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Enrichment of common carp (*Cyprinus carpio*) fingerlings diet with *Psidium guajava*: The effects on cutaneous mucosal and serum immune parameters and immune related genes expression

Seyed Hossein Hoseinifar[a], Abdolvahab Sohrabi[a], Hamed Paknejad[a], Valiollah Jafari[a], Marina Paolucci[b], Hien Van Doan[c,*]

[a] Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[b] Department of Biological, Geological and Environmental Sciences, Via Port'Arsa, 82100 Benevento, Italy
[c] Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand

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ABSTRACT

The present study aimed at evaluation of possible effects of *Psidium guajava* as immunostimulant on mucosal and serum immunity, as well as related genes expression in common carp (*Cyprinus carpio*) fingerlings. In an eight weeks feeding trial, fish were fed with experimental diets containing guava leaf powder [GLP] (0, 0.25, 0.5 and 1%). Then, skin mucus and serum immune parameters (total Ig, alkaline phosphatase activity, lysozyme activity), as well as immune related genes expression (TNF-alpha, IL1b, IL8) were measured. All levels of GLP inclusion in the diet brought about the increase in total Ig in the serum, while in the mucus only the highest level of GLP was effective. No significant differences were observed in mucus alkanine phosphatase activity, while fish fed 0.25% of GLP had noticeably higher serum lysozyme activity. The mucus total protein electrophoretic pattern revealed some differences between the control and the treated fish. Two high MW bands were present in the control group but not in the treated groups. Three bands with a low MW comprised between 35 and 17 mw were noticed in GLP fed carps and not in the control. Gene expression studies revealed no noticeable alterations in TNF-alpha gene between treated fish and control. However, the level of expression of IL-8 was strongly upregulated in fish fed 0.5 and 1% of GLP. Also, feeding on 0.5% of GLP significantly upregulated IL1b gene expression. Evaluation of growth performance revealed improved performance parameters in GLP treated groups. These results confirmed possible immunomodulatory and beneficial effects of GLP on common carp fingerlings.

1. Introduction

Aquaculture is a rapidly expanding economic sector worldwide. The 2016 FAO report estimates that the annual rate increases by an average value of 6.2% [1]. However, aquaculture is an economic activity with a high business risk. In fact, there are many factors that can cause adverse conditions that increase susceptibility to infections of the farmed species and that consequently pose a risk for the global production of aquaculture [2,3]. In particular, the intensive practices, based on fish cultured in confined and controlled areas such as ponds, or off-shore cages, with high reared fish density, negatively affect the health status of cultured fish, and make the control of the outbreak and spreading of potentially lethal diseases difficult [4,5]. Carps and other cyprinids are among the most cultured freshwater fish in the world, and according to the FAO statistics, their global production reached 28.22 million tons in 2014, a trend that is destined to increase [6]. As a consequence, the spread of diseases and infections could cause incalculable damage to this important economic aquaculture sector [7]. Once diseases and infections have occurred, it is very difficult to keep them under control [8]. This is partly due to the fact that there are no specifics antibiotics developed for fish and generally the same antibiotics used for terrestrial animals are employed [9]. Since the wide use of antibiotics causes insidious forms of bacterial resistance [10], the interest of research towards alternative solutions, such as bioactive molecules of plant origin, to be used as feed additives, has enormously increased [11]. Feed additives as health promoters are an important component of any strategy to prevent diseases in aquaculture [12]. Vaccination today is a very useful prophylactic measure for its effectiveness in controlling fish

* Corresponding author.
E-mail address: hienqbuni@gmail.com (H. Van Doan).

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diseases [13]. However, the vaccines are relatively expensive and present a rather narrow spectrum of action, being specific for defined pathogens (Dhar et al., 2014). A promising solution could be represented by the administration of immunostimulant substances as an alternative or simultaneously with the administration of vaccines and chemotherapeutics [14]. The strengthening of the fish defense mechanism by precautionary use of immunostimulants characterized by a broad spectrum activity could prove to be effective in terms of costs and ecocompatibility [15].

Medicinal plants have many beneficial actions such as stimulation of appetite and growth, stimulation of the immune system, antimicrobial and antifungal activities, as well as antistress effect in aquatic species [4,8,16,17]. The presence of many bioactive molecules in medicinal plants may explain the health promoting activity that in attributed to different chemical compounds such as phenolic acids, flavonoids, tannins, alkaloids, terpenoids, saponins, glycosides and others [18,19]. The presence of such bioactive plant components explains the enhancement of immunological parameters in fish fed medicinal herbs [8].

