Aquaculture Reports 24 (2022) 101180

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Combined effects of *Pediococcus acidi lactici* and natuzyme on growth performance, hematology and immunity indices in juvenile beluga (*Huso huso*)

Maryam Musavi [a,1], Shaghayegh Hasanpour [b,c,*,1], Roghieh Safari [a], Mohammad Reza Imanpour [a], Joaquim Gutiérrez [d]

[a] Department of Fisheries, Faculty of Natural Resources, University of Gorgan, Gorgan, Iran
[b] Department of Fisheries, Faculty of Natural Resources, University of Tehran, Karaj, Iran
[c] Development and Biosystematics Lab, Department of Fisheries and Animal Sciences, Faculty of Natural Resources, University of Tehran, P.C 31587-7787 Karaj, Iran
[d] Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

ARTICLE INFO

Keywords:
Pediococcus acidi lactici (PB)
Natuzyme (EN)
Growth hormone
Insulin-like growth factor and beluga

ABSTRACT

The present study was performed to compare the combined and individual effects of *Pediococcus acidi lactici* (PB) and natuzyme (a cocktail of protease, lipase as well as non-starch polysaccharidases) on the immune response and growth performance parameters of the juvenile beluga (*Huso huso*). To prepare the treatment diets, the basal diet was supplemented with either the exogenous natuzyme (at 0, 0.25 and 0.5 g kg^{-1}), PB (at 0% and 0.1%) or both of them. The six treatments were assigned to triplicate groups and the feeding trial lasted for two months. The results showed that PB treatment constrained the positive effect of EN (especially at the higher dose) on the FCR and final weight. However, IGF and GH expression not only increased following either PB or EN inclusion, but also, their simultaneous addition promoted their individual effects. Together, higher level of GH and IGF mRNA levels in this study was not associated with a significant growth enhancement, this can be due to the fact that more time should be considered to display their effects. In the light of these results, we recommend that the combined use of probiotics and exogenous enzymes especially at the higher dose can be inhibitory.

1. Introduction

To answer the growing global market demand for aqua-food and on the other hand, shortage of freshwater resources, aquaculture intensification seems unavoidable (Hassaan et al., 2019; Rodriguez et al., 2018; Yao et al., 2019). Nowadays disease incidence and nutritional constraints are the major challenges facing the aquaculture industry (Fuchs et al., 2015; Tarkhani et al., 2020). Intensification leading to stressful conditions suppresses immunity through cortisol level enhancement, thereby increasing the susceptibility to secondary infectious disease (Giri et al., 2019; Mohammadi et al., 2020). These issues can provoke a noticeable economic loss due to massive mortalities or a decrease in profit margin (Giri et al., 2019).

Dietary inclusion of antibiotics was traditionally used as prophylactic therapy. However, due to the emergence of antibiotic-resistant bacteria, disturbance of the natural microbial equilibrium in either environment or fish gut (Yu et al., 2018), antibiotic residue in aquaculture products, and low meat quality, their application was legally restricted and many efforts have been focused on their exclusion from the diets to reduce the abuse of antibiotics in aquaculture. Currently, the administration of probiotics as an environmental-friendly alternative to antibiotics has flourished to augment the immune system function (Fuchs et al., 2015; Wealleans et al., 2017; Yu et al., 2018). Indeed, probiotics confer health advantages by modulating the immune system, promoting the colonization of beneficial microbial flora as well as alleviating potential pathogens (Hassaan et al., 2019, 2020). Lactic acid bacteria including *Peducoccos acidilactici* (PB) are characterized by their ability to produce large amounts of lactic acid as the major end product of carbohydrate fermentation. Most of the Lactic acid bacteria species as the commensal fish intestinal bacterial flora, are extensively applied to

Abbreviations: PB, *Pediococcus acidi lactici*; EN, natuzyme.

* Corresponding author at: Department of Fisheries, Faculty of Natural Resources, University of Tehran, Karaj, Iran.
E-mail address: sh.hasanpour@ut.ac.ir (S. Hasanpour).

[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.aqrep.2022.101180
Received 8 January 2022; Received in revised form 17 May 2022; Accepted 18 May 2022
Available online 24 May 2022

reduce disease probability and improve growth performance (Ahmadifar et al., 2020; Hoseinifar et al., 2011; Tarkhani et al., 2020).

All fish diets contain a significant portion of plant source ingredients which have a wide variety of antinutritional factors such as protease-inhibitors, phytic acid, and non-starch polysaccharides (NSPs), that negatively impact fish health and its growth performance because fish cannot digest them efficiently compared with mammals due to the deficiency in the secretion of gastrointestinal enzymes required to break down these complex cellular structures (Ai et al., 2007; Fuchs et al., 2015; Hassaan et al., 2019; Zduńczyk et al., 2020; Zhou et al., 2013). To overcome the plant-by-product problems and expand their application, supplementation of exogenous enzymes like glucanase, phytase, xylanase, etc. has been proposed as a key solution for better nutrient digestibility of plant feedstuff in aquaculture diets (Diógenes et al., 2018; E Abd Elnabi et al., 2020; Hassaan et al., 2020; Rodriguez et al., 2018; Wickramasuriya et al., 2019).

