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Effects of combined application of nucleotides and β-glucan on intestinal health and flesh quality in grass carp (*Ctenopharyngodon idella*)

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A R T I C L E I N F O

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A B S T R A C T

Nucleotides and β-glucan have demonstrated positive effects on the growth and health of aquaculture species. However, little research has been done on the combined effects of using both supplements on the intestinal health and flesh quality of fish. In the present study, we evaluated the impact of nucleotides (0.01 %) or/and β-glucan (0.10 %) on parameters related to intestinal health and flesh quality in grass carp (69.97 ± 0.05 g) over a 60-day feeding period. The results showed that both nucleotides and β-glucan significantly increased catalase activity, enhanced mRNA expression level of *zonula occludens-3*, downregulated *interleukin-1β*, *interferon*, *Toll-like receptor-4*, and *Toll-like receptor-7* expression in the intestine ($P < 0.05$). Additionally, they also resulted in enhanced springiness and increased percentage of total unsaturated fatty acids, while reducing *Myostatin 1* expression and total saturated fatty acids content in the muscle ($P < 0.05$). This indicates that these supplements have similar effects in promoting oxidation resistance, suppressing inflammation, and improving intestinal barrier integrity and flesh quality in grass carp. Besides, the combination of nucleotides and β-glucan led to a decrease in Firmicutes abundance and an increase in Actinobacteria compared to the control group. We further observed the synergistic effects of nucleotides and β-glucan in decreasing the expression of inflammation related genes (*interleukin-1β*, *interferon*, *Toll-like receptor-4*, and *Toll-like receptor-7*) and improving parameters associated with intestinal morphology (villus height, villus width and gut wall thickness) and flesh quality (chewiness, springiness, total tasty free amino acids, total saturated fatty acids and total unsaturated fatty acids). Overall, our results suggest that the combined application of nucleotides and β-glucan is more effective in promoting intestinal health and improving flesh quality in grass carp compared to using either supplement alone.

1. Introduction

With the development of intensive farming, fish contacted increasing contaminants during the culture process. Recent studies have shown that contaminant-stimulated reactive oxygen species (ROS) production may lead to oxidative damage, immunosuppression, and even death in fish (Chowdhury and Saikia, 2020). Multiple tissues can be damaged by the generation and accumulation of ROS in living organisms. For example, the intestines are very susceptible to ROS, which can cause intestinal injury and inflammation, and increase intestinal permeability (Zheng et al., 2017; Wang et al., 2020). In addition, ROS can directly affect the quality of flesh by changing the structure of myofibrillar protein, intramuscular fat content and metabolism, and the formation of flavor precursors (Bekhit et al., 2013; Li et al., 2013). Therefore, it is

Abbreviation: Ala, alanine; CAT, catalase; FAA, free amino acids; GPX, glutathione peroxidase; IL-1β, interleukin-1β; INF, interferon; MSTN, Myostatin; OTUs, operational taxonomic units; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SFA, saturated fatty acids; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; TLR, Toll-like receptors; UFAs, unsaturated fatty acids; ZO-3, zonula occludens-3.

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important to select suitable supplements in fish feeds to minimize these deleterious effects.

nucleotides and β-glucan are probiotics and bioactive molecules that have anti-inflammatory and prebiotic effects through enhancing immune responses and promoting stress resistance on fish (Pogue et al., 2021). Nucleotides play essential roles in various physiological and biochemical processes (Krüger and van der Werf, 2018). The endogenously synthesized nucleotides could typically meet the demands of normal metabolism; however, during periods of rapid growth or in the presence of certain disease, they may not adequately fulfill the requirements of tissues and cells with high metabolic activity (Norton et al., 2001). Therefore, it is essential to provide additional nucleotides to support optimal cellular function and growth. Dietary nucleotides have been shown to have beneficial effects on fish growth and feed utilization (Li et al., 2007; Liu, 2016), immune response and stress resistance (Welker et al., 2011; Reda et al., 2018), intestinal health (Cheng et al., 2011), especially the balance of intestinal microbiota (Xu et al., 2015; El-Nokrashy et al., 2021), and flesh quality (Tie, 2018). The β-glucan is a highly conserved carbohydrate and chemically heterogeneous polysaccharide found in many natural sources. Numerous studies have reported the benefits of β-glucan for fish, including promoting growth (Aramli et al., 2015; Nieves-Rodríguez et al., 2018; Song et al., 2020), enhancing stress resistance (Cerezuela et al., 2012; Douxfils et al., 2017; Salah et al., 2017;), improving immune response (Yamamoto et al., 2020; Ching et al., 2021), optimizing intestinal health (Carballo et al., 2019; Kühlwein et al., 2014; Hisano et al., 2018). In addition, dietary glucan has been shown to improve the meat quality of pigs through increasing muscle pH and intramuscular fat content, reducing muscle drip losses, and changing the proportion of saturated fatty acid and unsaturated fatty acid (Luo et al., 2019a), yet the impact of β-glucan supplementation on fish flesh quality remains uncertain.

Combining two or more supplements has been shown to produce a synergistic effect, resulting in superior outcomes compared to using them individually (Hany et al., 2024). Previous research demonstrated that the combined application of nucleotides and β-glucan could improve the growth performance of pigs (Huang et al., 2023). However, the effects of this combination on intestinal health and flesh quality of fish have not been studied. The grass carp (*Ctenopharyngodon idellus*), the largest aquaculture species in China, faces various challenges under artificial breeding conditions, such as infectious diseases and decreased muscle quality, which urgently need to be addressed. This study was designed to determine the effects of β-glucan (0.1 %) (Huang et al., 2023) combined with nucleotides (0.01 %) (Douxfils et al., 2017) on intestinal health and flesh quality in grass carp.

2. Materials and methods

2.1. Experimental diets

The formulation and fundamental composition of the basal diet were shown in Table 1. Three other groups were formed, with individual application of 0.01 % nucleotides (NT) or 0.1 % β-glucan (Glu), or a combined application of 0.01 % nucleotides and 0.1 % β-glucan (NT×Glu), added based on basal diet (control). All constituents were ground and filtered through a 40-mesh screen grinding, then pelleted using an automatic granulator (2 mm × 2 mm). The feed was subsequently dried at 40 ℃ for 10 h in a ventilated oven and stored at −20 ℃ in an airtight bag until feeding.

2.2. Feeding trial and sample collection

The grass carps were brought from a fish farm in the Xidongting district of Changde, China. They were acclimated in a floating cage (2.0 m × 1.5 m × 1.5 m), and fed a basal diet for 2 weeks. After 24 h of fasting, healthy fish (69.97 ± 0.05 g) were randomly distributed into 12 cages (30 fish per cage) to ensure three replicates in each group. The fish were hand-fed to satiety three times a day (6:00, 12:00, and 18:00). The experiment lasted for 60 days during which the water quality parameters remained stable: temperature ranged between 24.5 and 30.3 ℃, pH ranged between 7.3 and 7.8, and dissolved oxygen averaged around 6.5–7.5 mg/L according to our previous study [Huang et al., (2023)]. Both the concentrations of NH_4^+-N and NO_2-N remained below 0.1 mg/L throughout the experimental period without significant fluctuations.