Psidium guajava L., commonly known as the guava tree, is a small tree belonging to the myrtle family (Myrtaceae) distributed through many tropical ans subtropical countries [20]. Well known by the traditional medicine, for the innumerable health promoting activities in humans [21,22], the guava leaf extract possess properties that can be exploited in aquaculture, such as antimicrobial, antioxidant effects. Guava leaf extracts were effective against infectious haematopoietic necrosis virus (IHNV), *Oncorhynchus masou* virus (OM V) and shrimp pathogenic virus, yellow-head virus (YHV), white spot syndrome virus (WSSV), and *Vibrio harveyi*, other than 24 strains of fish pathogenic bacteria *in vitro* [23]. The aqueous extract of guava leaf intraperitoneally injected in the *Cyprinus carpio*, caused the increase in lymphocyte proliferation, nitric oxide production and respiratory burst activity, indicating a general activation of the innate immune response of fish [24]. Oddly enough, the feasibility of guava leaf extracts as feed additives on mocusal immunity of aquatic species has been scarcely investigated. Therefore, this study was carried out to investigate the effects of the guava leaf powder in the diet of common carp fingerling on mucosal and serum immunity and related genes expression in common carp.

2. Materials and methods

2.1. Preparation of Guava leaf powder (GLP) and experimental diets

The fresh guava leaf was supplied from Nikshahr (Sistan & Baloochestan Province, Iran) and transferred to Gorgan University. Upon arrival, the leaf were oven dried at 40 °C for 12 h. Then the leaf were powdered using mill, passed through an 80-mesh sieve and stored in zippack at 4 °C until use [25]. A basal diet was formulated as described elsewhere [26] which served as control diet and experimental diets were prepared by inclusion of different levels (0.25, 0.5, and 1%) of GLP. The constituents were mixed carefully in a mixer and then made into pellets with suitable size by using a meat grinder. The prepared diets were kept in zippack at 4 °C until use.

2.2. Fish culture and feeding trial

The present study was performed at Aquaculture Laboratory of Gorgan University of Agricultural Sciences and Natural Resources (Iran). In this experiment, common carp fingerlings was used as experimental animal. The fish were adapted for 2 weeks and then they (15.88 ± 0.27 g) were stored in 12 fiberglass tanks assigned to four treatments with three replicates at rate of 14 fish per tank. The feeding trial lasted for 8 weeks and during this period, carps were fed three times daily and at rate of 3% of body weight. The amount of feed offered to fish in each experimental unit was corrected based on the data of biometry (every 10 days). The tanks were equipped with an air stone connected to aerator. Also, the tanks were cleaned daily and 50% of water was changed to maintain optimum quality. The physiochemical parameters of water were checked regularly and temperature, pH and dissolved oxygen were maintained at, 23 ± 1.2 °C, 7.6 ± 0.3 and 6.8 ± 0.2, respectively.

2.3. Evaluation of immune parameters

2.3.1. Serum and mucus collection

At the end of feeding trial)8-week) three fish were randomly selected from each tank and after anesthesia with 5 mg/l of clove powder, blood samples were obtained from the caudal vein. Then, the blood sample of each fish was discharged separately into sterile vials and kept at 4 °C for 4 h to clot. Blood serums were obtained by centrifuging samples and the supernatants were transferred to new vials and stored at −80 °C until use.

The skin mucus samples obtained according to the protocole suggested by Subramanian et al. [27]. Briefly, three fish were randomly selected from each tank and after anaesthesia, placed in separate zippacks having 10 ml of 50 mM NaCl (Sigma, Steinheim, Germany) for about 1 min. During this time, gentle rubbing in a downward motion was done. The collected mucus samples were instantly centrifuged (Centrifuge 5810 R; Eppendorf, Engelsdorf, Germany) at 1500 × *g* and 4 °C for 10 min and stored at −80 °C until use.

2.3.2. Skin mucus immune parameters

Skin mucus total Ig: Using the protocole explained by Lowry et al. [28] the total protein of skin mucus was determined. The total protein levels of samples were re-determined using the above mentioned method after the precipitation of immunoglobulins with 12% solution of polyethylene glycol (Sigma) The difference between the protein contents before and after immunoglobulin precipitation was calculated as the total Ig.

