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Synergies between vaccination and dietary arginine and glutamine supplementation improve the immune response of channel catfish against *Edwardsiella ictaluri*

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ABSTRACT

Channel catfish was used to investigate the enhancement of vaccine efficacy following dietary supplementation with arginine (ARG, 4% of diet), glutamine (GLN, 2% of diet), or a combination of both. After vaccination against *Edwardsiella ictaluri*, humoral and cellular immune responses, along with lymphoid organ responses were evaluated. *E. ictaluri*-specific antibody titers in plasma were higher ($P < 0.05$) in fish fed the supplemented diets compared to those fed the basal diet as early as 7 d post-vaccination (dpv). B-cell proportion in head-kidney was higher ($P < 0.05$) at 14 dpv in vaccinated fish fed the GLN diet. The responsiveness of spleen and head-kidney lymphocytes against *E. ictaluri* was enhanced ($P < 0.05$) by dietary supplementation of ARG or GLN at 14 dpv. Additionally, at 7 dpv, vaccinated fish fed the GLN diet had higher ($P < 0.05$) head kidney weights relative to the other dietary treatments, and vaccinated fish fed ARG-supplemented diets had higher ($P < 0.05$) protein content in this tissue. Results from this study suggest that dietary supplementation of ARG and GLN may improve specific cellular and humoral mechanisms, enhancing the acquired immunity in vaccinated channel catfish.

1. Introduction

Disease prevention is extremely relevant to all aquaculture ventures for profitability and sustainability. Impaired disease resistance will have a strong negative impact on growth and survival of fish, and potentially result in large economic losses [1]. Although vaccination has been proven as an effective prophylactic strategy for disease prevention, there is still a crucial need to increase vaccine efficacy for cultured fish [2,3].

Enteric septicemia of catfish (ESC) is caused by *Edwardsiella ictaluri*, a gram negative, rod-shaped, motile bacterium of the family Enterobacteriacae [4], which is capable of intra- and extracellular replication [5]. This disease is the most prevalent and economically devastating disease in farmed-raised channel catfish, costing the catfish industry $40–60 million annually in the United States [3]. A live-attenuated vaccine has been developed against ESC and made into the only commercially–licensed option for ESC control (AQUAVAC-ESC®).

Nutritional modulation of vaccine efficacy is an attractive strategy to enhance and extend protection against disease. Although nutritional factors are recognized as having major consequences on the immune response of fish [1], and great efforts have been expended in this area of research, the role of nutrition on the immune competence of fish is complex and still not totally elucidated. Generally such research has been conducted by feeding fish for a specific time period and then evaluating immune parameters or survival after a disease challenge [6]. However, few studies have combined immunization with nutrient supplementation [7,8]. Under this scenario, the amino acids glutamine (GLN) and arginine (ARG) may prove fundamental as they have been demonstrated not only to promote growth, but also to have an array of desirable immunological attributes in different animal species [9].

Adequate amino acid availability plays a key role in the performance of the immune system while combating invading pathogens.

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For example, GLN improves the secretion of important cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6 and IFN-γ [9–11], and plasma levels of complement proteins C3 and C4 [12]. In addition, GLN improves the phagocytosis and killing capacity of neutrophils and macrophages [13], and enhances the expression of major histocompatibility complex class-II molecules [11]. Also, lymphocyte proliferation is improved in the presence of this amino acid, and it plays an important role in plasma cell differentiation and immunoglobulin synthesis [9,11,14].

Similarly, ARG plays a role in several immunological functions. As the unique precursor for nitric oxide (NO), ARG has a key role in both the innate and adaptive immune systems. High concentrations of ARG has been shown to increase macrophage and natural killer cell cytotoxicity, synthesis of IL-2, and CD3 expression in T cells [9,15]. In addition, ARG increases phagocytosis and killing capacity of macrophages and neutrophils [14,15], modulates lymphocyte subsets and positively affects their adhesion molecules, chemotaxis and proliferation [14–16]. Dietary ARG supplementation has been shown to increase cell mediated immunity and antibody titers in poultry after vaccination [17–19]. In channel catfish, ARG-supplemented diets increased production of NO by macrophages, as well as resistance to experimental *E. ictaluri* infection [20,21].

Therefore we hypothesized that dietary ARG and GLN intervention in channel catfish may enhance vaccine effectiveness against ESC. The objective of the present experiments was to evaluate the immune performance of juvenile channel catfish fed supplemental levels of ARG, GLN and their combination after vaccination against *E. ictaluri* using a live-attenuated vaccine.

2. Materials and methods

2.1. Experimental diets

A basal diet was formulated to contain 32% crude protein from casein, gelatin and a crystalline L-amino acid premix. Dextrin was provided at 15.9% and lipids from corn and menhaden oil at 8%, on a dry-matter basis, for an estimated available energy level of 12 kJ g^{-1} (Table 1). This diet met minimum dietary requirements of channel catfish [22]. Based on better performance obtained in two separate experimental trials with each of these amino acids (not published), three experimental diets were formulated to provide either ARG at 4%, GLN at 2% or ARG + GLN (4 and 2%, respectively) by supplementing L-ARG (US Biochemical Corp., Cleveland, OH) and or GLN (US Biochemical Corp.) to the basal diet while maintaining them isonitrogenous by adjusting the levels of a 50:50 glycine-aspartate premix [23]. All diets were prepared as previously described [24] and maintained at −20 °C until used.