Application of exogenous enzymes in the poultry diet was associated with nutrient digestibility, energy availability, gut health improvement, growth performance promotion, and a decrease in phosphorous excretion into the environment (Wealleans et al., 2017; Wickramasuriya et al., 2019). Furthermore, the beneficial effects of protease supplementation in rainbow trout (Hassaan et al., 2020), Gibel carp (Liu et al., 2017), Caspian Salmon (ali Zamini et al., 2014) have also been reported. Regardless of the positive effects of exogenous enzymes on nutrient digestibility and feed efficiency, they may change the activity and community composition of intestinal microbiota, which play a key role in metabolic and immunologic functions (Ghodrati et al., 2021). Therefore, due to little work on the interaction between exogenous enzymes and probiotics in fish feed, the present study was conducted to compare the combined and individual effects of the PB and natuzyme (a cocktail of protease, lipase as well as non-starch polysaccharidases) on the immune response and growth performance parameters of the fish.

Juvenile beluga (*Huso huso*), one of the most commonly cultured sturgeon, has a unique gastrointestinal tract with pyloric stomach caeca, which can be easily adapted to commercial diets containing a high percentage of vegetable ingredients (Matani Bour et al., 2018). However, no previous investigation was done on the combination of exogenous enzymes and probiotics in belugas' diet. Hence, to follow the effects of treatments, the experiment was conducted on juvenile beluga.

2. Material and methods

2.1. Diets preparation

To prepare the treatment diets, the basal diet (Copenz, Germany; containing 54% crude protein, 15% lipid, 9.1% ash, 0.05% fiber and 1.25% Phosphorous) was supplemented with either the exogenous natuzyme (Bioproton, Australia; at 0, 0.25 and 0.5 g kg^{-1}), probiotic (*P. acidilactici* of Lameland, France; at 0% and 0.1% (10^7 CFU/g)) or both of them. *P. acidilactici* was supplied in a lyophilized form and cultured in de Man, Rogosa & Sharpe (MRS; Oxoid, Basingstoke, UK) broth and centrifuged. To make the experimental diets, EN, PB, or their complex were mixed with a 2% gelatin solution and sprayed onto the basal diet. The control diet was also sprayed with a 2% gelatin solution. The diets were then dried in the open air for two hours, and sealed in plastic bags at 4°C. The feeds were prepared as required every two days. The six treatments were assigned to triplicate groups and the feeding trial lasted for two months. The probiotic (PB) and enzyme (EN) supplemented diets were as follows:

(T1) basal diet (control),
(T2) basal diet + 0.25 EN,
(T3) basal diet + 0.5 EN,
(T4) basal diet + 0.1 PB,
(T5) basal diet + 0.1 PB + 0.25 EN,
(T6) basal diet + 0.1 PB + 0.5 EN.

2.2. Fish rearing

Following two weeks of acclimatization a total of 180 healthy juvenile beluga with an initial weight of 12.56 ± 0.48 g, were randomly assigned into 18 reservoirs of 300 Lit. The fish were hand-fed trice a day with the mentioned diets at 3% of the body weight per day. The amount of feeding was adjusted every two weeks following a 24 h starvation, and batch weighing. During the trial, the photoperiod was considered at 12 L: 12D. Water temperature, pH, and DO levels were monitored and maintained at 22 ± 2, 7–8, and 6–7 mg L^{-1} respectively.

2.3. Growth performance

At the end of the feeding trial (survival rate 100%), growth performance and feed utilization were assessed by measuring Specific Growth Rate (SGR), Body Weight Increase (BWI), Food Conversion Ratio (FCR), and Feed Efficiency Ratio (FER) using the following formula:

$$SGR = \frac{\text{Ln(Final weight)-Ln(Initial weight)}}{\text{Day}} \times 100$$

$$BWI = \text{Final weight-Initial weight}$$

$$FCR = \frac{\text{Feed in take (g)}}{\text{Weight gain (g)}} \times 100$$

$$FER = \frac{\text{Weight gain (g)}}{\text{Feed in take (g)}} \times 100$$

2.4. Sample collection

Following the sedation with clove powder (500 mg L^{-1}), blood samples from 9 fish per treatment were collected from the caudal vein, pulled, and divided into heparinized, and non-heparinized tubes for hematological and Serological assays respectively.

2.4.1. Hematological assays

Briefly, the red blood cells (RBCs) RBCs counts were determined using a Neubauer hemocytometer under a compound microscope at 100 × magnification after diluting the blood sample in Hayem. Hematocrit (Hct) was determined after centrifugation of heparinized capillary tubes at 6000 × *g* for 5 min with a microcentrifuge and reported as a percentage (Blaxhall and Daisley, 1973). Hemoglobin level (Hb) was spectrophotometrically assayed based on the cyanomethemoglobin method. The mean cell volume (MCV), the mean cell hemoglobin (MCH), and the mean-corpuscular hemoglobin concentration (MCHC) were calculated according to the method suggested by Drabkin (Drabkin and Austin, 1935).