Table 1
Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients (%)	Treatments			
	Control	Nucleotide (NT)	β-glucan (Glu)	Nucleotide+β-glucan (NT×Glu)
Fish meal	3.00	3.00	3.00	3.00
Soybean meal	24.00	24.00	24.00	24.00
Cottonseed meal	14.00	14.00	14.00	14.00
Rapeseed meal	29.00	29.00	29.00	29.00
DL-Met (98.5 %)	0.05	0.05	0.05	0.05
Wheat flour	22.9	22.89	22.8	22.79
$Ca(H_2PO_4)_2$	2.00	2.00	2.00	2.00
Fish oil	2.5	2.5	2.5	2.5
Soybean oil	1.25	1.25	1.25	1.25
Vitamin premix[a]	1.00	1.00	1.00	1.00
Choline chloride (50 %)	0.25	0.25	0.25	0.25
Ethoxyquin (30 %)	0.05	0.05	0.05	0.05
Nucleotides	0	0.01	0	0.01
β-glucan	0	0	0.1	0.1
Total	100	100	100	100
Nutrient contents				
Crude protein[b]	33.31	33.31	33.29	33.29
Crude lipid[b]	5.22	5.22	5.22	5.22

[a] Vitamin premix (g/kg): retinyl acetate (500,000 IU g−1), 0.386; cholecalciferol (500,000 IU g−1), 0.40; D, L-a-tocopherol acetate (50 %), 23.23; menadione (22.9 %), 0.83; cyanocobalamin (1 %), 0.94; D-biotin (2 %), 0.75; folic acid (95 %), 0.42; thiamine nitrate (98 %), 0.09; ascorhyl acetate (95 %), 9.77; niacin (99 %), 4.04; meso-inositol (98 %), 19.39; calcium-D-pantothenate (98 %), 3.85; riboflavin (80 %), 0.73; pyridoxine hydrochloride (98 %), 0.62. All ingredients were diluted with maize starch to 1 kg.
[b] Nutrient contents were calculated values.

The fish were anesthetized with 100 mg/L MS-222 after 8 h of fasting. Two fish from each cage were randomly selected to collect intestinal content samples for intestinal microbiota analysis. After a 24 h fast, four fish were randomly selected from each cage. Two fish were chosen and their anterior and distal intestines samples were collected for gene expression and enzyme activity analysis, respectively. Then, the mid intestines (1 cm) and dorsal muscles (1 cm × 1 cm × 1 cm) were collected and fixed in 4 % paraformaldehyde solution for histomorphometric analysis. Two other fish were used to analyze muscle texture, cooking loss, and pH using the right-side dorsal muscle, which were completed within 24 h. Dorsal muscle samples from the left side were collected for the analysis of gene expression and muscle flavor. These samples, which were used for examining intestinal microbiota, gene expression, enzyme activity, and muscle flavor, were immediately frozen in liquid nitrogen and then stored at −80 ℃ until further analysis.

2.3. Antioxidant analysis

The distal intestine tissues were homogenized with a 0.9 % saline solution and then centrifugated at 3500 × *g*, 4 ℃ for 10 min. The supernatant was collected for the analysis of enzyme activity. The total antioxidant capacity (T-AOC), catalase (CAT), glutathione peroxidase (GPX), and alkaline phosphatase in the distal intestine tissue were detected using commercial kits (Nanjing Jiancheng Biotechnic Institute, China).

2.4. Gene expression analysis

Total RNA extraction and reverse transcription were performed following our previous study (Luo et al., 2019b; Luo et al., 2020). The transcription level was analyzed by quantitative real-time PCR (qRT—PCR) with QuantStudio® 3 qRT—PCR (Applied Biosystems). The gene-specific primers used for qRT—PCR are listed in Table 2, of which the β-actin gene was chosen as an internal control. The mRNA expression level of target genes was conducted by the formula $2^{-\triangle\triangle Ct}$ ($\triangle\triangle Ct$ = ($Ct_{\text{gene of interest}}$ - $Ct_{\beta\text{-actin}}$) treated - ($Ct_{\text{gene of interest}}$ - $Ct_{\beta\text{-actin}}$) untreated). The PCR conditions were according to our previous study (Luo et al., 2019b).

2.5. Morphology analysis

The fixed intestines and muscle samples were examined with standard histological techniques (Wen et al., 2020). The gut wall thickness, villus height, and villus width were measured from at least 13 well-oriented crypt-villus units in each slice (n=3) via an optical microscope (Olympus, Tokyo, Japan). The number of myofibers in different areas of the transected muscle was measured from at least 22 well-oriented transected muscle units in each slice (n=3). The fiber diameters (≥ 10 μm) were calculated according to the formula $s = \pi r2$ (where "s" represents the muscle fiber area and "r" represents the muscle fiber radius). Muscle fiber density was calculated according to the number of muscle fibers/area selected (Tang et al., 2021).

Table 2
Primers used for quantitative real-time PCR.

Primer names	Sequence (5′—3′)	Accession numbers
β-actin	F: GAACACTGTGCTGTCTGGAGGTA	M25013
	R: CTTGGGTTGGTCGTTTGAATC	
SOD	F: CGCACTTCAACCCTTACA	GU901214
	R: ACTTTCCTCATTGCCTCC	
CAT	F: GAAGTTCTACACCGATGAGG	FJ560431
	R: CCAGAAATCCCAAACCAT	
GPX	F: GGGCTGGTTATTCTGGGC	E828796
	R: AGGCGATGTCATTCCTGTTC	
IL−1β	F: AGAGTTTGGTGAAGAAGAGG	JQ692172
	R: TTATTGTGGTTACGCTGGA	
TNF-α	F: TTGCCGAGGGTGATGGTG	HQ696609
	R: ACTTGTTGAGCGTGAAGCAGAC	
IFN	F: GGTGAAGTTTCTTGCCCTGACCTTAG	FJ695519
	R: CCTTATGTGATGGCTGGTATCGGG	
TLR4	F: TTCCACCTATTCATCTTTGC	EU699768
	R: ACTTTACGGCTGCCCATT	
TLR7	F: GGGTGATGAAGGAAATGTGC	XR003985123
	R: TTGCTCTTGTGGATGCTCTG	
OCC	F: TATCTGTATCACTACTGCGTCG	KF193855
	R: CATTCACCCAATCCTCCA	
ZO−1	F: CGGTGTCTTCGTAGTCGG	KJ000055
	R: CAGTTGGTTTGGGTTTCAG	
ZO−3	F: TTGTCATTTTGGGTCCTCTG	KF193854
	R: CTATCCGCCTAACCGTGTC	
CL−12	F: CCCTGAAGTGCCCACAA	KF998571
	R: GCGTATGTCACGGGAGAA	
MyoD	F: CCCTTGCTTCAACACCAACG	GU218462
	R: TCTCCTCTCCCTCATGGTGG	
Mrf4	F: TCATTCAACTTGTGCCCTCC	KT899334
	R: GCCCACTTTGCGATACCC	
Myf5	F: GGAGAGCCGCCACTATGA	AB012883
	R: GCAGTCAACCATGCTTTCAG	
MSTN1	F: GCAGGAGTCACGTCTTGGCA	KM874826
	R: GAGTCCCTCCGGATTCGCTT	

Abbreviations: IL-1β = interleukin-1β; TNFα = tumor necrosis factor-α; INF = interferon; TLR-4 = toll-like receptor 4; TLR-7 = toll-like receptor 7; OCC = occluding; ZO-1 = zonula occludens 1; ZO-3 = zonula occludens 3; CL-12 = claudin 12; MyoD = myoblast determination protein; Mrf4 = myogenic regulatory factor 4; Myf5 = myogenic factor 5; MSTN1 = myostatin 1.
F: stands for the forward primer.
R: stands for the reverse primer.

2.6. Intestinal microbiota analysis

The total DNA from the intestinal contents was extracted using either the cetyl trimethyl ammonium bromide or sodium dodecyl sulfat method, following the manufacturer's instructions. Next-generation sequencing of 16S rRNA was used to analyze the composition of the intestinal microbiota. The bacterial 16S rRNA gene was amplified using specific primers (515 F: 5'-GTGCCAGCMGCCGCGGTAA-3', 806 R: 5'-GGACTACHVGGGTWTCTAAT-3') and then sequenced on NovaSeq 6000 platform. The FLASH (V1.2.7) and QIIME (V1.9.1) software package was used to filter and assemble the resulting raw sequences (Bokulich et al., 2013). The filtered sequences were subsequently clustered into operational taxonomic units (OTUs) using Uparse (v7.0.1001), with a sequence identity of 97 %. To investigate bacterial richness and diversity, QIIME and R (Version 2.15.3) software were used to analyze the alpha and beta diversity, and species information with a similarity of more than 80 % was annotated accordingly. LEfSe sorftware (Version 1.0) was used to identify microbial species with significant differences between groups, with the parameter LDA Score set at 4.0. Based on the phylogenetic tree derived from OTUs in the Greengenes database, a Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was conducted to predict the metabolic functions of the microbial community. Finally, we calculated the sample distance matrix with weighted UniFrac distances and generated a clustering tree using the unweighted pair-group method with arithmetic means method (Wen et al., 2021).

