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Impact of dandelion polysaccharides on growth and immunity response in common carp *Cyprinus carpio*

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ABSTRACT

Natural products have gained considerable attention for improving fish growth performance and immunity to enhance disease resistance. This study evaluated the effect of dandelion polysaccharides (DP) on skin mucosal immune parameters, immune-related gene expression, and susceptibility to pathogen challenge in the Common carp *Cyprinus carpio*. Diets containing four different concentrations of DP (g Kg⁻¹): 0 g [basal diet], 0.5 g [D1], 1.5 g [D2], 2.5 g [D3], and 4.0 g [D4] were fed to the carp (average weight: 13.92 ± 0.83 g) for eight weeks. Growth parameters were analyzed four and eight weeks after feeding. Immunological, hematological, and antioxidant parameters were examined eight weeks post-feeding. Growth performance was significantly higher on D3, with a final weight gain of 71.48 ± 1.57 g and a specific growth rate of 3.06 ± 0.12. Among hematological parameters examined, erythrocyte hematocrit, and mean corpuscular volume (MCV) levels were significantly higher in D3. Skin mucosal immune parameters, such as lysozyme (31.04 ± 1.02 Unit mL⁻¹), alkaline phosphatase (122.6 ± 3.8 IU L⁻¹), and protein level (10.6 ± 0.74 mg mL⁻¹) were significantly higher in D3, while peroxidase activity was higher in D4. Furthermore, SOD activity was higher in D2–D3, whereas catalase activity was higher in D2–D4 ($P < 0.05$) than in the control. Malondialdehyde level decreased significantly in D3 (5.43 ± 0.36 nmol mL⁻¹); whereas, serum ALT and AST levels were significantly lower on D2–D4. Intestinal tight-junction-related genes *ZO-1* and *Claudin 7* were significantly higher in the DP-fed groups; however, DP had no significant effect on *claudin 3*. *Occludin* expression was higher ($p < 0.05$) on D3 only. Pro-inflammatory cytokines (*IL-1β* and *TNF-α*) and *IFN-γ* strongly upregulated in the head kidney at D3. Conversely, the expression of the anti-inflammatory cytokine *interleukin-10*, *HSP70*, and *TOR* were considerably downregulated in D3. Fish from D3 exhibited markedly higher relative post-challenge survival (66.67%) against *Aeromonas hydrophila* challenge. The results of the present study suggest that dietary supplements of DP at 2.5 g kg⁻¹ can significantly improve the growth performance, skin mucosal, and serum antioxidant parameters, and strengthen the immunity of *C. carpio*. Therefore, DP is a promising food additive for carp aquaculture.

1. Introduction

Fish are considered a vital protein source for humans. Aquaculture has been practiced for thousands of years, but it has now been transformed into an intensive practice [1]. Common carp *Cyprinus carpio* was the 4th most cultured finfish species in 2018, accounting for 7.7% of total finfish production [2]. Intensive culture often leads to stressful conditions that affect fish health and render them susceptible to various diseases. Oxidative stress in cells results in the oxidation of amino acids, unsaturated fatty acids, and nucleic acids, which are toxic to cells owing

to damage to proteins, lipids, nucleic acids, carbohydrates, and changes in the gene expression [3]. The extensive use of antimicrobial agents, prophylactically and metaphylactically, to control diseases in aquaculture has led to the emergence of antimicrobial-resistant (AMR) pathogens, accumulation of chemicals or residual antibiotics in tissues, and suppression of immune responses in cultured fish [4]. AMR genes from aquaculture species are transferred to humans [5], resulting in undesirable health implications in human through indirect assimilation. Therefore, an environment-friendly, non-toxic mitigation measure is necessary to control diseases in aquaculture.

Vaccination and functional feed supplementation are effective in

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Abbreviations

SOD	serum superoxide dismutase
DP	dandelion polysaccharides
AMR	antimicrobial-resistant
WGR	weight gain rate
FCR	feed conversion ratio
SGR	specific growth rate
WBC	white blood cell
ALT	alanine aminotransferase
AST	aspartate aminotransferase (AST)
HSP	heat-shock protein
AMR	antimicrobial-resistant
WBC	white blood cell
Hb	hemoglobin
MCV	mean corpuscular volume
CAT	Catalase
MDA	malondialdehyde
ALP	alkaline phosphatase
HSP	heat-shock protein
TOR	target of rapamycin
SEM	standard error of the mean

controlling and preventing diseases. However, their success rate differs because they are species-specific, and developing vaccines against intracellular pathogens is challenging [6]. Natural products have gained considerable attention for improving fish growth performance and immunity to enhance disease resistance [7]. Natural immunostimulants are readily available, biodegradable, biocompatible, and have few or no side effects on fish and humans. Many herbal and plant-based compounds can boost innate and adaptive immune responses in fish [8]. They provide extended protection compared with synthetic drugs. Immunostimulant plants and their by-products contain several alkaloids, phenolics, quinones, polyphenolics, lectins, terpenoids, and polypeptide compounds, many of which are effective alternatives to synthetic drugs, antibiotics, and vaccines. They also facilitate growth, stress reduction, environmental friendliness, and antimicrobial properties [9]. Whole plants, extracted compounds, or plant parts (leaves, seeds, or roots) have been used as feed supplements in aquaculture. Recently, we showed that dietary supplementation of ginger at 0.8% for 60 d [10], *Chlorophytum borvilianum* polysaccharide at 0.4% for 28 d [11], dietary turmeric at 15 g kg⁻¹ for eight weeks [12], and *Pandanus tectorius* extract 20 g kg⁻¹ for 8 weeks [13] could improve the growth performance, immune responses, and disease resistance of Indian major carp.

Fish mucosal surfaces act as barriers against various biological, physical, and chemical stressors. Goblet cells produce mucus that is scattered throughout the mucosal tissue with epithelial cells [14]. Skin mucus mainly consists of water (95%) and mucins, which are glycosylated proteins with high molecular weight. Mucins are structural proteins that play vital roles in viscosity [15]. Skin mucus is associated with important functions in fish, including respiration, osmoregulation, nutrition, and locomotion [16]. Therefore, the characterization of skin mucus immune parameters would be a useful approach for studying the impact of immunostimulants in fish. Dandelion (*Taraxacum officinale*) is a perennial herb widely distributed in the northern hemisphere [17]. Dandelion contains various phytochemicals, including polysaccharides, oligosaccharides, flavonoids, alkaloids, phenolic acids, and terpenes [18]. Traditionally, dandelion has been used to treat various health problems, including spleen and liver diseases, dyspepsia, gallbladder disorders, digestive problems, and inflammation [19]. Dandelion polysaccharides (DP) (400 mg kg⁻¹) inhibit hepatocellular carcinoma cell proliferation in vitro in human hepatocellular carcinoma cell lines and in vivo in a mouse model [20]. DP is an α -type polysaccharide composed

of glucose, galactose, arabinose, arabinose rhamnose, and glucuronic acid [20]. Further, dietary dandelion at 1.00 g kg⁻¹ for eight weeks could improve the growth performance, plasma biochemical parameters, and immune responses of golden pompano *Trachinotus ovatus* [18]. Furthermore, the dietary dandelion extract had beneficial effects on the antioxidant, immune, and physical barrier functions of juvenile golden pompano [21]. However, there are no studies on the effect of DP on skin mucosal immune or antioxidant responses in fish. Therefore, this study aims to evaluate the effects of DP on growth performance, hematological and serum antioxidant parameters, skin mucosal immune responses, and disease resistance in fish.