2.4.2. Serological assays

Non-heparinized blood samples were allowed to clot at 4 °C (12 h) and then centrifuged at 3600 rpm (5 min at 4 °C). The Serum samples were isolated and stored at − 80°C until use. The enzymatic activities of the liver including aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were determined based on the colorimetric method (Reitman and Frankel, 1957). An aliquot of the serum sample (0.5 mL) was incubated for 30 min at 37 °C with 0.5 mL substrate (sodium azide) and the solution was then mixed with 0.5 mL of 2,4-dinitrophenylhydrazine to arrest the reaction. After incubation for a minimum of 20 min at 37 ∘C, the mixture reaction was stopped with the sodium hydroxide, and the enzyme activities were spectrophotometrically determined by measuring the optical density at 546 nm. Alkaline phosphatase (ALP) was analyzed according to the enzyme-mediated conversion of *p*-nitrophenyl phosphate to nitrophenol in an alkaline buffer using commercial assay kits (Pars Azmcon company, Tehran) according to the manufacturer instructions and a biochemical Auto

analyzer (Prestige-24i) (Reitman and Frankel, 1957).

2.5. Mucus sampling

Nine Fish per treatment were randomly selected, anesthetized, and individually transferred into polyethylene bags containing 10 mL of 50 mm NaCl. After gently shaking for 2 min, the samples were collected and centrifuged (10 min at 4°C). The supernatants were kept frozen at − 80 °C until use (Sukumaran et al., 2016). Mucus lysozyme activity was measured using a turbidimetric method based on the lysis of the lysozyme-sensitive gram-positive bacterium *Micrococcus luteus* (Subramanian et al., 2007). Briefly, 50 µl of the mucus sample was added to 50 µl of buffer suspension containing *M. luteus,* and the reduction in absorbance was measured after 10 min, at 450 nm using a spectrophotometer (Biochrom Libra S12). Mucus IgM level was determined by ELISA method at 450 nm using a 96-well microplate reader.

2.6. GH and IGF gene expression

The liver and brain tissues of six juvenile beluga were dissected and immediately kept at liquid nitrogen and then transferred to − 80 °C until use. After sample homogenization, total RNAs were extracted using the RiboEx Kit according to the manufacturer's protocol. The quality and quantity of total RNAs were assessed by 1% agarose gel and spectrophotometer respectively. The first-strand cDNA was generated from 1 µg of total RNA by Supreme script RTase (GENET BIO). The real-time PCR was Performed (BlO-RAD, IQ5) using 2X-SYBR Green PCR Master Mix (Ampliqon) according to the manufacturer's instructions. The primers were as follows (Table 1) (Safari et al., 2020). Relative gene expression of each gene was quantified from threshold cycles for amplification, using the ΔΔCt method and normalized to b-actin levels (Livak and Schmittgen, 2001).

2.7. Data analysis

After the evaluation of the normality and homogeneity of data through Kolmogorov-Smirnov and Leven's test, they were analyzed using 2-way ANOVA, followed by the Tukey test. Pearson's correlation between variables was calculated as well. Differences were considered significant at a value of $P < 0.05$. Statistical analysis was done using the graph-pad prism and all the results were presented as mean ± SD.

3. Results

3.1. Growth performance

Meanwhile, exclusive administration of either EN or PB had no overall effect on growth performance parameters, a crossover interaction among the treatments was seen. Indeed, the effect of EN on the final weight, weight gain, FCR, and FER, as the dependent variables, was suppressed depending on the value of PB ($P < 0.05$-Fig. 1). In other words, PB treatment constrained the positive effect of EN (especially at the higher dose) on the FCR and final weight.

3.2. Hematobgical indices

The treatments were found not to have a significant effect on the Hb, RBC, and MCH. Furthermore, no interaction between PB and EN was observed ($P < 0.05$-Fig. 2**A, C, and E**). Feeding on an EN supplemented diet resulted in the neutralization of the PB effects on MCV, and MCHC ($P < 0.05$-Fig. 2**D, and F**). Only in the case Hct, where no interaction effect between treatments was seen, administration of PB was associated with a significant difference.

3.3. Serological parameters

The treatments exerted no significant effect on AST. However, dietary inclusion of PB significantly increased the ALP surface compared to the control group. But, PB's negative interaction with EN at the 0.05 level confined EN's positive effect on the ALP (Fig. 3A) i.e. the combined application of EN and PB attenuated their exclusive increasing effects on ALP ($P < 0.05$, Fig. 3C). A strong decrease in ALT level was observed after PB and or EN application. No significant interaction did not impact that relationship their exclusive effects(Fig. 3C).

3.4. Mucosal parameters

Dietary inclusion of exogenous EN had no effect on lysozyme content of mucus, while PB addition promoted that. The addition of EN did not intensify the positive effects of PB treatment ($P > 0.05$). No significant difference was observed in mucus Ig level among the treatments (Fig. 4).

3.5. GH and IGF gene expression

IGF and GH expression increased following either PB or EN inclusion. Besides, their simultaneous addition promoted their individual effects, therefore application of PB and EN at the higher level maximized the expression of IGF and GH genes (Fig. 5).

3.6. Evaluation of the correlation between variables

Pearson's correlation (r) was calculated between variables. As can be seen from the graph (Fig. 6), the changes in the weight gain were correlated positively with other growth performance parameters except the FCR ($P < 0.01$). In addition, the GH was positively correlated with IGF and lysozyme, (rIGF01 = 0.893, rLysozyme = 0.918, $P < 0.05$). An increase in the GH, IGF, and lysozyme corresponded with reductions in the ALT and Hct levels ($P < 0.05$).