2.7. Muscle pH, cooking loss, and texture

The pH value of the muscle samples stored at 4 ℃ for 8 h was determined by a pH meter (Testo-205, Testo AG, Germany) according to the method described in a previous study (Essid et al., 2020). The cooking loss was assessed by heat-treating 10 g of fish flesh in a high-temperature steamer (95–100 °C) for 15 min, followed by cooling and weighing. Muscle texture was evaluated using a TMS-PRO TPA device equipped with an 8 cm diameter, flat-bottomed, cylindrical probe and a 250 N load cell (Food Technology Corporation, USA), following the method used in a previous study (Tang et al., 2021).

2.8. Muscle free amino acids

The 0.2 g muscle samples were homogenized in 5 mL of 0.02 mol/L hydrochloric acid and then sonicated for 20 min at 4 ℃. Following centrifugation (6000 × *g*, 5 min, 4 ℃), the supernatant was collected and mixed with 8 % sulfosalicylic acid overnight at 4 ℃, and then centrifuged at 12,000 × *g* and 4 ℃ for 10 min. The resulting supernatant was filtered through a filtration membrane (0.45 μm) and analyzed using an Agilent 1260 equipped with a C18 column (diameter: 4.6 mm, length: 250 mm, particle size: 5 mm) (Wen et al., 2019). All determination results were expressed as mg/100 g.

2.9. Muscle fatty acids

The composition of fatty acids in muscle was determined using gas chromatography (Thermo Trace 1310 ISQ, USA) with a TG-5MS chromatography column (30 m × 0.25 mm × 0.25 μm). Briefly, the extraction procedure followed the method of Folch (Folch et al., 1957; Liu et al., 2015). The concentration of each individual fatty acid was quantified based on its peak area (Folch et al., 1957; Liu et al., 2015). The percentage of fatty acid (%) was calculated as the single fatty acid peak area divided by the total area of all fatty acid peaks multiplied by 100 %.

2.10. Statistical analysis

Statistical analyses were carried out using the GraphPad Prism 8.0

(GraphPad Software, USA), and the table data were presented as the mean ± SEM. An independent-samples t-test was used to assess significant difference between two given groups. The interactions between dietary nucleotides and β-glucan were examined using a two-way analysis of variance (ANOVA). The levels of significance were set at $P < 0.05$ for significant differences and $P < 0.01$ for highly significant differences.

3. Results

3.1. Effect of dietary nucleotides and β-glucan on intestinal antioxidant and inflammatory related genes in grass carp

There was no significant interaction between NT and Glu on antioxidant enzyme activity in the two-way ANOVA analysis (Fig. 1A-D). However, the two-way ANOVA revealed significant interactions between NT and Glu at the mRNA expression levels of *superoxide dismutase* (*SOD*), *GPX*, *interleukin-1β* (*IL-1β*), *interferon* (*INF*), *Toll-like receptors* (*TLR*)-4 (*TLR-4*), and *TLR-7* (Fig. 1E-M).

Comparatively, the Glu group showed a significant increase in the activities of T-AOC, AKP, and CAT as well as the mRNA expression of *CAT* ($P < 0.05$). The NT group also exhibited an obvious increase in CAT activity and the expression of *CAT* and *GPX* ($P < 0.05$) (Fig. 1A-G). Conversely, both the NT and Glu groups demonstrated a noticeable reduction in the expression of *IL-1β*, *INF*, *TLR-4*, and *TLR-7* ($P < 0.05$) (Fig. 1H-M). Moreover, when comparing the NT×Glu group with the NT group, there were significant increases in AKP activity and the expression of *SOD*, *CAT* and *GPX* ($P < 0.05$) (Figs. 1C, 1E-G), while the expression of *INF* decreased markedly ($P < 0.05$) (Fig. 1J). In addition, the overexpression of *SOD* and *GPX* was observed in the comparison between the NT×Glu group and the Glu group ($P < 0.01$) (Figs. 1E and 1G).

3.2. Effect of dietary nucleotides and β-glucan on intestinal tight junctions and morphology of grass carp

The two-way ANOVA analysis showed that there was an interaction between NT and Glu concerning villus height, villus width, and gut wall thickness (Fig. 2I-K).

The mRNA expression levels of intestinal *zonula occludens-1* (*ZO-1*) and *zonula occludens-3* (*ZO-3*) were significantly higher in both the NT and Glu groups compared to the control group ($P < 0.05$) (Figs. 2B and 2C). Additionally, there was also a notable increase of *ZO-3* in the NT×Glu group compared to both the NT ($P < 0.01$) (Fig. 2C). Moreover, *CL 12* (claudin 12) expression significantly decreased in the NT×Glu group compared to the Glu group ($P < 0.05$) (Fig. 2D). In terms of intestinal morphology parameters (Fig. 2I-K), the villus height and gut wall thickness significantly increased in the Glu group compared to the