2. Materials and methods

2.1. Diet preparation

The composition of the basal diet is shown in Table 1. It was formulated to contain 30.6% protein, 8.51% lipid, and 10.36% ash, and used as the control diet. The basal diet was mixed with 0.5, 1.5, 2.5, and 4.0 g kg⁻¹ of dandelion polysaccharide (DP, > 98% purity was purchased from Ci Yuan Biotechnology, Shanxi, Xian, China). All ingredients were thoroughly mixed, pelleted, air-dried, and sieved into appropriate pellet sizes. After drying, all diets were sealed in bags and stored at 4 °C, until used.

2.2. Experimental feeding

Healthy *C. carpio* (average weight: 13.92 ± 0.83 g) collected from a local farm were checked for health status using standard bacteriological techniques. Fish were disinfected with 5 ppm KMnO₄ for 5 min and acclimatized to laboratory conditions for 2 weeks. They were fed a basal diet ad libitum twice a day (09.00 and 16.00 h).

After acclimatization, the fish were equally divided into five groups, each with three replicates. Each group consisted of 60 fish (20 fish per tank × 3 tanks = 60 fish). The following groups were maintained: (I) control (fed basal diet); (II) diet supplemented 0.5 g kg⁻¹ DP (D1), (III) diet supplemented with 1.5 g kg⁻¹ DP (D2), (IV) diet supplemented with 2.5 g kg⁻¹ DP (D3), and (V) diet supplemented with 4.0 g kg⁻¹ DP (D4). Fish were fed twice a day at a rate of 3–4% of their body weight, and a feeding trial was conducted for eight weeks. The tank capacity was 200 L, and 30% of water was renewed daily in each tank. Almost 100% of water was exchanged once a week. Basic physicochemical parameters were measured during the trial period [22]. Ammonia concentration (0.03–0.06 mg L⁻¹), oxygen (6.2–7.3 mg L⁻¹), pH (7.0–8.0), and temperature (26 ± 2 °C) were maintained during the trial period, and maintained within the most favorable levels for common carp. All experiments complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were performed according to the National Institute of Health Guide for the Care and Use of Laboratory

Table 1
Formulation and composition of basal diet (g kg⁻¹ dry matter).

Ingredients	Concentrations (g kg ⁻¹)
Ground nut oil cake	400
Rice bran	350
Wheat floor	150
Fish meal	75
Vegetable oil	15
Fish oil (Menhaden)	5
Mineral and vitamin mixture ^a	5

^a Every 250 g of mineral-vitamin mixture (Supplevite-M, Sarabhai Zydus Animal Health Ltd., India) provides vitamin A, 500000 IU; vitamin D3, 100000 IU; vitamin B2, 0.2 g; vitamin E, 75 units; vitamin K, 0.1 g; calcium pantothenate, 0.25 g; nicotinamide, 0.1 g; vitamin B12, 0.6 mg; choline chloride, 15 g; calcium, 75 g; manganese, 2.75 g; iodine, 0.1 g; iron, 0.75 g; zinc, 1.5 g; copper, 0.2 g; and cobalt, 0.045 g.

Animals.

2.3. Growth performances

The body weight of the fish was measured at the beginning, after fourth week, and at the end of the feeding trial. The following growth parameters were calculated [12]:

Weight gain rate (WGR; %) = $[(W_t - W_0)/W_0] \times 100$

Specific growth rate (SGR; % day⁻¹) = $[(\ln W_t - \ln W_0)/t] \times 100$;

Feed conversion ratio (FCR) = [total dry feed intake (g) / wet weight gain (g)]

Survival rate (%) = (final number of fish/initial number of fish) × 100

where, W_t and W_0 were the final and initial weights (g) of the fish, respectively; and 't' is the duration of feeding (in day).

2.4. Sample collection

At the end of the feeding trial, 15 fish from each group were sampled to collect skin mucus. Fish were anesthetized with MS-222 (Sigma-Aldrich) and placed in a polyethylene bag containing 10 mL of 50 mM NaCl. The fish were rubbed gently inside the bags for 2 min. Thereafter, the solution was transferred into a 20 mL sterile tube and centrifuged at 1500 g at 4 °C for 10 min. The supernatant was filtered through a 0.45 µm millipore filter and stored at -80 °C, until further use.

Blood samples were collected from the caudal vein of anesthetized fish using a 2 mL syringe, for serum collection. Blood samples were transferred to centrifuge tubes (Eppendorf, Hamburg, Germany). Serum was collected by centrifugation (4000×g, 10 min, 4 °C) and stored at -20 °C, until further use.

2.5. Hematological parameters

Hematological parameters, including red blood cell count, white blood cell (WBC) count, hematocrit value (Hct, %), hemoglobin (Hb) level, and mean corpuscular volume (MCV), were measured using standard techniques. Total serum protein was estimated using a commercial kit (Sigma-Aldrich, USA) and absorbance was read spectrophotometrically (OD_{595 nm}).

2.6. Serum oxidative stress-related parameters

Serum superoxide dismutase (SOD) activity was determined by an enzymatic assay method using a reagent kit (Randox, Crumlin, UK), as described previously [23]. One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the NBT reduction rate measured at 550 nm.

Catalase (CAT) activity was measured as the rate of decrease in H₂O₂ absorbance at 240 nm, using a commercially available kit (Sigma-Aldrich, USA).

The malondialdehyde (MDA) content in serum was measured using commercial kit (Sigma-Aldrich) based on the thiobarbituric acid reaction [24]. MDA forms a chromophoric adduct with thiobarbituric acid. Reaction plates were analyzed in a plate reader at 532 nm.

