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L-glutamine *in vitro* supplementation enhances Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) leukocyte function

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ABSTRACT

Under appropriate conditions, glutamine (Gln) is an essential nutrient for immunological responses, acting as a metabolic substrate for proliferation of enterocytes and lymphocytes, and having positive effects on the function of stimulated immune cells. Thus, specific components of both innate and adaptive immune systems of Nile tilapia were evaluated after supplementing Gln to cell culture media. Primary cell cultures of kidney leukocytes were used for respiratory burst and phagocytic activity assessment. The ability of macrophages to kill *Streptococcus iniae* also was evaluated. Additionally, a proliferation assay was conducted with peripheral blood lymphocytes (PBL) exposed to non-specific mitogens. Results showed that macrophage phagocytosis, anion superoxide production, and bactericidal capacity were significantly ($P < 0.05$) enhanced by Gln supplementation to the culture media. The proliferation of lymphocytes upon mitogenic exposure also was significantly ($P < 0.05$) enhanced by Gln supplementation to the media. Our results suggest that *in vitro*, different levels of Gln were necessary for optimal immunological responses of leukocytes and lymphocytes. As such, Gln supplementation was able to enhance and modulate both innate and adaptive responses of Nile tilapia leukocytes, highlighting its potential application as an immunonutrient.

1. Introduction

Aquaculture has witnessed a significant growth throughout the past few decades. Tilapia culture is following the same trend, being the second largest farmed finfish group in the world, which in 2015 represented 5.4% of global aquaculture production [1]. The rapid industrialization of tilapia production is in part attributed to scientific and technological advancements, as well as changes in the farming practices so that extensive and semi-intensive systems have been substituted by intensive systems [2]. As a result, fish are commonly subjected to marginal environmental conditions, high densities and regular handling practices, thus increasing the likelihood of disease and other adverse health effects. Infectious diseases can be considered a significant bottleneck to aquaculture's continuous expansion, causing severe economic losses in commercial fish farming worldwide [3].

Addressing aquatic animal health issues has become an urgent requirement for sustaining the growth of aquaculture [4]. In this regard, essential research efforts have been made and must continue to prevent, control and mitigate the deleterious effects of infectious diseases. It is necessary to study and understand the fish immune system including components of both the specific and nonspecific immune responses, such as macrophages. These phagocytic cells belong to the first line of defense against invading organisms and act as a connecting bridge between the innate and adaptive immune system, by presenting the degraded components of pathogens as antigens to lymphocytes [5,6].

The activity of macrophages plays a vital role as part of the innate immune system through phagocytosis and consequently killing pathogens [7]. The functional capacities of those different cell types and humoral factors involved in fish defense have been investigated using a variety of *in vitro* assays. Such methods have been essential tools for

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understanding the complexity of the defense mechanisms, allowing for the assessment of potential effects of specific nutrients on the immune competence of fish [8]. However, information regarding how specific amino acids may modulate the functions of the fish immune system is still lacking [5].

These compounds have a central role in the defense mechanisms because they are not only the building blocks of immune-related proteins, but also participate in the control of key immune regulatory pathways. In animal nutrition, arginine (Arg), cysteine (Cys), and glutamine (Gln) have received considerable attention, though other amino acids such as alanine (Ala), Glycine (Gly), methionine (Met), proline (Pro), serine (Ser), threonine (Thr) and tryptophan (Trp) are also involved in sustaining immunocompetence and disease resistance [9]. These amino acids and their products can regulate cytokine production (Arg, Gln); participate in the activation of T lymphocytes, B lymphocytes, natural killer cells and macrophages (Gln); modulate cellular redox state and antioxidant status (Arg, Cys, Gly, Met, Pro, Ser, Trp), gene expression (Arg, Gln, Met, Pro) and lymphocyte proliferation (Ala, Gln, Pro, Ser, Thr). Amino acids are also precursors for the biosynthesis of molecules involved in protein and DNA synthesis (Arg, Gln, Met, Pro), as well as the production of antibodies, chemokines, and other cytotoxic and signaling substances (Arg, Cys, Gln) [10].

The effects of specific amino acids on the immune system of terrestrial species have been widely investigated, with proven immunomodulatory impact of Gln [11,12]. As a major energy substrate for cells of the immune system [13], Gln plays an important role in their function and homeostasis, participating in lymphocyte proliferation [14], enhancement of phagocytic cell activity and bacterial killing [15–17], immunoglobulin synthesis [16], cytokine production [10] and T-cell responses [18]. In fish, the role of this amino acid in leukocyte metabolism is limited, and, contrarily to mammalians, it seems to be species-specific [5].

A few studies have been undertaken to evaluate the effects of dietary Gln, either individually or combined with other amino acids, on the non-specific defense response of various fish species. Cheng et al. [19] reported that dietary Gln supplementation improved neutrophil oxidative radical production (NBT), superoxide anion production, and lysozyme activity in the kidney macrophages of red drum (*Sciaenops ocellatus*). Similar results were found in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) after an 8-week feeding trial [20]. Moreover, dietary Gln supplementation has been shown to increase lysozyme activity and serum levels of complement proteins C3 and C4 in juvenile hybrid sturgeon, *Acipenser schrenckii* × *Huso dauricus* [21], young mirror carp, *Cyprinus carpio* L [22]. and Jian carp, *Cyprinus carpio* var. Jian [23]. Assessing the effects of Gln *in vitro*, Buentello et al. [24] demonstrated its essentiality as a metabolic fuel for channel catfish (*Ictalurus punctatus*) immune cells. In this study, culture media supplementation with Gln enhanced nitric oxide (NO) production by activated channel catfish macrophages. As to proliferation of cultured lymphocytes, there are conflicting data. Rosenberg-Wiser and Avtalion [25] reported that lymphocyte proliferation of carp is Gln-dependent. More recent data have shown that proliferation of channel catfish peripheral blood lymphocytes to non-specific mitogens was positively modulated when Gln was supplemented to the culture media. A comparable pattern also was observed for B and T cell proliferation [5]. However, different results were found by Ganassin et al. [26] with rainbow trout (*Oncorhynchus mykyss*) and by McBride and Keast [27] with snapper (*Pagrus auratus*), showing Gln-independent responses.

Considering the limited literature and the contrasting results obtained on the effects of Gln supplementation to the culture media of fish immune cells, we conducted a series of *in vitro* studies evaluating the effect of Gln on Nile tilapia leukocyte function. For such, we investigated its influence on phagocytosis and bactericidal capacity of activated macrophages and the lymphocyte proliferative response upon non-specific mitogenic stimulation. In addition, the amino acid consumption in the culture media was also assessed after bactericidal and proliferation assays in order to better understand the immunomodulatory capacity of Gln in both branches of the Nile tilapia immune system.

