Acuabio 1 stimulates anaerobic metabolism and the immune system in goldfish and tilapia larvae

RESEARCH

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ABSTRACT

Acuabio 1 is a mixture of proteins and essential amino acids that exerts an important nutritional effect on early developmental stages of aquatic organisms. It synchronizes and accelerates larvae growth, promoting development, and subsequently increasing resistance to parasites. All these properties make Acuabio 1 very attractive to improve the production, survival and quality of the larvae of aquatic organisms, the most relevant developmental stage in aquaculture. Here, the stimulating activity of Acuabio 1 was demonstrated by obtaining goldfish and tilapia larvae 3.5-fold and 1.6-fold heavier than their respective negative controls. Their anaerobic metabolism and factors mediating the innate immune response were also improved. These experiments help to understand the molecular mechanisms triggered by this nutritional supplement.

Keywords: Acuabio 1, fish, growth, innate immune system

Biotecnología Aplicada 2006;23:294-299

RESUMEN

Acuabio 1 estimula el metabolismo anaerobio y el sistema inmune innato de las larvas de goldfish y tilapia. Acuabio 1 es una mezcla de proteínas y aminoácidos esenciales que ejercen un efecto nutricional importante en las etapas iniciales del desarrollo de los organismos acuáticos. Su efecto sincroniza y estimula el crecimiento de las larvas. Con su empleo se activa y acelera el mecanismo de crecimiento desde edades muy tempranas, así como el desarrollo y la talla de los animales, lo cual provoca, como efecto secundario, una mayor resistencia ante organismos parásitos. Estas propiedades del Acuabio 1 lo hacen muy atractivo para su utilización en el mejoramiento de la producción, sobrevida y calidad de las larvas de los organismos acuáticos, etapa crucial en el cultivo de esos animales. En esta investigación se evidenció la acción estimuladora de Acuabio 1. Se obtuvieron larvas de goldfish con un peso 3.5 veces mayor, y larvas de tilapia con un peso 1.6 veces mayor, comparando ambos resultados con el control negativo. A su vez, hubo un estímulo del metabolismo anaerobio y de los factores que median la respuesta inmune innata. A partir de este estudio, se comprendieron mejor los mecanismos moleculares que activa este suplemento nutricional.

Palabras clave: peces, crecimiento, sistema inmune innato

*I*ntroduction

Aquaculture is an alternative for protecting marine and freshwater ecosystems. However, the high fish densities obtained with aquaculture techniques increase stress and the outbreak of concurrent opportunistic infections, restricting productivity and thereby receiving special attention by researchers [1]. Therefore, increasing production indexes during the larval stage is one of the challenges in aquaculture. A large part of the losses occur during this growth period. Therefore any attempt to improve larvae survival and quality of life would enable a larger number of fish to reach the adult stage, leading to increased aquaculture productivity.

Several experimental aquatic biotechnological strategies have been developed to modify fish growth, such as, administering hormones (*e.g.* prolactin, insulin, growth hormone (GH) and steroid hormones)[2] and feeding specific protein-based nutritional supplements. Genetic improvement though gene transfer and chromosome manipulation has also had particular relevance. Salmon, trout, tilapia and other commercial fish species with increased growth rate and resistance to adverse environmental conditions and diseases have been obtained by applying these technologies [3], while also having significantly reduced nutritional requirements.

Hence, the Aquatic Biotechnology Department at the Center for Genetic Engineering and Biotechnology in Havana (CIGB) developed the nutritional supplement Acuabio 1, distributed by its commercial branch Heber Biotech. This product is a mixture of proteins and essential amino acids that exerts a positive nutritional effect at the initial developmental stages in aquatic organisms. It stimulates and accelerates the growth of larvae and further growth and development of the fish from their early stages, also increasing resistance to parasitic organisms as a secondary effect. Furthermore, Acuabio 1 stimulates the release of the endogenous GH into the blood stream and the transcription of the insulin-like growth factor in the liver.

It has been well documented that there is an interaction between immune and endocrine systems through hormones and cytokines, which is very important to adjust defense mechanisms in mammals

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and fish [4]. The immunological mechanisms protecting fish from pathogens have to be characterized, to improve the immunological state of culture fish.

