# Effects of Dietary Glutathione on the Growth Performance, Skin Mucus Antioxidant Capacity, and Immune Responses of Juvenile Taimen *Hucho Taimen*

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# Abstract

The effects of dietary glutathione on growth performance, skin antioxidant capacity, and immune responses and of juvenile taimen (*Hucho taimen*) were investigated. The experimental fish had a mean initial weight of 5.36 ± 0.13 g and were fed for eight weeks on diets containing graduated amounts of glutathione (0, 100, 200, 400, and 800 mg $kg^{-1}$). After the 7 d lipopolysaccharide challenge, the immune parameters of the skin mucus, and antioxidant ability were determined. The treatment groups (400 – 800 mg $kg^{-1}$) showed a higher survival rate and weight gain rate ($P < 0.05$). There were higher levels of skin mucus protein, lysozyme activity, and alkaline phosphate activity associated with dietary glutathione supplementation ($P < 0.05$). Dietary glutathione supplementation improved the minimal inhibitory concentration and antimicrobial activity of the skin mucus ($P < 0.05$). Fish in the treatment groups showed higher superoxide dismutase, glutathione peroxidase, and catalase activity ($P < 0.05$), whereas the malondialdehyde content was lower ($P < 0.05$) than those in the control group. Supplementary glutathione decreased the expression levels of TNF-α, IL1β, IL6, IL8 in the skin and ensured the relatively high expression levels of IκBα after lipopolysaccharide challenge. In conclusion, dietary glutathione (400 – 800 mg $kg^{-1}$) improved the growth performance, decreased lipopolysaccharide-induced skin inflammatory response, indicating that glutathione has the potential anti-inflammatory effects for preventing inflammation diseases in juvenile taimen.


# Introduction

Fish in intensive aquaculture are frequently subjected to a range of negative environmental stresses, including high temperatures, overcrowding, deteriorating water quality, pathogen invasion, and disinfectant and antibiotic abuse (Ming et al. 2015). This causes not just bacterial resistance and residues in aquatic products, but also immunosuppression in fish and increased susceptibility to different diseases (Liu et al. 2010), which can lead to significant economic losses. As a consequence, one of the methods is to improve fish immune and stress resistance through dietary approaches.

The feeding of immunostimulants for the promotion of fish welfare and disease prevention has been reported, and many forms of immunostimulants are available, such as synthetics, microbial materials, lactoferrin, vitamins, hormones, and animal and plant extracts (Faggio et al. 2015). Glutathione, the most abundant antioxidant in tues, is a tripeptide consisting of glutamate, cysteine, and glycine residues (Rahman and MacNee 2000). Glutathione can not only eliminate unnecessary free oxygen radicals from the cells, but it also has many physiological roles, such as increasing the activity of antioxidants (Doyotte et al. 1997), protecting liver cells (Ponsoda et al. 1996), maintaining DNA synthesis, enhancing immunity (Will et al. 1999), alleviating neuron intoxication (Raghunathan et al. 2007) and improving growth performance[9,10,11]. (Zambonino Infante et al. 1997; Ming et al. 2019; Ma et al. 2019). However, data on the function of glutathione in fish are currently rare.

Although immune response and disease tolerance records have been published for glutathione-fed European bass (*Dicentrarchus labrax*) (Zambonino Infante et al. 1997), grass carp (*Ctenopharyngodon*

*idellus*) Ming et al. 2019), Nile tilapia (*Oreochromis niloticus*) (Zhang et al. 2007), and Atlantic salmon (*Salmo salar*) (Ma et al. 2019), the effects of glutathione on fish skin mucosal parameters have not been published. Skin mucus is an essential part of the immune system response in fish. The epidermis covering the glandular dermal layer of the fish body comprises an abundance of glandular cells that secrete mucus to protect fish against chemical, physical, and biological stressors (Guardiola et al. 2016). Skin mucus is the first line of defense against pathogens, and it has multiple inherent immune components such as immunoglobulins, lysozymes, C-reactive and complementary proteins, proteases, lectins, and proteolytic enzymes, all of which play a significant role in inhibiting the induction of pathogens (Rao et al. 2015; Lauriano et al. 2016). Previous research has shown that dietary supplements enhance skin mucus immune reactions in different species of fish, including rainbow trout (*Oncorhynchus mykiss*) (Taee et al. 2017), barb (*Puntius sophore*) (Patel et al. 2020), Nile tilapia (Srichaiyo et al. 2020), and common carp (*Cyprinus carpio*) (Hoseinifar et al. 2018). Therefore, the immunity of fish skin may be enhanced by the addition of immune stimulants such as glutathione.

Taimen (*Hucho taimen*) is the most important cold-water fish farmed in China. However, skin ulcers frequently occur throughout fish production, which can be a significant hindrance to farming efficiency, therefore this study was conducted to investigate the role of glutathione in modulating the growth performance and skin mucus immune parameters, as well as the antioxidant capacity of juvenile taimen.

# Materials And Methods

### Experimental Diets

Experimental diets with five glutathione supplementation levels (0, 100, 200, 400, and 800 mg $kg^{-1}$) were prepared (Table 1). According to spectrophotometric analysis, dietary glutathione concentration were 0.25 (G1), 98.35 (G2), 201.36 (G3), 398.66 (G4), and 796.98 (G5) mg $kg^{-1}$ diets, respectively. The key protein sources in the experimental diets were casein and gelatine, with fish oil as a lipid source, and dextrin and starch as carbohydrate sources. The ingredients were finely ground before mixing (< 250 μm) and then blended with vitamins and minerals. All the ingredients were thoroughly mixed for 15 min and mixed again for 10 min after the addition of the lipid source. The resultant dough was made into feed pellets with a diameter of 1.2 mm and dried in a ventilated oven at 45 °C for approximately 12 h. The pellets were then frozen at −20 °C until required. Normal procedures were used to determine the crude lipid, crude protein, moisture, ash, and gross energy of feed (AOAC 2012).

### Feeding experiment

The experimental fish were adapted in a laboratory for 14 d before the experimental feed was introduced. The fish were starved for 24 h and pooled at the start of the trial. A total of 4500 fish (initial weight 5.36 ± 0.13 g) were distributed into each of 15 tanks (size: 500 L). The tanks were divided into five treatment groups with three replicates per treatment. The fish in each tank were weighed as a batch. Fish challenged by intraperitoneally injecting with 100 μL of lipopolysaccharide (3.00 mg $kg^{-1}$ of body weight;

Sigma, USA) diluted in sterile phosphate buffer (PBS) on day 49 (Haukenes and Barton 2004). During the 56 d feeding trial, the fish were manually fed until evident satiation, four times a day (08:00, 11:00, 14:00, and 17:00). The fish were cultured in spring water (flow rate: 1.0 L $s^{-1}$) and a YSI 6600 v2-2 water quality monitoring system (YSI, Yellow Springs, OH, USA) was used for daily monitoring of water temperature (11.3–15.8 °C), pH (7.2–7.5), dissolved oxygen (7.6–9.6 mg $L^{-1}$), and ammoniacal nitrogen (< 0.2 mg $L^{-1}$) in the tanks. After a 24 h starvation period at the end of the feeding trial, the fish from each tank were again weighed as a batch, and the fork length and body weight of a random sample of 30 fish from each tank were determined to calculate the condition factor. The fish were handled in compliance with the Chinese Law on the Protection of Animal Health and the Instructions for the Granting of Permits for Scientific Experimental Animals (Ethics approval number: SCXK (YU) 2005-0001).