2. Material and methods

2.1. Fish

Healthy Nile tilapia with an average weight of 539 ± 41 g (mean ± SE) were held in an 1100-L round fiberglass tank as part of a recirculation system (flow of 1.1 L min^{-1}) provided with a standard mechanical and biological filtration system. Fish were fed a commercial omnivorous fish diet with 32% crude protein and 5% crude fat, and sampled as needed. Before drawing blood, fish were anesthetized with tricaine methane sulfonate (MS-222, 100 mg L^{-1}, Tricaine-S, Western Chemical Inc., Ferndale, WA, USA) according to Popovic et al. [28]. Blood of six fish was drawn from the caudal vasculature with a heparinized syringe and used for peripheral blood lymphocyte (PBL) proliferation assays. Six more fish were euthanized with an overdose of anesthetic (MS-222, 250 mg L^{-1}) and head and trunk kidneys were aseptically excised and placed in cold incomplete culture media (sterile, described below) and pooled into a composite sample for the isolation of leukocytes.

2.2. Culture media

The culture media was prepared according to Pohlenz et al. [5]. Complete culture medium (CCM) consisted of Gln-free Leibowitz cell culture (L-15, cat #L5520, Sigma Aldrich) media, 50 units mL^{-1} of penicillin and 0.05 mg mL^{-1} of streptomycin plus 5% (cat #P0781, Sigma Aldrich) of bovine calf serum (BCS) (cat # 12133C, Sigma Aldrich). The control medium consisted of CCM without Gln supplementation. Based on previous studies with catfish macrophages [24,29], CCM was supplemented with L-glutamine (cat #G5763, Sigma Aldrich) at 0.5, 1.0, 1.5, 2.0 or 2.5 mM mL^{-1}.

2.3. Leukocyte primary culture

Leukocytes were isolated from head and trunk-kidney as previously reported [30], with slight modifications. Briefly, head and trunk-kidney tissues were stored in L-15 2% and homogenized with a glass Potter-Elvehjem tissue grinder. The homogenized tissues were filtered through a 100-µm sterile nylon mesh, and the resulting cell suspension was centrifuged and washed with cold, sterile phosphate buffer saline (PBS, cat #P4417, Sigma Aldrich). The isolated cells were layered on a Percoll (cat #P1644, Sigma Aldrich) gradient (51% v/v) and centrifuged at 400 × *g* for 30 min. The cell layer at the interface was collected and washed two times with ice-cold phosphate buffer saline at 200 × *g* for 10 min. Leukocytes were enumerated using a hemocytometer, and viability assessed by Trypan blue staining and only used when cell survivability was higher than 95%. The cell suspension was adjusted to 2×10^7 cells mL^{-1} in L-15 with 0.1% FCS and 100 µl of leukocyte suspension was added per well in a sterile flat bottom 96-well microplate. The microplates were incubated at 27 °C for 2 h to allow cell attachment. The primary culture media was then removed and substituted with Gln-supplemented media and incubated overnight for further use during respiratory burst and bactericidal assays. For the phagocytosis assay, five hundred microliters of the leukocyte suspension were added to microtubes and the aforementioned protocols for media exchange and incubation period were applied.

2.4. Respiratory burst

Respiratory burst activity of leukocytes was measured by extracellular and intracellular superoxide anion production as described by Secombes [30]. Briefly, 100 µl of cytochrome *c* solution (cat #C2506,

Sigma Aldrich) (1.5 mg mL^{-1}, in phenol red-free PBS) containing phorbol 12-myristate 13-acetate (PMA, cat #P8139, Sigma, 1 µg mL^{-1}) was added to 12 wells per treatment. As a control, 100 µl of cytochrome *c* solution containing PMA and superoxide dismutase (SOD, 300 U mL^{-1}) (cat #S2515, Sigma Aldrich) was added to four wells per treatment. Readings were taken from a plate reader spectrophotometer every 15 min at 545 nm. The final concentration of extracellular O_2^- produced was calculated according to the formula presented by Pick and Mizel [31] with slight modifications: nmol of anion superoxide O_2^- = [(Δ Absorbance after 45 min × 100) ÷ 6.3].

The intracellular production of superoxide anion was estimated by the formation of formazan granules. A 100-µl aliquot of nitro blue tetrazolium (NBT, cat #N6876, Sigma Aldrich) (0.1% in PBS) was pipetted into 12 replicate wells and incubated at room temperature for 45 min. Leukocytes were washed twice with 100% methanol (MeOH) and fixed with 70% methanol. Formazan crystals were dissolved by adding 120 µl of 2 M KOH and 140 µl Dimethyl sulfoxide (DMSO, cat #D8418, Sigma Aldrich). After the re-suspension of the turquoise-blue-colored solutions, absorbance values were read in a plate reader spectrophotometer operating at 620 nm.

2.5. Phagocytosis assay

Phagocytosis of stained yeast cells (*Saccharomyces cerevisiae*) by Nile tilapia leukocytes cultured with different Gln concentrations was evaluated according to the procedures of Ainsworth and Chen [32], with modifications. First, 1.5 g of dried yeast was hydrated in 10 ml of PBS and then autoclaved for 15 min to sterilize the medium and kill the yeast. The cell suspension was mixed with 0.1% Trypan blue in a 1:1 ratio, and was allowed to stain for 15 min at room temperature. The resulting solution was washed several times with PBS, adjusted to 2×10^8 cells mL^{-1} and stored at 4 °C until used. Five hundred microliters of the leukocyte suspension were mixed with 50 µl of autoclaved dyed yeast suspension (providing yeast cells:leukocytes ratio of 4:1) and incubated in a microtube at room temperature for 60 min. A light microscope and a hemocytometer were used to enumerate the cells and the phagocytes with at least one yeast. The total number of engulfed bacteria per phagocyte was recorded as well. Phagocytic index (PI = total engulfed yeast ÷ total phagocytes) and (PA = number of phagocytes with engulfed yeast × 100 ÷ total phagocytes) were computed for both chambers of the hemacytometer and their average value used in phagocytic activity equations. This procedure was performed with six replicates for each treatment.

2.6. Bactericidal assay

The *Streptococcus iniae* primary culture was kindly donated by the Aquatic Diagnostic Laboratory (Department of Pathobiological Sciences, School of Veterinary Medicine - Louisiana State University) and stored frozen in 20% glycerol prior to use. The bacteria was cultured and incubated in brain heart infusion broth (BHI, cat # 53286, Sigma Aldrich) for 18 h at 27 °C. The bacterial broth was centrifuged at 200 × *g* for 10 min, and then the bacteria pellet was washed once in phenol-free Hank's balanced salt solution (HBSS, cat # 55037C, Sigma Aldrich) (pH 7.4) for 10 min and re-suspended in 5 mL of HBSS. Bacteria solution was then adjusted to 0.5 optical density at 540 nm.

The ability of leukocytes to kill *S. iniae* was evaluated at different Gln media concentrations according to the procedures of Secombes [30] and modified by Shoemaker et al. [33]. The leukocyte primary culture was incubated for 2 h, then washed twice with 200 µL of CCM, and supplemented with corresponding treatments into sets of 12 wells. The bacterial suspension was added to the plate (20 µL to each well) and then centrifuged at 150 × *g* for 5 min. The combined cell cultures (leukocyte + bacteria) were incubated at 27 °C for 0 h (control) and 2.5 h. After each incubation period, supernatants were removed and leukocytes lysed with 50 µL of 0.2% Tween 20 (cat #H285, Mallinckrodt) solution. Fresh BHI was added (100 µL) to each well, and the plate was further incubated for 18 h at 27 °C in an orbital incubator. After the incubation period, 20 µL of thiazolyl blue tetrazolium bromide (MTT, cat #M2128, Sigma Aldrich, 5 mg mL^{-1}) were added per well and the plate incubated for an additional 15 min. The plate was then read at 620 nm. Bacterial concentrations were calculated by comparing the absorbance (ABS) obtained for each well to a standard curve previously constructed. Bactericidal capacity (% killing = bacteria 0 h - bacteria 2.5 h × 100 ÷ bacteria 0 h) was computed for each well and presented as mean % killing per treatment.

