differences were noted final body weight in groups P1 (107.07 ± 7.66 g) and P2 (111.98 ± 1.93 g) in comparison to the control group (99.73 ± 2.71 g) (P < 0.05; Table 1). Significant (P < 0.05) differences were also noted in the daily (DGR) and specific growth rate (SGR) (Table 1). Feed conversion ratio (FCR) was significantly lower in group P2 (1.74) in comparison to the control group (1.94 ± 0.07) and to group P1 (1.95 ± 0.03) (Table 1). Higher protein efficiency ratio (PER) values were noted in group P2 in comparison to P1 and the control group (P < 0.05; Table 1). The value of the hepatosomatic index (HSI) in group P2 was significantly higher than other feeding treatments. No differences were noted in the values of the viscerosomatic index (VSI). No fish mortality was noted in any of the groups during the experiment.

3.2. Body composition

The proximate analysis of chemical composition for whole-body and fillets are presented in Table 2. No significant (P > 0.05) differences were noted in the content of crude protein, lipid, or moisture in whole fish or in filets in treatments groups.

3.3. Digestive enzymes

Digestive enzymes in anterior and posterior intestinal fish fed different levels of papain are presented in Table 3. No significant (P > 0.05) in α-amylaseand leucine aminopeptidase of anterior intestine of fish fed different levels of papain. However, trypsin activities in anterior intestine of fish decreases with increasing level of papain. The activity of lipase of anterior in group P2 was significantly (P < 0.05) higher than other treatments groups. The activities of α-amylase, trypsin, lipase and leucine aminopeptidase was significantly higher in posterior intestine of fish fed group (P2) than other treatment groups.

3.4. Oxidative stress

The effect of papain on the activity of antioxidant enzymes is presented in Table 4. The activity of total antioxidant status (TAS), superoxide dismutase (SOD) glutathione peroxidase (GPx) significantly (P < 0.05) increased in groups P1 and P2 compared with control group. The highest activities of TAS and GPx were obtained in group P2, while the highest level of SOD was observed in P2 group. The activities of glutathione reductase was decreased with increasing levels of papain.

3.5. Histological analysis of the gastrointestinal tract

The results of the liver and middle gut histological measurements are presented in Table 5. Papain had no significant (P > 0.05) effect on the size of hepatocytes or their nuclei, the intestinal muscle thickness, or the intestinal mucosa size. However, the highest of intestinal enterocytes was observed in group P2, and the highest supranuclear surface of the intestinal enterocytes was found in groups P1 and P2. There were also no pathological changes in either the liver or the intestines.

3.6. Non-specific humoral immunity

Non-specific humoral immunity indices are presented in Table 6. No significant differences were found in lysozyme and total protein level among treatment group. While, significant (P < 0.05) increased in ceruloplasmin and total Immunoglobulin (Ig) were confirmed in groups P1 and P2 compared to control group. The highest level of blood ceruloplasmin was noted in group P2 (59.54 ± 5.88 IU) that was significantly higher in comparison to the control group (52.57 ± 4.38 IU) and P1 (54.61 ± 4.39 IU) (P < 0.05; Table 6). Significantly higher values of immunoglobulin (16.75 ± 4.05 g L-1) were noted in group P1 in comparison to the other groups.

3.7. Non-specific cellular immunity and disease resistance

Table 6 presented the non-specific humoral immunity for fish fed different level of papain. Statistically (P < 0.05) significant differences in the metabolic activities of splenic macrophages (PMA) and splenic phagocyte potential killing activity (PKA) were noted in the groups fed feed supplemented with papain in comparison to the control group. The proliferative activity of spleen T lymphocytes stimulated by mitogen concanavaline A (ConA) and B lymphocytes stimulated by lipopolysaccharide (LPS) were significantly (P < 0.05) higher in group P2 in comparison to the control and group P1.

3.8. Challenge test

At the end of the challenge trial, the fish survival of fish fed diet supplemented with 20 g papain kg-1 diet was significantly higher (*P* < 0.05) than that of the other diets (Figure 1).