2.2. Feeding trial

A general overview of the experimental trial is presented in Fig. 1. Four hundred and eighty disease-free and *E. ictaluri* naïve juvenile channel catfish, with an average weight of 18.8 ± 0.6 g were placed into 24, 110-L aquaria, at a density of 20 fish per aquarium. Before stocking fish into the experimental system, they were surveyed for antibody titers and PCR detection of *E. ictaluri* [25], with no positive results. The aquaria were arranged as a recirculating system equipped with a sand filter for mechanical filtration and biofilter for ammonia removal. A constant flow of 1 L · min^{-1} was maintained in all aquaria. Dissolved oxygen and water temperature were maintained close to 90% of air saturation and 27 ± 1 °C, respectively. A 12:12 h light:dark cycle was provided through fluorescent lights regulated with a timer. Water quality was monitored weekly for pH, hardness, alkalinity, nitrite, ammonia, temperature and dissolved oxygen and kept within acceptable levels known to channel catfish culture. Fish were acclimated to the experimental conditions for a period of 2 weeks and fed the basal diet. Thereafter, each dietary treatment was randomly assigned to six aquaria, and fish were fed the experimental or basal diets for a period of 4 weeks. Feeding rate was set at a level approaching satiation (4% of biomass) and provided in two daily feedings (a.m. and p.m.). Fish were weighed once a week and the feed ration was adjusted accordingly. Procedures used in this study were approved by the Texas A&M University System Animal Care and Use Committee (AUP # 2007-188).

Table 1
Formulation and proximate composition of the basal diet.

Ingredient	g 100 g^{-1} dry weight
Casein[a]	16.2
Gelatin[a]	3.7
Amino acid premix[a,b]	7.1
Dextrin[a]	15.9
Celufil[a]	29.3
Corn oil[c]	4.0
Menhaden oil[c]	4.0
Vitamin premix[d]	3.0
Mineral premix[e]	4.0
$Ca(PO_4)$[f]	1.0
Carboxymethyl cellulose[a]	2.2
Aspartate:Glycine premix[a]	8.2
L-ARG[a]	0.2
L-GLN[a]	0.0
Analyzed proximate composition (% dry weight)	
Dry matter	90.2
Crude protein	33.5
Crude lipid	7.9
Ash	3.8

[a] U.S. Biochemical Corp., Cleveland, OH, USA.
[b] Buentello and Gatlin [23]. Consisted of (% of diet): L-histidine, 0.14; L-isoleucine, 0.19; L-leucine, 0.06; L-lysine, 0.64; DL-methionine, 0.32; L-phenylalanine, 0.42; L-serine, 1.57; L-threonine, 0.13; L- tryptophan, 0.02; L-valine, 0.11; L-proline, 1.57; L-alanine, 1.57.
[c] Omega Protein, Reedville,VA, USA.
[d] Moon and Gatlin [24].
[e] Same as Moon and Gatlin [24] but prepared by MP Biomedicals, Solon, OH, USA.
[f] Fisher Scientific, Waltham, MA, USA.

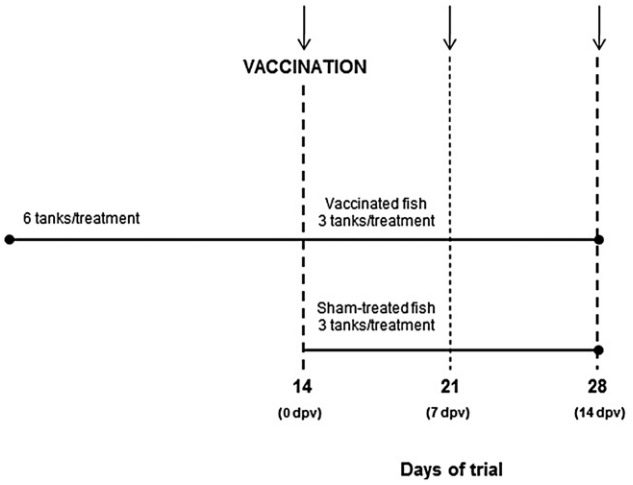

Fig. 1. Experimental design overview. The trial lasted 28 d and was composed of pre-vaccine (14 d), and post-vaccine (14 d) periods. At 14 d of the trial three tanks (17 fish/tank) per treatment were vaccinated with AQUAVAC-ESC®, three more tanks (17 fish/tank) were sham-treated with water. Arrows (↓) represent sampling points (n = 9/treatment). dpv, days post-vaccination.

2.3. Vaccination

After 2 weeks of the feeding trial, fish from three aquaria from each treatment were vaccinated against *E. ictaluri* using the commercial vaccine Aquavac-ESC® (Merck Animal Health, Summit, NJ). Vaccination protocol followed the manufacturer's instructions. Briefly, one vaccine vial was dissolved with water to reach a dosage sufficient to immunize 1 kg of fish biomass. Fish from three aquaria per treatment were removed and placed in previously cleaned plastic containers with an appropriate amount of water and oxygen supply, and exposed to the vaccine solution for 30 min. After vaccination, fish were returned to their appropriate aquarium. Fish in the remaining aquaria were sham-treated by placing them in a container with tank water and no vaccine. The feeding trial continued for 2 additional weeks.