2.3.2.1. The skin mucus protein profile. The skin mucus protein profile was determined using SDS-PAGE as described in our previous publication [29]. Briefly, the samples were boiled at 98 °C for 10 min in loading buffer (50 mM TrisHCl pH 6.8, 100 mM β-mercaptaethanol, 2% SDS, 0,1% blue bromophenol, 10% glycerol). The proteins were separated on a 12% SDS-polyacrylamide gel electrophoresis with 4% stacking gel in 1% Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS [pH 8.3]) in a miniprotean cell (Bio-Rad) at 130 V for 2 h. Molecular weight markers (Invitrogen) without reducing agents were employed as standards. After electrophoresis, gels were stained with 0.25% Comassie Brillant Blue R-250 in 50% methanol and 10% acetic acid.

2.3.2.2. Skin mucus alkaline phosphatase activity. The alkaline phosphatase activity was measured by using a commercial kit (Pars Azmoon, Iran) as described elsewhere [30].

2.3.3. Serum immune parameters

Serum total Ig: As described in case of skin mucus, the total Ig levels in serum samples was measured after Siwicki and Anderson [31].

Serum lysozyme activity: The turbidimetric method was used, as we explained elsewhere [32]. Briefly, 50 µL suspension of a lysozyme-sensitive Gram-positive bacterium *Micrococcus luteus* (Sigma) was prepared and added to equal amount of mucus sample. Then, the samples were incubated for 15 min (at 30 °C) in a 96 well plate. The changes in absorbance was monitored and recorded for 50 min. A unit of activity was defined as the amount of enzyme absorbance decreases of 0.001 min^{-1} at 450 nm [33].

Serum alternative complement pathway hemolytic activity (ACH50): The serum ACH50 activity were measured according to the method suggested by Yano [34] as described in our previous publication [32].

The serum volume producing 50% haemolysis (ACH50) was measured and used to calculate the complement activity.

2.4. Evaluation of immune related genes expression

2.4.1. RNA extraction, cDNA synthesis

Intestinal samples were collected from three fish per replicate (i.e 9 per treatment), quickly frozen in liquid nitrogen and then stored at −80 C until further analysis [35]. The total RNA was isolated using BIOZOL RNA extraction kit (Bioflux-Bioer, China) as described in our previous publication [35]. Total RNA was treated with DNase I (Fermentas, Lithuania) to avoid contamination with genomic DNA. The concentration and quality of the obtained RNA were checked by 1.5% Agarose gel running (to verify the integrity of 28S and 18S rRNA), as well as measuring absorbance at 260/280 nm, using a nanodrop spectrophotometer (Nanodrop technology, Wilmington, DE, USA). cDNA was prepared from the extracted RNA using cDNA synthesis kit (Fermentas, Lithuania) according to the manufacturer's protocol.

2.4.2. Primer design and real time PCR

The qPCR primers for selected cytokine gene were designed based on the conserved regions of the common carp Gene Bank sequences by Oligo7 (Table 1). Quantitative real-time PCR (qPCR) assays was performed to study the effects of WBMP on the expression of the cytokines TNF-α, IL-1b, and IL-8 in the intestine of the common carp as described in our previous publication [35]. β-actin gene was used as the housekeeping gene to normalize the expression of the target genes. The iQ5 optical system software (Bio-Rad) and $\Delta\Delta C_t$ method were used for data analysis. qPCR was performed in triplicate.

2.5. Growth performance

All fish were straved for 24 h and biometry was performed and fish weight was measured to calculate growth performance using following formula:

Weight gain = [(W_2 (g) − W_1 (g)]; W_1 is the initial weight, W_2 is the final weight

Specific growth rate (SGR) = 100 × [(ln W_2 − ln W_1) / T]; W_1 is the initial weight, W_2 is the final weight and T is the duration of the feeding trial; Feed conversion ratio (FCR) = feed intake (g) / weight gain (g); Survival = (final number of fish / initial number of fish) × 100

2.6. Statistical analysis

The normality of obtained data was confirmed and then significant difference ($P < 0.05$) among treatments was determined by one-way ANOVA followed by Tukey test. All statistical analysis was done by SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Table 1
Primers sequences and amplification efficiencies.