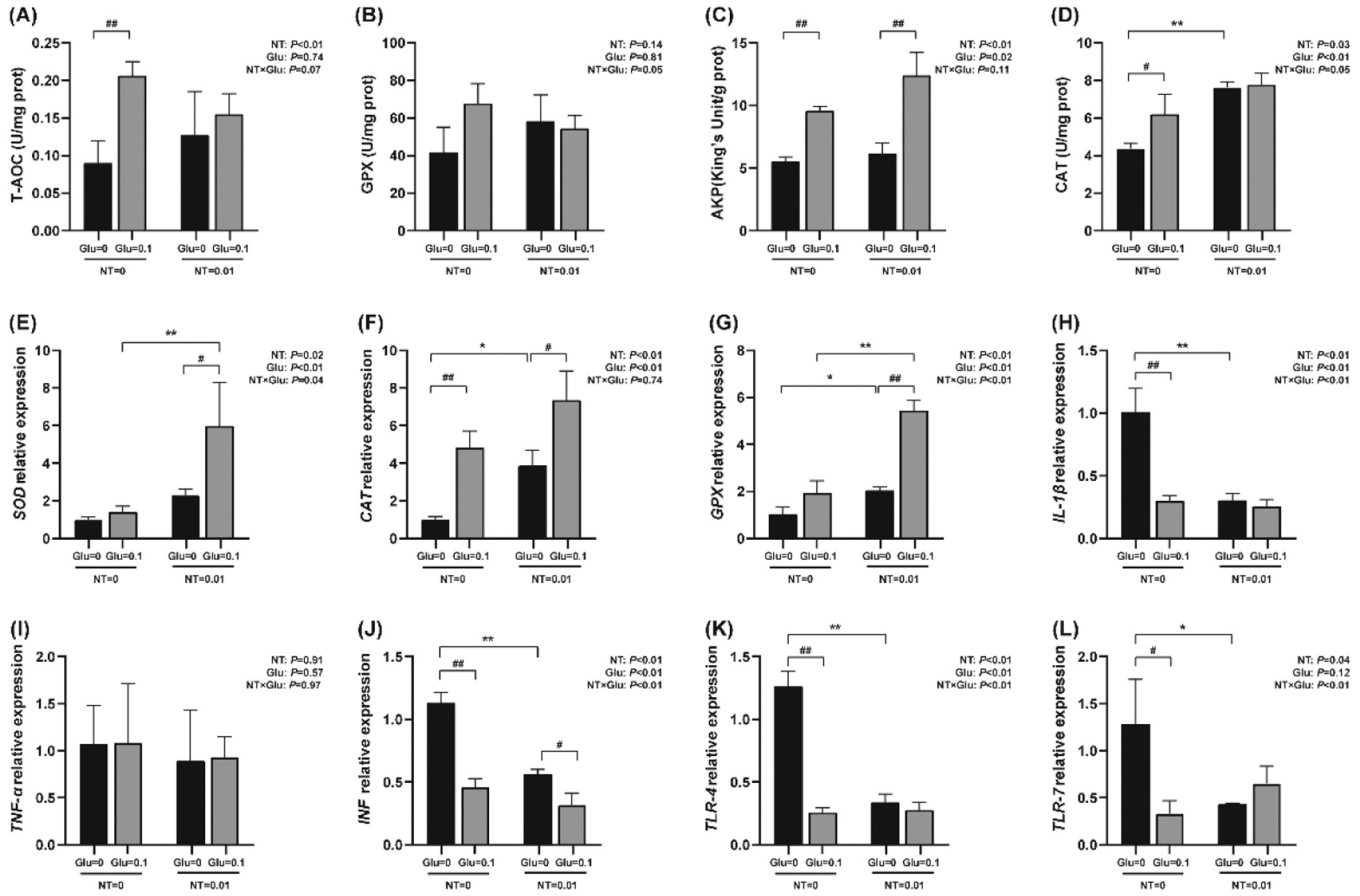

Fig. 1. Effect of dietary nucleotides and β-glucan on intestinal antioxidant and inflammatory in grass carp.(A-D): the activity of antioxidant enzymes of intestinal. Abbreviations: T-AOC = total antioxidant capacity; GPX = glutathione peroxidase; AKP = alkaline phosphatase; CAT = catalase. (E-M): the relative expression levels of intestinal antioxidant and inflammatory-related genes. Abbreviations: IL-1β = interleukin-1β; TNFα = tumor necrosis factor-α; INF = interferon; TLR-4 = toll-like receptor 4; TLR-7 = toll-like receptor 7. Data are presented as mean ± SEM (n = 3). # and ## indicate a significant difference ($p < 0.05$) and a very significant difference ($p < 0.01$), respectively, between different β-glucan levels within the same dietary nucleotide levels; * and ** indicate a significant difference ($p < 0.05$) and a very significant difference ($p < 0.01$), respectively, between different nucleotide levels within the same β-glucan levels. In the nucleotide level 0 group, the black column refers to control treatment, and the gray column refers to the Glu treatment. In the nucleotide level 0.01 group, the black column refers to the NT treatment, and the gray column refers to the NT×Glu treatment. The same as below.

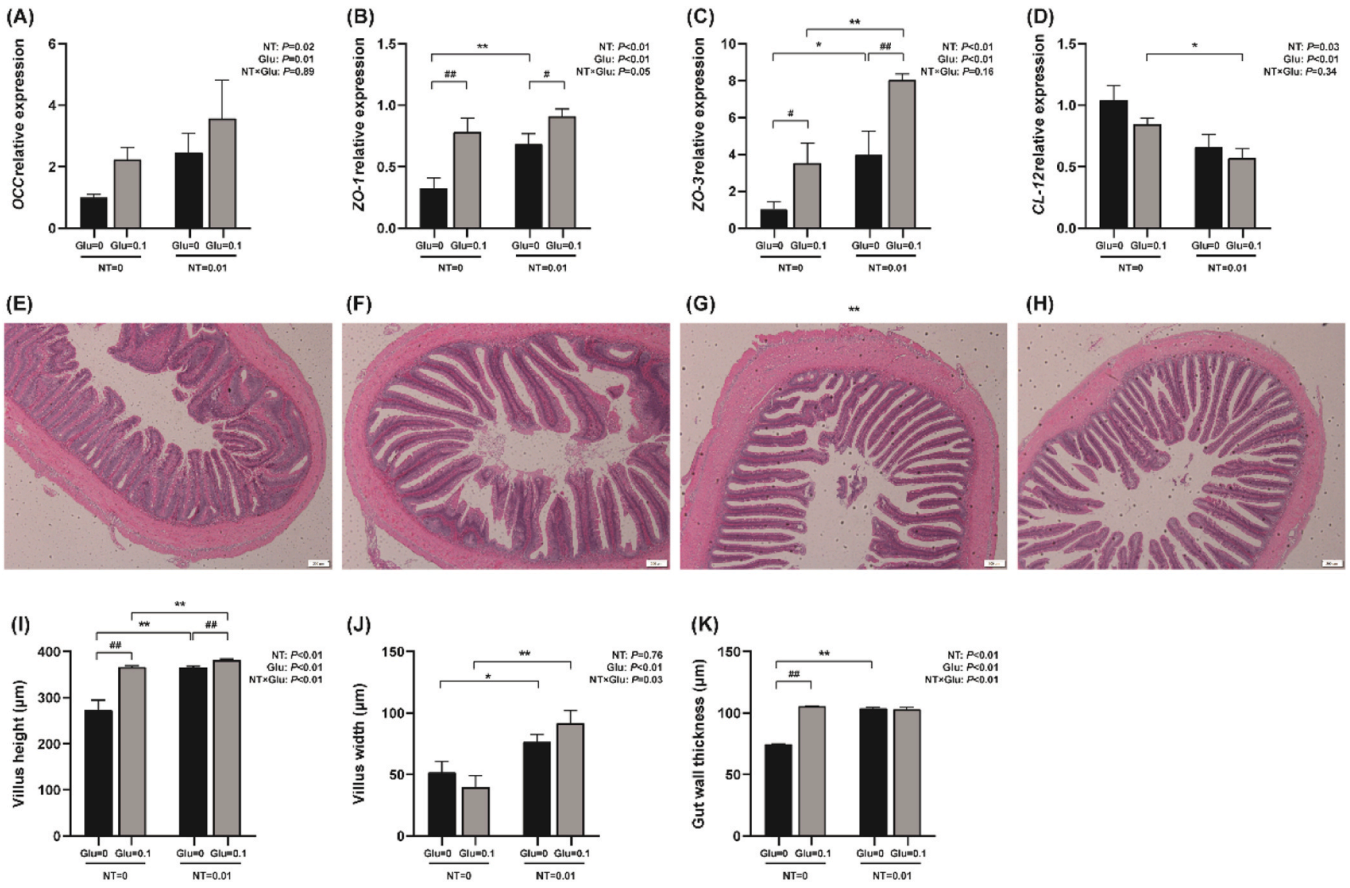

Fig. 2. Effect of dietary nucleotides and β-glucan on intestinal tight junctions and morphology in grass carp.(A-D): the expression levels of tight junctions related genes of intestinal. Abbreviations: OCC = occluding; ZO-1 = zonula occludens 1; ZO-3 = zonula occludens 3; CL-12 = claudin 12. (E-H): intestinal histological sections of control group (E), β-glucan group (F), nucleotide group (G), and nucleotide+β-glucan group (H) under 10 × magnification (Scale bars, 200 μm). (I): villus height. (J): villus width. (K): gut wall thickness.

control group ($P < 0.01$). Villus width also exhibited a noticeable increase in the NT group ($P < 0.05$). Furthermore, in the NT×Glu group, both the height and width of the villus were substantially higher than those in the NT group ($P < 0.01$), with only the villus height being remarkably higher than that in the Glu group ($P < 0.01$).

3.3. *Effect of dietary nucleotides and β-glucan on intestinal microbiota composition of grass carp*

The community richness analysis indicated that the sequencing depth was sufficient for all four samples (Fig. 3A), with the NT×Glu group showing the highest number of OTUs and the Glu group having the lowest. A Venn diagram illustrated that 125 unique OTUs were present in the NT group, 61 in Glu, 237 in NT×Glu, and 129 in the control group, with 596 OTUs shared across all groups (Fig. 3B). In Fig. 3C, Firmicutes were the most abundant bacteria, followed by Proteobacteria and either Bacteroidota or Fusobacteriota. Compared to the control group, a decrease in the relative abundance of Firmicutes and an increase in Fusobacteriota were observed in the NT×Glu group. Moreover, the bacterial phyla composition of NT and Glu groups exhibited a closer similarity than that of the control or NT×Glu groups. f_Rhodobacteraceae. o_Rhodobacterales, c_Alphaproteobacteria, and o_Rhizobiales were enriched in NT×Glu group, 0_Lactobacillales, f_Streptococcaceae, g_Streptococcus, s_Streptococcus_sp_M_S_TSA_29, f_Neisseriaceae, g_Stenoxybacter and g_Neisseria were enriched in NT group, f_Enterobacteriaceae was enriched in Glu group (Fig. 3D). Compared with NT or Glu group, the predicted function of Porphyrin and chlorophyll metabolism, Arginine and proline metabolism, and Pyruvate metabolism were enriched in NT×Glu group (Fig. 3E).