Serum aspartate aminotransferase (AST) was measured by colorimetric analysis (A_{450 nm}) using a commercial kit (Jiancheng Bioengineering Ltd.) in accordance with the manufacturer's instructions. Serum alanine aminotransferase (ALT) level was determined using a commercial assay kit (Jiancheng Bioengineering Ltd.), and the absorbance was measured at 510 nm.

2.7. Skin mucus immune parameters

Lysozyme activity of skin mucus was determined using a

turbidimetric method based on lysis of the lysozyme-sensitive gram-positive bacterium *Micrococcus lysodeikticus* (Sigma) [25]. Skin mucus total immunoglobulin (Ig) levels were measured using the method described by Hoseinifar et al. [26]. The mucus protein level was using commercial kit (Sigma-Aldrich, USA). The alkaline phosphatase (ALP) activity in mucus was measured using a commercial kit (Sigma-Aldrich, USA). The samples were prepared according to the manufacturer's protocol, and the absorbance was measured at 405 nm.

The skin mucosal peroxidase activity was measured according to the method described by Guardiola et al. [27]. One unit was defined as the amount that produced an absorbance change of 1, and the activity was expressed as units mL⁻¹.

2.8. Expression of immune-related genes

At the end of the feeding period, the head-kidney, and intestine were dissected from nine fish per group. Total RNA was extracted from the tissues (kidney and intestine) using TRIZOL reagent (Invitrogen, USA). The RNA concentration and purity were quantified by spectrophotometer, and the quality was checked by 1% agarose gel containing 0.5 µg mL⁻¹ ethidium bromide. Complementary DNA (cDNA) was synthesized using a SuperScript® cDNA synthesis kit (Life Technologies) as per the manufacturer's instructions. Real-time PCR analyses of *ZO-1*, *occludin*, *claudin 3*, *claudin 7*, *IL-10*, *IL-8*, *IL-1β*, *TNF-α*, *HSP70*, *TOR*, *TLR22*, *IFN-γ*, and *β-actin* (housekeeping gene) were performed using a CFX96™ Real-Time PCR system (Bio-Rad, Laboratories, Inc.) following standard protocols with the primers and thermocycling conditions indicated in Table 2. Intestinal samples were used to study the expression of *ZO-1*, *occludin*, *claudin 3*, and *claudin 7*. A melt curve analysis was performed after amplification to verify the accuracy of each amplicon. All samples were run parallel with the housekeeping gene to normalize cDNA loading. Gene expression results were analyzed using the 2^{-ΔΔCT} method after verifying that the primers were amplified with an efficiency of approximately 100% [31]. The data for all treatment groups were compared to those of the control group.

2.9. Challenge study

After eight weeks of experimental feeding, 12 fish from each tank (i.e., 3 × 12 = 36 fish per group) were selected for the challenge test. All fish were intraperitoneally injected with 100 µL of phosphate buffered saline (PBS) containing 1 × 10⁷ live *A. hydrophila* cells [12]. Another group of 10 fish (fed the basal diet) was intraperitoneally injected with 100 µL of PBS to test if there was any adverse effect of PBS on fish. The challenged fish were kept under observation for one month and fed a basal diet. The mortality of the fish in each group was recorded daily over the course of 4 weeks. Relative percent survival (RPS, %) was calculated using the following formula:

$$RPS (\%) = \left[1 - \left(\frac{\text{mortality in treated group} (\%)}{\text{mortality in control group} (\%)} \right) \right] \times 100.$$

2.10. Statistical analysis

The obtained data were analyzed by one-way analysis of variance, and Tukey's test was used to assess the differences between treatments. All statistical analyses were performed using OriginPro software (version 8; OriginLab Corporation, Northampton, MA, USA). A *p*-value of less than 0.05 was considered statistically significant. The results are expressed as mean ± standard error of the mean (mean ± SEM).

3. Results

3.1. Growth performance

The effects of DP on the growth parameters of the fish are presented

Table 2

Real-time primer sequences and thermocycling conditions.

Target gene	Primer sequence (5' to 3')	Thermocycling conditions	Reference/Accession no.
<i>IL-1β</i>	CTCTACCTTGTCTGTACCCAG AGCTGTGCTAATAAACCATCCAG	94 °C 5 min, 45 cycles of 94 °C 15 s, 60 °C 15 s and 72 °C 20 s	[13]
<i>TNF-α</i>	TGGCTTGAAATTAGTGGACAG TAGATGCCGAAGAAATCAGAG	94 °C 5 min, 45 cycles of 94 °C 15 s, 60 °C 15 s and 72 °C 20 s	[13]
<i>IL-10</i>	AACCATTACTGGACGAA CGAACTCAAGGGATT	94 °C 5 min, 45 cycles of 94 °C 15 s, 60 °C 15 s and 72 °C 20 s	[13]
<i>HSP70</i>	GGC AGA AAG TTT GAT GAC CCA GCA ATC TCC TTC ATA TTC ACC	95 °C 30 s, 40 cycles of 95 °C 5 s, 61.1 °C 30 s and 72 °C 30 s	[13]
<i>TLR22</i>	TCAAGGTTTGTCTCTCTTGG TTTGGTTAGCTCGAAATGG	94 °C 1 min, 40 cycles of 94 °C 20 s, 59 °C 20 s and 72 °C 50 s	[13]
<i>TOR</i>	ATCATACGCATCCAGTCCATTG GGTCATTAGCCAGTAGAGTGTC	94 °C 1 min, 40 cycles of 94 °C 20 s, 59 °C 20 s and 72 °C 50 s	[28]
<i>IFN-γ</i>	TGC-ACT-TGT-CAG-TCT-CTG-CT TGT-ACT-TGT-CCC-TCA-GTA-TTT	95 °C 10 min, 40 cycles at 94 °C 15 s, 60 °C 30 s and 72 °C 30 s	AM261214
<i>Occludin</i>	GACGCCATGGATGAGTACAA GTGGTTGAGTTTGGCTTTTCAG	95 °C for 30 s, 40 cycles of 5 s at 95 °C and 34 s at 60 °C	[29]
<i>ZO-1</i>	CCGAAGCTTTGACAGCAAAC GGTTGATCTTCTCCACTGACTC	95 °C for 30 s, 40 cycles of 5 s at 95 °C and 34 s at 60 °C	[29]
<i>Claudin 3</i>	GCACCAACTGTATCGAGGATG GGTTGTAGAAGTCCCAGATGG	95 °C for 30 s, 40 cycles of 5 s at 95 °C and 34 s at 60 °C	[30]
<i>Claudin 7</i>	CTTCTATAACCCCTTCACACCAG ACATGCCTCCACCCATTATG	95 °C for 30 s, 40 cycles of 5 s at 95 °C and 34 s at 60 °C	[30]
<i>β-actin</i>	GACTTCGAGCAGGAGATGG CAAGAAGGATGGCTGGAACA	95 °C 30 s, 40 cycles of 95 °C 5 s, 62.4 °C 30 s and 72 °C 30 s	[13]

in Table 3. The final weight gain was considerably higher ($p < 0.05$) only in the D3 group (71.48 ± 1.57 g) compared to the control after 8 weeks of feeding trial. The WGR was higher ($p < 0.05$) in D2 and D3, with the highest in the D3 ($434.28 \pm 4.9\%$). The DP supplementation increased SGR in the fish groups, but the difference was significant only in the case of D3. FCR values were slightly lower in D2–D4; however, the differences were not significant compared to the control. No mortality was recorded during the feeding trial.