Gene name	Sequences of primers	Accession no	efficiency
b-Actin	Forward: AGACATCAGGGTGTCATGGTTGGT Reverse: CTCAAACATGATCTGTGTCAT	M24113.1	97%
IL1B	Forward: ACCAGCTGGATTTGTCAGAAG Reverse: ACATACTGAATTGAACTTTG	AB010701.1	98%
IL8	Forward: GTCTTAGAGGACTGGGTGTA Reverse: ACAGTGTGAGCTTGGAGGGA	AB470924.1	98%
TNF-alpha	Forward: GGTGATGGTGTCGAGGAGGAA Reverse: TGGAAAGACACCTGGCTGTA	AJ311800.1	97%

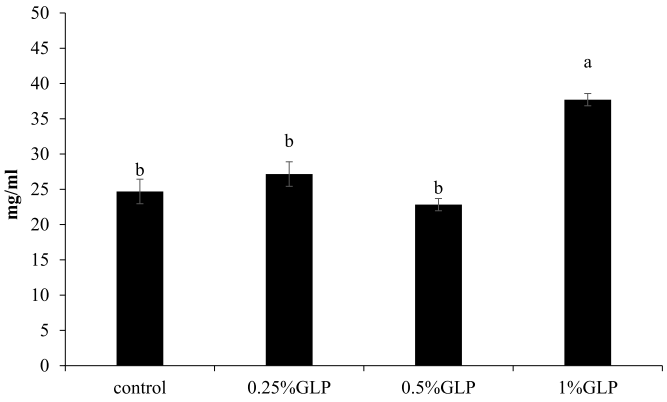

Fig. 1. Skin mucus total Ig levels in common carp (*Cyprinus carpio*) fingerlings fed different dietary guava leaf powder levels. The bars assigned with different letter denote significant difference between treatments ($P < 0.05$).

3. Results

3.1. Skin mucus immune responses

Fig. 1 shows the effects of feeding on GLP supplemented diets on skin mucus total Ig levels. The highest total Ig level was noticed in carps treated with 1%. GLP ($P < 0.05$). No significant differences in Ig levels were detectable in fish fed lower levels of inclusion of GLP. No significant differences were observed in mucus ALP at any level of GLP inclusion when compared with those of fish fed with control diet (Fig. 2).

3.2. Non-specific immune parameters of serum

The serum total Ig levels of serum in fish fed with different experimental diet are presented in Fig. 3. Regardless of inclusion levels, feeding on GLP supplemented diets resulted in significant increase of serum total Ig with respect to the fish fed the basal diet ($P < 0.05$). The serum total Ig increment was dose dependent up to 0.5%, while no significant difference was observed between fish fed 0.5 and 1% of GLP levels of inclusion. Serum lysozyme activity is reported in Fig. 4. Lysozyme activity, significantly increased only in 0.25% GLP treatment compared to the control group ($P < 0.05$). GLP administration changed the SDS-PAGE pattern of the mucus total proteins with respect to the control (Fig. 5). In particular, in fish fed all levels of inclusion of GLP the two bands with mw of about 150 kDa and 70 kDa well evident in the control fish are absent. On the contrary, in fish fed GLP three bands with a mw of about 35, 25 and 20 kDa are present that are

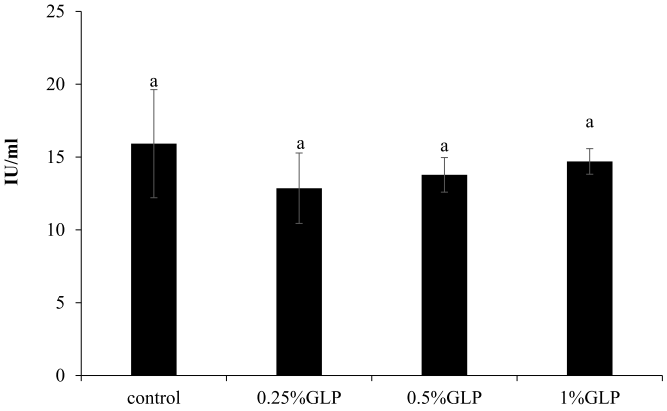

Fig. 2. Mucus ALP of common carp (*Cyprinus carpio*) fingerlings fed different dietary guava leaf powder levels. Bars assigned with same superscripts are not significantly different ($P > 0.05$).