4. Discussion

In the current experiment, addition of papain at 10 g or 20 g kg-1 diet had a positive effect on growth performance and feed utilization of starlet, which fish was fed feed supplemented with the papain enzyme had higher weight gain and specific growth rate than the control group. Particularly, significant (P < 0.05) differences were noted in the group fed feed supplemented with 20 g papain kg-1 diet. In addition, positive correlation was observed between endogenous enzymes trypsin, lipase and leucine aminopeptidase and FCR of fish fed the experimental diet. The improvement of performance in fish fed diets supplemented with papain may be due to the exogenous enzyme could be decreased the anti-nutritional factors of plant protein ingredients (Singh et al., 2011). Proteolytic properties of papain have a significant impact on the hydrolysis of proteins into short-chain peptides that increases the digestibility of feed (Manosroi et al., 2014; Nilsang et al., 2005; Sawant and Nagendran, 2014), subsequently, improve growth performance of fish. In this context, addition of papain or a mixture of papain and bromelain in diets of iridescent shark (*Pangasianodon hypophthalmus*) significantly increased the WG and improved FCR (Rostika et al., 2018). In addition, Carter et al. (1994) showed that higher efficiency ratio for Atlantic salmon (*Salmo salar*) in fish fed diet supplemented with enzymes, which selected to hydrolyze proteins and carbohydrates. From this point, exogenous enzymes could be decreased or eliminate the anti-nutritional factors, subsequently, improved the nutrient efficiency (Cowieson et al., 2005; Hassaan et al., 2021a). Furthermore, Nile tilapia fed diets containing feather meal and supplemented with papain significantly improved the digestibility (Munguti et al., 2014). In addition, tilapia diets containing high level of palm kernel meal while pre-treatment with enzyme, improved the net protein utilization for tilapia (Boonyaratpalin et al., 2000). Extracted enzymes from pineapple wastes significantly improved SGR and PER of *Labeo rohita* than the control, and this application could be suitable for reducing production costs (Deka et al., 2003). While, WG, SGR and FCR of juvenile sterlet (*Acipenser ruthenus*) significantly improved by supplementation with 10 or 20 g exogenous bromelain kg-1 diet than control group (Wiszniewski et al., 2019).

The digestion of feeds in fish mainly are influenced by secretion of digestive enzymes from intestine as well as the topography of intestine and habits of feeding (Hassaan et al., 2021b; Ribeiro et al., 2008). The current results showed that papain supplementation did not have significant effect on intestinal α-amylase or trypsin activity, however it significantly increase the activities of endogenous pepsin and lipase (Table 4). Furthermore, addition of papain significantly improved leucine aminopeptidase (LAP) activity in posterior intestine, but no significant effect on LAP enzyme in anterior intestine affected by papain addition. Interestingly, papain enzyme have the identic way of action on proteases and trypsin secretion which may be decreased the secretion of endogenous enzyme particularly trypsin. It may be explain why the endogenous trypsin was decreased in diet with addition of papain (Wiszniewski et al., 2019). Kvåle et al. (2007) and He et al. (2012) indicated that LAP enzyme activity used as a marker of enterocyte development. Digestive enzymes in animals are present in the gastrointestinal lumen and are associated with the intestinal epithelial cell brush‐border membrane (Bakke et al., 2010). Digestive and metabolic functions of fish clearly correlated with digestive and brush‐border enzyme activities (Hakim et al., 2006). In this context, Rachmawati et al. (2019) evaluated the effects of papain addition to diets of *C. gariepinus* on the activity of endogenous protease*,* which showed the higher activity of intestinal protease after addition of papain. Compared with control diet, endogenous amylase significantly increased in Nile tilapia fed diet supplemented with mixed enzymes (protease neutral, ß-glucan, and xylanase) (Lin et al., 2007). Contrary, Liu et al. (2018) noted that endogenous protease activity of Gibel carp (*Carassius auratus gibelio*) did not affect by supplementation of exogenous protease enzyme. While, the highest values of pepsin were obtained in snakehead (*Channa argus*) fed diet supplemented with mixture of enzymes compared with control (Dai et al., 2019).

In the present results, examination of hepatocytes sizes, nuclei sizes and hepatonuclei index cleared no significant changes for fish fed diets supplemented with papain enzymes, thus, this results indicated that the addition of papain to with 10 or 20 g kg-1 diet have not pathological hepatic tissues. On the other hand, morphometric of gut tissue significantly improved by addition of papain enzyme (Table 4). The improvement in intestinal morphology displayed in current study could have been the result of complementary enhancement to meet the increased rate of digestion and assimilation after the intake of the diets. The enterocyte absorptive area of sterlet in this study, which fed diets supplemented with papain was larger than control diets, which resulted in higher nutrient utilization in diets supplemented with 10 or 20 g papain kg-1 diet. Similarly, Dang et al. (2018) noted that intestinal mucosal topography of grass carp (*Ctenopharyngodon idella*) was improved by addition of protease in feed. In this context, Smirnov et al. (2004) and Hassaan et al. (2021a) cleared that intestinal barrier function and mucosal function as well as absorption of nutrients.