## Skin mucus sampling

After starvation for 24 h, sixty specimens were randomly selected from each tank. These fish were anesthetized using a 5 mg $L^{-1}$ clove oil solution before collection of their skin mucus following the method of Ross et al. (2000) The individuals were then moved to a 10 mL sodium chloride polyethylene container (50 mmol). After 2 min, the mucus was collected, placed in a sterile tube (15 mL), and centrifuged for 10 min at 1500 *g* and 4 ℃. The surfactant was collected and stored at −80 °C until further use.

## Skin mucus protein and enzyme activity

For skin mucus from each of the five treatments, the total mucus protein was determined using the method of Lowry et al. (1951). The standard was bovine serum albumin, and the spectrophotometer absorbance was determined at 750 nm. Spectrophotometric kits were bought from the Chinese Nanjing Jiancheng Institute of Bioengineering and were used to analyze the malonaldehyde content (MDA; cat. no. A003-1), and the activity of the enzymes: lysozyme (cat. no. A059-2), alkaline phosphatase (ALP; cat. no. A059-2), glutathione peroxidase (GSH-px; cat. no. A006-2), superoxide dismutase (SOD; cat. no. A001-1), and catalase (CAT; cat. no. A007-1) in the mucus from each treatment.

## Skin gene expression

RNAiso Plus Reagent (TaKaRa, Dalian, China) was used to extract total RNA from the skin of three specimens that were randomly selected from each tank according to the manufacturer's instructions. A spectrophotometer was used to examine the absorbance at 260 nm to determine the RNA concentration. Using agarose gel electrophoresis, the RNA integrity was determined, and the absorbance ratio at A260 nm/A280 nm ranged from 1.8 to 2.0. Tumor necrosis factor (TNF-α), interleukin 6 (IL-6), interleukin 8 (IL-8), NF-kappa-B inhibitor alpha-like (IκBα), mucins-2 (MUC-2), claudin-1, zonula occludens-1 (ZO-1), occludin, and β-actin expression levels were determined using quantitative real-time PCR (ABI 7500, USA) with a reaction volume of 20 μL, including 10 μL of 2 SYBR® Premix Ex Taq (TaKaRa, Dalian, China), 0.8 μL for quantitative real-time PCR, specific primers were constructed based on sequences

cloned and published in the *O. mykiss* gene bank (Table 2). $2^{-\Delta\Delta Ct}$ was used to measure the expression values.

### Skin mucus antibacterial activity

Two bacterial strains (*Yersini ruckeri* and *Aeromonas hydrophila*) were used to test the antimicrobial properties of the fish skin mucus from each treatment. A traditional disc diffusion approach was used for assessing antimicrobial properties (Hellio et al. 2002). In brief, the bacteria were cultivated for 24 h at 37 °C, and then 0.1 mL of each broth culture medium ($1.5 \times 10^8$ CFU $mL^{-1}$) was cultured on nutrient agar. Paper discs (6 mm in diameter) were impregnated with 100 µL of the mucus samples, mounted on the medium, and incubated for 24 h at 37 °C. The diameter of the growth inhibition region was measured using a ruler. Antimicrobial activity was indicated by a clear zone around the disks. Serial dilution was used to assess the minimum inhibitory concentration (MIC) of the mucus samples, which was determined using the visual observation method (Hellio et al. 2002).

### Calculations and statistical analysis

Weight gain rate (WGR, %) = 100 × (weight gain, g) / (initial weight, g); Survival rate (%) = 100 × (final amount of fish) / (initial amount of fish); Condition factor (CF) = 100 × [(body weight, g) / (total length, cm) $^3$]; Feed conversion ratio (FCR) = (dry dietary intake, g) / (weight gain, g).

After normality and homogeneity checking, One-way variance analysis (ANOVA) and Duncan multi-multiple-range analyses were used to examine the data. $P$ values < 0.05 were considered significantly different. The SPSS statistical package 23.0 was used for statistical analysis (SPSS Inc., Chicago, IL, USA). The GraphPad Prism software version 9.0 is used to draw column graphs.

# Results

### Growth performance

There was no evidence to suggest that dietary glutathione enhanced juvenile taimen growth (Table 3) as there were no significant differences in weight gain among the groups ($P > 0.05$). In addition, no differences were observed in the feed conversion rate and condition factor among the groups ($P > 0.05$). However, the survival rate of fish in the treatment groups was higher than in the control group ($P < 0.05$).

### Skin immunity

The skin mucus antimicrobial activities are presented in Table 4–5. Compared to the control group, the skin mucus MIC was significantly lower in the dietary glutathione supplementation groups ($P < 0.05$), indicating significant improvements in antibacterial activity against *Y. ruckeri* and *A. hydrophila* through dietary glutathione supplementation ($P < 0.05$).

Regarding the mucus protein content and enzyme activity, the amount of skin mucus protein, and the ALP and lysozyme activity in the treatment groups were higher than in the control group. These findings suggest a positive effect of dietary glutathione supplementation on protein content and the activity of certain enzymes ($P < 0.05$; Table 6; Figure 1).

### Antioxidant capacity

The skin mucus of juvenile taimen in the treatment groups exhibited significant antioxidant activity (Table 6). In contrast to that in the control group, the skin mucus obtained from the treatment groups showed higher SOD and GSH-px activity ($P < 0.05$). The CAT activity in the mucus from the 400 and 800 mg $kg^{-1}$ glutathione supplementation treatments was significantly higher than that in the control and 200 mg $kg^{-1}$ groups. The amount of MDA was reduced by dietary glutathione supplementation ($P < 0.05$).

### Skin gene expression

Compared to that of the control group, skin TNF-α, IL-1β, IL-6, and IL-8 gene expression showed a significant decrease in fish fed 400 – 800.0 mg $kg^{-1}$ glutathione ($P < 0.05$; Figure 2). However, a significant increase in IκBα was observed as the amount of glutathione was increased to 200 g/kg ($P < 0.05$; Figure 2). There were no significant differences in the MUC-2, claudin-1, occludin, and ZO-1 gene expression ($P > 0.05$; Figure 3).