2.4. Sample collection

Samples from nine randomly selected fish per treatment (three per aquarium) were taken at 0, 7 and 14 d post-vaccination (dpv). Prior to all sampling, fish were euthanized with tricaine methanesulphonate (Western Chemical Inc., Ferndale, WA, USA, 300 mg L^{-1}). Whole-body, spleen and head-kidney weight were recorded for six fish. Blood samples (~1 mL) were obtained from the caudal vasculature with heparinized needles (1-mL syringe, 23-ga needle). Heparinized blood was used for peripheral blood lymphocyte (PBL) isolation ($n = 3$), and plasma ($n = 6$) obtained by centrifuging whole blood at 3800 × *g* for 10 min. Intestinal mucus samples ($n = 6$) were obtained by placing both sides of a 1-cm^2 filter paper (No 2., Whatman International Ltd., Kent, ME, UK) in contact with the enteric mucosa for 1 min each side. Protein was eluted by shaking filter papers in 1 mL of 7.2 pH phosphate buffer solution (PBS, Sigma, St. Louis, MO, USA) for 2 h [26]. Bile samples ($n = 6$) were collected using non-heparinized needles, and centrifuged at 2000 × *g* for 10 min [27]. Spleen and head kidney ($n = 3$) were placed in sterile Hank's buffered salt solution (HBSS, Sigma) and used for lymphocyte isolation. In addition, six more spleen and head kidney ($n = 6$) were frozen and kept at −80 °C for protein analysis. Finally, plasma, intestinal mucus and bile samples were kept at −20 °C for specific antibody determination.

2.5. Lymphoid organs weight and protein content

To determine the responses of lymphoid organs after vaccination against ESC, relative spleen weight (spleen weight * 100/whole-body weight) and relative head-kidney weight (head-kidney weight * 100/whole-body weight) were computed. In addition, the protein content (%) was evaluated in spleen and head-kidney tissues, following established methodology [28].

2.6. Anti-E. ictaluri antibody response

To evaluate specific humoral responses against *E. ictaluri*, specific antibody titers in plasma, intestinal mucus and bile were measured by enzyme linked immunosorbent assay (ELISA). Plates were prepared as described by Waterstrat et al. [29], and stored at room temperature until used.

Based on previous titration assays of positive and negative samples (data not shown), PBS was used to dilute (v/v) plasma 1:160, and bile 1:20. Intestinal mucus was not diluted. In order to detect specific antibodies against *E. ictaluri*, 50 μL of sample were dispensed into duplicate wells of the ELISA plate. After 1 h incubation, the plate was washed three times with PBS. Mouse anti-channel catfish IgM was used as a primary antibody, adding 50 μL (1:10 dilution) to each well; repeating incubation and washing steps. The secondary antibody consisted of peroxidase-conjugated sheep anti-mouse IgG (Sigma), adding 50 μL (1:1000 dilution) to each well, incubated and washed as above. Substrate solution (1 tetramethylbenzidine tablet + 1 mL dimethyl sulfoxide + 9 mL citric acid buffer + 2 mL H_2O_2, Sigma) was added (100 μL) to each well. Reaction was stopped after 5 min with 50 μL of 1M H_2SO_4 (VWR, Radnor, PA, USA). An ELISA plate reader (iMark™, Biorad, Hercules, CA, USA) was used to read the absorbance at 450 nm. Inter-plate differences were prevented by standardizing raw values to a positive control value.

2.7. Lymphocyte isolation from blood and tissues

To study cellular immune responses, lymphocytes were isolated from peripheral blood as described by Miller et al. [30], and from spleen and head-kidney following the procedures of Secombes [31], with modifications. Briefly, heparinized blood was diluted 1:2 with PBS. Each tissue was mechanically disaggregated using a glass tissue homogenizer and filtered through a 100 μm nylon mesh. Then cell suspensions were layered over Lymphoprep™ (Axis-Shield PoC AS) and centrifuged at 350 × *g* for 20 min. Lymphocytes were recovered from the interface. Cells were washed three times with PBS at 600 × *g* for 10 min, and red blood cell lysing buffer (0.15 M NH_4Cl + 0.01 M $KHCO_3$ + 0.0003 M ethylenediaminetetraacetic acid, Sigma) was used to lyse remnant erythrocytes. Cells were re-suspended in 1 mL ice-cold PBS and enumerated using a hemocytometer and viability was assessed by trypan blue (Sigma) staining. Viability was >95% in all cases. Lymphocytes for flow cytometry were kept in cold PBS; whereas, cells intended for responsiveness assays were transferred to complete channel catfish medium [CCM, L-15 (Sigma) + AIM-V (Invitrogen™, Carlsbad, CA, USA) + water (cell culture grade, Hyclone® Laboratories Inc., Logan, UT, USA), 45:45:10 v/v; 50 units/0.05 mg mL^{-1} of penicillin/streptomycin (Sigma); 0.02 mg mL^{-1} gentamicin (Sigma); 0.05 mM of 2-mercaptoethanol (Sigma), 0.09% $NaHCO_3$ (Sigma); 5% v/v pooled heat-inactivated channel catfish serum].

2.8. Flow cytometry for B lymphocytes

To evaluate modulation of lymphocyte populations, the proportion of B lymphocytes (IgM^+ cells) was analyzed. Isolated lymphocytes were transferred (5×10^5) to a flow cytometry tube (VWR) and washed once with 1 mL of PBS. Supernatant was decanted and the tube blotted on paper towel. Cells were re-suspended in the remaining PBS. Mouse anti-channel catfish IgM (1:10 dilution) was added (10 μL) and incubated in ice for 1 h. Cells were then washed twice with 2 mL of PBS. After the second wash, supernatant was decanted and tube blotted on paper towel, and the cells re-suspended in remaining PBS. Secondary antibody (FITC conjugated goat anti-mouse IgG, Sigma, 1:100 dilution) was added (5 μL) and incubated for 1 h in ice. After washing the cells twice they were re-suspended in 300 μL of PBS. Stained cells were counted in a cell-coulter (FACSCalibur, Becton–Dickinson, San Jose, CA, USA). Results are presented as the mean percentage of IgM^+ cells per 10,000 events.