3.2. Hematological parameters

As shown in Table 4, erythrocyte count was significantly higher in the D3 ($1.94 \pm 0.09 \times 10^6 \text{ mm}^{-3}$). Significantly higher WBC counts were recorded only in D3. Dietary DP had no significant effect on the Hb or total protein content. Dietary DP had no significant effects on the Hb and total protein levels; however, both were higher in D3. The hematocrit level was higher in the DP-fed groups, and the difference was significant in the D3 group ($25.81 \pm 0.94\%$) compared to that in the control. The MCV level was higher ($p < 0.05$) in all treatment groups

Table 3Growth performance of *Cyprinus carpio* that were fed a supplementary dandelion polysaccharide (DP) diet for 8 weeks.

Parameters	Control	D1	D2	D3	D4
0–4 weeks					
Initial weight (g)	13.57 $\pm$ 0.68	13.08 $\pm$ 0.82	13.41 $\pm$ 0.66	13.26 $\pm$ 0.61	14.02 $\pm$ 0.81
FWG (g)	29.36 $\pm$ 1.22 ^a	29.86 $\pm$ 0.87 ^{ab}	32.17 $\pm$ 1.33 ^{ab}	32.98 $\pm$ 1.06 ^{ab}	35.77 $\pm$ 1.13 ^b
WGR (%)	115.63 $\pm$ 2.07 ^a	128.13 $\pm$ 1.44 ^{ac}	138.94 $\pm$ 2.46 ^{bc}	147.54 $\pm$ 1.73 ^b	153.02 $\pm$ 2.38 ^b
SGR (% day ⁻¹)	2.75 $\pm$ 0.12 ^a	2.73 $\pm$ 0.09 ^a	3.11 $\pm$ 0.13 ^{ac}	3.24 $\pm$ 0.08 ^{bc}	3.32 $\pm$ 0.11 ^{bc}
Survival rate (%)	100 ^a	100 ^a	100 ^a	100 ^a	100 ^a
0–8 weeks					
Initial weight (g)	13.57 $\pm$ 0.68	13.08 $\pm$ 0.82	13.41 $\pm$ 0.66	13.26 $\pm$ 0.61	14.02 $\pm$ 0.81
FWG (g)	59.86 $\pm$ 1.16 ^a	62.13 $\pm$ 0.79 ^a	67.83 $\pm$ 0.88 ^{ab}	71.48 $\pm$ 1.57 ^b	66.19 $\pm$ 1.01 ^{ab}
WGR (%)	341.17 $\pm$ 4.02 ^{ad}	379.71 $\pm$ 3.98 ^{ab}	402.46 $\pm$ 6.1 ^b	434.28 $\pm$ 4.9 ^c	367.26 $\pm$ 6.04 ^{bd}
SGR (% day ⁻¹)	2.63 $\pm$ 0.08 ^a	2.79 $\pm$ 0.11 ^{ab}	2.905 $\pm$ 0.07 ^{ab}	3.06 $\pm$ 0.12 ^b	2.74 $\pm$ 0.09 ^{ab}
FCR	2.97 $\pm$ 0.11 ^a	3.01 $\pm$ 0.08 ^a	2.86 $\pm$ 0.09 ^a	2.59 $\pm$ 0.13 ^a	2.78 $\pm$ 0.06 ^a
Survival rate (%)	100 ^a	100 ^a	100 ^a	100 ^a	100 ^a

Note: FWG = final weight gain; WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion ratio; values in the same column with different superscripts letters are significantly different ($P < 0.05$); values are presented as mean $\pm$ SEM ($n = 3$).

than in the control group, and the highest MCV level was measured in the D3 group ($137.83 \pm 5.3 \text{ g dL}^{-1}$).

3.3. Serum antioxidant parameters

Administration of DP had a positive effect on the serum antioxidant parameters of the fish (Table 5). Serum SOD active was significantly higher in D2 and D3, with the highest in the D3 group ($18.23 \pm 1.04 \text{ U mg protein}^{-1}$). The CAT activity was significantly higher in the D2–D4 groups, with the strongest activity recorded on D3. MDA activity was lower in the treatment groups, but the differences were only significant in the D3 group than in the control.

Dietary DP significantly lowered serum ALT levels compared to the control. However, the serum AST levels were significantly lower in the D2–D4 groups. The lowest ALT ($77.08 \pm 1.03 \text{ U mL}^{-1}$) and AST ($27.33 \pm 0.91 \text{ U mL}^{-1}$) levels were recorded in D3 (Table 5).

3.4. Skin mucosal immune parameters

Skin mucosal immune parameters are presented in Table 6. The skin mucosal lysozyme activity was significantly higher in the DP-fed groups than in the control group. The highest lysozyme activity was recorded in D3 ($31.04 \pm 1.02 \text{ Unit mL}^{-1}$). Dietary DP had no significant effect on the Ig levels. Supplementation of DP at $1.5\text{--}4.0 \text{ g kg}^{-1}$ increased ($p < 0.05$) the skin mucosal ALP activity in fish, with the highest activity ($122.6 \pm 3.8 \text{ IU/L}$) recorded in D3 (Table 6). Skin mucosal protein levels increased in the DP-fed groups, but the difference was significant only on D3 ($10.6 \pm 0.74 \text{ mg mL}^{-1}$). Peroxidase activity was significantly higher in the D3 and D4 groups than in the control group.

3.5. Effects of DP on gene expression

Expression of *Zo-1* and *claudin 7* in fish intestinal tissues increased significantly in the D1–D4 groups compared to that in the control (Fig. 1A). The expression of *occludin* increased significantly only in the D3 group (1.3 fold higher than that in the control group). Dietary DP had

Table 4Effects of various levels of DP on haematological parameters in *C. carpio*.