2.7. Lymphocyte primary culture

Lymphocytes were isolated from peripheral blood (PBL), as previously described by Miller and Clem [34]. Briefly, Nile tilapia blood (~10 mL) was diluted in a ratio of 1:2 with PBS, then 5 mL were layered over 4 mL of Lymphoprep™ (cat # AXS-1114544, Cosmo Bio USA), centrifuged at 350 × *g* for 25 min at room temperature, and lymphocytes were recovered from the interface. Cells were washed with sterile PBS at 600 × *g* for 10 min. The resulting cell pellet was re-suspended in 1 mL of CCM, enumerated and checked for viability (> 95%). Lymphocyte concentration was adjusted to 2.5×10^6 cells mL^{-1} and 190 µL were added to eight replicate wells of a sterile round-bottom 96-well microplate.

2.8. Proliferation assay

The proliferation of PBL upon stimulation with non-specific mitogen was evaluated using the colorimetric assay based on the tetrazolium salt MTT (3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl tetrazolium bromide), as described by Mosmann [35], with modifications. Briefly, supplemented media were added to appropriate wells (sets of eight for each treatment) of PBL primary culture plates as indicated earlier. Lipopolysaccharide solution (LPS, cat #L2630, Sigma Aldrich, 10 mg mL^{-1}) was used for the PBL proliferation. A set of eight wells did not receive the mitogen and served as control. After an 18-h incubation at 27 °C, cell proliferation was determined: 10 µl of 5 mg MTT mL^{-1} solution were added to each well and incubated at 27 °C for 4 h. The precipitated formazan was dissolved with dimethyl sulfoxide and the optical density was measured at 570 nm (SpectraMax 250 plate reader). Peripheral blood lymphocyte proliferation capacity was computed and presented as stimulation index (SI = ABS stimulated cells ÷ ABS non-stimulated [control] cells).

2.9. Amino acid utilization

Amino acid levels in CCM were evaluated as indirect assessments of amino acid utilization during bactericidal and proliferation assays. Supernatants of cell culture media were sampled at 0 h and after the bactericidal (2.5 h) and proliferation (18 h) assays. The samples were frozen and kept at −80 °C until further analysis. Amino acid levels in culture media were determined using ultra performance liquid chromatography (UPLC-Acquity system®, Waters™, Milford, MA) and the commercial kit MassTrak™ (cat # 186004094, Waters™) following established methodology [36]. The MassTrak kit uses pre-column derivatization of AAs with a 6-aminoquinolyl-Nhydroxysuccinimidyl carbamate tag (AccQTag), followed by reversed-phase UPLC on a C18 column (1.7 µm; 2.1 × 150 mm) and UV detection at 260 nm. Samples were deproteinized with 1.5 M $HClO_4$ (cat # 9552–05, J.T. Baker, Phillipsburg, NJ) and neutralized with 2 M K_2CO_3 (cat #P5833, Sigma Aldrich) before derivatization.

2.10. Statistical analysis

All analyses were conducted using the Statistical Analysis System (SAS®, 9.2v, SAS Institute Inc. Cary, NC, USA) software. Data were

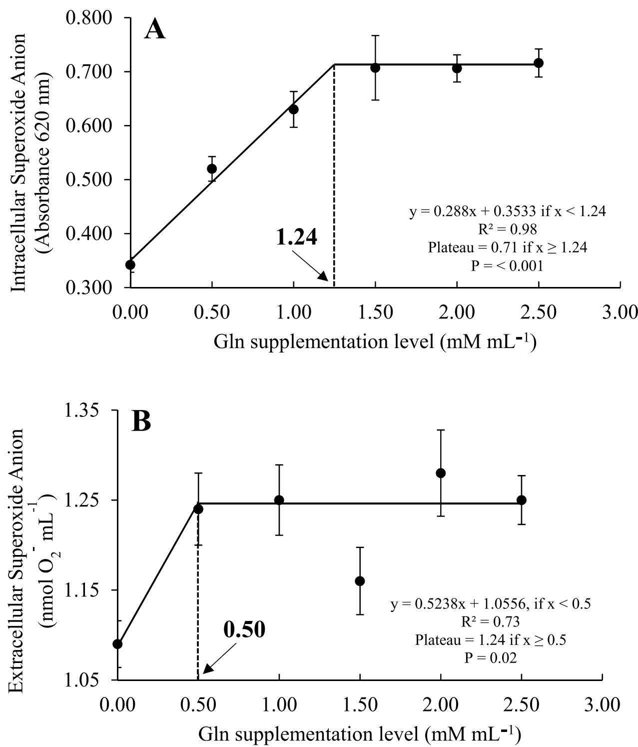

Fig. 1. Broken-line regression of Nile tilapia leukocyte intracellular (A) and extracellular (B) superoxide anion production in response to glutamine (Gln) supplementation to the culture media. Linear trends were found according to the orthogonal polynomial contrast analysis. Error bars represent standard error (SE).

subjected to an analysis of variance (ANOVA) to determine whether the inclusion of Gln significantly ($P < 0.05$) affected the observed responses; in addition, a follow-up trend analysis using orthogonal polynomial contrasts was performed to determine whether the significant effects were linear and/or quadratic. Treatment effects were considered significant at $P \leq 0.05$. Data also were subjected to regression analysis using the linear broken-line model [37] (PROC NLIN) to estimate the minimum level of Gln supplementation in the culture media for Nile tilapia leukocytes that provided optimal respiratory burst (intracellular and extracellular), phagocytic, bactericidal and proliferative responses.

3. Results

3.1. Respiratory burst

The intracellular anion superoxide production increased 2.2-fold upon Gln supplementation at 1.5 mM, but further addition of the amino acid to the media did not elicit any additional improvement (Fig. 1A). Extracellular anion superoxide production by Nile tilapia leukocytes ranged from 1.09 to 1.29 nmol mL^{-1} and also showed statistical differences among the treatments (Fig. 1B). On subjecting the respiratory burst data to a linear broken-line regression model, breakpoints were found at 1.24 and 0.5 mM Gln for intracellular and extracellular superoxide anion production, respectively (Fig. 1).

3.2. Bactericidal activity

The ability of leukocytes to kill *S. iniae* was responsive to Gln supplementation in the culture media (Fig. 2). Unlike the other parameters, the bactericidal activity followed an upward linear pattern achieving the best result with the highest Gln level (2.5 mM).