2.9. Lymphocyte responsiveness against E. ictaluri

In order to evaluate formation of memory cells, proliferation of lymphocytes upon exposure to *E. ictaluri* was analyzed. The concentration of isolated lymphocyte was adjusted to a concentration of 2.77×10^6 cell · mL^{-1} with CCM. Lymphocytes were seeded into nine wells by adding 180 μL/well of a sterile round bottom 96-well microplate (Nunc, Roskilde, Denmark). Formalin-killed *E. ictaluri* (bacteria cultured with 1% formalin for 24 h) was added (20 μL) to three wells to achieve 1×10^5 bacteria/well. Three more

wells received CCM to serve as control (non-stimulated) cells. Finally the last set of triplicate wells with cells received Concanavalin-A (MP Biomedicals, Solon, OH, USA) to serve as a positive control for the assay. The cells were cultured at 27 °C in a humidified environment with a 5% CO_2 atmosphere for 72 h and then pulsed with 0.5 μCi of ^{3}H-thymidine (MP Biomedicals) for 18 h before harvesting. Incorporation of the radionucleotide was measured with a liquid scintillation β−counter (Wallac MicroBeta TriLux, Perkin Elmer, Waltham, MA, USA). Results are presented as stimulation index (SI = counts per min stimulated cells/counts per min control cells). The positive control cells validated the assay as proliferation was detected (data not-shown).

2.10. Statistical analysis

Data was subjected to two-way ANOVA with diet and vaccine as main effects and the interaction of both. The Statistical Analysis System (SAS, 9.2 v) software was used for all analyses. A $P \leq 0.05$ was taken to indicate statistical significance among treatment means, which were separated using Duncan's multiple-range test.

3. Results

3.1. Lymphoid organ responses

The lymphoid-organosomatic indices and protein content are presented in Table 2. The relative size of lymphoid organs was affected by vaccination only at 14 dpv. However, at 7 dpv, vaccinated fish fed the GLN diet had significantly higher head-kidney size than any other treatment. Protein content in lymphoid organs was more readily affected in head kidney, although a diet effect, regardless of vaccination status, was observed in spleen at 7 dpv. At this time also, among vaccinated fish, those fed the ARG-supplemented diets had significantly higher tissue protein in the head kidney than those fed the basal or the GLN diet. At 14 dpv, although no factor interaction was observed, protein content in this tissue was significantly higher in vaccinated fish than non-vaccinated, and in fish fed amino acid-supplemented diets compared to those fed the basal.

3.2. Antibodies titers against E. ictaluri

Low absorbance was detected in plasma [optical density (O.D.) = 0.22 avg.], bile (O.D. = 0.20 avg.) and intestinal mucus (O.D. = 0.08 avg.) before vaccination, with no significant differences among treatments. These values were interpreted as negative for specific antibodies against *E. ictaluri*, and remained similar in non-vaccinated fish throughout the experiment. Vaccination significantly increased antibody titers in all three fluids analyzed (Fig. 2). Among vaccinated fish, those fed the ARG + GLN diet had significantly higher plasma titers at 7 dpv, whereas, at 14 dpv, titers were significantly higher in all fish fed supplemented diets (Fig. 2).

3.3. Lymphocyte population

No significant changes were observed in IgM^+ cells from peripheral blood (Fig. 3) as influenced by dietary treatment. In contrast, dietary GLN supplementation significantly increased the proportion of IgM^+ cells (1.5-fold from basal levels) in the head-kidney of vaccinated fish at 14 dpv. Arginine supplementation, although not significantly different from the basal fish, produced marked differences in vaccination status within the same diet group (Fig. 3). The latter case was similar in spleen for the GLN and ARG treatments (Fig. 3). In contrast, vaccinated fish fed the ARG + GLN diet had significantly decreased proportion of these cells at both sample periods in the spleen (Fig. 3).

3.4. Lymphocyte response to E. ictaluri

The proliferation capacity of lymphocytes, prompted by the presence of formalin-killed *E. ictaluri*, was significantly increased by vaccination as indicated by higher SI in immunized fish of all dietary treatments. In PBL, this was the only difference found at both 7 and 14 dpv (Fig. 4). In contrast, at 14 dpv, proliferation of spleen- and head-kidney-derived lymphocytes was affected by both diet and vaccination. Vaccinated fish fed the ARG or GLN diets had significantly higher SI than fish fed the basal or the ARG + GLN diets (Fig. 4).

4. Discussion

The objective of the present study was not to test the efficacy of the commercial vaccine used, which has been tested elsewhere [3], but to test whether dietary ARG and/or GLN supplementation enhanced immune responses of channel catfish upon *E. ictaluri* vaccination. The data generated supports the expected outcome. As evaluated in the classical way − measurement of antibody titers [2] − results from the present experiment indicate an improvement

Table 2
Post-vaccination lymphoid organs relative weight and protein content of channel catfish fed supplemented levels of GLN, ARG and GLN + ARG.