3.4. *Effect of dietary nucleotides and β-glucan on muscle texture, morphology and growth factor of grass carp*

Interactions between nucleotides and β-glucan were observed regarding muscle chewiness, springiness, and the expression of *myostatin1* (*MSTN1*), analysed using two-way ANOVA (Figs. 4B, 4C, and 4P).

The effects of nucleotides and β-glucan on muscle texture are presented in Fig. 4A-F. The NT groups exhibited a significant decrease compared to the control group ($P < 0.05$), while NT×Glu groups exhibited a significant increase in muscle chewiness compared to the Glu group ($P < 0.05$), while only the NT group showed a notable rise in muscle chewiness compared to the control group ($P < 0.01$) (Figs. 4B and 4C). The NT×Glu group demonstrated significantly higher muscle hardness, chewiness, and gumminess compared to the NT and Glu groups ($P < 0.05$) (Figs. 4A, 4B, and 4D). In addition, there were no significant differences observed among the groups in terms of muscle cooking loss and pH value ($P > 0.05$) (Figs. 4E and 4F). The effects of nucleotides and β-glucan on muscle morphology are illustrated in Fig. 4G-L. The myofiber density remarkably increased, while the fibers diameter significantly decreased in both the comparison between the NT group and the control group and the NT×Glu group and Glu group ($P < 0.05$) (Figs. 4G, 4H). The influence of dietary nucleotides and β-glucan on the expression of muscle growth factor-related genes is presented in Fig. 4M-P. Compared to the control group, the expression of *myogenic regulatory factor 4* (*Mrf4*) and *myogenic factor 5* (*Myf5*) significantly increased in the Glu group ($P < 0.01$), while *MSTN1* decreased notably

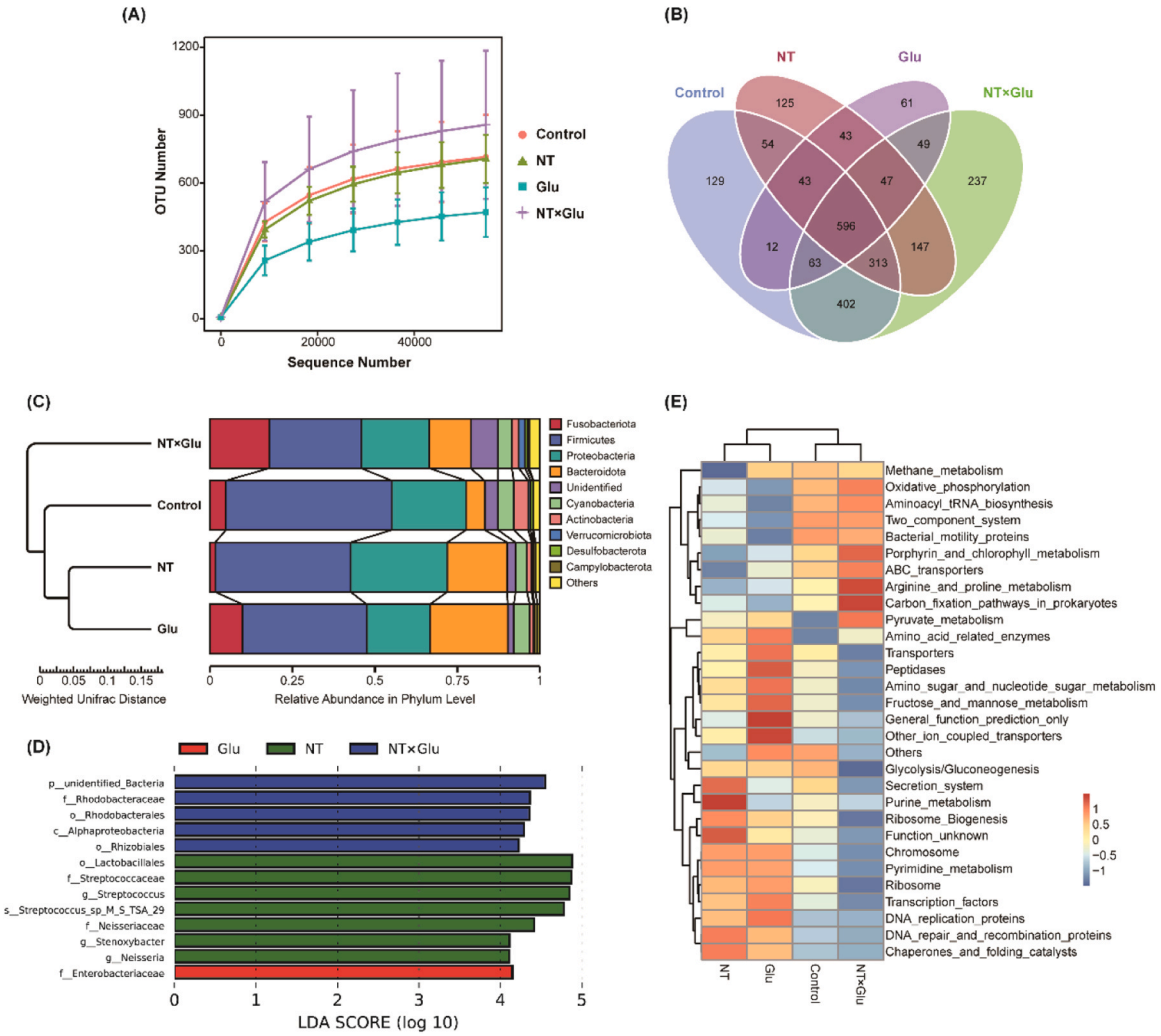

Fig. 3. Effect of dietary nucleotides and β-glucan on intestinal microbiota composition in grass carp.(A): rarefaction curves of observed species number for all the intestinal microbiome samples. The rarefied curves for observed species number tended to approach the saturation plateau. (B): Venn diagram of unique and shared OTUs. Every circle in the Venn diagram represents one group. The value from the overlapping part of different circles represents the shared OTUs between groups, and the value from the nonoverlapping part of one circle represents the unique OTUs of that group. (C): the UPGMA clustering tree for all the intestinal microbiome samples. Only the top 10 most abundant bacterial phyla or genera (based on relative abundance) are shown in the figures, while other phyla or genera are assigned as 'Others'. (D): Bar chart of LDA value distribution. (E): PICRUSt-predicted enrichment of microbial functions at KEGG level 3.

in both the NT and Glu groups ($P < 0.01$). Furthermore, the NT×Glu group exhibited higher expression of *myoblast determination protein* (*MyoD*) and *Myf5* than the NT group ($P < 0.05$), along with an elevated level of *Mrf4* compared to both the NT group and Glu group ($P < 0.01$).

3.5. *Effect of dietary nucleotides and β-glucan on muscle free amino acids and fatty acids composition of grass carp*

The interactions between nucleotides and β-glucan were observed for threonine, glutamic acid, proline, Σtasty free amino acids (FAA), C16:0, Σsaturated fatty acids (SFAs), and Σunsaturated fatty acids (UFAs) in two-way ANOVA analysis (Tables 3–4).

The FAA composition of grass carp muscle is detailed in Table 3. Compared to the control group, there was a significant increase in the percentage of Σtasty FAA in the NT group ($P < 0.01$), while a remarkable decrease in the percentage of Σbitter FAA was observed in the Glu group ($P < 0.05$). In the NT×Glu group, significant increases were noted in the percentages of ΣFAA and Σtasty FAA compared to the Glu group ($P < 0.05$). In addition, when compared with the NT group, a notable increase in Σtasty FAA and a decrease in Σbitter FAA were observed ($P < 0.01$). The fatty acids composition of grass carp muscle is listed in Table 4. Compared to the control group, the percentages of docosahexaenoic acid, ΣUFA, and Σn-3 polyunsaturated fatty acid (PUFA) significantly increased in the NT group ($P < 0.05$), while alanine and ΣUFA dramatically elevated in the Glu group ($P < 0.01$). In contrast, both the NT group and Glu group significantly decreased the percentage of ΣSFA ($P < 0.05$). Additionally, the NT×Glu group exhibited a significantly higher percentage of Ala than the Glu group ($P < 0.01$).