Antioxidant enzymes such as SOD can accelerate decomposition of reactive oxygen species to H2O2− as well as is consider an indicator of aquatic animal defense against oxidative stress (Ruas et al., 2008). The performance of SOD as antioxidant enzymes depends on their collaboration with the other antioxidant agents, such as CAT and Gpx (Burgos-Aceves et al., 2018; Faggio et al., 2016). In the present study, sterlet fed the two experimental diets displayed higher SOD and GPx activities than those fed the control diet, which indicated that papain could improve enzymatic antioxidant protection in this fish. The effect of papain on fish antioxidant enzymes have been poorly understood to date. Enzymatic antioxidant GPx and SOD have main role in primary antioxidant protection against free radicals in organisms (Yang et al., 2019b, 2019a). Fish have evolved systems to protect cells from these highly toxic radicals. The production of detoxifying SOD and catalase enzymes, which decompose superoxide and peroxide radicals, respectively, are reported to contribute to the disposal of many pathogens (Lefebre and Valvano, 2001; Lynch and Kuramitsu, 2000; Uzzau et al., 2002). Dietary protease improved immune system of shrimp (Song et al., 2017). In addition, protease supplementation increased scavenge the free radicals of white shrimp, *Litopenaeus vannamei*, and tilapia, *Oreochromis niloticus* × *O. aureus* (Li et al., 2016).Xu et al. (2016) and Wu et al. (2020) showed digestible dietary protein of diet of grass carp and tilapia was increased by the addition of protease indicating that optimal digestible dietary protein may protect fish from damage in the intestine.

Earlier reports have demonstrated that papaya enzyme might have a positive impact on the immune system (Otsuki et al., 2010). Non-specific immunity in fish plays a significant role in body defense mechanisms (Anderson, 1992). Previous studies have been conducted to the possibilities of using various substances, including plant-based enzymes preparations, to stimulate immune response. These studies showed that plant-based enzymes have a wide spectrum of activity on body physiology (Bricknell and Dalmo, 2005; Dügenci et al., 2003). The present study revealed that addition of papain could be stimulated the immune system of fish. Natural killer (NK) cells and immune responses of T and B-lymphocytes in the blood activated by exogenous enzymes supplementation Chandran and Nachimuthu, 2018). Supplementing feed with 20 g papain kg-1 diet led to statistically significantly increased ceruloplasmin levels and proliferative activity of T and B-lymphocytes of sterlet, while supplementing the feed with 1 % of the enzyme resulted in increased immunoglobulin levels. Furthermore, either 10 or 20 g papain kg-1 diet significantly increased the metabolic activities of splenic macrophages (PMA) and splenic phagocyte potential killing activity (PKA) of Sterlet in the current study. The role of ceruloplasmin is similar to those of interferon and transferrin in that is inhibits the growth of bacteria by depriving them of essential nutrients, i.e., copper ions (Alexander and Ingram, 1992). Concentrations of blood ceruloplasmin increase with fish growth and liver weight (Kolman, 2001). In the current experiment, distinctly higher hepatosomatic indices in the group of fish stimulated with papain confirmed this dependency. Chandran and Nachimuthu (2018) demonstrated that papain stimulated the proliferative activity of T lymphocytes, which was also, observed in the sterlet. Following stimulation with papain, the phagocytic activity of granulocytes and macrophages increased. Higher values of PMA indicated that the phagocytic cells were more efficient and were capable of more effective respiratory burst, which mean that the elimination of pathogenic factors was more effective. The stimulatory effects of the enzyme were also apparent in the ability of phagocytes to kill bacteria intracellularly.

Fish survival after the challenge of pathogenic bacteria is a key indicator of fish health status (Ringø et al., 2010). Unfortunately, little reports found to the effect of dietary supplementation enzymes on resistance of fish disease. In the present study noted, that 56-day of dietary supplementation of papain increased the fish survival of starlet after challenge. In the present study, it was noted that feeding the sterlet juveniles with diets supplemented with papain for 56 days, increased the fish survival after the challenge test. Similarly, dietary exogenous protease increased the fish survival of Nile tilapia from 51.11 % (PE0) to 64.44 % (PE5) after challenge with *S. agalactiae* (Wu et al., 2020)*.*

5. Conclusion

The current study indicated that supplementing feed with doses of 10 g and/or 20 g papain kg-1 for a period of 56-day improved growth efficiency and feed utilization, and stimulated the immunity of juvenile sterlet in aquaculture conditions. Additionally, this method was safe for both the fish and the natural environment. Few studies investigated cleared the effects of enzyme papain addition on fish, which provides the impetus to conduct further experiments specifically focused on its immunostimulatory effects.