# Discussion

### Growth performance

In this study, the fish that were fed a semi-purified glutathione diet showed an increase in weight gain and survival. Similar results were reported for European bass (Zambonino Infante et al. 1997), grass carp (Ming et al. 2019), and Atlantic salmon (Ma et al. 2019). This may be related to glutathione is involved in the transfer of amino acids in vivo and in raising the gene mRNA expression levels (IGF-1, etc.) in fish cells to promote development (Ming et al. 2015). Moreover, dietary glutathione functions as an antioxidant, enhancing antioxidant capacity, immunity, and antistress processes, keeping the body healthy and finally leading to a rise in growth performance (Zhou et al. 2013). In addition, previous studies such as Atlantic salmon (Ma et al. 2019), grass carp (Zhou et al. 2013), and Nile tilapia (Zhou et al. 2013) showed that excess glutathione in the diet has a negative influence on growth performance. Excess glutathione in the body can react with halogenated olefins and anthraquinones, causing cytotoxicity (Monks et al. 1990). Meanwhile, excess glutathione may induce DNA damage and oxidative stress (Thomas et al. 1998). In the present study, There were no significant changes in the fish fed 400 to 800 mg $kg^{-1}$ dietary glutathione. Inconsistent findings in the literature about the impact of glutathione may be attributed to variation among studies in organisms, fish size, age, climate, life cycle, dosage, and types (Merrifield et al. 2010).

### Skin immunity

Previous studies have shown that fish skin mucus components can be improved by including immunostimulants in the diet (e.g., immunoglobulin, complements, lysozyme, and lectin) (Sheikhzadeh et al. 2012). For example, the antibacterial skin mucus activity of probiotic-fed black swordtail (*Xiphophorus helleri*) was improved ($P < 0.05$) (Hoseinifar et al. 2015). In this study, an improvement in skin mucus antibacterial activity against *Y. ruckeri* and *A. hydrophila* was detected in association with dietary glutathione supplementation. The possible underlying antimicrobial mechanism is that glutathione has a structure similar to the *Penicillium* antibiotic precursors, which act as inhibitors of the D-alanyl-D-alanine-carboxypeptidase enzyme that catalyzes the conversion of glycopeptides (D-alanyl-alanine) to peptidoglycan in cell walls (Mustikaningtyas et al. 2021).

It has been shown that fish have a large amount of skin mucus protein (Rajan et al. 2011), and findings of the present study indicate that the secretion of mucus-soluble skin protein may be increased by dietary glutathione supplementation. A high soluble protein level has been identified as a marker for mucus secretion, one of the defense mechanisms of fish (Taoka et al. 2006). In salmonids, skin mucus protein is known to increase the level of a protein involved in oxidative defense, motility, and general stress responses (Ræder et al. 2006). In the present study, the high level of skin mucus protein is associated with dietary glutathione supplementation. Meanwhile, the activity of skin mucus lysozyme and ALP in treatment groups was also affected ($P < 0.05$). Similarly, in a study on rainbow trout fed with Ergosan (Sheikhzadeh et al. 2012). Increased skin mucus secretion or induction of a skin mucus immune reaction may have caused an increase in lysozyme and ALP activity triggered by dietary glutathione supplementation (Hoseinifar et al. 2014). The increased levels of skin mucus protein, lysozyme, and ALP suggest that dietary glutathione may trigger such an innate immune response in taimen (Biller et al. 2018).

## Skin antioxidant capacity

In the scavenging of free radicals, oxidative destruction, SOD, CAT, GSH-px, and cellular structure have a major role to play. These antioxidant enzymes are stimulated as a cellular defense mechanism and are a significant safety mechanism for cellular oxidative damage reduction (Tabrez et al. 2009). Increased activation of SOD, CAT, and GSH-Px is more likely to mitigate oxidative damage, which is one of the more successful and effective methods of preventing peroxidative damage to the body. In the present study, the skin mucus of juvenile taimen fed dietary glutathione demonstrated an improved antioxidant ability, and the skin mucus antioxidant reaction was obvious in the treatment groups that received dietary glutathione levels of 100–800 mg $kg^{-1}$ ($P < 0.05$). Malonaldehyde is an oxidation metabolite in the body that reflects tissue and cell damage caused by free radicals. Similar findings were found in studies of grass carp (Ming et al. 2015), Nile tilapia (Zhou et al. 2013), and Atlantic salmon (Ma et al. 2019). Glutathione, as a nonenzymatic antioxidant, can eliminate free radicals and peroxides in the body(Martínez-Álvarez et al. 2005). A lower MDA content and increased SOD activity in mucus from the treatment groups in the present study suggests that dietary glutathione has a positive effect on the taimen's antioxidant capacity under the combined activity of an enzymatic and nonenzymatic antioxidant system. In addition, in this study, dietary glutathione level of 800 mg $kg^{-1}$ did not affect the body's

antioxidant metabolism balance, resulting in antioxidant malfunction, and the mechanism by which excess glutathione reduces taimen's antioxidant ability deserves more investigation.

### Skin gene expression

The pro-inflammatory cytokines including TNF-a, IL-1β, IL-6, and IL-8 are involved in the initiation and regulation of inflammatory response (Peng et al. 2020). Indeed, chemokines were first identified to be involved in the recruitment of leukocytes during early inflammation, but they are now recognized to modulate many stages of the immune response and to regulate cell migration during growth and development (Bird and Tafalla 2015). In our study, after the 7 d lipopolysaccharide challenge, skin TNF-α, IL-1β, IL-6, and IL-8 gene expression showed a significant decrease by dietary glutathione supplementation (100–800 mg $kg^{-1}$). These suggested that dietary glutathione had the potential anti-inflammatory effects. Similar results were found in the study of common carp (Hoseinifar et al. 2019). The nuclear transcription factor (NF-κB) signaling pathway is important in the regulation of non-specific immunity. The NF-κB inhibitor (IκB) is a critical component of the NF-B complex, and its breakdown can cause the production of proinflammatory cytokines (Seifried et al. 2007). In this study, dietary glutathione inhibited the breakdown of IκBα and down-regulated the expression levels of TNF-α, IL1β, IL6, and IL8. The findings suggested that the anti-inflammatory effects of dietary glutathione in the skin may be due to its suppression of the NF-κB signaling system.

Tight junctions, which are made up of the membrane proteins claudins, occludin, and cyclin ZOs, are the primary connectors between mucosal epithelial cells and perform critical functions in maintaining the mechanical integrity and the mucosal barrier function (Pummi et al. 2004). Pro-inflammatory factors such as TNF-α, IL-1β, IL-6, and IL-8 play a key role in triggering the chain reaction of tight junction damage (Al-Sadi et al. 2013). In this study, the claudin-1, occludin, and ZO-1 gene expression levels had no significant differences among the treatment groups. This implies that dietary glutathione supplementation may not impact the expression activity of skin mucosal cell tight junction structural protein genes. Therefore, dietary glutathione supplementation may be one potential possibility for affecting skin by decreasing the production of inflammatory cytokines rather than tight junctions.