Fish group	Erythrocyte ($\times 10^6 \text{ mm}^{-3}$)	WBC ($\times 10^3$)	Hb (g dL ⁻¹)	Haematocrit (%)	MCV (fl) (g dL ⁻¹)	Total protein (mg/mL)
Control	1.58 $\pm$ 0.08 ^a	12.89 $\pm$ 1.26 ^{ac}	6.62 $\pm$ 0.27 ^a	18.36 $\pm$ 0.78 ^a	115.8 $\pm$ 7.46 ^a	3.09 $\pm$ 0.22 ^a
D1	1.51 $\pm$ 0.06 ^a	12.94 $\pm$ 1.18 ^{ab}	6.49 $\pm$ 0.18 ^a	20.03 $\pm$ 1.16 ^a	123.43 $\pm$ 4.16 ^b	3.14 $\pm$ 0.12 ^a
D2	1.72 $\pm$ 0.10 ^{ab}	13.37 $\pm$ 1.48 ^{bc}	6.82 $\pm$ 0.15 ^a	22.26 $\pm$ 0.86 ^{abc}	129.07 $\pm$ 7.1 ^b	3.26 $\pm$ 0.17 ^a
D3	1.94 $\pm$ 0.09 ^b	14.76 $\pm$ 0.87 ^b	7.14 $\pm$ 0.23 ^a	25.81 $\pm$ 0.94 ^b	137.83 $\pm$ 5.3 ^c	3.87 $\pm$ 0.19 ^a
D4	1.69 $\pm$ 0.07 ^{ba}	14.08 $\pm$ 1.06 ^{bc}	6.53 $\pm$ 0.11 ^a	21.98 $\pm$ 1.01 ^{ca}	128.08 $\pm$ 6.14 ^b	3.19 $\pm$ 0.13 ^a

Where, D1 = 0.5 g kg⁻¹ DP group, D2 = 1.5 g kg⁻¹ DP group, D3 = 2.5 g kg⁻¹ DP group, and D4 = 4.0 g kg⁻¹ DP group.Values in the same column with different superscripts letters are significantly different ($p < 0.05$); values are presented as mean $\pm$ SEM ($n = 3$).**Table 5**Serum oxidative stress and antioxidant activities of *C. carpio* after 8 weeks feeding with experimental diets containing various levels of DP.

Fish group	SOD (U mg protein ⁻¹)	CAT (U mg protein ⁻¹)	MDA (nmol mL ⁻¹)	ALT (U mL ⁻¹)	AST (U mL ⁻¹)
Control	13.72 $\pm$ 0.68 ^a	22.06 $\pm$ 1.13 ^a	7.68 $\pm$ 0.81 ^a	88.4 $\pm$ 1.36 ^a	32.76 $\pm$ 0.87 ^a
D1	13.79 $\pm$ 0.36 ^a	21.64 $\pm$ 0.76 ^a	6.97 $\pm$ 0.47 ^{ac}	83.26 $\pm$ 1.91 ^{bd}	33.08 $\pm$ 1.14 ^a
D2	17.44 $\pm$ 0.86 ^{bc}	25.38 $\pm$ 1.22 ^{bd}	6.64 $\pm$ 0.79 ^{ac}	81.91 $\pm$ 0.84 ^{cd}	27.91 $\pm$ 0.67 ^b
D3	18.23 $\pm$ 1.04 ^b	29.14 $\pm$ 1.06 ^c	5.43 $\pm$ 0.36 ^{bc}	77.08 $\pm$ 1.03 ^d	27.33 $\pm$ 0.91 ^b
D4	15.31 $\pm$ 0.79 ^{ac}	26.87 $\pm$ 0.83 ^{cd}	6.29 $\pm$ 0.42 ^{ac}	82.16 $\pm$ 0.91 ^{cd}	30.02 $\pm$ 0.73 ^b

Where, D1 = 0.5 g kg⁻¹ DP group, D2 = 1.5 g kg⁻¹ DP group, D3 = 2.5 g kg⁻¹ DP group, and D4 = 4.0 g kg⁻¹ DP group.Values in the same column with different superscripts letters are significantly different ($p < 0.05$); values are presented as mean $\pm$ SEM ($n = 3$).**Table 6**Skin mucosal immune parameters of *C. carpio* after 8 weeks feeding with experimental diets containing various levels of DP.

Fish group	Lysozyme (Unit mL ⁻¹)	Ig (Unit-mg/mL)	ALP (IU/L)	Protein level (mg/mL)	Peroxidase activity (U mL ⁻¹)
Control	22.18 $\pm$ 0.73 ^a	5.8 $\pm$ 0.29 ^a	87.63 $\pm$ 4.07 ^a	0.741 $\pm$ 0.83 ^a	0.13 $\pm$ 0.001 ^a
D1	25.31 $\pm$ 0.48 ^b	5.3 $\pm$ 0.44 ^a	93.87 $\pm$ 5.61 ^a	0.744 $\pm$ 0.62 ^{ac}	0.15 $\pm$ 0.003 ^{ac}
D2	27.97 $\pm$ 0.93 ^{bd}	6.4 $\pm$ 0.46 ^a	104.1 $\pm$ 5.17 ^b	9.71 $\pm$ 0.68 ^{ac}	0.16 $\pm$ 0.001 ^{ac}
D3	31.04 $\pm$ 1.02 ^c	6.8 $\pm$ 0.32 ^a	122.6 $\pm$ 3.8 ^c	10.6 $\pm$ 0.74 ^{bc}	0.22 $\pm$ 0.002 ^b
D4	26.93 $\pm$ 0.86 ^d	5.9 $\pm$ 0.47 ^a	116.4 $\pm$ 6.73 ^c	8.8 $\pm$ 0.59 ^{ac}	0.23 $\pm$ 0.003 ^{bc}

Where, D1 = 0.5 g kg⁻¹ DP group, D2 = 1.5 g kg⁻¹ DP group, D3 = 2.5 g kg⁻¹ DP group, and D4 = 4.0 g kg⁻¹ DP group.Values in the same column with different superscripts letters are significantly different ($p < 0.05$); values are presented as mean $\pm$ SEM ($n = 3$).no significant effect on *claudin 3* expression (Fig. 1A).

The expression of inflammatory cytokines in the head kidney is shown in Fig. 1B. Dietary DP had no significant effect on *IL-8* expression. TNF- α expression increased in the DP1 – DP4 groups, with the highest expression observed in D3 (1.9 fold higher than the control). IL-1 β levels were higher in the DP group, but the difference was significant only for D3 compared to the control. The expression of the anti-inflammatory cytokine IL-10 decreased significantly only on D3.