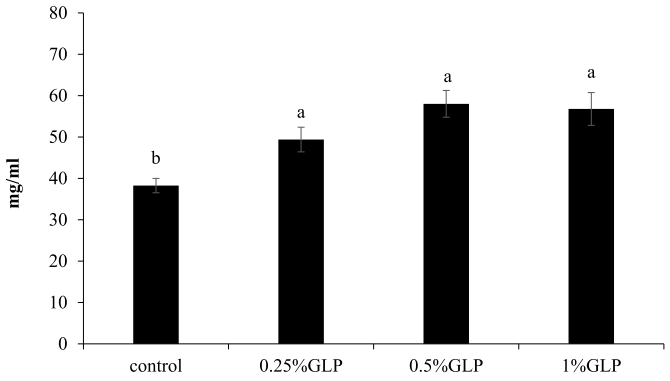

Fig. 3. Serum total Ig of common carp (*Cyprinus carpio*) fingerlings fed different dietary guava leaf powder levels. Bars assigned with different superscripts are significantly different ($P < 0.05$).

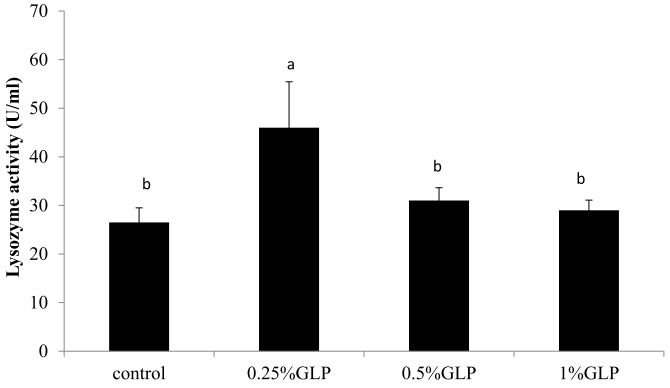

Fig. 4. Serum lysozyme activity of common carp (*Cyprinus carpio*) fingerlings fed different dietary guava leaf powder levels. Bars assigned with different superscripts are significantly different ($P < 0.05$).

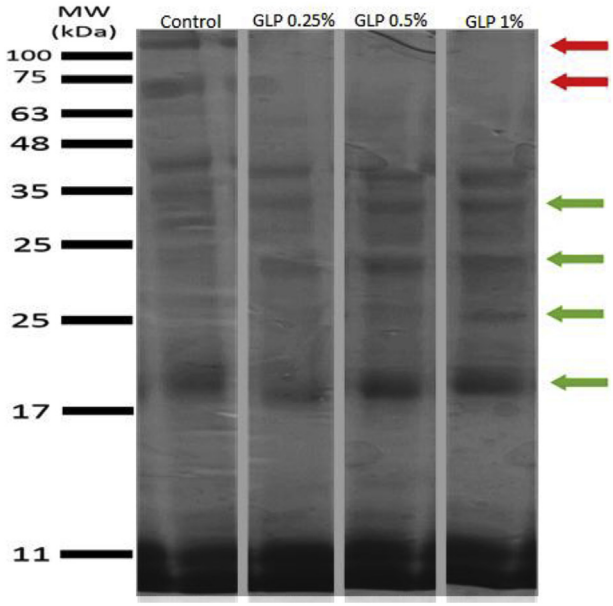

Fig. 5. Evaluation of skin mucus protein profile using SDS-PAGE. Green arrows indicate bands with enhanced intensity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