## Conclusion

In conclusion, dietary glutathione (400 – 800 mg $kg^{-1}$) improved the growth performance and protected the skin of juvenile taimen from oxidative damage by enhancing the SOD, GSH-Px, and CAT activities. Skin non-specific immunity was also enhanced by mucus protein, lysozyme, alkaline phosphate, and antimicrobial activity. Moreover, supplementary glutathione significantly up-regulated IκBα expression level while down-regulating TNF-α, IL1β, IL-6, and IL-8 expression levels. These results showed that glutathione may be utilized as a functional component in fish feed to avoid inflammatory disorders in juvenile taimen.

## Declarations

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### Data availability

The data will be provided upon direct request to the authors.

### Ethics approval

The study was approved by the ethics committee on animal use of the Protection of Animal Health and the Instructions for the Granting of Permits for Scientific Experimental Animals (Ethics approval number: SCXK (YU) 2005-0001).

### Authors' contributions

CW designed the study and wrote the manuscript. SL assisted with sampling. ZF tested the samples and analyzed the results. DW tested the samples and analyzed the results. JL tested the samples. LW analyzed the results. YZ analyzed the results. HJ analyzed the results. ZL tested the samples. SH carried out the rearing work. YL assisted with sampling. HL provided the guidance. YY provided the guidance. All the authors read and approved the final manuscript.

### Consent for publication

All authors agree with submit the paper for publication in the Journal of Fish Physiology and Biochemistry.

### Conflict of interest

The authors declare no conflict of interest.

## References

1. Al-Sadi R, Guo S, Ye D, Ma TY (2013) TNF-α modulation of intestinal epithelial tight junction barrier is regulated by ERK1/2 activation of Elk-1. The American Journal of Pathology 183(6), 1871-1884. https://doi.org/10.1016/j.ajpath.2013.09.001

2. AOAC (2012) Official Methods for Analysis, 19th edn. Association of Official Analytical Chemists, Washington, DC.
3. Biller JD and Takahashi LS (2018) Oxidative stress and fish immune system: phagocytosis and leukocyte respiratory burst activity. Anais da Academia Brasileira de Ciências 90: 3403-3414. https://doi.org/10.1590/0001-3765201820170730
4. Bird S and Tafalla C (2015) Teleost chemokines and their receptors. Biology 4(4), 756-784. https://doi.org/10.3390/biology4040756
5. Doyotte A, Cossu C, Jacquin M, Babut M, Vasseur P (1997) Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. Aquat Toxicol 39(2), 93-110. https://doi.org/10.1016/S0166-445X(97)00024-6
6. Faggio C, Fazio F, Marafioti S, Arfuso F, Piccione G (2015) Oral administration of gum arabic: effects on haematological parameters and oxidative stress markers in *Mugil cephalus*. Iran J Fish Sci 14(1), 60-72.
7. Guardiola FA, Cuesta A, Esteban MÁ (2016) Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immun 59, 323-330. https://doi.org/10.1016/j.fsi.2016.11.005
8. Haukenes AH and Barton BA (2004) Characterization of the cortisol response following an acute challenge with lipopolysaccharide in yellow perch and the influence of rearing density. J Fish Biol 64(4), 851-862. https://doi.org/10.1111/j.1095-8649.2004.00354.x
9. Hellio C, Pons AM, Beaupoil C, Bourgougnon N, Le Gal Y (2002) Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus. Int J Antimicrob Ag 20(3), 214-219. https://doi.org/10.1016/S0924-8579(02)00172-3
10. Hoseinifar SH, Mirvaghefi A, Amoozegar MA, Sharifian Ma, Esteban MÁ (2015) Modulation of innate immune response, mucosal parameters and disease resistance in rainbow trout (*Oncorhynchus mykiss*) upon synbiotic feeding. Fish Shellfish Immun 45(1), 27-32. https://doi.org/10.1016/j.fsi.2015.03.029
11. Hoseinifar SH, Sharifian M, Vesaghi MJ, Khalili M, Esteban MÁ (2014) The effects of dietary xylooligosaccharide on mucosal parameters, intestinal microbiota and morphology and growth performance of Caspian white fish (*Rutilus frisii kutum*) fry. Fish Shellfish Immun 39(2), 231-236. https://doi.org/10.1016/j.fsi.2014.05.009
12. Hoseinifar SH, Sohrabi A, Paknejad H, Jafari V, Paolucci M, Van Doan H (2019) Enrichment of common carp (*Cyprinus carpio*) fingerlings diet with *Psidium guajava*: the effects on cutaneous mucosal and serum immune parameters and immune related genes expression. Fish Shellfish Immun 86, 688-694. https://doi.org/10.1016/j.fsi.2018.12.001
13. Hoseinifar SH, Zou HK, Van Doan H, Kolangi Miandare H, Hoseini SM (2018) Evaluation of some intestinal cytokines genes expression and serum innate immune parameters in common carp

(*Cyprinus carpio*) fed dietary loquat (*Eriobotrya japonica*) leaf extract. Aquac Res 49(1), 120-127. https://doi.org/10.1111/are.13440

14. Lauriano ER, Pergolizzi S, Capillo G, Kuciel M, Alesci A, Faggio C (2016) Immunohistochemical characterization of Toll-like receptor 2 in gut epithelial cells and macrophages of goldfish *Carassius auratus* fed with a high-cholesterol diet. Fish Shellfish Immun 59, 250-255. https://doi.org/10.1016/j.fsi.2016.11.003
15. Liu B, Ge X, He Y, Xie J, Xu P, He Y, Zhou Q, Pan L, Chen R (2010) Effects of anthraquinones extracted from *Rheum officinale* Bail on the growth, non-specific immune response of *Macrobrachium rosenbergii*. Aquaculture 310(1-2), 13-19. https://doi.org/10.1016/j.aquaculture.2010.09.020
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin-phenol reagent. Biol Chem 193, 265-275.
17. Ma J, Zhang J, Sun G, Lou Y, Li Y (2019) Effects of dietary reduced glutathione on the growth and antioxidant capacity of juvenile Atlantic salmon (*Salmo salar*). Aquacult Nutr 25(5), 1028-1035. https://doi.org/10.1111/anu.12920
18. Martínez-Álvarez RM, Morales AE, Sanz A (2005) Antioxidant defenses in fish: biotic and abiotic factors. Rev Fish Biol Fisher 15(1), 75-88. https://doi.org/10.1007/s11160-005-7846-4
19. Merrifield DL, Dimitroglou A, Foey A, Davies SJ, Baker RT, Bøgwald J, Castex M, Ringø E (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302(1-2), 1-18. https://doi.org/10.1016/j.aquaculture.2010.02.007
20. Ming J, Ye J, Zhang Y, Xu P, Xie J (2015) Effects of dietary reduced glutathione on growth performance, non-specific immunity, antioxidant capacity and expression levels of IGF-I and HSP70 mRNA of grass carp (*Ctenopharyngodon idella*). Aquaculture 438, 39-46. https://doi.org/10.1016/j.aquaculture.2014.12.038
21. Ming J, Ye J, Zhang Y, Yang X, Shao X, Qiang J, Xu P (2019) Dietary optimal reduced glutathione improves innate immunity, oxidative stress resistance and detoxification function of grass carp (*Ctenopharyngodon idella*) against microcystin-LR. Aquaculture 498, 594-605. https://doi.org/10.1016/j.aquaculture.2018.09.014
22. Monks TJ, Anders MW, Dekant W, Stevens JL, Lau SS, van Bladeren PJ (1990) Glutathione conjugate mediated toxicities. Toxicol Appl Pharm 106(1), 1-19. https://doi.org/10.1016/0041-008X(90)90100-9
23. Mustikaningtyas D, Widyarti S, Rifa I M, Widodo N (2021) Proposed mechanism of antibacterial activity of glutathione by inhibition of the D-alanyl-D-alanine carboxypeptidase enzyme. Int J Pept Res Ther 27(2), 843-849. https://doi.org/10.1007/s10989-020-10124-5
24. Patel M, Ashraf MS, Siddiqui AJ, Ashraf SA, Sachidanandan M, Snoussi M, Adnan M, Hadi S (2020) Profiling and role of bioactive molecules from *Puntius sophore* (freshwater/brackish fish) skin mucus with its potent antibacterial, antiadhesion, and antibiofilm activities. Biomolecules 10(6), 920. https://doi.org/10.3390/biom10060920