There are no previous biochemical studies addressing the stimulatory effect of Acuabio 1 on the immune system of treated animals. Therefore, the purpose of this paper is to study some biochemical parameters (lactate dehydrogenase (LDH), creatine kinase (CK), aspartate aminotransferase (ASAT), triacylglycerides (TAG)) and mediators of the innate immune response (lysozyme and lectin levels, and antiprotease activity) in larvae of tilapia (*Oreochromis sp.*) and goldfish (*Carasius auratus*) treated with Acuabio 1. Tilapia (*Oreochromis sp.*) is one of the most relevant species in aquaculture, due to its high protein content, low production cost and requiring only a six-month-period to gain the 250 g standard commercial weight [5]. Therefore, it is ideal for growth studies. Additionally, there is an increasing interest in ornamental fish worldwide [6], and also their growth stimulation. These studies could represent a model to alternative future applications in economically relevant fish.

Materials and methods

Growth experiments

The experiments were conducted at the Center for Genetic Engineering and Biotechnology (CIGB), under stable temp erature (28 ºC) and controlled day-night cycle (14 hrs of light and 10 hrs of darkness), conditions in goldfish and tilapia larvae provided by the Center for Aquaculture of Mampostón (CPAM) of the Ministry of Fishing.

Larvae were distributed into experimental groups of 150 individuals and fed a pulverized commercial feed (Center for the Production of Laboratory Animals, CENPALAB), twice a day at 40% of their body weight.

Fish were fed Acuabio 1 at a 0.01 g/L concentration (dosage recommended by the manufacturer), applied by immersion there time a week for one hour each time. The negative controls received placebo feed at the same concentration. After the one hour treatment, ponds containing goldfish were oxygenated by dropping, and those containing tilapia by artificial oxygenation.

Animals were anesthetized with ethyl 3-aminobenzoate (methanesulfonic acid salt) (Sigma Chemical Co.), sized, weighed, and frozen at -70 ºC until use.

A 19-day growth experiment was conducted to study the effect of Acuabio 1 in goldfish (*Carasius auratus*) larvae. A group of 15 fish was initially weighed, at starting 0.0012 g. At the end of the experiment (day 19), 30 animals from each group were sized and weighed. The conditioning factor (k) was calculated from size (mm) and weight (g) data, according to Rhaman and Maclean [7], by the formula:

$$k = \frac{\text{weight (g)}}{\text{length (cm)}^3} \bullet 1{,}000$$

The specific growth rate (SGR) was calculated according to Gill and coworkers[8], by the formula:

$$SGR\ (\%/day) = \frac{\ln (\text{final weight}) - \ln (\text{initial weight})}{t} \bullet 1{,}000$$

Where t is the time of the experiment.

Preparation of homogeneous larvae extracts

Tilapia larvae were homogenized with the Polytron ULTRA-TURRAX T25 (IKA Labortechnik), by using a ratio of 10 ml 1X PBS/g of larval tissue. Afterwards, the mixture was centrifuged at 5 000 rpm for 15 min at 4 °C. The supernatant was collected and stored at -70 °C until use, 48 hrs later.

Determination of LDH, ASAT, CK, TAG and total protein parameters

One and a half milliliters of the crude extract were centrifuged at 12 500 pm for 4 min at 4 °C. The supernatant was collected for determining the enzymatic activity of LDH, ASAT, CK and TAG, and total proteins. The enzyme specific activity (ESA) of LDH, ASAT, CK and triacylglycerides concentration were determined by using commercial kits supplied by HELFA Diagnostics, EPB "Carlos J. Finlay", Quimefa, Cuba. Determinations were carried out as recommended by the manufacturer. The total protein content was assessed by the bicinchoninic acid method, using a kit by Pierce.