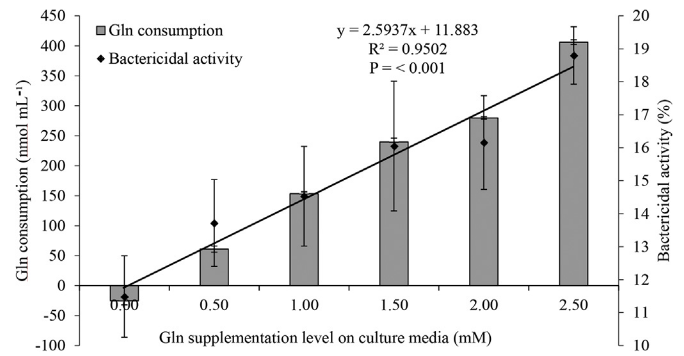

Fig. 2. Glutamine (Gln) consumption and killing capacity of Nile tilapia leukocytes isolated from the head kidney and incubated with complete culture media (CCM) either without or with supplemental levels of Gln. A linear trend was found according to the orthogonal polynomial contrast analysis. Error bars represent standard error (SE).

3.3. Phagocytosis assays

Phagocytic index (PI) and phagocytic activity (PA) were positively affected by Gln supplementation levels as compared to the control media. Phagocytic activity increased 62% upon Gln supplementation at 1.5 mM, but further supplementation did not elicit additional improvements. According to the linear broken-line regression model, the minimum Gln requirements for maximum PI and PA of Nile tilapia isolated leukocytes were 0.94 and 1.29 mM, respectively (Fig. 3).

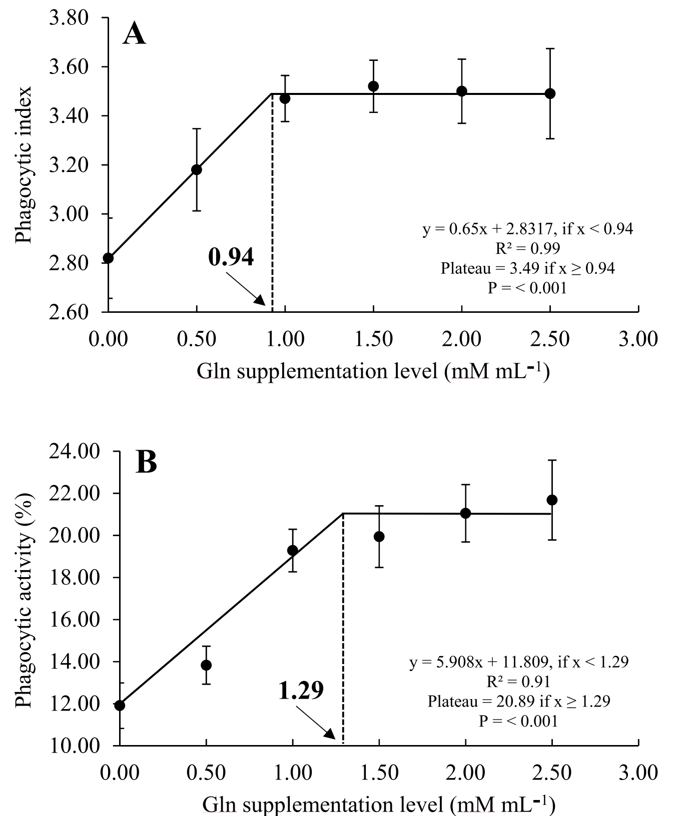

Fig. 3. Linear broken-line regression of Nile tilapia leukocyte phagocytic index (A) and phagocytic activity (B) in response to glutamine (Gln) supplementation to the culture media. Linear trends were found according to the orthogonal polynomial contrast analysis. Error bars represent standard error (SE).

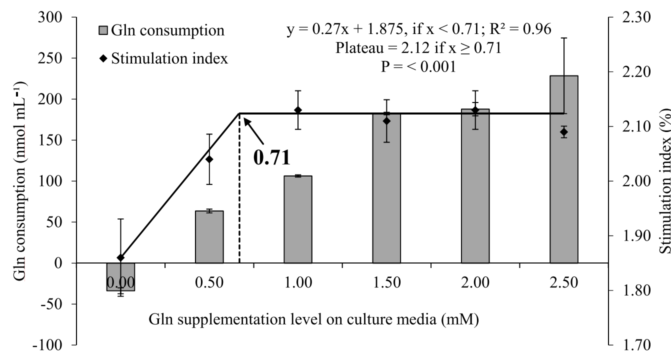

Fig. 4. Glutamine (Gln) consumption and proliferation capacity upon non-specific stimulation of Nile tilapia lymphocytes isolated from peripheral blood and incubated with complete culture media (CCM) either without or with supplemented levels of Gln. A linear trend was found according to the orthogonal polynomial contrast analysis. Error bars represent standard error (SE).

3.4. Cell proliferation assay

The proliferation of Nile tilapia PBL to lipopolysaccharides was positively modulated when Gln was supplemented to the culture media regardless of the amino acid concentration. On subjecting the PBL proliferation data to a linear broken-line regression model, a breakpoint was found at 0.71 mM Gln (Fig. 4).

3.5. Amino acid utilization

The amino acid utilization by Nile tilapia immune cells was affected by both the bactericidal and proliferation assays. As to the bactericidal assay after the incubation period with *S. iniae*, the total amino acids in the culture media significantly decreased by 30% when compared to the initial composition. The differences observed were mainly driven by four amino acids (Table 1). Gln concentration decreased from the original levels by 70%. Together with Gln, histidine and phenylalanine experienced remarkable reductions in concentration (71 and 46%, respectively). On the other hand, lysine increased 129% although this level was not significantly ($P = 0.059$) different from the initial value. Fig. 2 illustrates Gln consumption and the dose-response correlation between Gln supplementation level and the killing capacity of Nile tilapia leukocytes against *S. iniae* after the 2.5-h incubation period.

The changes in amino acid levels in the media after PBL proliferation assays followed a similar pattern as that observed for the bactericidal assay. The total amino acids in the culture media significantly decreased by 26% from the original level after PBL proliferation, and it was also driven by four amino acids, namely histidine, Gln, methionine and phenylalanine (Table 2). Similar to the bactericidal assay, Gln significantly decreased from the original levels, presenting a 30% reduction. Fig. 4 illustrates the dose-response correlation between Gln consumption and the proliferation of Nile tilapia PBL after the 18-h incubation period.

Table 1
Amino acid profile (nmol mL^{-1}) of the culture medium before and after bactericidal assay with Nile tilapia leukocytes and *S. iniae*.

Amino acid	0 h	2.5 h	Change	Δ	*P*-value	P.S.E.
Histidine	252.8	72.3	–	180.5	0.006	8.1
Serine	220.9	167.2	–	53.7	0.267	12.5
Glutamine	140.4	41.1	–	99.3	0.008	4.9
Arginine	242.3	207.3	–	35.1	0.506	14.7
Glycine	523.5	330.7	–	192.8	0.091	24.8
Threonine	184.0	141.1	–	43.0	0.291	10.6
Alanine	325.6	209.2	–	116.3	0.123	17.3
Lysine	48.5	111.5	+	63.0	0.059	6.7
Methionine	67.9	48.8	–	19.1	0.104	2.6
Valine	108.5	75.8	–	32.7	0.162	5.6
Isoleucine	86.7	74.0	–	12.7	0.525	5.6
Leucine	100.4	72.4	–	28.0	0.092	3.6
Phenylalanine	91.9	49.7	–	42.1	0.036	3.7
Tryptophan	14.8	12.6	–	2.1	0.744	1.9
Proline	7.5	10.1	+	2.6	0.262	0.6
Ornithine	0.1	1.7	+	1.6	0.011	0.1
Glutamate	206.9	215.4	+	8.5	0.744	7.5
SUM	2622.8	1840.9	–	781.8	0.026	31.0

Values represent means of 12 replicate wells. P.S.E., pooled standard error.