Factor		Relative weight (% of whole body)				Protein (% of tissue)			
		Spleen		Head kidney		Spleen		Head kidney	
Diet	Vaccine	7 dpv	14 dpv	7 dpv	14 dpv	7 dpv	14 dpv	7 dpv	14 dpv
Basal	Yes	0.06	0.08	0.65	0.84	11.2	15.8	13.9	13.3
	No	0.06	0.08	0.76	0.58	10.7	16.4	11.8	13.1
GLN	Yes	0.08	0.13	1.01	0.98	14.7	16.4	13.7	14.3
	No	0.06	0.08	0.71	0.78	14.9	15.8	13.6	13.8
ARG	Yes	0.05	0.11	0.66	0.90	15.4	17.3	14.3	14.5
	No	0.06	0.09	0.65	0.69	15.1	15.3	13.5	13.5
GLN + ARG	Yes	0.07	0.07	0.56	0.79	15.3	17.2	14.7	14.8
	No	0.07	0.08	0.90	0.88	14.9	15.1	13.8	13.6
Pooled std. error		0.01	0.02	0.13	0.14	1.1	0.8	0.3	0.3
Pr > F									
Diet		0.064	0.135	0.199	0.284	0.006	0.388	0.002	0.014
Vaccine		0.580	0.017	0.715	0.029	0.614	0.407	0.001	0.030
Diet × vaccine		0.290	0.332	0.022	0.256	0.968	0.427	0.034	0.388

Values represent the mean of six randomly sampled fish.
Significance probability associated with the F-statistic.

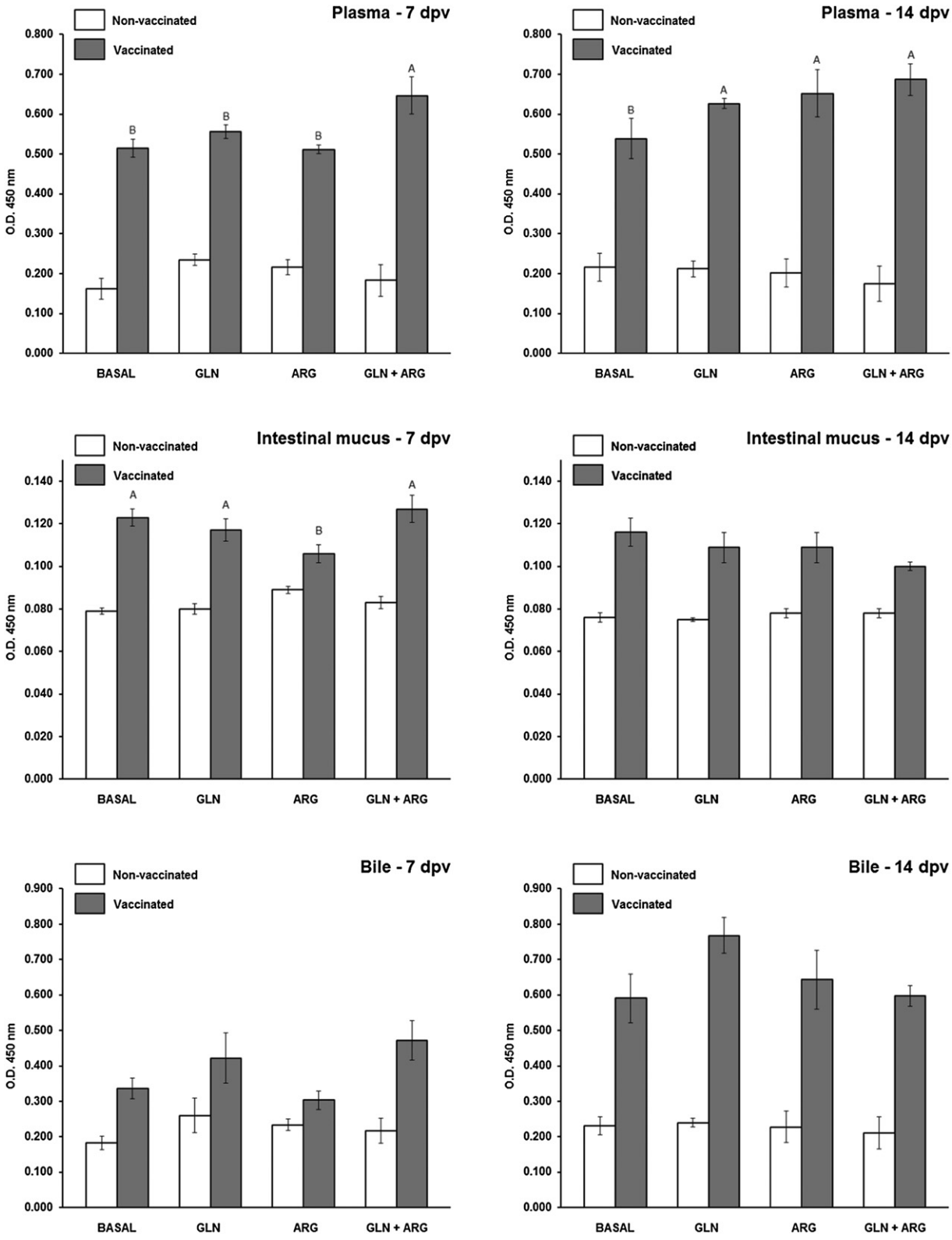

Fig. 2. Antibody titers after *E. ictaluri* vaccination in all fluids evaluated. Antibodies titers were estimated from absorbance obtained through indirect ELISA. Data represent the mean O.D. at 450 nm of six randomly sampled fish (±standard error). Titers were significantly different ($P < 0.05$) between vaccinated and non-vaccinated groups throughout the pv period. A,B: Significant difference ($P < 0.05$) among groups with *diet* × *vaccination* interaction.