The expression of HSP70, *TOR*, *TLR22*, and *IFN- γ* in the head kidney is shown in Fig. 1C. Dietary DP significantly reduced the expression of *HSP70* on D3 compared to that in the control, but a slightly higher expression was recorded on D4. However, DP treatment had no significant effect on *TLR22* expression. The expression of *IFN- γ* increased ($p < 0.05$) by 1.6 and 1.7 folds in D3 and D4, respectively. *TOR* expression

was gradually downregulated ($p < 0.05$) up to D3, except for D1.

3.6. Challenge study

Dietary supplementation with DP increases the resistance of fish to pathogen challenges. The highest post-challenge relative percent survival (RPS) was recorded in D3 (66.67%), followed by D2 (57.58), D4 (51.51%), and D1 (33.32%). No mortality was observed in the negative control group. The highest mortality (91.66%) was recorded in the control group.

4. Discussion

Plant extracts/compounds have been used to promote growth, enhance immunity and appetite, and increase disease resistance in fish. The use of plant extracts has become routine in fish farming. Very few studies have reported the application of dandelion in aquaculture, and there are no reports on the use of dandelion polysaccharides (DP) as feed additives in fish. Dietary dandelion extract (1 g kg⁻¹) increased SGR, WGR, and feed intake of golden pompano during an 8-week feeding trial, but these parameters decreased significantly with further increase in dandelion extract levels [18]. Similarly, significantly higher FWG, WGR, and SGR values were recorded in the D3 group, and a further increase in DP had a negative effect on growth performance. FCR was lower on D3, indicating that fish utilized feed more efficiently on D3. The primary purpose of feed supplements is to achieve rapid growth and the lowest FCR, both of which were achieved in D3. Higher DP did not result in higher growth performance, which is consistent with the results of *C. carpio* [12] or *L. rohita* [11] fed herbal diets. Excess DP might lead to higher energy consumption for metabolism, which results in unfavorable effects (growth and feed utilization) and stress in fish [18]. Tan et al. [18] used dandelion extract as a feed additive which had 50% polysaccharide content, which might be responsible for the enhanced growth performance of D3. Dietary supplementation with DP improves the growth performance of rabbits [32]. In this study, WGR was significantly higher on D2 and D3, but not D4. This is due to the fact that supplementation of the higher amount of DP in the long-term might have a negative impact on fish health.

The hematological profile is an important tool for measuring the physiological and functional status of fish [33]. In the present study, significantly higher WBC, erythrocyte, hematocrit (%), and MCV levels were recorded in D3. WBC count is commonly used to diagnose medical conditions and the host's immune system. An increase in WBC in the normal range may indicate the stimulation of innate immune responses and defense against invading pathogens [34]. Dietary peppermint supplementation (4 g/kg) for 8 weeks increased the hematological indices ($p < 0.05$) in Caspian roach [34]. Similarly, dietary dandelion flower extract (2 or 3 g/kg) supplementation for eight weeks increased hematological parameters in rainbow trout [35]. The exact mechanism by which plant compounds can increase hematological indices is unclear. However, the variation in hematological indices mostly depends on a wide range of nutritional and non-nutritional factors.

Skin mucus secretion is the first line of defense against stressful or diseased conditions in fish. The skin mucus secretes goblet cells which

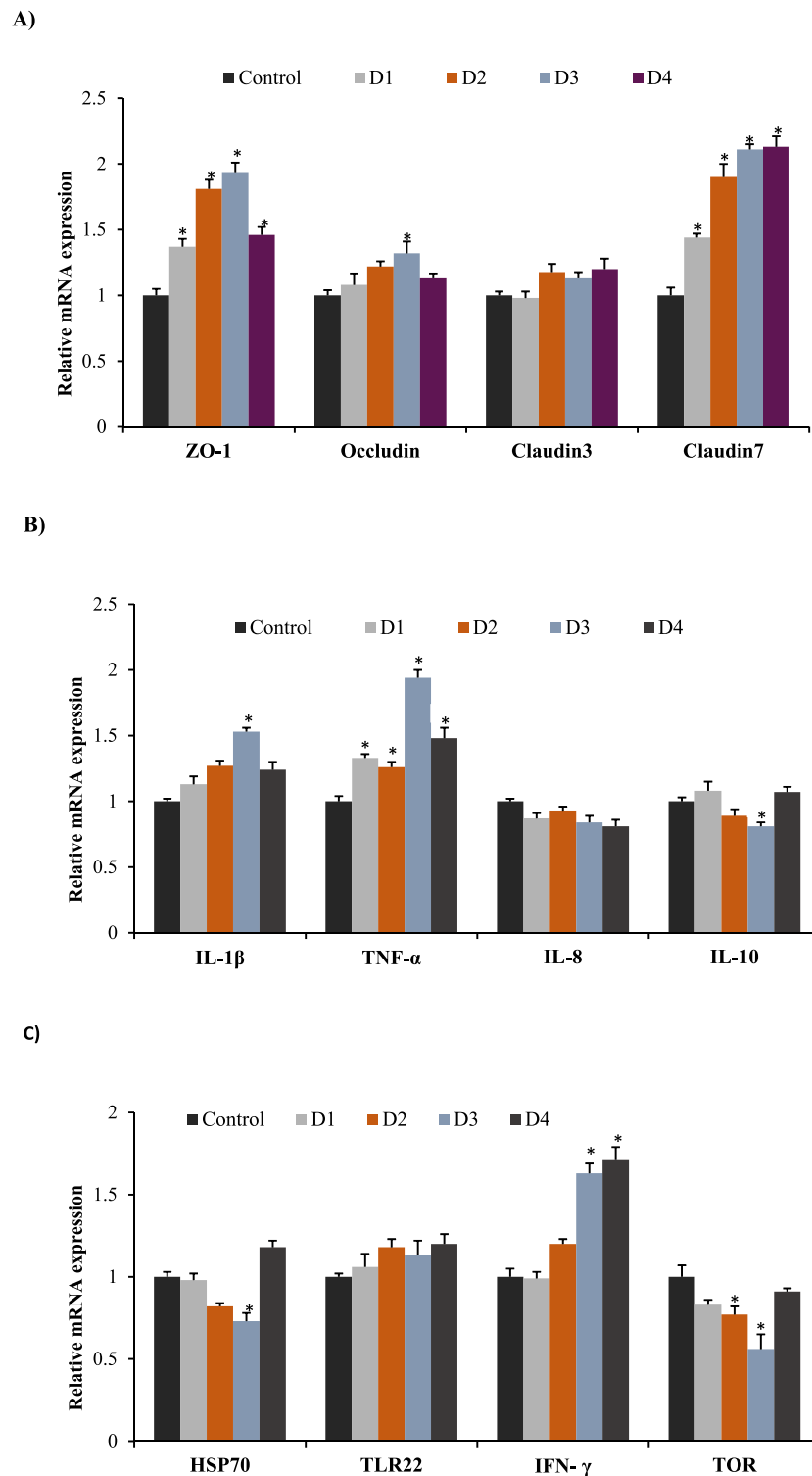


Fig. 1. Expression of cytokine-related genes in tissues of fish fed different concentrations of dandelion polysaccharide supplemented diets. Data are shown as mean $\pm$ SEM ($n = 3$). Column bearing asterisk (*) differs significantly from the control group at $p < 0.05$.