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Table 1. Effect of dietary papain levels 10 g kg-1 (P1) and 20 g kg-1 (P2) rearing parameters of sturgeon for 56 days (mean ± SD, n = 30)

|  |  |  |  |
| --- | --- | --- | --- |
|  | Dietary treatment | | |
| C | P1 | P2 |
| Initial body length (cm fish-1) | 21.16 ± 0.51 | 21.74 ± 0.39 | 20.99 ± 0.89 |
| Final body length (cm fish-1) | 29.45 ± 0.37 | 29.67 ± 0.53 | 29.56 ± 0.43 |
| Initial body weight (g fish-1) | 36.92 ± 1.41 | 38.38 ± 3.05 | 36.22 ± 1.03 |
| Final body weight (g fish-1) | 99.73 ± 2.71c | 107.07 ± 7.66b | 111.98 ± 1.93a |
| Daily growth rate (DGR; g day-1) | 1.12 ± 0.04c | 1.23 ± 0.08b | 1.35 ± 0.02a |
| Specific growth rate (SGR; % day-1) | 1.77 ± 0.06c | 1.83 ± 0.03b | 2.02 ± 0.02a |
| Initial condition factor | 0.39 ± 0.018 | 0.37 ± 0.02 | 0.39 ± 0.04 |
| Final condition factor | 0.39 ± 0.01b | 0.40 ± 0.011a | 0.41 ± 0.01a |
| Feed conversion ratio (FCR) | 1.94 ± 0.07a | 1.95 ± 0.03a | 1.74 ± 0.02b |
| Protein efficiency ratio (PER) | 092 ± 0.03b | 0.91 ± 0.03b | 1.03 ± 0.01a |
| Viscerosomatic index VSI (%) | 5.59 ± 1.10 | 5.29 ± 0.91 | 5.51 ± 0.81 |
| Hepatosomatic index HSI (%) | 0.92 ± 0.35c | 1.16 ± 0.23b | 1.42 ± 0.50a |

Means followed by different letters in the same row are significantly different (P < 0.05).

Table 2. Effect of dietary papain levels 10 g kg-1 (P1) and 20 g kg-1 (P2) on proximate composition (%) of sturgeon for 56 days (mean ± SD, n = 3)

|  |  |  |  |
| --- | --- | --- | --- |
|  | Dietary treatment | | |
|  | C | P 1 | P 2 |
| *Proximate of whole fish composition (g kg -1 ww)* |  |  |  |
| Protein | 168.0 ± 4.8 | 168.4 ± 3.7 | 168.7 ± 2.2 |
| Lipid | 98.9 ± 3.1 | 100.1 ± 1.5 | 98.1 ± 1.7 |
| Moisture | 685.5 ± 10.0 | 701.3 ± 4.5 | 702.0 ± 8.3 |
| *Proximate of fillet composition (g kg -1 ww)* |  |  |  |
| Protein | 200.7 ± 4.3 | 195.01 ± 3.5 | 193.8 ± 4.7 |
| Lipid | 33.4 ± 2.1 | 29.3 ± 1.3 | 29.9 ± 2.2 |
| Moisture | 746.0 ± 18.1 | 793.7 ± 9.3 | 758.9 ± 3.2 |

Means followed by different letters in the same row are significantly different (P < 0.05).

Table 3. Effect of dietary papain levels 10 g kg-1 (P1) and 20 g kg-1 (P2) on digestive enzymes activity of sturgeon for 56 days

|  |  |  |  |
| --- | --- | --- | --- |
|  | Dietary treatment | | |
| C | P1 | P2 |
|  | *Anterior intestine* | | |
| α-amylase1 (IU g-1) | 35.24 ± 15.11 | 36.66 ± 11.96 | 26.77 ± 14.22 |
| Trypsin1 (IU g-1) | 3.59 ± 1.13a | 1.86 ± 0.22b | 2.32 ± 0.45b |
| Lipase1 (IU g-1) | 142.42 ± 63.94a | 47.75 ± 7.85b | 123.81 ± 29.40a |
| Leucine aminopeptidase LAP1 (IU g-1) | 19.43 ± 4.29 | 20.31 ± 5.43 | 19.40 ± 6.24 |
|  | *Posterior intestine* | | |
| α-amylase1 (IU g-1) | 27.04 ± 8.93b | 19.42 ± 9.00b | 47.90 ± 17.41a |
| Trypsin1 (IU g-1) | 1.31 ± 0.66 | 1.85 ± 0.79 | 1.96 ± 1.08 |
| Lipase1 (IU g-1) | 146.42± 18.23c | 170.63 ± 21.14b | 194.53 ± 13.91a |
| Leucine aminopeptidase LAP1 (IU g-1) | 27.57 ± 7.53b | 18.60 ± 8.10c | 36.52 ± 10.42a |

Means followed by different letters in the same row are significantly different (P < 0.05).