Table 2
Amino acid profile (nmol mL^{-1}) of the culture medium before and after proliferation assay with Nile tilapia peripheral blood lymphocytes.

Amino acid	0 h	18 h	Change	Δ	*P*-value	P.S.E.
Histidine	252.8	87.78	–	165.1	0.005	6.8
Serine	220.9	161.91	–	59.0	0.070	6.7
Glutamine	140.4	98.37	–	42.0	0.050	4.2
Arginine	242.3	217.97	–	24.4	0.353	7.0
Glycine	523.5	390.23	–	133.2	0.067	15.0
Threonine	184.0	149.96	–	34.1	0.157	5.7
Alanine	325.6	221.14	–	104.4	0.060	11.2
Lysine	48.5	46.25	–	2.3	0.769	2.2
Methionine	67.9	51.66	–	16.2	0.024	1.2
Valine	108.5	76.97	–	31.5	0.064	3.5
Isoleucine	86.7	71.15	–	15.6	0.275	3.7
Leucine	100.4	75.35	–	25.1	0.107	3.5
Phenylalanine	91.9	51.79	–	40.1	0.011	2.3
Tryptophan	14.8	16.80	+	2.1	0.724	1.7
Proline	7.5	9.03	+	1.5	0.317	0.4
Ornithine	0.0	0.68	+	0.7	0.223	0.1
Glutamate	206.9	225.01	+	18.1	0.632	10.8
SUM	2622.7	1952.1	–	670.6	0.047	32.0

Values represent means of 12 replicate wells. P.S.E., pooled standard error.

4. Discussion

The immune system has a complex set of defense mechanisms that involve multiple cellular and humoral components of both innate and adaptive immune components [10,38]. Different from higher vertebrates, the immune system of fish relies more on the innate response to constantly combat and kill invading microorganisms [39]. In this scenario, the macrophages are immune cells that play a key role during the early phase of infection, because they not only modulate the activation and migration of other leukocytes by secreting cytokines, but they also play pivotal roles in the phagocytosis process and antigen presentation [40–43].

In the present study, phagocytic activity was modulated by Gln supplementation to the culture media. This finding is consistent with previous studies with mammals that found positive responses when Gln was added to the media. It is known that Gln can promote macrophage and monocyte phagocytosis by acting as an energy source and increasing cellular adenosine triphosphate (ATP) [44]. Monocytes cultivated under reduced Gln allowances have shown low ATP levels. The depletion of ATP levels can modify the organization of the cell structure, which possibly affects antigen expression and phagocytosis [45]. A protective effect of Gln on intracellular ATP levels has already described in endothelial cells during oxidant injury [46]. A sufficient supply of extracellular Gln is also required for the maximal production of interleukin-6 (IL-6) and IL-8 by human monocytes and of IL-1 and TNFα by murine macrophages [47], as well as major histocompatibility complex (MHC) class II expression and antigen presentation to T-lymphocytes [45]. In addition, phagocytic uptake of complement opsonized *Escherichia coli* and sheep red blood cells, as well as unopsonized zymosan, seems to be directly influenced by Gln availability [15,45,48]. On the other hand, results obtained by Pohlenz et al. [5] found that an enhanced phagocytic activity by channel catfish cells was only observed when Gln worked synergistically with arginine, demonstrating a

potential species-related response. Therefore, it is possible that the increased phagocytic activity observed in the present study was driven by an adequate supply of energy to the cells, as well as an enhanced expression and release of key cytokines, however further research is needed to evaluate the latter notions.

Based on the elevated phagocytic activity provided by Gln supplementation, a higher production of oxygen free radicals via respiratory burst and, consequently, an increased bactericidal activity were expected. The respiratory burst involves a sudden stimulus-induced increase in non-mitochondrial oxidative metabolism that results in the production of the superoxide anion and associated reactive oxygen species [49]. Indeed, the results found in the present study showed a 2.2-fold increase in the absorbance values for intracellular superoxide anion production when the culture media was supplemented with at least 1.5 mM Gln, and a dose-dependent increase in the bactericidal activity against *S. iniae*.

During the synthesis of reactive species and nitric oxide, the enzymes responsible for their production require NADPH. Glutamine can generate considerable amounts of NADPH for basal cellular requirements through catabolic metabolism involving $NADP^+$-dependent malate dehydrogenase, as demonstrated by Costa Rosa et al. [50]. In this study, the authors observed an inhibition of glucose 6-phosphate dehydrogenase in rat macrophages by adrenaline, but NADP + -dependent malate dehydrogenase was activated under the same conditions. Therefore, a considerable proportion of NADPH generating capacity was provided via Gln oxidation, which may have shifted some of the burden of NADPH production from the pentose phosphate pathway to Gln metabolism. An alternative hypothesis is an energy sparing effect of macrophages by not expressing Gln synthetase (GS). An inhibitory feedback of Gln synthetase mRNA expression is found by muscle cells when extracellular levels of Gln is higher than 2 mM [51]. Moreover, GS was found to be expressed by some specialized macrophages for different mammal's species [52,53], but no published information to date described GS on fish macrophages.

In addition, Gln carbon skeleton can be used for amino acid synthesis *de novo*, required by macrophages and monocytes for NO production via arginine synthesis. Considering that arginine derived from Gln seems to be essential for the activity and killing capacity of murine macrophages [54], its supplementation to the medium may have contributed to higher NO^- production and, consequently, higher percentage of bactericidal activity. Further studies addressing the production of NO^- by stimulated Nile tilapia leukocytes under different levels of Gln in the culture media may help validate this hypothesis.

In the present study, Gln was highly utilized by tilapia leukocytes during the immunological events. This result is in agreement with previous metabolic investigations in which monocytes and macrophages presented high demand for Gln [5,44,45]. Besides its role as an energy source for these cells, Gln provides intermediates in the biosynthesis of purine and pyrimidine nucleotides, which are required in the synthesis of nucleic acids for the production of DNA and RNA. Spittler et al. [45] described that the synthesis of RNA by human macrophages was significantly influenced by the concentration of Gln available in culture medium. The synthesis of RNA in the macrophage and monocytes is important for the production of specific proteins, including secretory proteins such as IL-l, lysosomal enzymes, and membrane proteins, being essential to the efficient functioning of the primary immune response [15].