Lysozyme determinations

Lysozyme activity was determined by the turbidimetric method, based on the lysis of *Micrococcus lysodeikticus* lyophilized particles (Sigma) [9] and using chicken egg white lysozyme (Boehringer Mannheim, Germany) as the positive control. Lysozyme standards were prepared at 0.5, 1, 2, 4, 6, 8, and 10 µg/ml in 0.05 M phosphate-buffered solution, pH 6.2. One-hundred microliters of each sample were dispensed in 96-well plates, by duplicates. One-hundred microliters of microorganism suspension was added to each well, to a final volume of 200 ml. The optical density (OD) was measured at 450 nm, at 0, 2, 5, 15, 30 and 60 min. The variation of 0.001 OD units per min was established as one lysozyme unit (U).

Determination of lectins

For this assay, rabbit blood was collected in a solution containing 0.11 M D-glucose, 37 mM sodium citrate, 72 mM NaCl and 2.9 mM citric acid in a 1:1 (v/v) proportion, and stored at 4 °C for the assay. The blood collected was washed twice with 1X PBS, pH 7.2 (136mM NaCl, 2.6 mM KCl, 8.0 mM Na_2HPO_4, 1.47 mM KH_2PO_4), by centri-fugation at 1 500 rpm during 5 min. The assay was carried out in 2% (v/v) erythrocyte suspension in 1X PBS, pH 7.2, with samples serially diluted with 1X PBS, pH 7.2, in 96-well U-bottom plates (COSTAR, 96 Well Serocluster U-Bottom) with 100 ml of total volume per well. One-hundred microliters of 2% erythrocyte suspension were added to each well and incubated at 28 °C for 1 hr. One-hundred microliters of 1X PBS, pH 7.2 and 100 ml of 2% erythrocyte suspension were included as the negative control. The hemagglutination titer was determined to each sample as the higher dilution with complete hemagglutination.

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Antiprotease activity

The method was employed as described by Magnadottir *et al.* [10], by incubating 20 ml of homogenized larvae extracts with 20 ml of 5 mg/ml trypsin solution at 22 °C for 10 min. Afterwards, 200 ml of 0.1 M phosphate-buffered saline solution, pH 7.0, and 250 ml of 2% azocasein, were added and incubated at 22 °C for 1 hr. Five-hundred microliters of 10% trichloroacetic acid (TCA) were added and mixtures were incubated at 22 °C for 30 min, and centrifuged at 6,000 rpm for 5 min. One-hundred microliters of each supernatant were transferred to a 96-well plate filled with 100 ml of 1N NaOH per well. Optical density was measured at 450 nm. A buffer solution was added in the 100% control group to replace the homogenized larvae extracts. The homogenized larvae extracts and trypsin were substituted by phosphate-buffered solution, as negative control. The percentage of inhibition was calculated for each sample by comparing it to the 100% control group. All the samples were analyzed by duplicates.

Statistical analyses

Fish treated or untreated with Acuabio 1 were grown under the same environmental conditions, to minimize environmental effect and variation [11]. Data were presented as the mean ± standard error. Means of treated and untreated animals were compared by the Student's t test, with differences considered significant for $p < 0.05$.

Results

Growth of fish larvae

Results of goldfish larvae experiments are shown in table 1 and figures 1 and 2, respectively.

A significant difference in size, weight and conversion factor between larvae treated and untreated with Acuabio 1 (growth rate of 2.38 mg/day and 0.61 mg/day, respectively) was observed at the end of the trial. These results indicated that fish fed Acuabio 1 grew 3.9-fold faster than untreated control fish.

Goldfish Biochemical parameters

To correlate the growth-stimulating capacity of Acuabio 1 in goldfish with certain biochemical parameters, the ESA of anaerobic metabolism enzymes (LDH and CK), amino acid metabolism (ASAT) and triacylglycerides (TAG) and lysozyme were determined in homogenized fish extracts. Lytic enzymes (*e.g.* lysozyme) are relevant defensive elements, particularly against bacteria. Results are shown in table 2.

Table 1. Conditioning factor and specific growth rate of goldfish (Carasius auratus) larvae treated with Acuabio

Experimental group	Conditioning factor (k)	SGR (%/day)
NC	10.13 ± 1.03	11.19 ± 0.722
Acuabio 1	13.23 ± 0.78 *	18.77 ± 0.435 *

Data are expressed as the mean ± standard error.