4. Discussion

The present study found that in grass carp dietary nucleotide (0.01 %) or β-glucan (0.1 %) had beneficial effects on antioxidant activity and barrier function, while simultaneously decreasing inflammatory response in intestine and improving fatty acids composition in muscle. Interestingly, the combined application of nucleotides (0.01 %) and β-glucan (0.1 %) exhibited a superior effect by significantly reducing inflammation response, improving intestinal morphology, and enhancing flesh-related parameters, which indicate a synergistic effect in promoting intestinal health and enhancing flesh quality in grass carp.

Inflammation and antioxidant processes are intricately linked, with

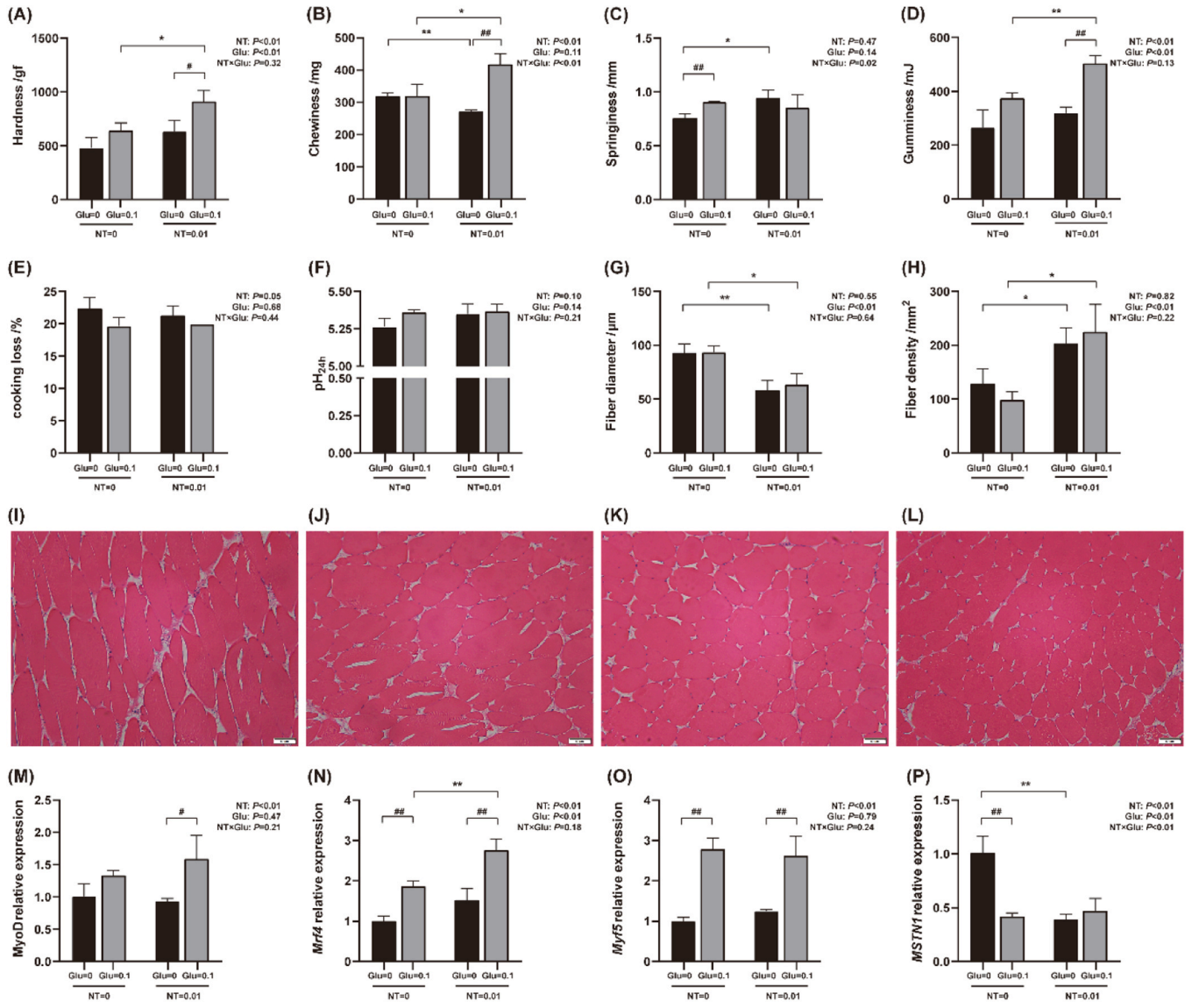

Fig. 4. Effect of dietary nucleotides and β-glucan on muscle texture, morphology and growth factor of grass carp. (A): hardness of muscle. (B): chewiness of muscle. (C): springiness of muscle. (D): gumminess of muscle. (E): cooking loss of muscle. (F): pH 24 h of muscle. (G-L): (G): average diameter of muscle fiber (μm). (H): muscle fibers density (mm2). (I-L): muscle histological sections of control group (I), β-glucan group (J), nucleotide group (K), and nucleotide+β-glucan group (L) under 40 × magnification (Scale bars, 50 μm). (M-P): the expression levels of muscle growth factor-related genes. Abbreviations: MyoD = myoblast determination protein; Mrf4 = myogenic regulatory factor 4; Myf5 = myogenic factor 5; MSTN1 = myostatin 1.

each influencing the other in various physiological and pathological conditions (Thirumagal and Chris, 2015). Antioxidant enzymes form the first barrier of defence against oxidative stress, with their activity mainly depend on the cellular oxidative equilibrium (Cristina et al., 2015). Inflammation in intestinal tissue can result in increased intestinal permeability, heightening the vulnerability to pathogen invasion (Yang et al., 2021). Antioxidants can mitigate the oxidative stress that often accompanies inflammation, while inflammation can induce the production of reactive oxygen species (ROS) and other free radicals (Nitish et al., 2024). Studies have shown that exogenous nucleotides and β-glucan play significant roles in the immune response and antioxidant defence of fish (Zhao et al., 2017). In yellow catfish (*Pelteobagrus fulvidraco*), dietary nucleotides significantly increase the activities of *SOD* and *CAT* (Zhao et al., 2017). Diet with β-glucan remarkably enhanced CAT and GPX activity in the intestinal tissue of grass carp (Yang et al., 2021). In this study, we determined the activities of key antioxidant enzymes and examined their gene expression levels in grass carp. Remarkably, the NT×Glu group exhibited a significant upregulation in the mRNA expression levels of *SOD* and *GPX* compared to the NT and Glu groups, with noticeable combined effects of nucleotides and β-glucan on them. Antioxidants can help to neutralize ROS, thereby reducing inflammation and potentially preventing or alleviating the severity of inflammatory conditions (Juan and Kristine, 2022). These findings highlight the synergistic effects of nucleotides and β-glucan on the intestinal antioxidant status.

β-glucan, which acts as a potent adjuvant, can effectively stimulate both natural and adaptive host immune responses (Qi et al., 2011) and attenuate inflammation response through suppressing TLRs and reducing the production and releasement of proinflammatory cytokines, such as IL-1β and tumor necrosis factor-α (TNF-α) (Kankkunen et al., 2010). The immunomodulatory properties of nucleotides have also been proved in fish, as evidenced by their ability to down-regulate the inflammatory tone in the zebrafish intestine when supplemented, (Guo et al., 2017), as well as inhibit the mRNA expression level of *TNF-α* in grass carp (Tie et al., 2021). Accordingly, the observations in the present study not only reveal that the combination of nucleotides and β-glucan

Table 3
Effect of dietary nucleotides and β-glucan on the muscle amino acid composition (mg/100 g) in grass carp.