25. Peng K, Wu N, Cui Z, Zhang X, Lu X, Wang Z, Zhang Y (2020) Effect of the complete replacement of dietary fish meal by soybean meal on histopathology and immune response of the hindgut in grass carp (*Ctenopharyngodon idellus*). Vet Immunol Immunop 221, 110009. https://doi.org/10.1016/j.vetimm.2020.110009
26. Ponsoda X, Bort R, Jover R, Gómez-Lechón MJ, Castell JV (1999) Increased toxicity of cocaine on human hepatocytes induced by ethanol: role of GSH. Biochem Pharmacol 58(10), 1579-1585. https://doi.org/10.1016/S0006-2952(99)00249-X
27. Pummi KP, Heape AM, Grénman RA, Peltonen JT, Peltonen SA (2004) Tight junction proteins ZO-1, occludin, and claudins in developing and adult human perineurium. J Histochem Cytochem 52(8), 1037-1046. https://doi.org/10.1369/jhc.3A6217.2004
28. Ræder ILU, Paulsen SM, Smalås AO, Willassen NP (2007) Effect of fish skin mucus on the soluble proteome of *Vibrio salmonicida* analysed by 2-D gel electrophoresis and tandem mass spectrometry. Microb Pathogenesis 42(1), 36-45. https://doi.org/10.1016/j.micpath.2006.10.003
29. Raghunathan VK, Ellis EM, Tettey JN, Grant MH (2007) Involvement of reduced glutathione and glutathione reductase in the chronic toxicity of hexavalent chromium to monocytes in vitro. Toxicology 231(2-3), 105-106. http://dx.doi.org/10.1016/j.tox.2006.11.030
30. Rahman I and MacNee W (2000) Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. Free Radical Bio Med 28(9), 1405-1420. https://doi.org/10.1016/S0891-5849(00)00215-X
31. Rajan B, Fernandes JM, Caipang CM, Kiron V, Rombout JH, Brinchmann MF (2011) Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. Fish Shellfish Immun 31(2), 224-231. https://doi.org/10.1016/j.fsi.2011.05.006
32. Rao V, Marimuthu K, Kupusamy T, Rathinam X, Arasu MV, Al-Dhabi NA, Arockiaraj J (2015) Defense properties in the epidermal mucus of different freshwater fish species. Aquaculture, Aquarium, Conservation & Legislation 8(2), 184-194.
33. Ross NW, Firth KJ, Wang A, Burka JF, Johnson SC (2000) Changes in hydrolytic enzyme activities of naive Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Dis Aquat Organ 41(1), 43-51. https://doi.org/10.3354/dao041043
34. Seifried HE, Anderson DE, Fisher EI, Milner JA (2007) A review of the interaction among dietary antioxidants and reactive oxygen species. The Journal of Nutritional Biochemistry 18(9), 567-579. https://doi.org/10.1016/j.jnutbio.2006.10.007
35. Sheikhzadeh N, Pashaki AK, Nofouzi K, Heidarieh M, Tayefi-Nasrabadi H (2011) Effects of dietary *Ergosan on cutaneous* mucosal immune response in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immun 32(3), 407-410. https://doi.org/10.1016/j.fsi.2011.11.028
36. Srichaiyo N, Tongsiri S, Hoseinifar SH, Dawood MA, Jaturasitha S, Esteban MÁ, Ringø E, Van Doan H (2020) The effects Gotu Kola (*Centella asiatica*) powder on growth performance, skin mucus, and

serum immunity of Nile tilapia (*Oreochromis niloticus*) fingerlings. Aquacult Rep 16, 100239. https://doi.org/10.1016/j.aqrep.2019.100239

37. Tabrez S and Ahmad M (2009) Effect of wastewater intake on antioxidant and marker enzymes of tissue damage in rat tissues: implications for the use of biochemical markers. Food Chem Toxicol 47(10), 2465-2478. https://doi.org/10.1016/j.fct.2009.07.004
38. Taee HM, Hajimoradloo A, Hoseinifar SH, Ahmadvand H (2017) Dietary myrtle (*Myrtus communis* L.) improved non-specific immune parameters and bactericidal activity of skin mucus in rainbow trout (*Oncorhynchus mykiss*) fingerlings. Fish Shellfish Immun 64, 320-324. https://doi.org/10.1016/j.fsi.2017.03.034
39. Taoka Y, Maeda H, Jo J, Jeon M, Bai SC, Lee W, Yuge K, Koshio S (2006) Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. Fisheries Sci 72(2), 310-321. https://doi.org/10.1111/j.1444-2906.2006.01152.x
40. Thomas S, Lowe JE, Hadjivassiliou V, Knowles RG, Green IC, Green MH (1998) Use of the comet assay to investigate the role of superoxide in glutathione-induced DNA damage. Biochem Bioph Res Co 243(1), 241-245. https://doi.org/10.1006/bbrc.1997.8000
41. Will O, Mahler H, Arrigo A, Epe B (1999) Influence of glutathione levels and heat-shock on the steady-state levels of oxidative DNA base modifications in mammalian cells. Carcinogenesis 20(2), 333-337. https://doi.org/10.1093/carcin/20.2.333
42. Zambonino Infante JL, Cahu CL, Peres A (1997) Partial substitution of di-and tripeptides for native proteins in sea bass diet improves *Dicentrarchus labrax* larval development. The Journal of Nutrition 127(4), 608-614. https://doi.org/10.1093/jn/127.4.608
43. Zhang GL, Zhao HH, Zhou ZW, He ZJ, Guo H, Chen J, Liu L (2007) The effects of reduced glutathione on growth rate and antioxidation capacity of tilapia. Journal of South China Agricultural University 28(3), 90-93. https://doi.org/10.3969/j.issn.1001-411X.2007.03.020
44. Zhou TT, Cao JM, Huang YH, Chen B, Wang G, Sun Z, Liu QF, Liu XL (2013) Effects of dietary glutathione on growth performance, tissue bio-chemical indexes and non-specific immune related enzymes of GIFT *Oreochromis niloticus*. Journal of Fisheries of China 37, 742-750. https://doi.org/10.3724/sp.j.1231.2013.38161