The adaptive immune defense mechanism of fish is based on humoral and cell-mediated responses similar to higher vertebrate systems, including analogous T- and B-lymphocyte populations capable of eliciting specific immune responses against a diversity of antigens. This system can develop immunological memory, which is correlated to the initial activation and expansion of naïve lymphocytes after antigen presentation, cytokine stimulation and co-stimulation by membrane receptors [35,55]. In the present study, lymphocyte proliferation capacity upon non-specific mitogenic stimulation was modulated by Gln supplementation to the culture media. Our results are in agreement with previous *in vitro* proliferative assays using rat [43,56–58] and human lymphocytes [48,59]. As an essential precursor for the synthesis of purine and pyrimidine nucleotides, Gln is required for proliferation of lymphocytes [16,18,57,60]. Also, stimulated rat lymphocytes showed that protein and RNA synthesis are dependent upon the Gln concentration in the culture medium [61]. It is also worth noting that the release of IL-2 by activated T-lymphocytes plays a central role in the control of T-cell proliferation [62]. According to Calder and Newsholme [63], the presence of IL-2 in the culture medium of stimulated rat lymphocytes seems to be Gln-dependent. This result suggests that Gln may stimulate lymphocyte proliferation by promoting IL-2 production. However, the essentiality of Gln for mammalian lymphocyte cultures is already established, and conflicting results were found among fish species. In the present study, Nile tilapia lymphocytes responded to Gln supplementation in a similar fashion as previously reported for channel catfish [5] and common carp [25], but differently from snapper [27], or rainbow trout [26], which do not seem to require Gln to proliferate lymphocytes.

Besides the aforementioned effects of Gln on the fish immune parameters, other amino acids also may have marked influences on the ability to kill pathogens and proliferate. Indeed, the amino acid analysis performed on culture media showed that Gln, histidine and phenylalanine were highly used up by Nile tilapia leukocytes during the bactericidal assay. A similar pattern was observed after the proliferation assay; however, the methionine levels also decreased after mitogen exposure. The significant consumption of amino acids after bactericidal activity and PBL proliferation observed in the present study (Tables 1 and 2) are consistent with earlier studies on mammalian [17,64] and fish cells [5]. According to these reports, 15 amino acids are necessary for optimal *in vitro* proliferation, DNA synthesis and survival of lymphocytes, including Gln, arginine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine, tyrosine, histidine, cysteine, phenylalanine, serine and alanine [17].

Histidine may have been consumed in high amounts due to its ability to regulate several biological processes such as cell adhesion and migration, complement activation, immune complex clearance and phagocytosis of apoptotic cells [65]. Additionally, after decarboxylation, histidine gives rise to histamine, a primary mediator of inflammatory reactions and cell signaling [66,67]. However, only a limited number of *in vitro* studies have shown the effects of histidine on immune response. According to Duval et al. [68], the supplementation of 2 mM-histidine to the culture medium prevented apoptosis, increased cell growth and promoted antibody production in murine B-lymphocytes. Phenylalanine also was consumed in high amounts by both Nile tilapia's macrophages and lymphocytes. This amino acid has a pivotal role in NO synthesis by leukocytes because it up-regulates the expression and activity of GTP cyclohydrolase I, which is the first and rate-controlling enzyme for the synthesis of tetrahydrobiopterin, an essential cofactor for NO synthase [69,70]. Activated macrophages produce nitric oxide to eliminate pathogens during the respiratory burst. Because Gln supplementation enhanced bacterial killing and the production of oxygen free radicals, phenylalanine also could have been highly consumed to support the enhanced phagocytic activity observed in the present study. In addition to its role in NO^- synthesis, phenylalanine degradation generates tyrosine, an immediate precursor for the synthesis of catecholamine hormones, thyroid hormones, as well as dopamine and melanin [71]. These hormones can stimulate Th1 cells and B cell proliferation [72], regulate the metabolism and differentiation of leukocytes and monocytes [73], induce the production of anti-inflammatory cytokines by leukocytes, and regulate the phagocytic activity of neutrophils [74,75]. As to methionine, it is known that a sufficient intake of this amino acid is necessary for the synthesis of proteins of the immune system [76]. Methionine concentration also presented a significant decline in the culture medium after PBL mitogen exposure. This amino acid is essential for the proliferation and

differentiation of lymphocytes because it is a donor of the methyl group that participates in the methylation of DNA and proteins, the synthesis of polyamines, and regulation of gene expression [77,78]; thus, it plays a role beyond just being a protein constituent [10]. Furthermore, methionine is a substrate for the synthesis of choline and thus phosphatidylcholine and acetylcholine that are essential for leukocyte metabolism [71].

In conclusion, results of the present study corroborate and extend understanding on the influences of Gln on Nile tilapia immune cell function. Different levels of glutamine supplementation were required for optimal responses of both branches of the immune system, enhancing the phagocytic activity and bacterial killing capacity of activated macrophages and the proliferation of mitogen-induced lymphocytes *in vitro*. Finally, the amino acid analysis performed on culture media after cell stimulation showed that both bactericidal and proliferative capacities were responsive to Gln supplementation. These positive results are promising for fish immunonutrition and renders Gln as a potential functional amino acid for aquafeeds. However, further research is still necessary to evaluate the effects of glutamine supplementation *in vivo*, especially under intensive farming conditions, where stress usually leads to immunosupression and economical losses.