Items (mg/100 g)	Treatments				P-value		
	Control	β-glucan	Nucleotide	Nucleotide+β-glucan	NT	Glu	NT×Glu
Aspartic acid	1.4±0.25	2.07±0.03	2.90±0.12**	3.33±0.55	0.11	<0.01	0.71
Threonine	93.00±2.08	116.00±12.49	126.67±8.82*	220.00±10.0$^{\#\#}$**	<0.01	<0.01	<0.01
Serine	8.10±1.15	15.67±0.33$^{\#\#}$	12.00±1.53	14.33±6.23$^{\#\#}$**	<0.01	<0.01	0.54
Glutamic acid	18.00±0.58	28.00±1.73$^{\#\#}$	23.67±2.03	38.33±7.06$^{\#\#}$**	<0.01	<0.01	<0.01
Glycine	76.00±8.50	82.00±4.36	90.67±6.01	130.00±11.55$^{\#}$*	0.02	<0.01	0.07
Alanine	47.67±4.84	58.67±10.17	78.00±9.29*	55.67±5.61	0.49	0.12	0.07
Cysteine	3.47±0.26	2.67±0.26	1.63±0.09**	1.23±0.09$^{\#}$**	0.02	<0.01	0.33
Valine	8.10±1.21	4.47±0.22$^{\#}$	6.03±1.02	3.77±0.52	<0.01	0.14	0.44
Lysine	223.33±16.67	123.33±8.82$^{\#\#}$	176.67±6.67	119.33±10.67$^{\#}$	<0.01	0.06	0.18
Histidine	363.33±28.48	313.33±3.33	373.33±14.53	256.67±28.48$^{\#}$	<0.01	0.31	0.16
Arginine	55.00±2.31	69.33±2.67$^{\#}$	80.67±5.70*	146.67±20.28$^{\#}$*	<0.01	<0.01	0.44
Proline	12.63±1.37	20.33±0.30$^{\#\#}$	17.00±1.00	40.33±4.98$^{\#}$*	<0.01	<0.01	0.02
∑FAA	910.63±40.04	835.87±40.92	989.23±33.39	1074.67±50.00*	0.85	<0.01	0.09
∑tasty FAA	312.40±2.95	392.07±28.96	431.57±13.94*	693.67±48.47$^{\#\#}$**	<0.01	<0.01	0.01
∑bitter FAA	598.23±41.19	443.8±12.02$^{\#}$	557.67±21.61	381.00±25.53$^{\#\#}$	<0.01	0.09	0.69

Abbreviations: FAA = free amino acids; ∑tasty FAA = aspartic acid, threonine, serine, glutamic acid, glycine, alanine, arginine, proline; ∑bitter FAA = cysteine, valine, lysine, histidine.
For amino acid composition, results were expressed as mean ± SEM (n = 3, dry matter).
$^{\#}$ and $^{\#\#}$ indicate a significant difference ($p < 0.05$) and a very significant difference ($p < 0.01$), respectively, between different β-glucan levels within the same dietary nucleotide levels; * and ** indicate a significant difference ($p < 0.05$) and a very significant difference ($p < 0.01$), respectively, between different nucleotide levels within the same β-glucan levels.

Table 4
Effect of dietary nucleotides and β-glucan on the muscle fatty acid composition in grass carp (% of total FA methyl esters).

Items	Treatments				P-value		
	Control	β-glucan	Nucleotide	Nucleotide+β-glucan	NT	Glu	NT×Glu
C14:0	2.89±2.89	2.83±0.06	2.81±0.08	2.77±0.05	0.51	0.36	0.88
C16:0	25.44±0.38	222.41±0.03$^{\#\#}$	23.39±0.38*	22.71±0.10*	<0.01	0.01	<0.01
C16:1n7	7.07±0.49	7.23±0.25	7.61±0.36	7.33±0.48	0.89	0.46	0.41
C18:0	5.49±0.33	4.41±0.23	4.55±0.20	4.26±0.19	0.02	0.06	0.14
C18:1n9	28.98±0.35	31.78±1.09	29.67±0.66	29.68±0.98	0.13	0.41	0.13
C18:2n6	15.20±0.61	17.08±0.53	16.34±0.24	17.33±0.67	0.03	0.23	0.43
C18:3n3 (ALA)	1.56±0.04	1.93±0.06$^{\#\#}$	1.80±0.02	1.96±0.08**	<0.01	0.04	0.09
C20:4n6	3.42±0.28	2.05±0.20$^{\#}$	2.67±0.12	2.37±0.34	<0.01	0.41	0.07
C20:5n3	2.65±0.02	2.53±0.20	2.65±0.14	3.05±0.03$^{\#}$	0.28	0.07	0.07
C22:5n3	1.19±0.06	1.02±0.02	1.05±0.03	1.04±0.02	0.03	0.15	0.15
C22:6n3 (DHA)	6.11±0.12	6.74±0.36	7.47±0.05**	7.51±0.32	0.21	<0.01	0.26
ΣSFA	33.83±0.7	29.65±0.20$^{\#\#}$	30.75 ±0.33*	29.74±0.20	<0.01	<0.01	<0.01
ΣUFA	66.17±0.7	70.35±0.20$^{\#\#}$	69.25±0.33*	70.26±0.22	<0.01	<0.01	<0.01
ΣMUFA	36.06±0.63	39.01±1.31	37.27±0.33	37.01±1.39	0.22	0.71	0.15
ΣPUFA	30.12±0.73	31.35±1.12	31.97±0.023	33.25±1.17	0.20	0.07	0.98
Σn3 PUFA	11.51±0.15	12.22±0.55	12.96±0.12**	13.56±0.3	0.09	<0.01	0.88
Σn6 PUFA	18.61±0.68	19.12±0.65	19.01±0.13	19.69±0.89	0.39	0.48	0.90
Σn3/Σn6	61.97±2.13	63.92±1.94	68.19±1.08	69.05±2.41	0.49	0.02	0.79

Abbreviations: ALA = gamma linolenic acid, DHA = docosahexaenoic acid; SFA = saturated fatty acid; UFA = unsaturated fatty acids; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; n-6 PUFA = n-6 poly-unsaturated fatty acid; n-3 PUFA = n-3 poly-unsaturated fatty acid.
For fatty acid composition, results were expressed as mean ± SEM (n = 3, dry matter).
$^{\#}$ and $^{\#\#}$ indicate a significant difference ($p < 0.05$) and a very significant difference ($p < 0.01$), respectively, between different β-glucan levels within the same dietary nucleotide levels; * and ** indicate a significant difference ($p < 0.05$) and a very significant difference ($p < 0.01$), respectively, between different nucleotide levels within the same β-glucan levels.

effectively suppresses the inflammatory response but also imply a synergistic effect of these two supplements on reducing the expression of proinflammatory genes in the intestinal tissue of grass carp. Understanding the intricate relationship between inflammation and antioxidants is vital for developing therapeutic strategies that target inflammation-related disorders.

The intestinal barrier plays a pivotal role in maintaining host health (Natalia et al., 2021). Studies have shown that dietary nucleotides or β-glucan can increase villus height and gut wall thickness in chicken (Aya et al., 2024). This effect extends to fish species, with nucleotide-supplemented diets improving intestinal morphology in juvenile largemouth bass (Chen et al., 2022), Nile tilapia (Robson et al., 2022), grass carp (Huang et al., 2023), and *hybrid tilapia* (Xu et al., 2015). Moreover, dietary β-glucan has been found to increase villus length in pacu (Cerozi et al., 2017) and pompano (Hoang et al., 2024). Our results indicate that dietary supplementation of nucleotides and β-glucan, either individually or in combination, effectively improves intestinal histomorphology in grass carp, further supporting the positive impact of these supplements on fish intestinal health. In addition, dietary nucleotides and β-glucan not only improved intestinal morphology but also enhanced the tight junction barrier. Tight junction proteins, such as claudins, occluding, and ZO, are crucial for epithelium formation, tissue integrity maintenance, and cell permeability regulation in vertebrates (Kolosov et al., 2014). Previous research has proved that

β-glucan supplementation can improve intestinal barrier function by upregulating the expression of tight junction proteins-related genes (Yang et al., 2021). In the present study, our investigation into the expression of tight junction-related genes revealed a significant enhancement in the expression level of *ZO-3* when grass carp were treated with a combination of nucleotides and β-glucan, surpassing the effects of individual treatments, which indicates that these supplements collectively contribute to fortifying the intestinal barrier in fish.