Table 4. Effect of dietary papain levels 10 g kg-1 (P1) and 20 g kg-1 (P2) on oxidative stress activity of sturgeon for 56 days

|  |  |  |  |
| --- | --- | --- | --- |
|  | Dietary treatment | | |
| C | P1 | P2 |
| Total Antioxidant Status TAS (mmol g-1) | 4.02 ± 2.64a | 2.15 ± 0.586b | 1.93 ± 0.77b |
| Superoxide dismutase SOD (IU g-1) | 198.11 ± 71.092c | 686.93 ± 589.40a | 648.35 ± 452.34b |
| Glutathione peroxidase GPX (IU g-1) | 62.08 ± 32.67c | 65.95 ± 24.06b | 101.91 ± 30.50a |
| Glutathione reductase GLURED (IU g-1) | 16.70 ± 8.38a | 12.13 ± 3.627b | 13.35 ± 5.55b |

Means followed by different letters in the same row are significantly different (P < 0.05)

Table 5. Histological morphometrics of liver and gut samples of sturgeon fed dietary papain levels 10 g kg-1 (P1) and 20 g kg-1 (P2) (mean ± SD, n=7)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Morphometric data | Dietary treatments | | | |
| C | P1 | | P2 |
| Size of hepatocyte (μm) | 16.01 ± 0.82 | | 16.23 ± 0.29 | 15.97 ± 0.66 |
| Size of nuclei (μm) | 4.68 ± 0.28 | | 4.85 ± 0.38 | 4.79 ± 0.16 |
| Hepatonuclei index | 0.29 ± 0.08 | | 0.30 ± 0.05 | 0.30 ± 0.04 |
| Muscularis thickness (μm) | 177.11 ± 40.16 | | 180.21 ± 52.17 | 186.47 ± 46.87 |
| Height of mucosal fold (μm) | 605.89 ± 64.21 | | 621.93 ± 39.25 | 614.76 ± 31.85 |
| Height of enterocytes (μm) | 41.12 ± 4.11c | | 43.32 ± 3.27b | 47.81 ± 2.68a |
| Height of supranuclear zone (μm) | 12.98 ± 0.89b | | 14.27 ± 0.44a | 14.99 ± 0.37a |
| Size of nuclei (μm) | 4.81 ± 0.27 | | 4.71 ± 0.33 | 4.88 ± 0.61 |

Means followed by different letters in the same row are significantly different (P < 0.05).

Table 6. Effect of dietary papain levels 10 g kg-1 (P1) and 20 g kg-1 (P2) on the non-specific cellular and humoral defense mechanisms in sturgeon for 56 days (mean ± SD, n = 10)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Dietary treatment | | |  | |
| C | P1 | P2 |  | |
| *Non-specific humoral immunity* |  |  |  |  | |
| Lysozyme activity (mg L-1) | 2.73 ± 1.05 | 2.67 ± 0.63 | 2.61 ± 0.95 |  | |
| Ceruloplasmin (IU) | 52.57 ± 4.38b | 54.61 ± 4.39b | 59.54 ± 5.88a |  | |
| Total protein level (g L-1) | 31.05 ± 4.01 | 33.43 ± 5.28 | 34.18 ± 6.26 |  | |
| Total Immunoglobulin (Ig) level (g L-1) | 11.93 ± 3.97b | 16.75 ± 4.05a | 15.86 ± 5.06ab |  | |
| *Non-specific cellular immunity* |  | | | |
| Metabolic activity of splenic macrophages (PMA) | 0.33 ± 0.08 b | 0.41 ± 0.11a | 0.47 ± 0.13a |  | |
| Potential killing activity of splenic phagocytes (PKA) | 0.33 ± 0.07b | 0.40 ± 0.13a | 0.41 ± 0.17a |  | |
| Proliferative response of lymphocytes T  stimulated by mitogen concanavaline A (ConA) | 0.09 ± 0.01b | 0.09 ± 0.01b | 0.12 ± 0.01a |  | |
| Proliferative response of lymphocytes B  stimulated by lipopolysaccharide (LPS) | 0.08 ± 0.06b | 0.07 ± 0.002b | 0.11 ± 0.01a |  | |

Means followed by different letters in the same row are significantly different (P<0.05).

Figure 1. Effect of dietary papain levels 10 g kg-1 (P1) and 20 g kg-1 (P2) on the cumulative mortality of sturgeon after challenge test with *Yersinia ruckeri* at a dose of 0.2 mL; 1 × 107.