4. Discussion

One of the objectives of this study was to explore the synergy between enzyme and probiotic supplementation. Not only, dietary supplementation of either EN or PB had no effects on growth performance parameters, but also, the use of EN, in the presence of PB, could not affect them positively, i.e. their simultaneous administration resulted in an antagonistic effect on final weight, weight gain, FCR, and FER. Likewise, Mass et al. showed that there are no synergistic effects between enzymes (phytase and xylanase) and probiotic supplementation

Table 1
The sequences of primers used in the experiment.

Sequence of primers	Connection temperature (°C)	Length of the piece (bp)	Gene	Primer performance
F: AGACATCAGGGTGTCATGGT R: CTCAAACATGATCTGTGTCAT	58	224	Beta-actin (GenBank: MK771092.1)	99%
F: TTCATGATGAGTGCTCCGTTC R: GTCAGAATTCAAGTGGCGAATC	58	210	GH (GenBank: AB517597.1)	99%
F: CAAACATGATCTGTATGTG R: AGAATTCAAGTGGCGACATG	58	220	IGF (GenBank: AB512770.1)	99%

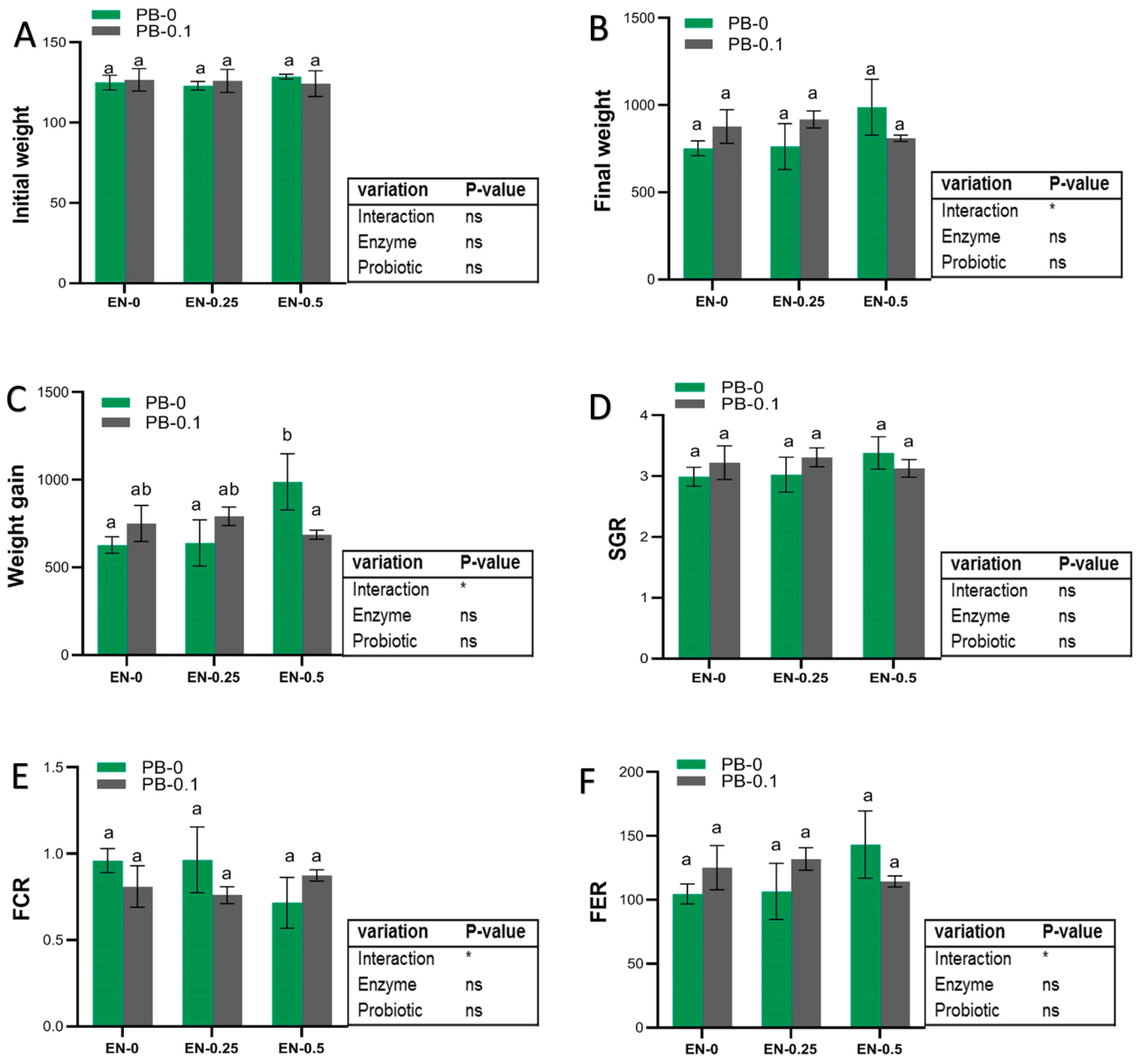

Fig. 1. Growth performance and nutrient utilization of juvenile beluga (*Huso huso*) fed experimental diets supplemented with either PA probiotic (PB), natuzyme (EN) or both over 2 months. Data are mean ± SD. Letters indicate significant differences in treatments, according to Tukey test ($P < 0.05$).