The intestinal microbiota exerts a role in maintaining intestinal morphology and barrier function (Kaisa et al., 2018), with disruptions potentially leading to inflammatory responses (Przewłócka et al., 2020). Previous researches have indicated that the addition of β-glucan in fish diets can influence the composition of the intestinal microbiota. For example, dietary β-glucan triggered a shift in the microbiota profile of senegalese sole (*Solea senegalensis*), particularly decreasing the relative proportion of Vibrio in the intestine (Carballo et al., 2019). Similarly, the β-glucan supplemented diets in sea bass significantly decreased the abundance of the *dysgonomonas* genus within the Bacteroidetes phylum (Carda-Diéguez et al., 2014). In another study, dietary β-glucan was linked to a decrease in the number of OTUs and species richness of the microbiota in carp (Kühlwein et al., 2013). These findings align with the outcomes of our current investigation, where the nucleotides and β-glucan supplementations in grass carp led to a significant reduction in the abundance of *Bacteroidetes* genus. Besides, upon feeding grass carp nucleotide+β-glucan diets, we observed a remarkable increase in Fusobacteria and Bacteroidetes. Recent studies showed that intestinal bacteria could promote the fermentation of carbohydrates to produce short-chain fatty acids (SCFAs), which serve as the primary energy source for intestinal epithelial cells (Douglas and Tom, 2016). These SCFAs also modulate the proliferation and differentiation of intestinal epithelial cells, thereby strengthening intestinal barrier function and shielding the intestine from inflammation (Przewłócka et al., 2020). Specifically, the microbial communities responsible for producing SCFAs mainly include *fusobacterium* (Fusobacteriia), known for butyric acid production, and *bacteroidaceae* (Bacteroides), which produce succinic acid, acetic acid, formic acid, and propanoic acid. Therefore, our results indicated that the combination of nucleotides and β-glucan may promote intestinal health in grass carp by fostering the growth of SCFA-producing colonies.

The quality of fish flesh depends on various physicochemical parameters, including flesh texture (hardness, chewiness, springiness, and gumminess), water-holding capacity, and pH (Jiang et al., 2016). Previous studies have shown that both nucleotides and β-glucan supplements can improve flesh quality. For instance, dietary β-glucan has been found to enhance flesh quality in broilers (Cho et al., 2013) and pigs (Luo et al., 2019a) by reducing cooking loss. Similarly, dietary nucleotides have been reported to improve flesh quality in grass carp by reducing muscle cooking loss and inhibiting the decline in muscle pH (Tie, 2018). In our current study, an increase in muscle springiness was observed. Furthermore, our results demonstrated that the combination of nucleotides and β-glucan increased muscle hardness, chewiness, and gumminess compared to individual treatments alone. Notably, interactions between nucleotides and β-glucan were observed in terms of muscle chewiness and springiness, further supporting their synergistic potential to enhance flesh quality through combined application.

Nutritional status influences muscle growth, leading to changes in fiber type, size, and number (Zeng et al., 2014). In rainbow trout (*Oncorhynchus mykiss*), muscle tissues texture parameters were positively correlated with fiber density but negatively correlated with fiber diameter (Lefevre et al., 2015). Similarly, in our study, the combined application of nucleotides and β-glucan led to a reduction in muscle fiber diameter, an increase in fiber density, and an improvement in muscle texture. Regarding the expression of muscle growth-related genes, the NT×Glu group exhibited an upregulation expression of *Myf4*, *Myf5*, and *MyoD*. These regulatory factors are necessary for the determination of skeletal myoblasts and play a vital role in the development and maturation of skeletal muscle (Tomohiko et al., 2022). MSTN negatively regulates protein turnover rate in muscle, as well as the hyperplasia and hypertrophy of muscle cells (Zeng et al., 2014). Accordingly, mRNA expression of *MSTN1* was remarkably reduced after adding nucleotides or β-glucan, with a synergistic inhibitory effect observed with the combined application of nucleotides and β-glucan. This suggests that the combined use of nucleotides and β-glucan may improve grass carp muscle morphology by regulating the expression levels of muscle growth genes.

FAA play an important role in shaping the flavor profile of fish flesh, which can be classified into three taste categories: sweet (Gly, Ser, Ala, Pro, Thr, Cys, and Met), bitter (Arg, His, Ile, Leu, Phe, Lys, Tyr, and Val), and umami (Asp, Glu) (Simopoulos, 2006b). It has been reported that dietary nucleotides improved the percentage of muscle ΣFAA in grass carp (Tie et al., 2019). In the current study, we found that the independent use of nucleotides or β-glucan did not yield a significant impact, but a noticeable increase was observed when they were applied in combination. Moreover, dietary nucleotides significantly raised the percentage of muscle Σtasty FAA but had no effect on Σbitter FAA. Conversely, dietary β-glucan notably reduced the percentage of Σbitter FAA without affecting Σtasty FAA. Interestingly, the combined application group showed simultaneous increases in Σtasty FAA and decreases in Σbitter FAA. We also found a significant interaction between nucleotides and β-glucan that affected the Σtasty FAA content in muscle. Hence, it can be inferred that the combined application of nucleotides and β-glucan has a more pronounced effect than their individual applications in enhancing flesh flavor by optimizing the amino acids composition.

The fatty acid profile serves as another critical determinant of flesh flavor. A high percentage of long-chain PUFAs in muscle systems often initiates phospholipids oxidation (Higgins et al., 1998). Elevated levels of SFAs can lead to softer and more acidic carcass fat (Luo et al., 2019a). Previous research showed that β-glucan supplementation can alter the proportions of SFA and SUFA, thereby improving the flesh flavor of finishing pigs (Luo et al., 2019a). In Beluga sturgeon juveniles (*Huso huso*), dietary nucleotides have been found to significantly decrease the SFA/TUFA ratio and potentially influence the conversion of short-chain fatty acids to their long-chain derivatives (Abtahi et al., 2013). Additionally, research has indicated that dietary nucleotides increased the percentage of ΣUFA while decreasing the percentage of ΣSFA in grass carp muscle (Tie et al., 2019), consistent with our own findings. Furthermore, we observed a synergistic impact of the combined application of nucleotides and β-glucan in enhancing the ΣUFA percentage and reducing the ΣSFA percentage. Researches showed that n-3 PUFAs inhibited chronic diseases, while n-6 PUFAs boosted inflammation (Simopoulos, 2006). Animals fed a high n-6/n-3 PUFAs ratio are more prone to inflammation (Yu et al., 2013). Therefore, our current findings suggest that dietary nucleotides could significantly increase the percentage of Σn-3 PUFAs in grass carp, underscoring their great benefits to animal health. In brief, these observations indicate that the combined application of nucleotides and β-glucan can improve flesh quality by modulating the fatty acid composition in grass carp muscle.

5. Conclusion

In conclusion, the combined application of nucleotides and β-glucan may offer an enhanced benefits compared to supplementation with either nucleotides or β-glucan alone in potentially promoting intestinal health and improving flesh quality in grass carp. These findings offer a more comprehensive insight into the strategy of multiple supplementations in aquaculture nutrition and feed.

Ethics approval

The study involving animal care and experiments was complied with the Guide for Care and Use of Laboratory Animals. The experimental

protocol was approved by the ethics committee of Hunan Normal University under permit No. 20200012.

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CRediT authorship contribution statement

Lina Zhang: Formal analysis. **Wenjie Luo:** Writing – original draft, Methodology, Investigation, Data curation. **Jianzhong Li:** Writing – review & editing, Formal analysis. **Conghui yang:** Writing – review & editing, Validation, Methodology, Investigation, Funding acquisition, Data curation. **Chunyan Li:** Formal analysis. **Zhongtian Tang:** Formal analysis. **Qinbo Qin:** Formal analysis. **Wei Huang:** Formal analysis. **Yating Zhu:** Formal analysis.

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No Generative AI and AI-assisted technologies were used in the writing process.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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Data Availability

Data will be made available on request.

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