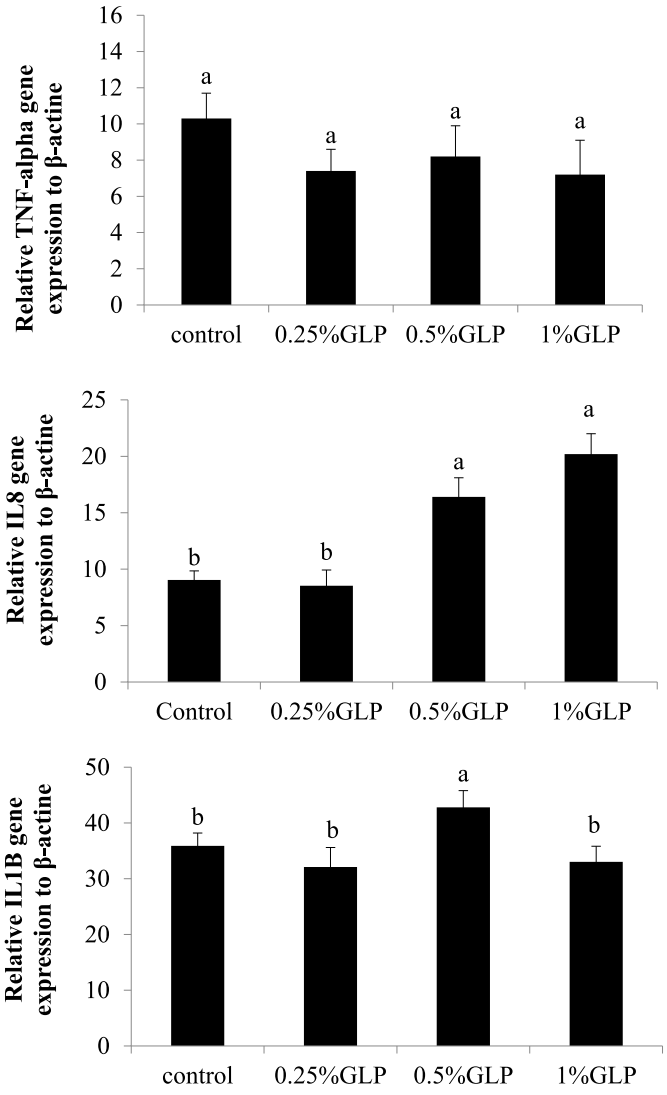

Fig. 6. Intestinal cytokines gene expression including TNF-α (a), IL8 (b), and IL1β (c) in *C. carpio* fingerlings fed different dietary guava leaf powder levels. The bars assigned with different letter denote significant difference between treatments ($P < 0.05$).

instead missing in skin mucus of carps fed the control diet. The intensity of such bands increased according to the level of GLP inclusion in the diet.

3.3. Immune related genes expression in intestine

Fig. 6 presents the effects of inclusion of GLP in common carp diet on the expression of selected immune related genes including TNF-alpha, IL1b and IL8 in the intestine. Evaluation of TNF-alpha gene expression in different treatments revealed no significant alterations when compared with control group ($P > 0.05$). However, the IL-8 gene expression was strongly upregulated in fish fed 0.5 and 1% of GLP ($P < 0.05$). The expression level of IL1b gene in fish fed 0.5% GLP was significantly increased compared other treated groups and control ($P < 0.05$).

3.4. Growth performance

The growth performance parameters are presented in Table 2. While at the beginning of the feeding trial there were no significant difference between treatment, at the end of the trial treatment with GLP resulted

Table 2
The effects of different levels of dietary *Psidium guajava* on growth performance, feed utilisation and survival rate of common carp fingerling.

	Psidium guajava level (%)			
	0.00 (control)	0.25	0.50	1.00
Final weight (g)	23.87 ± 0.10^{c}	26.26 ± 0.26^{a}	24.22 ± 0.21^{b}	25.02 ± 0.13^{b}
WG (g)	13.03 ± 0.12^{c}	15.28 ± 0.18^{a}	13.75 ± 0.28^{b}	14.42 ± 0.40^{b}
SGR (% day^{-1})	1.41 ± 0.01^{c}	1.55 ± 0.02^{a}	1.49 ± 0.03^{b}	1.53 ± 0.04^{b}
FCR	1.53 ± 0.08^{b}	1.39 ± 0.03^{a}	1.51 ± 0.04^{b}	1.46 ± 0.05^{b}
Survival (%)	100	100	100	100

Values are mean ± S.D and in each row with different letters denote significant differences ($P < 0.05$).

in alteration of growth performance. Supplementation of diet with GLP significantly increased weight gain and SGR, regardless of inclusion level *($P < 0.05$)*. Regarding FCR, the lowest rate was observed in fish fed 2.5% GLP which was significantly lower that other treatments ($P < 0.05$). No mortality was observed during the feeding trial, and survival was 100% in all treatment.