(*) indicate significant differences between fish treated with Acuabio 1 or placebo for a value of $p < 0.05$ (Student's t test).

SGR: Specific growth rate.

NC: Negative control group treated with placebo.

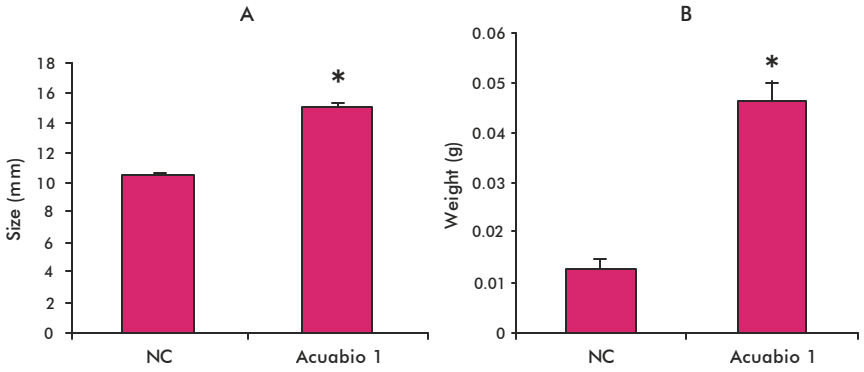

Figure 1. Effect if the nutritional supplement Acuabio 1 on goldfish (*Carasius auratus*) larvae growth. Size (A) and weight (B) data obtained at the end of the experiment are shown.
Legend:
*- Indicates significant differences between Acuabio 1-treated and placebo-treated (NC) groups; $p < 0.05$ (Student's t test).
n = 30 fish per group. Data are represented as the mean ± standard error.

Significant differences were detected in the ESA of LDH and lysozyme concentration between extracts from goldfish larvae treated with Acuabio 1 (n = 9) and those treated with placebo (n = 8). In contrast, there were no significant differences between groups in CK ESA and ASAT ESA. Similar results were obtained for TAG concentration analyses.

Growth of Tilapia larvae

The growth of tilapia (*Oreochromis sp.*) larvae was followed for 27 days to study the effect of the Acuabio 1 nutritional supplement. Fish were sized and weighed on days 0, 15 and 27 (end of the trial).

The mean size and weight of the larvae at the beginning of the trial were of 15.22 ± 0.5 mm and 0.065±0.01 g (results are shown in table 3 and figure 3, respectively). Differences in weight and ESA between treated and untreated animals were evidenced after 15 days of treatment, with a 1.21-fold higher SGR in fish treated with Acuabio 1 than in placebo-treated animals (table 4).

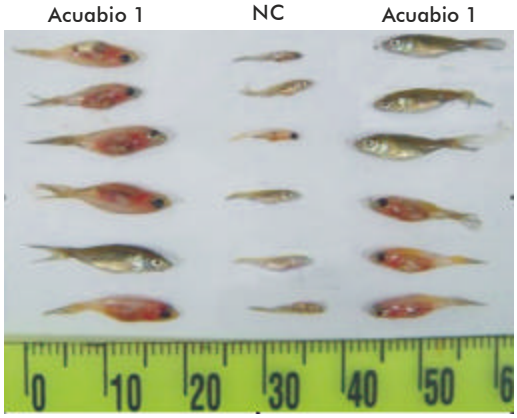

Figure 2. Goldfish larvae phenotype at the end of the experiment.
Legend:
Acuabio 1: Larvae treated with the nutritional supplement Acuabio 1.
NC: Larvae treated with placebo.

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Table 2. Effect of the nutritional supplement Acuabio 1 on the biochemical parameters studied in goldfish (Carasius auratus) larvae

Parameters	NC	Acuabio 1
LDH (U/mg prot.)	0.466 ± 0.056 (n = 8)	0.814 ± 0.084* (n = 9)
CK (U/mg prot.)	0.094 ± 0.024 (n = 8)	0.136 ± 0.037 (n = 10)
ASAT (U/mg prot.)	0.127 ± 0.017 (n = 8)	0.123 ± 0.016 (n = 8)
TAG (mmol/l)	0.218 ± 0.025 (n = 9)	0.288 ± 0.040 (n = 10)
Lysozyme (µg/g of tissue)	28.25 ± 1.05 (n = 4)	33.48 ± 0.00* (n = 4)

Data are expressed as the mean ± standard error.