(*Bacillus amyloliquefaciens*) on growth parameters in Nile tilapia (Maas et al., 2021). Following the same pattern, the probiotic, *B. amyloliquefaciens*, and the multi-enzyme complex did not enhance the growth performance of snakehead (Dai et al., 2019). These negative interactions can be ascribed to probiotic functions. Probiotics alter the gut environment in various ways (physiology, gut microbiota, production of metabolites, pH, etc.) by releasing many metabolic compounds, which in turn, lead to a less favorable condition for the exogenous and endogenous enzymes and decrease their activity (Dai et al., 2019). Furthermore, based on Maas et al. enzymes had a strong effect on nutrient digestibility and retention of N, energy, and P, which were higher for the enzyme- treated group, compared to the probiotic one (Maas et al., 2021). If enzymes and probiotics compete for the same substrate, this might also explain the lack of additivity. Besides, the antagonistic effect of PB on EN could partly be due to the control diet with a good FCR (0.9), and a high nutritional value which already limits any further improvement in diet quality through additional additives, such as other enzymes and probiotics (Maas et al., 2019). Therefore, it could be expected that under challenging conditions, the combination could lead to better growth performance.

Hematological indices were measured to evaluate general health status as well as nutritional and environmental conditions influencing fish (Hoseinifar et al., 2011). Based on our findings, either EN, PB or their combination made no significant effect on these parameters. Similar results have been reported earlier (Adeoye et al., 2016; ali Zamini et al., 2014). However dietary inclusion of papain proteinase (Prabjeet et al., 2011) provoked a significant increase in RBC, in Nile tilapia (*Oreochromis niloticus*). Aside from a few beneficial reports in this context, the health status of fish has not been affected negatively in this experiment as well.

A rise in the hepatic enzymes' levels including AST, and ALT, as the biomarkers of hepatocellular dysfunction, elucidates a liver injury. Indeed, a wide range of AST-containing organs such as kidneys or muscles makes it a relatively less specific indicator of liver damage

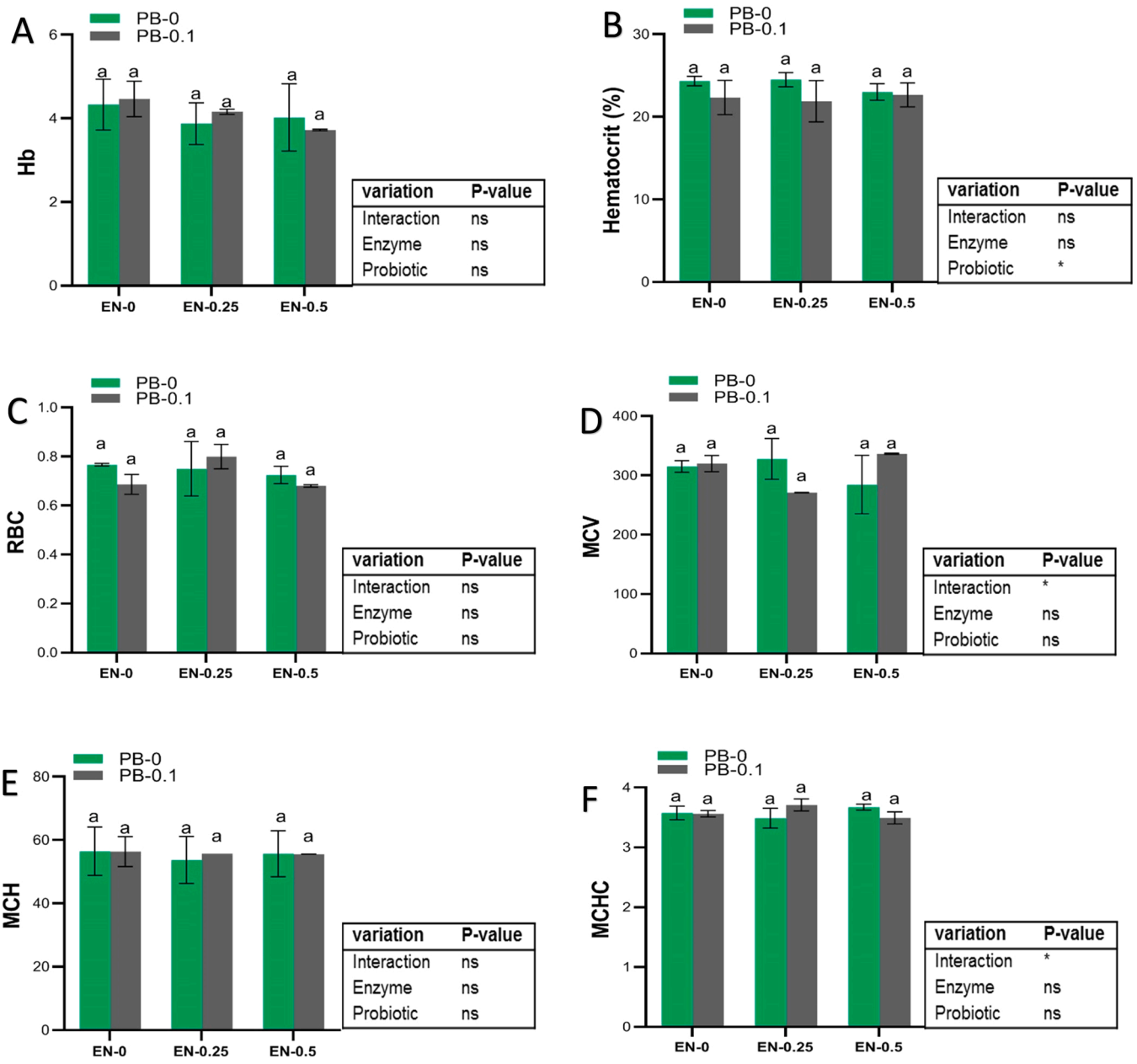

Fig. 2. Hematological parameters of juvenile beluga (*Huso huso*) fed experimental diets supplemented with either PA probiotic (PB), natuzyme (EN) or both over 2 months. Data are mean ± SD. Letters indicate significant differences in treatments, according to Tukey test ($P < 0.05$).