in the catfish humoral response upon vaccination. Increases in antibody titers were observed in all examined body fluids of immunized fish. Moreover, dietary treatment appeared to have a fluid-specific effect. Differences in sample processing prevented comparison of titer levels among fluids but, titer tendencies in each fluid appeared to be representative of both diet and/or vaccine effects. In line with the current results are those reports for common carp [32], where plasma antibody titers were more responsive to

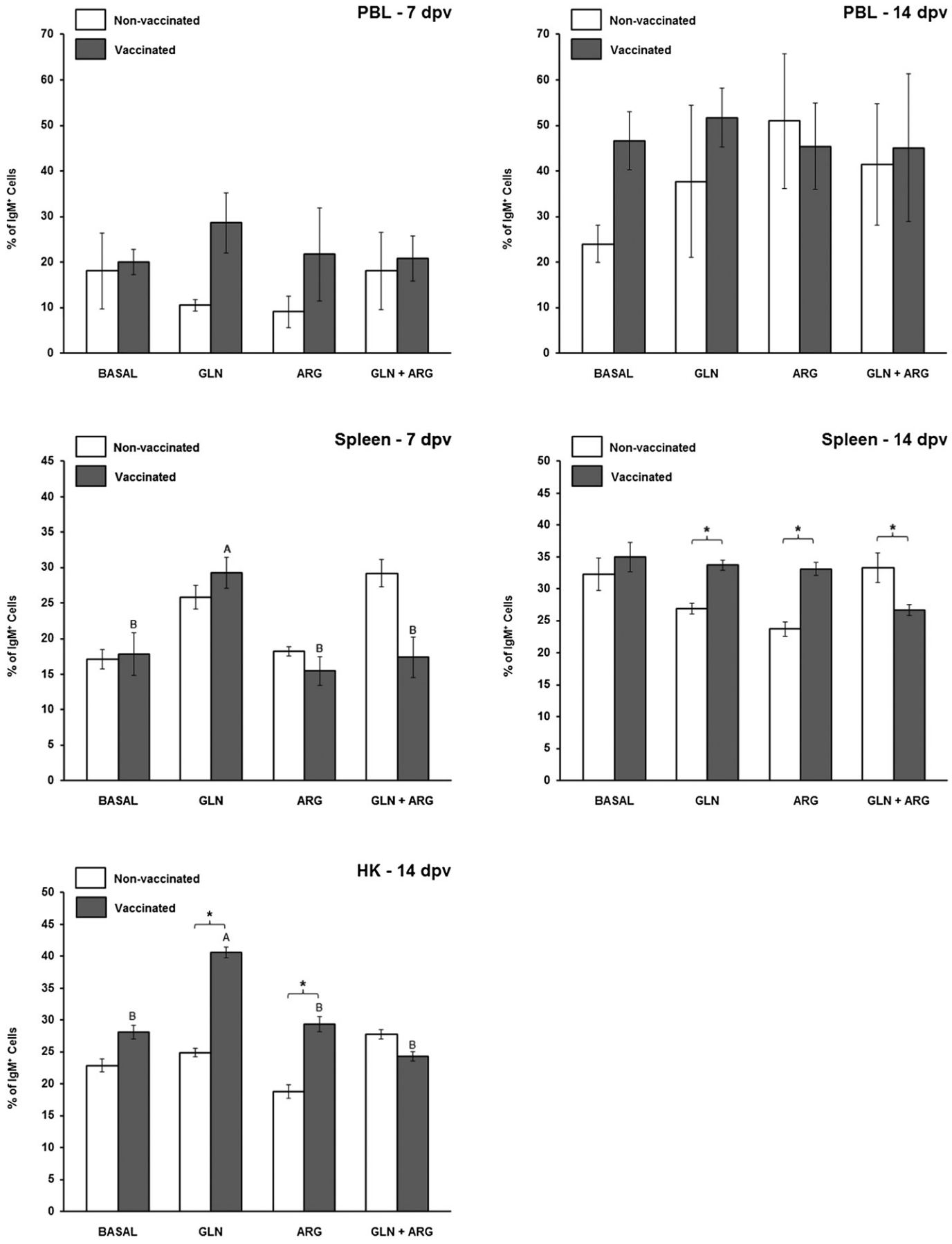

Fig. 3. IgM$^+$ cells proportion in different tissues after *E. ictaluri* vaccination. Data represent the mean % cells of three randomly sampled fish (±standard error). A,B: significance difference ($P < 0.05$) among groups with *diet* × *vaccination* interaction. *: significance difference ($P < 0.05$) between vaccinated and non-vaccinated fish. PBL, peripheral blood lymphocytes; HK, head-kidney lymphocytes.