play a vital role as biological, physiological, and immunological barriers against foreign particles, stressors, and pathogens [36,37]. Lysozyme, proteases, immunoglobulins, peroxidases, and phosphatases are important non-specific immune parameters of the skin [36]. The present study revealed that the administration of DP (D3) considerably improved lysozyme, ALP, protein levels, and peroxidase activity in the skin mucus. Due to its bactericidal activity, lysozyme helps neutralize

invading bacteria by hydrolyzing their peptidoglycan layer [38]. The effect of DP on the skin mucus of fish is unknown. Improved skin mucosal immune responses have been reported in various fish species: Nile tilapia fed on diets supplemented with berberine powder [39]; *C. carpio* fed on diets supplemented with orange peels [33], curcumin [12], and date palm fruit extracts [26]; Caspian roach fed on a diet supplemented with peppermint [34]. The increased protein level in the

DP-supplemented groups also indicated a rise in antibody levels in the fish skin [40]. In fish, the predominant antibody type is Ig, which is often referred to as IgM or IgM-like antibodies. The distribution of Ig was not uniform. The Ig level in channel catfish was lowest in the caudal fin and ventral skin but highest in the lateral skin [41]. IgM plays a crucial role as an immunomodulatory molecule in fish blood, and is a vital part of fish skin [42]. DP had no significant effect on Ig levels; however, it was higher in the D3 group. Dietary supplementation with dandelion flower extract (DFE) at 3 g/kg for eight weeks increased skin mucosal lysozyme, IgM, ALP, and total protein levels in rainbow trout [35]. Previous studies revealed that Ig stimulation is a short-term phenomenon attributable to immunostimulants. Furthermore, peroxidase in skin mucus is essential for mucosal immunity and skin defense. They effectively eliminate H_2O_2 and maintain the redox balance of the immune system [43]. The increase in skin mucus peroxidase activity in D3 and D4 was in agreement with earlier studies that reported higher skin peroxidase activities in fish-fed herbal supplemented diets [12,39,43]. The increase in skin mucosal enzymatic parameters may be due to enhanced mucus secretion and immunomodulatory effects of DP on mucosal immune responses in fish [44].

In this study, SOD and CAT activities were markedly higher in the D2 and D3 groups than in the control group but decreased in D4. SOD, catalase, and peroxidases form the first line of defense against free radicals [3]. SOD first catalyzes the reaction between superoxide radicals and produces H_2O_2 and oxygen [45]. CAT converts H_2O_2 to H_2O and molecular oxygen. Since CAT is absent in mitochondria, glutathione peroxidases convert H_2O_2 into H_2O and lipid oxides into water [46]. Similarly, dietary grape seed proanthocyanidin [3] or curcumin [12] increased serum and skin antioxidant parameters (SOD, CAT, and peroxidases) in common carp. *Labeo rohita* fed on diets supplemented with 200 or 300 mg kg^{-1} of grape pomace flour showed considerably increased antioxidant activities [47]. In this study, SOD, CAT, and peroxidase activities were declined at higher doses. A similar trend was noted in liver SOD, CAT, and glutathione peroxidase activities in *C. carpio* fed with GSPE [3]. In view of earlier studies, we assumed that decreased antioxidant activities in higher DP-fed groups indicate the scavenging capability of DP in removing more free radicals from cells. Therefore, DP may have a positive effect on antioxidant enzymes and the scavenging of free radicals. Hence, an appropriate DP dose must be used to achieve optimal antioxidant effects. Furthermore, dietary DP reduced ($p < 0.05$) the serum MDA in *C. carpio*. Similarly, dietary astaxanthin markedly SOD and MDA levels in *Channa argus* where decreased the MDA level [48]. Dietary curcumin decreases serum MDA levels in *C. carpio* [12]. Similarly, dietary dandelion extract at 1 g kg^{-1} increased ($p < 0.05$) hepatic antioxidant enzymes in golden pompano, whereas MDA levels were considerably reduced [18]. Main components of lipid peroxidation. It has strong biological toxicity and can damage cells [49]. A lower MDA content indicates an increase in the endogenous antioxidant defense system in fish fed the DP diet.

Variations in ALT and AST activities have been utilized extensively as biomarkers of health status and stress responses in fish [40]. Elevated levels of AST and ALT often indicate liver necrosis, degeneration, and destruction due to liver cell damage. Golden pompano-fed dandelion extract showed lower plasma ALT and AST activity [18]. In the present study, dietary DP at 2.5 g kg^{-1} (D3) considerably decreased the levels of serum ALT and AST in fish. Similarly, dietary astaxanthin reduced serum ALT and AST levels in sea bass [40]. Furthermore, ALT and AST levels were inversely correlated with the serum protein levels. Lower protein levels were recorded in the control fish, with markedly higher AST and ALT levels. Therefore, AST and ALT levels may play a role in protein metabolism [40].

Cytokines play a pivotal role in immune mechanisms and trigger a cascade of events that lead to immunostimulation, enhancement, or reticence of cytokine-related genes. Cytokines primarily mediate inflammatory responses. Changes in the expression levels of inflammatory cytokine-related genes can reflect the immune status of the fish. Multiple