4. Discussion

In the present study, we fed carp fingerlings with guava leaf powder (GLP) in an eight-week feeding trial and analyzed the innate immune responses at the skin, blood serum as well as intestinal levels. The active ingredients of plant origin have been widely used for centuries as functional foods, due to their positive effects in fighting infections and stimulating the immune system [17,36]. In recent years, guava leaves have attracted a certain interest in their effectiveness to fight different pathological conditions in humans [19,21]. Evidences on fish are however still scarce. At the best of our knowledge, this is the first time that guava leaf powder is employed in a fish feeding trial aimed at evaluation of possible effects on mucosal immunity and related genes expression.

A variety of elements extracted from plants, have been identified as compounds with potential immune stimulant properties [15,37]. The functional activities of guava leaves are attributed to different classes of compounds, such as essential oils, tannins, alkaloids, phenolic acids, flavonoids, carotenoids and vitamin C [20,22].

Among the most frequently tested immune parameters in diets supplemented with immunostimulants, are immunoglobulins, key elements in the genesis of the immune response [38,39]. Therefore the evaluation of the total immunoglobulins in the skin mucus and in the circulating blood is an important element of evaluation of the immune response. In this study, total Ig increased in both the mucus and serum, although the increase in the mucus occurred only at the highest inclusion level of GLP in the diet. In agreement, previous studies revealed elevation of the total Ig in Indian major carp (*Labeo rohita*) fed root (*Achyranthes aspera*) extract containing diet [40], common carp fry fed Persian hogweed (*Heracleum persicum*) (Hoseinifar et al., 2015), Ferula (*Ferula assafoetida*) [41], loquat (*Eriobotrya japonica*) [42], Labeo rohita (Hamilton) fed *Ocimum sanctum* Linn. (Tulsi) [43]. Elevation of total Ig hint at possible immunomodulatory effects of GLP.

Recently, the fish skin mucus composition has attracted much attention as a biomarker of stress condition and health status [44]. While in the past the studies focused on one or more molecules at a time, in recent years the -omics approach has become an instrument of choice for the contemporary study of many molecules [45]. The proteomic analysis conducted on the Atlantic cod (*Gadus morhua*) reveals that proteins can be grouped into several clusters, many of which are composed of proteins that play role in the mocusal immunity (Rajan et al., 2011). Also, proteomics study of farmed gilthead seabream (*Sparus aurata*) profile skin mucus revealed overall structural and metabolic proteins (Jurado et al., 2015). The studies conducted on Atlantic salmon (*Salmo salar*) also revealed that the composition of the mucus protein pattern may change in relation to infections (Easy and Ross, 2009). The same results, regarding the changes of skin mucus protein profile have been reported following using feed additives such as pro- and prebiotics [29,46]. In our study, we evaluated the protein profile of carp skin mucus by using SDS-PAGE. Despite the limits of this technique, it has been possible to highlight differences in the protein pattern between the control and the GLP enriched diet fed carp fingerlings. In treated carps, high mw bands present in the control were absent, while more low mw bands appeared and were more evident in the group fed the highest GLP concentration. Further studies will be needed to identify the nature of these proteins.

It is now generally accepted that lysozyme play an important role in the innate immune defence [47]. A widely distributed enzyme, it can breaks b-1,4 glycosidic acids and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls [48]. The lysozyme acts either directly or indirectly in conjunction with complement on the cell wall of Gram positive bacteria and Gram negative bacteria [47]. Lysozyme is a widely distributed enzyme at the different surfaces of skin, gills, intestinal tract and serum. It has been widely reported that herbal extracts can enhance lysozyme activity [8]. Dietary administration of Chinese herbal extracts increased lysozyme activity in crucian carp (*Carassius carassius*) [49] and common carp [50,51]. Also, similar results was obtained on common carp fingerlings fed Persian hogweed (*Heracleum persicum*) (Hoseinifar et al., 2015), Ferula (*Ferula assafoetida*) [41], loquat (*Eriobotrya japonica*) [42].

In this study significant increase of serum lysozyme activity was detected only in the group fed with GLP at the lowest level of inclusion. This outcome may be explained hypothesizing that the lack of increased activity of lysozyme in the experimental groups fed with higher concentrations of GLP, may be due to the time-dependant increase, and therefore it escaped observation. In fact, in Nile tilapia (*Oreochromis niloticus*) the increase in lysozyme is massive at 14 and 21 days, while it returns to the initial levels after 28 days [52]. In common carp fed with herbal immunostimulant diets, the lysozyme activity decreased after 45 days of treatment [53].