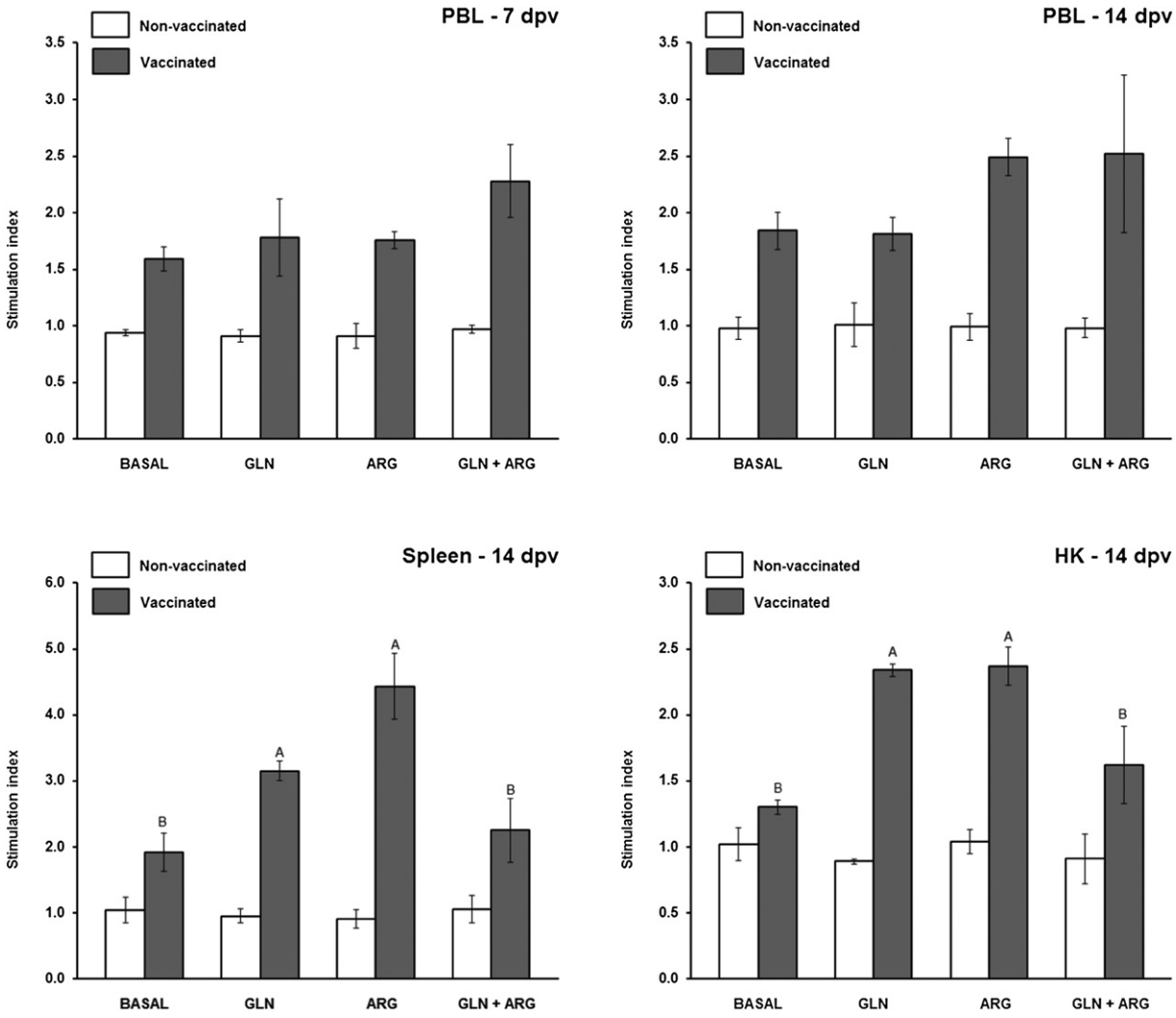

Fig. 4. Lymphocyte, from specific tissues, responsiveness to formalin-killed *E. ictaluri* after vaccination with the same pathogen. Data represent the mean SI of three randomly sampled fish (±standard error). Stimulation index was significantly different ($P < 0.05$) between vaccinated and non-vaccinated groups throughout the pv period. A,B: Significant difference ($P < 0.05$) among groups with *diet* × *vaccination* interaction. PBL, peripheral blood lymphocytes; HK, head-kidney lymphocytes.

diet and vaccination than intestinal mucus or bile. Interestingly, plasma titers observed at 7 and 14 dpv suggest that high levels of ARG and GLN in the diet accelerate antibody production. Reports in higher vertebrates have demonstrated similar results. Broiler chickens fed supplemental levels up to three times the ARG dietary requirement had highest serum-specific antibody titers when vaccinated and challenged against hydropericardium syndrome virus [33] and infectious bursal disease virus [17–19,34], or when inoculated with sheep red blood cells [19]. Likewise, the production of specific antibodies against *Pseudomonas aeruginosa* significantly increased in mice fed supplemental levels of ARG or GLN [10,35]. Also in agreement with results observed herein for GLN supplementation, Bartell and Batal [36] found an increase in non-specific IgG and IgA in serum, intestine and bile of broiler chickens fed diets supplement with this amino acid. A positive specific-antibody response to *E. ictaluri* does not correlate well with protection against ESC unless very high titers are achieved [37]. Therefore, based on the current results, supplementing dietary ARG and GLN to fish appears to be a valid management tool to increase the correlates of protection after vaccination against this disease.