aspects of the immune response are regulated by *IL-1 β* and *TNF- α* [50]. *TNF- α* is associated with the mobilization and stimulation of phagocytes. In the present study, *IL-1 β* , *TNF- α* , and *IFN- γ* levels were considerably higher in D3. Dietary Radix Rehmanniae Preparata polysaccharide (RRPP) at 0.2% for 60 d upregulated the expression of *IL-1 β* , *TNF- α* , and *IFN- γ* in *Luciobarbus capito* [51]. *IL-1 β* and *TNF- α* expression were higher in *L. rohita* fed grape pomace flour (GPF) supplemented diets for 60 d [47]. Dietary curcumin supplementation for eight weeks increased the expression of *IL-1 β* and *TNF- α* in *C. carpio* [12]. Dietary DP had no significant effect on *IL-8* expression in kidneys. *IL-8* expression is related to the inflammatory response, and it activates phagocytes [52]. Various studies have reported an inverse expression pattern of pro-and anti-inflammatory cytokines in fish following dietary feeding with herbal immunostimulants [12,53]. In the present study, the *IL-10* levels were markedly lower in the D3 group. Dietary jujube fruit extract increased *TNF- α* and *IL-1 β* gene expression and decreased *IL-10* expression in *C. carpio* [53]. Dietary dandelion extract of juvenile golden pompano at 1 or 4.0 g kg^{-1} for eight weeks increased the mRNA levels of *TNF- α* , but decreased *IL-10* and *TOR* mRNA levels in fish intestines [21]. The *TOR* pathway plays an important role in inflammatory responses in fish, and its inhibition could increase the production of pro-inflammatory cytokines and decrease the anti-inflammatory cytokine *IL-10* [54]. In this study, *IL-1 β* and *TNF- α* expression was strongly upregulated on D3, whereas the *TOR* level was lowest on D3. A previous study demonstrated that *TNF- α* mRNA levels were inversely correlated with *TOR* mRNA levels, whereas *IL-10* mRNA levels were positively correlated with *TOR* mRNA levels in the intestine of grass carp [55]. The presence of bioactive compounds in polysaccharides may regulate cytokine gene expression. However, the exact mechanism of inflammatory cytokine regulation should be investigated in future studies.

The intestinal physical barrier is a type of intestinal mucosal barrier that enhances immune responses and protects the fish from pathogen invasion. It is dependent on the integrity of intercellular junctions, which in turn depend on tight junction proteins (TJPs). TJPs, such as ZO-1, occludin, and claudins, are the most apical components of the junctional complex. This provides a form of cell-to-cell adhesion in enterocytes [56]. In the present study, dietary DP positively modulated the expression of ZO-1, occludin, claudin 3, and claudin 7 in the fish intestines. In fish, approximately 63 genes encoding claudins have been reported in 16 teleost species [57]. Upregulation of TJ genes might be an indicator of improved gut health and integrity after eight weeks of feeding with DP. Claudins are the major structural and functional components of TJ proteins that largely determine TJ permeability [58]. Grass carp fed a tryptophan-supplemented diet exhibited higher levels of ZO-1, occludin, and claudin-c mRNA compared to those observed in the control group of fish [59]. Similarly, dietary administration of olive leaf extract for four weeks increased ($p < 0.05$) the mRNA levels of occludin, claudin 3, and claudin 7 in *C. carpio*; however, after eight weeks, only claudin 7 expression was considerably higher [30]. These authors suggested that the mechanism by which short-and long-term influences the expression of TJ needs to be elucidated. Furthermore, dietary DE upregulated ZO-1 and occludin mRNA levels in the intestines of golden pompano [21]. The increased mRNA levels of ZO-1, occludin, claudin 3, and claudin 7 in fish intestines may improve the intestinal mucosal barrier function.

HSPs are expressed for the purpose of stress tolerance and promote cell survival. Monitoring the effect of aquatic environments on fish is a major concern. In this regard, HSP70 could be used as a biomarker for different types of environmental stresses, such as hypoxia, microbial invasion, and starvation [60]. Dietary DP at 2.5 g kg^{-1} (D3) considerably reduced HSP70 mRNA levels in the head-kidneys of fish. Similarly, dietary dandelion extract decreased HSP70 expression levels in golden pompano [21]. However, slightly higher HSP70 expression was recorded in D4, which might be due to the fact that excessive amounts of DP increase stress in fish. The downregulation of HSP70 may be due to enhanced tolerance to stresses during fish culture. In the present study,

DP supplementation had no significant effect on toll-like receptor 22 (*TLR22*) expression in fish. Among various *TLRs*, *TLR22* is unique to fish, can detect infections with dsRNA, and is associated with antiviral protection in fish [61].

The vital goal of immunostimulant supplementation is to boost disease resistance in fish. Bacterial challenge studies are key in evaluating the effectiveness of dietary treatments in terms of protection against pathogen attack and are often regarded as a final indicator of fish health status after immunostimulant supplementation [18]. Many plant polysaccharides are used as immunostimulants to control fish diseases. Dietary supplementation with *Astragalus* polysaccharides (200 mg kg⁻¹) for 8 weeks increased the resistance of *C. catla* against edwardsielliosis [6]. Dietary administration of *C. borvilianum* polysaccharide for eight weeks improved the disease resistance of *L. rohita* against *A. hydrophila* infection [11]. Additionally, dietary RRPP at 0.2% improved the disease resistance of *L. capito* against *A. hydrophila* challenge [51]. Similarly, in the present study, fish fed DP exhibited markedly higher survival rates than those in the control group after challenge with *A. hydrophila*. The highest survival rate of 66.67% was observed in D3 (2.5 g kg⁻¹). Therefore, DP enhanced protection against *A. hydrophila* challenge. The observed result might be due to the DP-mediated enhanced elevation of skin mucosal innate immune parameters, increased serum antioxidant activity, upregulation of pro-inflammatory cytokines, and down-regulation of anti-inflammatory cytokines.

Dietary DP at 2.5 g kg⁻¹ had no marked impact on FCR in fish, but it could increase SGR and WGR. Dietary DP at 2.5 g kg⁻¹ for eight weeks modulated skin mucosal immune parameters, hematological parameters, and serum antioxidant activities. DP attenuated serum oxidative stress, which may be due to its higher antioxidant activities. Moreover, DP increased the expression of pro-inflammatory cytokines and down-regulated the expression of anti-inflammatory cytokines and HSP. Moreover, it reduced fish mortality and enhanced the resistance of fish to *A. hydrophila* challenge. Hence, dandelion polysaccharides (2.5 g kg⁻¹) may be recommended for application in carp. However, further studies are required to elucidate the pharmacokinetic mechanisms and safety issues.

CRedit authorship contribution statement

Sib Sankar Giri: Conceptualization, design, and experiment. **Sang Guen Kim:** Formal analysis, manuscript revision, Data curation. **Kang Jeong Woo:** Formal analysis, manuscript preparation, Validation. **Won Joon Jung:** Formal analysis, manuscript preparation, Validation. **Sung Bin Lee:** Manuscript formatting, revision. **Young Min Lee:** Formal analysis, manuscript preparation, Validation. **Su Jin Jo:** Formal analysis, manuscript preparation, Validation. **Ji Hyung Kim:** Formal analysis, manuscript revision, Data curation. **Se Chang Park:** Supervision, Funding acquisition.

Declaration of competing interest

Authors do not have any competing interest to declare.

Data availability

All data for the study are presented with the article & its associated files

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