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References

[1] Food and Agriculture Organization of the United Nations (FAO), Food and agriculture organization of the united nations FishStatJ: a tool for fishery StatisticsAnalysis,Version1.0.1, http://www.fao.org/fishery/statistics/software/fishstatj/en, (2016) , Accessed date: 4 September 2017.
[2] A.F.M. El-Sayed, Tilapia Culture, second ed., CABI Publishing, CABI International, Willingford, Oxfordshire, 2006.
[3] D.M. Gatlin III, Nutrition and Fish Health, in: J.E. Halver, R.W. Hardy (Eds.), Fish Nutrition, Academic Press, San Diego, CA, 2002, pp. 671–702.
[4] R. Subasinghe, Fish health and quarantine, A Review of the State of the World Aquaculture, Vol. 886, FAO, Fisheries circular, Rome, 2003, pp. 45–49.
[5] C. Pohlenz, A. Buentello, W. Mwangi, D.M. Gatlin III, Arginine and glutamine supplementation to culture media improves the performance of various channel catfish immune cells, Fish Shellfish Immunol. 32 (2012) 762–768.
[6] C. Pohlenz, D.M. Gatlin III, Interrelationships between fish nutrition and health, Aquaculture 431 (2014) 111–117.
[7] C.J. Secombes, The nonspecific immune system: cellular defenses, in: G. Iwama, T. Nakanishi (Eds.), The Fish Immune System. Organism, Pathogen and Environment, Academic Press, 1996, pp. 63–101.
[8] A.J. Villena, Applications and needs of fish and shellfish cell culture for disease control in aquaculture, Rev. Fish Biol. Fish. 13 (2003) 111–140.
[9] V. Kiron, Fish immune system and its nutritional modulation for preventive health care, Anim. Feed Sci. Technol. 173 (2012) 111–133.
[10] P. Li, Y.L. Yin, D. Li, S.W. Kim, G. Wu, Amino acids and immune function, Br. J. Nutr. 98 (2007) 237–252.
[11] G.E. Lobley, S.O. Hoskin, C.J. McNeil, Glutamine in animal science and production, J. Nutr. 131 (2001) 2525–2531.
[12] A. Adams, K.D. Thompson, Biotechnology offers revolution to fish health management, Trends Biotechnol. 24 (2006) 201–205.
[13] G. Wu, Z.L. Wu, Z.L. Dai, Dietary requirements of "nutritionally nonessential amino acids" by animals and humans, Amino Acids 44 (2013) 1107–1113.
[14] P. Newsholme, R. Curi, T.C. Pithon Curi, C.J. Murphy, C. Garcia, M. Pires de Melo, Glutamine metabolism by lymphocytes, macrophages, and neutrophils: its importance in health and disease, JNB (J. Nutr. Biochem.) 10 (1999) 316–324.
[15] C. Wallace, D. Keast, Glutamine and macrophage function, Metabolism 41 (1992) 1016–1020.
[16] P. Newsholme, Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? J. Nutr. 131 (2001) 2515–2221.
[17] J. Muhling, M. Fuchs, C. Fleck, A. Sablotzki, M. Krull, M.G. Dehne, Effects of arginine, L-alanyl-L-glutamine or taurine on neutrophil (PMN) free amino acid profiles and immune functions *in vitro*, Amino Acids 22 (2002) 39–53.
[18] E. Roth, Immune and cell modulation by amino acids, Clin. Nutr. 26 (2007) 535–544.
[19] Z. Cheng, A. Buentello, D.M. Gatlin III, Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*, Aquaculture 319 (2011) 247–252.
[20] Z. Cheng, D.M. Gatlin III, A. Buentello, Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*), Aquaculture 43 (2012) 362–363.
[21] Q. Zhu, Q.Y. Xu, H. Xu, C.A. Wang, D.J. Sun, Dietary glutamine supplementation improves tissue antioxidant status and serum non-specific immunity of juvenile Hybrid sturgeon (*Acipenser schrenckii* x *Huso dauricus*), J. Appl. Ichthyol. 27 (2011) 715–720.
[22] X. Hong, Z. Qing, W. Chang-an, Z. Zhi-gang, L. Ling, W. Lian-sheng, X. Qi-you, Effect of dietary alanyl-glutamine supplementation on growth performance, development of intestinal tract, antioxidant status and plasma non-specific immunity of young mirror carp (*Cyprinus carpio* L.), J. NE Agric. Univ. 21 (2014) 37–46 (English Edition).
[23] K. Hu, J.X. Zhang, L. Feng, W.D. Jiang, P. Wu, Y. Liu, X.Q. Zhou, Effect of dietary glutamine on growth performance, non-specific immunity, expression of cytokine genes, phosphorylation of target of rapamycin (TOR), and anti-oxidative system in spleen and head kidney of Jian carp (*Cyprinus carpio* var. Jian), Fish Physiol. Biochem. 41 (2015) 635–649.
[24] J.A. Buentello, D.M. Gatlin III, Nitric oxide production in activated macrophages from channel catfish (*Ictalurus punctatus*): influence of dietary arginine and culture media, Aquaculture 179 (1999) 513–521.
[25] S. Rosenberg-Wiser, R.R. Avtalion, The cells involved in the immune response of fish. III. Culture requirements of PHA-stimulated carp (*Cyprinus carpio*) lymphocytes, Dev. Comp. Immunol. 6 (1982) 693–702.
[26] R.C. Ganassin, J. Barlow, N.C. Bols, Influence of glutamine on phytohemagglutinin stimulated mitogenesis of leucocytes from the rainbow trout head kidney, Fish Shellfish Immunol. 8 (1998) 561–564.
[27] S. McBride, D. Keast, Mitogenesis of snapper lymphocytes and their requirement for glutamine, Dev. Comp. Immunol. 21 (1997) 91.
[28] N.T. Popovic, I. Strunjak-Perovic, R. Coz-Rakovac, J. Barisic, M. Jadan, A. Persin Berakovic, R. Sauerborn Klobucar, Tricaine methane-sulfonate (MS-222) application in fish anaesthesia, J. Appl. Ichthyol. 28 (2012) 553–564.
[29] J.A. Buentello, M. Reyes-Becerril, M.J. Romero-Geraldo, F.J. Ascencio-Valle, Effects of dietary arginine on hematological parameters and innate immune function of channel catfish, J. Aquat. Anim. Health 19 (2007) 195–203.
[30] C.J. Secombes, Isolation of salmonid macrophages and analysis of their killing activity, in: J. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, W.B. Van Muiswinkel (Eds.), Techniques in Fish Immunology, Fair Haven: SOS Publications, 1990, pp. 137–154.
[31] E. Pick, D. Mizel, Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader, J. Immunol. Meth. 46 (1981) 211–226.
[32] A.J. Ainsworth, D.X. Chen, Differences in the phagocytosis of four bacteria by channel catfish neutrophils, Dev. Comp. Immunol. 14 (1990) 201–209.
[33] C.A. Shoemaker, P.H. Klesius, J.A. Plumb, Killing of *Edwardsiella ictaluri* by macrophages from channel catfish immune and susceptible to enteric septicemia of catfish, Vet. Immunol. Immunopathol. 58 (1997) 181–190.
[34] N.W. Miller, L.W. Clem, A culture system for mitogen-induced proliferation of channel catfish (*Ictalurus punctatus*) peripheral blood lymphocytes, Meth. Cell Sci. 11 (1988) 69–73.
[35] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol Meth. 65 (1983) 55–63.
[36] A. Schuhmacher, C. Wax, J.M. Gropp, Plasma amino acids in rainbow trout (*Oncorhynchus mykiss*) fed intact protein or a crystalline amino acid diet, Aquaculture 151 (1997) 15–28.
[37] K.R. Robbins, A.M. Saxton, L.L. Southern, Estimation of nutrient requirements using broken-line regression analysis, J. Anim. Sci. 84 (2006) 155–165.
[38] C. Secombes, Will advances in fish immunology change vaccination strategies? Fish Shellfish Immunol. 25 (2008) 409–416.
[39] B. Magnadottir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2006) 137–151.
[40] C.J. Bayne, L. Gerwick, The acute phase response and innate immunity of fish, Dev. Comp. Immunol. 25 (2001) 725–743.
[41] C.A. Shoemaker, P.H. Klesius, C. Lim, Immunity and disease resistance in fish, in: C. Lim, C.D. Webster (Eds.), Nutrition and Fish Health, Food Products Press, Binghamton, New York, 2001, pp. 149–162.
[42] A. Spittler, S. Holzer, R. Oehler, G. Boltz-Nitulescu, E. Roth, A glutamine deficiency impairs the function of cultured human monocytes, Clin. Nutr. 16 (1997) 97–99.
[43] P. Yaqoob, P.C. Calder, Glutamine requirement of proliferating T-lymphocytes, Nutrition 13 (1997) 646–651.
[44] S. Furukawa, H. Saito, T. Inoue, T. Matsuda, K. Fakatsu, I. Han, S. Ikeda, A. Hidemura, Supplemental glutamine augments phagocytosis and reactive oxygen intermediate production by neutrophils and monocytes from postoperative patients *in vitro*, Nutrition 16 (2000) 323–329.
[45] A. Spittler, S. Winkler, P. Gotzinger, R. Oehler, M. Willhiem, C. Tempfer, G. Weigel, R. Fugger, G. Boltz-Nitulescu, E. Roth, Influence of glutamine on the phenotype and function of human monocytes, Blood 86 (1995) 1564–1569.