compared to ALT (Giannini et al., 2005). Although our results displayed no significant difference in serum AST concentration in different treatments, application of EN or PB lowered ALT level compared to the control group and their combined administration had no additive effect on the ALT decrease. The results were in line with previous reports following the application of *Pediococcus acidilactici* in rainbow trout (Ferguson et al., 2010) and rockfish (Rahimnejad et al., 2018). Although present knowledge about the combined effects of dietary probiotics and exogenous enzymes on serum liver enzymes is scant in fish, previous studies reported the lower serum level of liver enzymes related to probiotics function (Ghodrati et al., 2021). Thus, combined administration of PB and EN provided at least a liver care in beluga juveniles by decreasing ALT concentration.

Alkaline phosphatase is a lysosomal enzyme, which acts as a potential defensive component against parasite invasion and other stressful statuses (Fast et al., 2002). Therefore, improved ALP activity can lead to better immunity function (Hoseinifar et al., 2018). Based on the results, increased ALP activity may be a result of improved mucosal immunity over feeding by either EN or PB treated groups, and combined EN and PB application intensified the ALP enhancement.

Fish skin mucus contains various components of the innate immune system, like Ig and lysozyme, to combat pathogens entry. The skin mucus parameters can be extensively affected by environmental and nutritional modifications (Caipang and Lazado, 2015), like the dietary combination with probiotics. According to our results, dietary inclusion of EN made no significant differences in mucus lysozyme level, while the PB application significantly stimulated skin mucus innate immunity by lysozyme enhancement that might be ascribed to modulation of the intestinal microbiota in favor of the lactic acid bacteria population, stimulating bactericidal activity of skin-associated lymphoid tissue through lysozyme (Ashouri et al., 2018; Tarkhani et al., 2020). In addition, the lysozyme concentration in groups treated by EN and PB combination was not significantly higher than those of fed PB singularly. Therefore, no synergistic effect was observable. Indeed probiotic