# Tables

**Table 1** Formulation and chemical proximate composition of the experimental diets

| Ingredients (g $kg^{-1}$) | Contents | | | | |
|---|---|---|---|---|---|
| Gelatin[1] | 50 | 50 | 50 | 50 | 50 |
| Casein[2] | 450 | 450 | 450 | 450 | 450 |
| Dextrin | 250 | 250 | 250 | 250 | 250 |
| Fish oil[3] | 90 | 90 | 90 | 90 | 90 |
| Starch | 120.5 | 120.5 | 120.5 | 120.5 | 120.5 |
| Calcium dihydrogen phosphate | 20 | 20 | 20 | 20 | 20 |
| Butylated hydroxytoluene | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin premix[4] | 3 | 3 | 3 | 3 | 3 |
| Mineral premix[5] | 6 | 6 | 6 | 6 | 6 |
| Glutathione (mg $kg^{-1}$) | 0 | 100 | 200 | 400 | 800 |
| Glycine | 10 | 9.9 | 9.8 | 9.6 | 9.2 |
| Nutrient levels (measured value) | | | | | |
| Glutathione (mg $kg^{-1}$) | 0.25 | 98.35 | 201.36 | 398.66 | 796.98 |
| Crude protein | 460.2 | 459.6 | 461.1 | 460.2 | 460.5 |
| Crude lipid | 94.5 | 94.8 | 93.3 | 94.2 | 93.6 |
| Crude ash | 24.65 | 24.66 | 24.32 | 24.61 | 24.22 |
| P | 0.62 | 0.61 | 0.60 | 0.61 | 0.62 |

[1]Zhiyuan Chemical Agent Company, Tianjin, China

[2]Vitamin-free casein, Sigma, America

[3]Huludao Chia Tai Feed Corporation, Huludao, China

[4]Vitamin premix (mg $kg^{-1}$): alpha-tocopherol 100, ascorbic acid 200, retinol acetate 5.2, menadione sodium bisulfate 5, thiamin 25, cholecalciferol 0.07, pyridoxine 25, riboflavin 40, nicotinic acid 275, cyanocobalamin 0.8, biotin 5, folic acid 8, pantothenic acid 100

[5]Mineral premix (mg $kg^{-1}$): KCl 1 500, $MgSO_4 \cdot 7H_2O$ 2 000, $CuSO_4 \cdot 5H_2O$ 20, $FeSO_4 \cdot 7H_2O$ 1 000, ZnSO4·$7H_2O$ 150, $MnSO_4 \cdot 4H_2O$ 100, NaCl 500, KI 3, $Na_2SeO_3$ 3, $CoCl_2$ 5

**Table 2** Primers used for quantitative real-time PCR

| Genes | Forward primer sequences (5'–3') | Reverse primer sequences (5'–3') | PCR fragment length (bp) |
|---|---|---|---|
| TNF-α | GTACCAGACGCTGCTCAACT | CAGGCTAAACACTGCTCCCA | 115 |
| IL-1β | CCGGTGGACATGTGTCAGAA | GTGTCACAGTGTTTGCGACC | 116 |
| IL-6 | CTTCCATCCCCACACACCTC | ACACCTGCAGACCATGACTG | 96 |
| IL-8 | TGAGACGGAGAGCAGACGTA | AGCGCTGACATCCAGACAAA | 136 |
| IκBα | AAGTGGTCGCATCACATGGT | CCAGACTTCCATCCCGATCG | 184 |
| MUC2 | CTCAGGGTATGGTGGAGGGA | CCAGCCTCTCCTCTCTGTCT | 87 |
| Claudin-1 | GGACAACATCATAACGGCGC | CTGCACCATCCCCGTTAGTT | 124 |
| ZO-1 | CCGCCTTACGCCTACTGTAG | GGTTGTCGTAGTGCAGGTGA | 97 |
| Occludin | GCAGGAACGTGTGGACTACA | TCCCTCCTGCAGATCATCCA | 133 |
| β-actin | CTCTGGTGATGGTGTGACCC | CGATGTCACGCACGATTTCC | 176 |

[a] *Hucho taimen* β-actin gene as a housekeeping gene was used as an internal reference.

**Table 3** Growth performances of taimen fed the experimental diets

| Indicies | Dietary glutathione level | | | | |
|---|---|---|---|---|---|
| | Control | 100 | 200 | 400 | 800 |
| IBW[1] (g) | 5.33±0.12 | 5.40±0.10 | 5.38±0.23 | 5.29±0.11 | 5.38±0.16 |
| FBW[2] (g) | 12.76±0.35 | 13.17±0.95 | 12.87±0.51 | 13.34±0.17 | 13.48±0.08 |
| WGR[3] (%) | 139.24±1.56[a] | 144.04±5.30[ab] | 143.53±6.88[ab] | 152.26±2.12[b] | 150.46±6.23[b] |
| CF[4] | 0.66±0.02 | 0.68±0.03 | 0.70±0.04 | 0.69±0.01 | 0.68±0.05 |
| FCR[5] | 1.12±0.01 | 1.15±0.06 | 1.11±0.02 | 1.16±0.05 | 1.12±0.01 |
| SR[6] | 90.61±0.61[a] | 93.07±1.25[b] | 93.53±0.38[b] | 93.82±0.27[b] | 94.19±0.87[b] |

Values are means ± SD of three replicates. Data in a raw assigned with different letters denote significant different ($P < 0.05$).

[1]IBW, initial body weight

[2]FBW, final body weight

[3]WGR, weight gain rate

[4]CF, condition factor

[5]FCR, feed conversion rate

[6]SR, survival rate

**Table 4** The antibacterial activity of the skin mucus (growth inhibition zone diameter [mm]) of taimen

| | Dietary glutathione level | | | | |
|---|---|---|---|---|---|
| | Control | 100 | 200 | 400 | 800 |
| *A.hydrophila* | 7.19±0.11$^{a}$ | 7.82±0.65$^{b}$ | 9.11±0.15$^{c}$ | 9.04±0.22$^{c}$ | 9.01±0.20$^{c}$ |
| *Y.ruckeri* | 6.32±0.35$^{a}$ | 7.93±0.08$^{b}$ | 7.86±0.09$^{b}$ | 7.84±0.22$^{b}$ | 7.72±0.11$^{b}$ |

Values are means ± SD of three replicates. Data in a raw assigned with different letters denote significant different ($P < 0.05$).