In our study, we examined the expression levels of two proinflammatory cytokines TNF-α and IL-1β and the chemokine IL-8. We did not report statistically significant changes in TNF-α and IL-1β expression, although in the latter an increase was observed in the experimental group of carp fingerlings fed with GLP at 0.5% level of inclusion. TNF-α is a proinflammatory cytokine, and is expressed at an early stage of infection in fish [54]. It plays a key role in the regulation of inflammation, as well as the cytokine IL-1β, whose functions widely overlap with those of TNF-α [55,56]. The two cytokines are also linked, since IL-1β administered directly into the carp intestine induces severe intestinal inflammation and TNF-α expression [57]. The absence of upregulation of the two cytokines reported in this study, seems to indicate that following the administration of guava leaves, no inflammatory phenomena occurred in the digestive system. The use of natural substances and/or the use of plant extracts containing high concentrations of bioactive molecules, to protect the intestine from inflammation or infection, is increasingly widespread and has proved to be effective even in cases of serious diseases such as inflammatory bowel disease [58,59]. However, although the use of plant products is

considered safe by most consumers and sector operators, it is necessary to support this assumption with experimental evidence.

In our study, the expression of IL-1 β shows an increase in carp fingerlings fed with 0.5% GLP in the diet. It is difficult to explain why only the concentration of 0.5% has been active in increasing the expression of IL-1 β. Additional studies are needed to discover the mode of action of GLP on the expression of IL-1 β. It is also strange that the increase in IL-1 β does not parallel that of TNF- α. Although speculative, we may hypothesize a role for IL-1 β beyond that closely linked to immune defense. Indeed, in addition to its role in immune regulation, IL-1 β has been reported to be involved in the regulation of other physiological processes. Indeed, recent studies show that the metabolism of fish muscle is influenced by IL-1 β. Primary trout muscle cells incubated with IL-1 β showed greater expression of genes related to growth and metabolism [60].

In this study, the expression of IL-8, a chemokine with potent chemotropic activity for neutrophils, monocytes, basophils, resting T cells, and stimulated eosinophils [56], is induced in a dose dependent manner. In agreement with our finding, in an study with common carp fed Ferula, Safari et al. [41] reported elevation of IL-8 expression. Similar results were noticed in case of Astragalus polysaccharides [61] and Jujube (*Ziziphus jujube*) fruit extract [62], loquat (*Eriobotrya japonica*) [42] administration in common carp diet. The main function of IL-8 is the recruitment of monocytes and neutrophils, the cells of the acute inflammatory response [63]. The inflammatory cells follow the increase in the concentration of chemokines and head towards their source. The increase in IL-8 could be considered as an induction by GLP that prepares for a possible immune defense. In fact, although chemokines were initially discovered to be involved in the recruitment of leukocytes during early inflammation, they are now known to regulate various passages of the immune response and to direct cell migration during growth and development [64]. On the other hand, however, we must consider that the IL-8 gene is an inflammatory gene typical of the acute phase and its expression is sensitive to oxidative stress [65], thus we cannot rule out that the extracts of GLP may cause a condition of intestinal stress with increased chemokines. Again, if this condition is the consequence of an ongoing inflammatory process or the implementation of a physiological condition that improves the response of the tissue/organ to a possible establishment of a pathological condition cannot be affirmed.

The present results showed that feeding on GLP improved growth performance parameters and the best results were obtained when fish were treated with 0.25% GLP. Improving wight gain and decreasing FCR is of high importance for commercial aquaculture due to he high costs of feeding. In accordance with our finding, Giri et al. [66] reported that GLP increased growth performance of Labeo rohita. However, they obtained best results in 0.5% GLP treatment. Also, the same growth enhancing effects were reported for GLP when administered in black tiger shrimp (*Penaeus monodon*) [67]. However, the exact mode of action of GLP on growth enhancement and improving feed utilisation remained unclear and merit further research.

The results of this study confirm the potential of guava leaf powder as stimulatory agent of the immune system as well as growth enhancer. Nevertheless, further studies are needed to correctly identify the time and dose to be used to obtain a better immune response and general improvement in health in carp fingerlings.

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