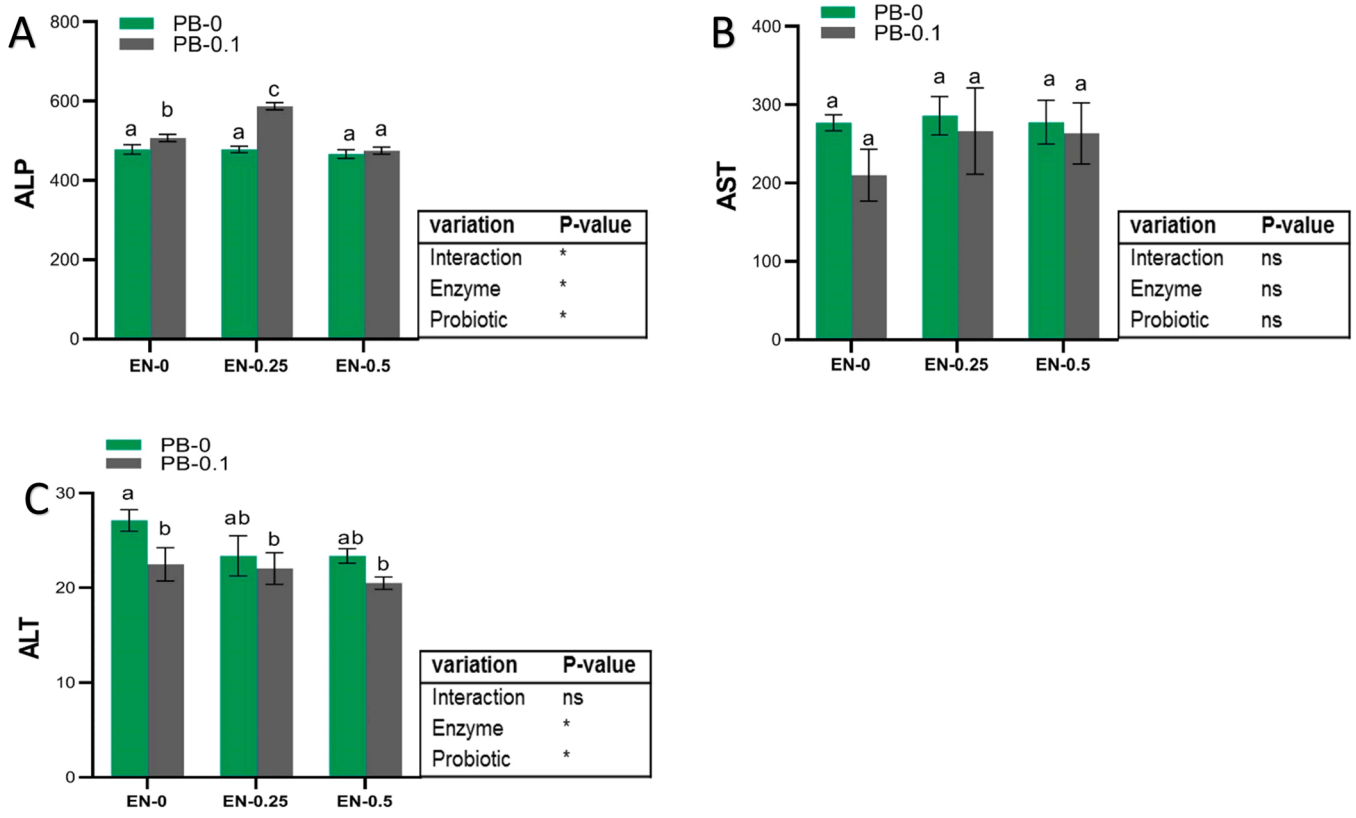

Fig. 3. Serological parameters of juvenile beluga (*Huso huso*) fed experimental diets supplemented with either PA probiotic (PB), natuzyme (EN) or both over 2 months. Data are mean ± SD. Letters indicate significant differences in treatments, according to Tukey test ($P < 0.05$).

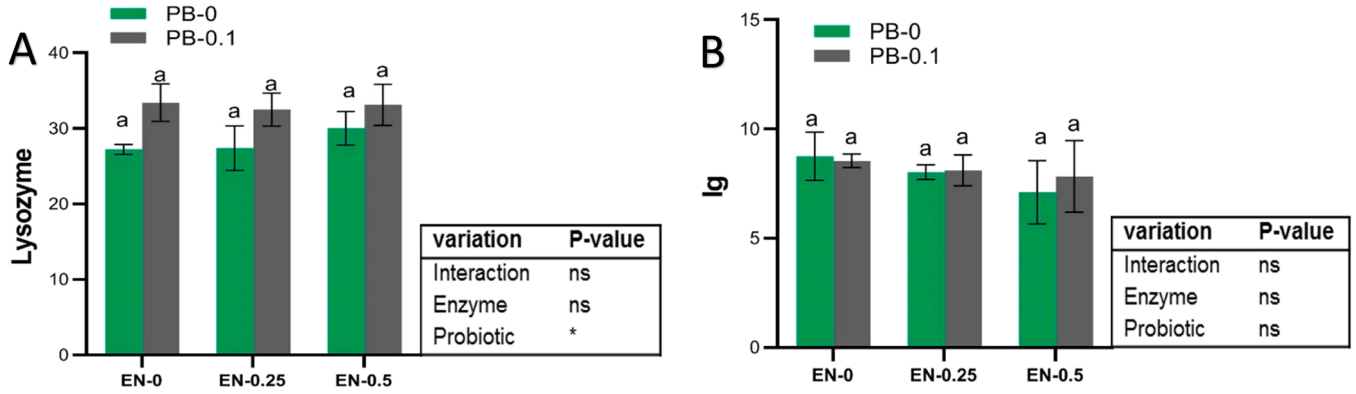

Fig. 4. Mucosal immunity parameters of juvenile beluga (*Huso huso*) fed experimental diets supplemented with either PA probiotic (PB), natuzyme (EN) or both over 2 months. Data are mean ± SD. Letters indicate significant differences in treatments, according to Tukey test ($P < 0.05$).

bacteria and gut-associated lymphoid tissue interaction stimulated mucosal immunity in the host whether in the gut or its skin mucus (Jami et al., 2019) and put the body on alert condition to fight against pathogens. The increase in skin mucus lysozyme level has been reported in a variety of studies following the probiotic application in agreement with our results (Dawood et al., 2015; Jami et al., 2019). Altogether, it seems that probiotic administration strengthens the host immune system and it can be considered an eco-friendly alternative for antibiotics in the aquaculture industry.

Ig protecting fish against pathogens showed no significant differences in the skin-associated lymphoid tissues among the different treatments. In contrast, serum Ig enhancement has been reported in various studies following either probiotic or multienzyme treatments. These discrepancies might be attributed to the fish species, size, experimental design, etc. (Ashouri et al., 2018).

Dietary inclusion of either PB or EN significantly increased the expression of GH and IGF genes as observed in gilthead sea bream (*Sparus aurata*) fed with either *Shewanella Puterfaciens* (Guzmán-Villanueva et al., 2014) or *Lactobacillus plantarum* and *Bacillus licheniformis*-TsB27 (Bahi et al., 2017), also in common carp fed with *L. casei* (Safari et al., 2017), and in zebrafish fed with royal jelly (Aksakal et al., 2021; Vural et al., 2021). The combination of EN and PB further enhanced GH and IGF, compared to the EN or PB alone treatment. Indeed, impaired food digestion in juvenile fish because of the non-developed digestive system and insufficient endogenous enzymes production, was associated with lower GH and IGF mRNA levels. But exogenous enzymes administration underpinned the endogenous enzymes and nutrient bioavailability (ali Zamini et al., 2014) which in turn