**Table 5** The minimum inhibitory concentration (μl mL$^{-1}$) of the skin mucus of taimen

| | Dietary glutathione level | | | | |
|---|---|---|---|---|---|
| | Control | 100 | 200 | 400 | 800 |
| *A.hydrophila* | >200 | 200 | 200 | 200 | 150 |
| *Y.ruckeri* | >200 | 200 | 200 | 150 | 100 |

**Table 6** Skin mucus parameters of taimen fed the experimental diets

| Indicies | Dietary glutathione level | | | | |
|---|---|---|---|---|---|
| | Control | 100 | 200 | 400 | 800 |
| [1]SOD (U L$^{-1}$) | 7.51±1.10$^{a}$ | 9.45±0.07$^{b}$ | 9.51±0.27$^{b}$ | 9.52±0.11$^{b}$ | 9.49±0.02$^{b}$ |
| [2]CAT (U L$^{-1}$) | 0.05±0.01$^{a}$ | 0.08±0.02$^{ab}$ | 0.10±0.02$^{b}$ | 0.10±0.01$^{b}$ | 0.10±0.02$^{b}$ |
| [3]GSH-px (U L$^{-1}$) | 8.68±0.50$^{a}$ | 9.68±0.49$^{b}$ | 9.96±0.02$^{b}$ | 9.91±0.12$^{b}$ | 10.11±0.08$^{b}$ |
| [4]MDA (nmol L$^{-1}$) | 0.23±0.01$^{b}$ | 0.±0.01$^{a}$ | 0.19±0.01$^{a}$ | 0.20±0.01$^{a}$ | 0.20±0.02$^{a}$ |
| [5]ALP (U L$^{-1}$) | 4.50±0.43$^{a}$ | 5.09±0.14$^{b}$ | 5.34±0.19$^{b}$ | 5.29±0.09$^{b}$ | 5.27±0.07$^{b}$ |
| Lysozyme (U mg$^{-1}$ prog) | 2.11±0.06$^{a}$ | 2.74±0.11$^{c}$ | 2.47±0.16$^{b}$ | 2.65±0.18$^{bc}$ | 2.67±0.08$^{bc}$ |

Values are means ± SD of three replicates. Data in a raw assigned with different letters denote significant different ($P < 0.05$).

[1]SOD, superoxide dismutase

[2]CAT, catalase

[3]GSH-Px, glutathione peroxidase

[4]MDA, malondialdehyde

[5]ALP, alkaline phosphatase

# Figures

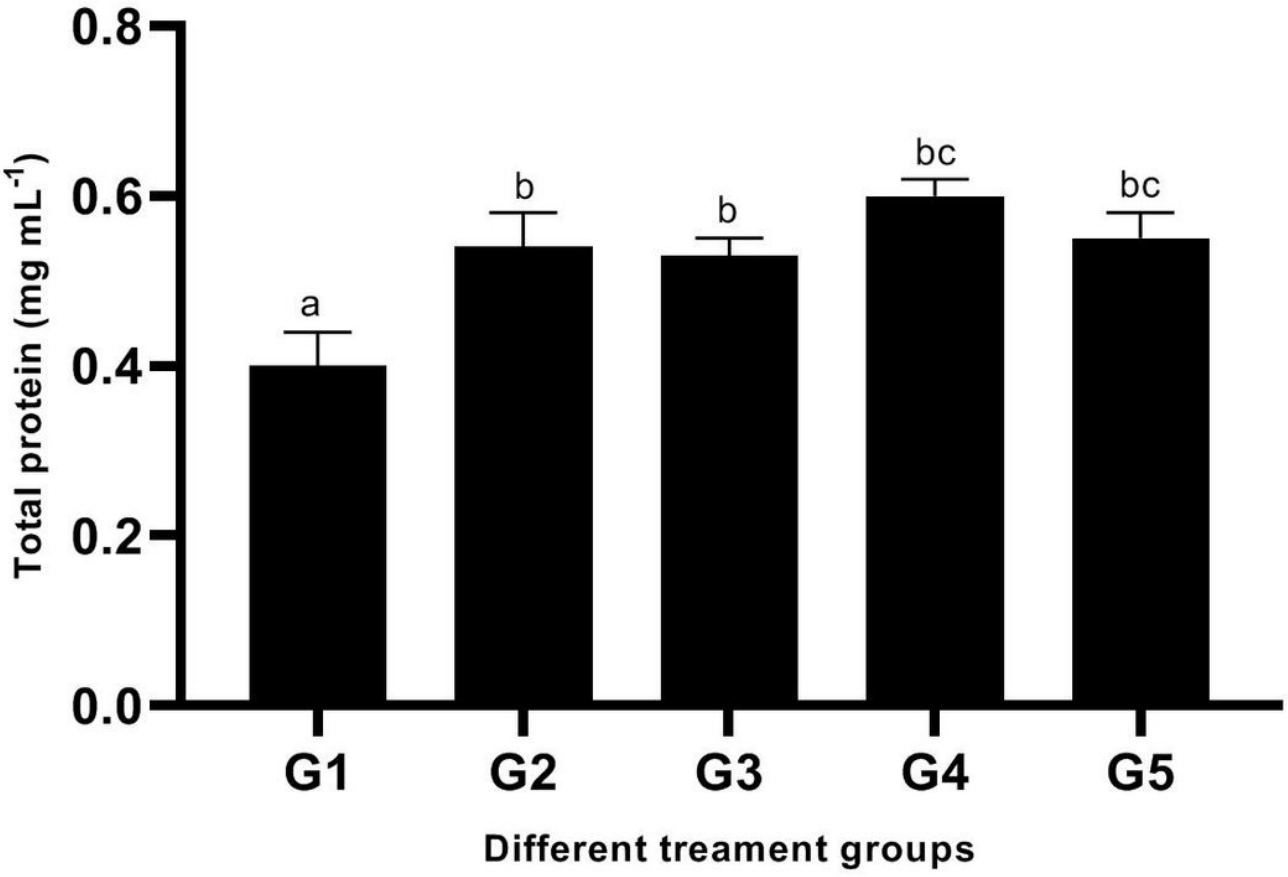
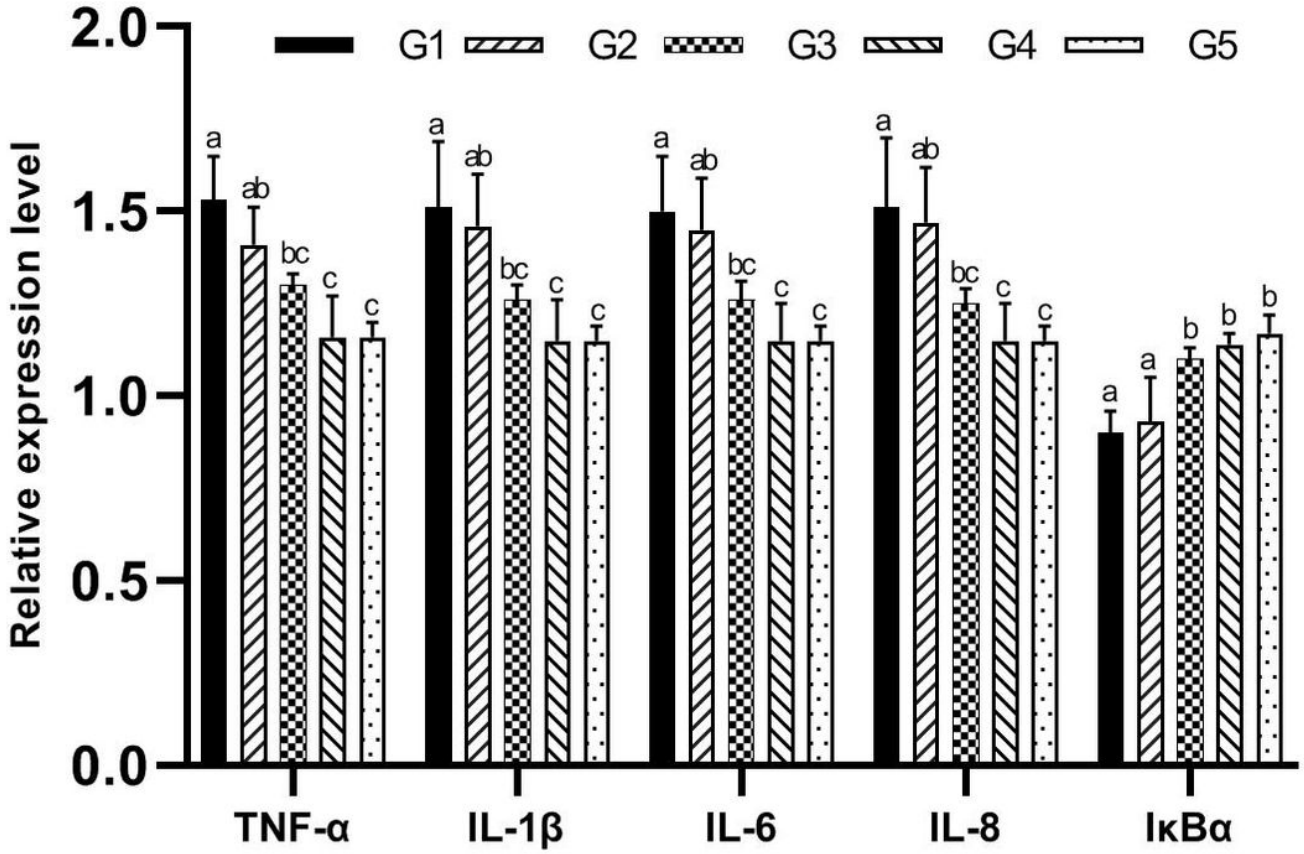