To further elucidate the effects of ARG and GLN on immune responses of channel catfish upon vaccination, cellular responses were analyzed. The lack of modulation of circulating IgM^+ cells observed in the current experiment contrasts with the findings of Abdukalykova et al. [16], where feeding high levels of ARG increased the proportion of circulating B and T cells in vaccinated fish. On the other hand, what appeared to be the positive modulation of IgM^+ cells in the head kidney and spleen observed in vaccinated fish fed the GLN or ARG diets agrees with the reported results of high yields of B and T lymphocytes from Peyer's patches in mice fed GLN and treated with LPS [38], as well as with the murine need of ARG for proper B lymphocyte maturation and lymphoid organ development and function [39]. Nonetheless, the opposite findings in spleen IgM^+ cell population from fish fed ARG + GLN observed in this experiment cannot be explained with the current data. In addition, the biological implications of the B lymphocyte sorting results presented herein are limited, mainly because we did not assess the T lymphocytes population. Future utilization of different lymphocyte surface antigens for cell identification and sorting should provide a clearer picture of the modulatory effects of ARG and GLN on this type of cells, which seemed to have a direct impact of channel catfish survival to *E. ictaluri* infection [40]. However, to date, the lack of specific antibodies against these cell markers is a limiting factor for the study of lymphocyte subsets in most teleosts.

Memory cells are the most unique and advantageous characteristic of the adaptive immune system. Confronting lymphocytes to a known antigen will trigger proliferation of those cells with specific receptors to that antigen. Lymphocyte responsiveness against whole formalin-killed *E. ictaluri* was positively modulated by vaccination, however, dietary ARG or GLN supplementation enhanced vaccination efficacy only in lymphocytes residing in spleen and head kidney. That is consistent with the reported effects of these two amino acids on IL-2 signaling [9], and confirmed, *in vivo,* previous *in vitro* findings of an increased non-specific lymphocyte proliferation rate when media were supplemented with ARG and GLN [14]. Also, in line with these results, Tayade et al. [34] found higher proliferation rates of tissue-residing lymphocytes when chicks were fed a diet supplemented with ARG. To our knowledge, there are no reports relating dietary GLN supplementation, vaccination and lymphocytes responsiveness. However, supplementation of dietary GLN did enhance non-specific mitogen proliferation of spleen lymphocytes in mice [41] and swine [42]. Contrasting to the results obtained with circulating lymphocytes, Tayade et al. [17] and Munir et al. [33] reported higher antigen-specific and non-specific PBL proliferation, respectively, in vaccinated chicks fed supplemental ARG in the diet. The finding of an increased number of memory cells in vaccinated fish fed ARG or GLN is noteworthy as the protective immunity in catfish against *E. ictaluri* is largely influenced by a cell-mediated response [14,43]. In addition, the latter together with the current findings of improved antibody titers, and previously reported modulation of phagocytosis and bacterial killing by this amino acids [14], should result in augmented chances of pathogen clearance and host survival [40,44].

Moreover, reports exist for positive association between lymphoid organ size and immune response or disease resistance in teleost fish [45]. The lymphoid-organosomatic indices presented herein (Table 2) are in line with the reported key immune roles of the head kidney and spleen in the channel catfish response to *E. ictaluri* infection [44], and other fish species to other pathogens. For instance, Ronsholdt & McLean [46], and Harun et al. [47], found an increased spleen index after inoculating Atlantic salmon with *Aeromonas salmonicida* and rainbow trout exposed to *Yersinia ruckeri*. Similarly, Tayade et al. [17] and Ruiz-Feria & Abdukalykova [19] found augmentation in spleen and other immune organ weights between vaccinated and non-vaccinated chicks, but they did not find differences among vaccinated chicks fed a control or ARG-supplemented diet.

Interestingly, the finding of lymphoid organs of a significantly larger mass may be due to the increased cellularity, which can be associated with the multiplication of B-cell as well as the increased populations of memory cells found in the present study in vaccinated fish fed supplemented diets. This effect may be also related to the reported roles of these amino acids as metabolic fuels or proliferative compounds for cells of the immune system [11]. On the other hand, the protein metabolism in lymphoid organs is presumably affected during activated states such as *E. ictaluri* infection. This aspect was recently documented by Pridgeon et al. [43] who documented numerous important up-regulated genes in head kidney of channel catfish vaccinated with Aquavac-ESC®. From these genes 21% were related to lymphoid organ metabolism whereas 28% were related to the immune response. It is noteworthy that both areas of up-regulation encompass protein metabolism, thus, the increased protein content of the head kidney may indicate an improved capacity of this organ to respond to the artificial immunization [43,47].

Results from the present experiment highlight the important roles of ARG and GLN on the immune response of channel catfish immunized against *E. ictaluri*. However, special consideration must be taken in what seems a counter-productive or non-synergistic effect observed when the diet was supplemented with ARG + GLN (e.g., Fig. 4). A similar pattern was observed in previous *in vitro* studies with these two amino acids and channel catfish immune cells [14]. The reasons for such effects appear to be related to the compartmentalization of some biochemical reactions of these two amino acids at the organ or subcellular levels. Thus, the effects of combining ARG and GLN may vary from synergy to inhibition or may be neutral, according to the target organ and to the pathophysiological conditions of the animal [48,49].

5. Conclusion

Dietary supplementation with ARG or GLN had beneficial immunological effects on channel catfish vaccinated against ESC. Although the evidence from this study is promising as related to vaccinology and immunonutrition fields for aquatic species, more research is needed before recommending supplementing aquafeeds with elevated levels of both ARG and GLN. This is mainly because of what appeared to be an antagonist effect of feeding high levels of these amino acids at the same time on specific immune responses.

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