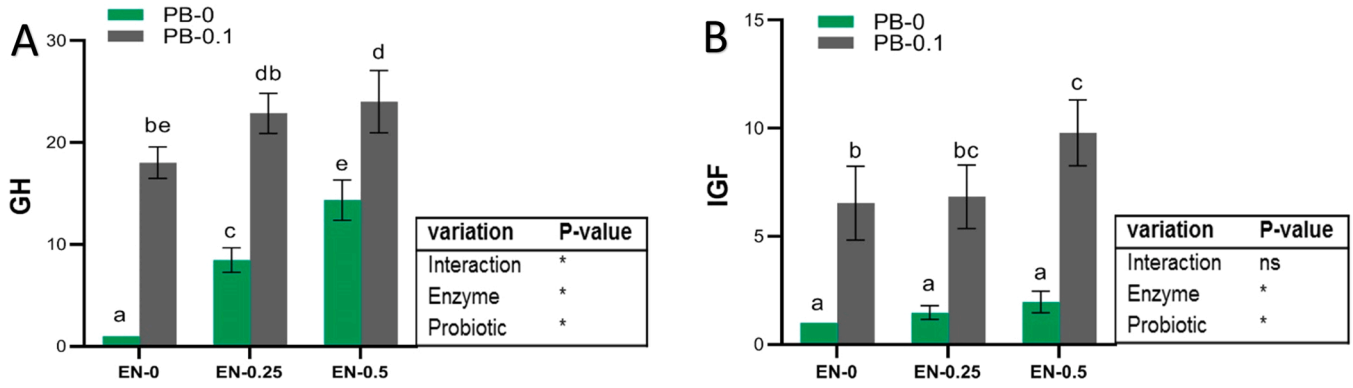

Fig. 5. Expression of growth-relevant genes [(A) GH and (B) IGF] determined by real-time PCR in liver and brain of *H. huso* fed experimental diets supplemented with either PA probiotic (PB), natuzyme (EN) or both over 2 months. Data are mean ± SD. Letters indicate significant differences in treatments, according to Tukey test ($P < 0.05$).

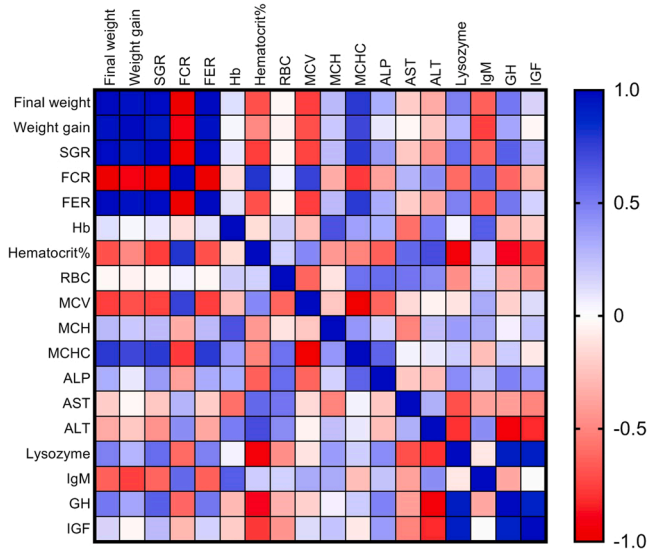

Fig. 6. Heat map shows Pearson's correlation between variables following different diet exposures. The bar is the color key. Red signifies down-regulation and blue upregulation.

triggered GH & IGF gene expression. Additionally, probiotic bacteria increased nutrient assimilation by their exogenous enzymes and stimulation of host digestive enzymes secretion (Irianto and Austin, 2002; Tarkhani et al., 2020). Notwithstanding, higher level of GH and IGF mRNA levels in this study was not associated with a significant growth enhancement, this can be due to the fact that higher levels of GH and IGF mRNAs do not necessarily mean higher levels of those proteins, and or maybe more time should be considered to display their effects.

Based on the results, the positive correlation between GH, IGF, and lysozyme was significant. Likewise, an increase in lysozyme activity was found in olive flounder fed a diet containing 20 mg GH Kg^{-1} (Lee et al., 2008). In another study, a dose-dependent increase in plasma lysozyme level in rainbow trout was observed by implantation of a cholesterol pellet containing GH (Yada et al., 2004). Indeed, it has been determined that GH stimulates the expression and secretion of IGF, which is responsible for sulfate uptake, thereby increasing the synthesis of proteins, RNA, and DNA (Aksakal et al., 2021; Triantaphyllopoulos et al., 2020; Vural et al., 2021). Therefore, due to the promotion of liver health, GH and IGF showed negative correlation with ALT concentration.

5. Conclusion

Together, while PB treatment constrained the positive effect of EN (especially at the higher dose) on the FCR and final weight, IGF and GH expression increased following either PB or EN inclusion, besides, their simultaneous addition promoted their individual effects. However, higher level of GH and IGF mRNA levels in this study was not associated with a significant growth enhancement, this can be due to the fact that more time should be considered to display their effects. In the light of these results, we recommend that the combined use of probiotics and exogenous enzymes especially at the higher dose can be inhibitory.

CRediT authorship contribution statement

Maryam Musavi: Conceptualization, Methodology. **Shaghayegh Hasanpour**: Data curation, Writing – original draft preparation, Software, visualization. **Roghieh Safari**: Supervision investigation. **Mohammad Reza Imanpour**: Validation. **Joaquim Gutiérrez:** Editing.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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