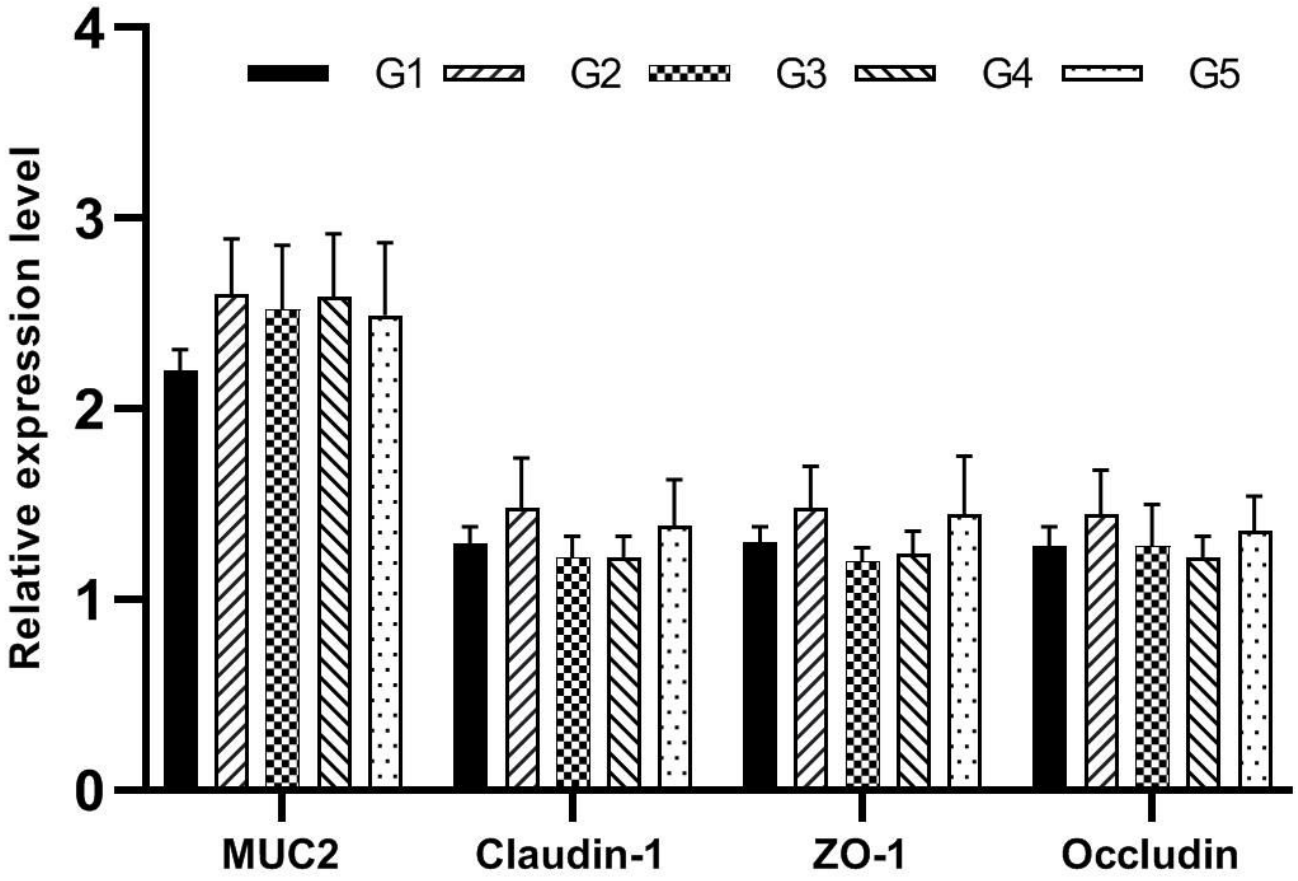

**Figure 1**

The skin mucus total protein levels (mg mL-1) of juvenile taimen fed diet supplemented with different levels of glutathione for 56 days. Values are means ± SD of three replicates. Data in a raw assigned with different letters denote significant different ($P < 0.05$).




## Figure 2

The cytokine gene expression of juvenile taimen fed diet supplemented with different levels of glutathione for 56 days. Values are means ± SD of three replicates. Data in a raw assigned with different letters denote significant different ($P < 0.05$).




## Figure 3

The mucin and tight junction gene expression of juvenile taimen fed diet supplemented with different levels of glutathione for 56 days. Values are means ± SD of three replicates. Data in a raw assigned with different letters denote significant different ($P < 0.05$).