[46] Hinshaw, D. B., Burger, J. M., Miller, M. T., Adams, J. A., Beals, T. F., Omann, G. M. 1993. ATP depletion induces an increase in the assembly of a labile pool of polymerized actin in endothelial cells. American Journal of Physiology-Cell Physiology 264: 1171-1179.Field, C.J., Johnson, I.R., Schley, P.D. 2002. Nutrients and their role in host resistance to infection. J. Leukoc. Biol. 71: 16–32.

[47] C.J. Field, I.R. Johnson, P.D. Schley, Nutrients and their role in host resistance to infection, J. Leukoc. Biol. 71 (2002) 16–32.

[48] M. Parry-Ballings, J. Evans, P.C. Calder, E.A. Newsholme, Does glutamine contribute to immunosuppression after major burns? Lancet 336 (1990) 522–525.

[49] G.M. Rosen, S. Pou, C.L. Ramos, M.S. Cohen, B.E. Britigan, Free radicals and phagocytic cells, Faseb. J. 9 (1995) 200–209.

[50] L. Costa-Rosa, R. Curi, C. Murphy, P. Newsholme, Effect of adrenaline and phorbol myristate acetate or bacterial lipopolysaccharide on stimulation of pathways of macrophage glucose, glutamine and O2 metabolism. Evidence for cyclic AMP-dependent protein kinase mediated inhibition of glucose-6-phosphate dehydrogenase and activation of NADP+-dependent'malic'enzyme, Biochem. J. 310 (1995) 709–714.

[51] G. Wu, Amino Acids: Biochemistry and Nutrition, CRC Press, 2013.

[52] J.G. Bode, T. Peters-Regehr, R. Kubitz, D. Häussinger, Expression of glutamine synthetase in macrophages, J. Histochem. Cytochem. 48 (2000) 415–421.

[53] F. Chretien, A.V. Vallat-Decouvelaere, C. Bossuet, A.C. Rimaniol, R. Le Grand, G. Le Pavec, C. Creminon, D. Dormont, F. Gray, G. Gras, Expression of excitatory amino acid transporter-2 (EAAT-2) and glutamine synthetase (GS) in brain macrophages and microglia of SIVmac251-infected macaques, Neuropathol. Appl. Neurobiol. 28 (2002) 410–417.

[54] C.J. Murphy, P. Newsholme, The importance of glutamine metabolism in murine macrophages and human monocytes to L-arginine biosynthesis and rates of nitrite or urea production, Clin. Sci. 95 (1998) 397–407.

[55] C. Uribe, H. Folch, R. Enriquez, G. Moran, Innate and adaptive immunity in teleost fish: a review, Vet. Med. 56 (2011) 486–503.

[56] M. Griffiths, D. Keast, The effect of glutamine on murine splenic leukocyte responses to T and B cell mitogens, Immunol. Cell Biol. 68 (1990) 405–408.

[57] M.S.M. Ardawi, E.A. Newsholme, Glutamine metabolism in lymphocytes of the rat, Biochem. J. 212 (1983) 835–842.

[58] Z. Szondy, E.A. Newsholme, The effect of glutamine concentration on the activity of carbamoyl-phosphate synthase II and on the incorporation of [^{3}H] thymidine into DNA in rat mesenteric lymphocytes stimulated by phytohaemagglutinin, Biochem. J. 261 (1989) 979–983.

[59] W.K. Chang, K.D. Yang, M.F. Shaio, Lymphocyte proliferation modulated by glutamine: involved in the endogenous redox reaction, Clin. Exp. Immunol. 117 (1999) 482–488.

[60] G. Wu, C.J. Field, E.B. Marliss, Enhanced glutamine and glucose metabolism in cultured rat splenocytes stimulated by phorbol myristate acetate plus ionomycin, Metabolism 41 (1992) 982–988.

[61] P.C. Calder, Glutamine and the immune system, Clin. Nutr. 13 (1994) 2–8.

[62] K.A. Smith, Interleukin-2: inception, impact, and implications, Science 240 (1988) 1169–1176.

[63] P.C. Calder, E.A. Newsholme, Glutamine promotes interleukin-2 production by concanavalin A-stimulated lymphocytes, Proc. Nutr. Soc. 51 (1992) 105.

[64] G.B. Segel, Amino acid transport in lymphocytes, in: M.S. Kilberg, D. Häussinger (Eds.), Mammalian Amino Acid Transport: Mechanisms and Control, Plenum Press, New York, New York, 1992, p. 319.

[65] A.L. Jones, M.D. Hulett, C.R. Parish, Histidine-rich glycoproteins: a novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems, Immunol. Cell Biol. 83 (2005) 106–118.

[66] M. Dy, E. Schneider, Histamine–cytokine connection in immunity and hematopoiesis, Cytokine Growth Factor Rev. 15 (2004) 393–410.

[67] S. Tanaka, A. Ichikawa, Recent advances in molecular pharmacology of the histamine systems: immune regulatory roles of histamine produced by leukocytes, J. Pharmacol. Sci. 101 (2006) 19–23.

[68] D. Duval, C. Demangel, K. Munierjolain, S. Miossec, I. Geahel, Factors controlling cell proliferation and antibody production in mouse hybridoma cells: 1. Influence of the amino acid supply, Biotechnol. Bioeng. 38 (1991) 561–570.

[69] G. Wu, C.J. Meininger, Regulation of nitric oxide synthesis by dietary factors, Annu. Rev. Nutr. 22 (2002) 61–86.

[70] W. Shi, C.J. Meininger, T.E. Haynes, K. Hatakeyama, G. Wu, Regulation of tetrahydrobiopterin synthesis and bioavailability in endothelial cells, Cell Biochem. Biophys. 41 (2004) 415–433.

[71] S.W. Kim, R.D. Mateo, Y.L. Yin, G. Wu, Functional amino acids and fatty acids for enhancing production performance of sows and piglets, Asian-Australas. J. Anim. Sci. 20 (2007) 295–306.

[72] N.W. Kin, V.M. Sanders, It takes nerve to tell T and B cells what to do, J. Leukoc. Biol. 79 (2006) 1093–1104.

[73] K. Dorshkind, N.D. Horseman, The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency, Endocr. Rev. 21 (2000) 292–312.

[74] S. Basu, P.S. Dasgupta, Dopamine, a neurotransmitter, influences the immune system, J. Neuroimmunol. 102 (2000) 113–124.

[75] N. Mohagheghpour, N. Waleh, S.J. Garger, L. Dousman, L.K. Grill, D. Tuse, Synthetic melanin suppresses production of proinflammatory cytokines, Cell. Immunol. 199 (2000) 25–36.

[76] R.F. Grimble, The effects of sulfur amino acid intake on immune function in humans, J. Nutr. 136 (2006) 1660–1665.

[77] N.E. Flynn, C.J. Meininger, T.E. Haynes, G. Wu, The metabolic basis of arginine nutrition and pharmacotherapy, Biomed. Pharmacother. 56 (2002) 427–438.

[78] G. Wu, F.W. Bazer, J.M. Wallace, T.E. Spencer, Intrauterine growth retardation: implications for the animal sciences, J. Anim. Sci. 84 (2006) 2316–2337.