(*) indicate significant differences between fish treated with Acuabio 1 or placebo (NC) for a value of $p < 0.05$ (Student's t test).

n indicates the number of goldfish larvae of approximately 250 mg analyzed in the trials.

U/mg prot.: Units per milligram of total proteins.

µg/g of tissue: Micrograms per gram of larvae tissue.

Tilapia biochemical parameters

Biochemical parameters were studied, which included ESA of anaerobic metabolism (LDH and CK) or amino acid metabolism (ASAT) enzymes, and also TAG, as shown in table 5. Significant differences were observed in ESA data from LDH and CK anaerobic enzymes between treated and untreated fish with Acuabio 1.

Fish larvae do not develop specific immunity at early stages, but show active innate immune responses, including lectins. Lectins are glycoproteins identified in several fish species and functionally involved in host-defense mechanisms. Increased hemagglutination activity was observed in homogenized extracts from larvae treated with Acuabio 1, showing hemagglutination titers 2.5-fold higher than untreated animals (figure 4).

Another factor characterizing the innate response was the anti-protease activity, protecting against the proteolytic activity of bacterial toxins in host tissues. When measured in this experiment, the anti-protease activity was 1.8-fold higher in homogenize extracts of treated larvae, compared to levels of the untreated negative controls (figure 5).

Discussion

Most fish loss occur during the larval stage. Therefore, an attempt to improve larvae survival and their quality of life would increase the number of fish reach the adult stage thereby increasing aquaculture productivity.

Rations including concentrated protein hydrolysates have been reported in stimulating the growth rate of common carp and sea bass larvae [12]. The feeding method can exert temporary effects on the development

Table 3. Growth experiment with the nutritional supplement Acuabio 1 in tilapia (Oreochromis sp.)

Time (days)	Size of larvae (mm) NC	Size of larvae (mm)Acuabio 1
0	15.22 ± 0.5 (n = 23)	15.22 ± 0.5 (n = 23)
15	24.00 ± 1.12 (n = 11)	27.10 ± 0.94 (n = 10)
27	27.92 ± 0.53 (n = 51)	31.69 ± 0.38* (n = 162)

Data are expressed as the mean ± standard error.

Asterisks (*) indicate significant differences between fish treated with Acuabio 1 or placebo (NC) for a value of $p < 0.05$ (Student t test).

n: indicates the number of individually analyzed fish.

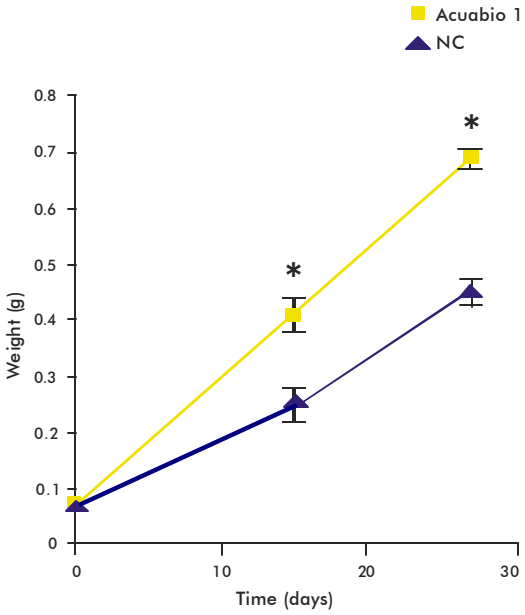

Figure 3. Weight of tilapia larvae after the growth experiment with the nutritional supplement Acuabio 1.
Legend: Data are expressed as the mean ? standard error. Asterisks (*) indicate significant differences between animals treated with Acuabio 1 or placebo (NC); $p < 0.05$ (Student's t test); n=162.

of fish larvae, probably related to the ontogeny of the digestive tract [13], denoting the relevance of timing and route for protein feed administration.

Here, the growth of goldfish and tilapia larvae was stimulated by administering the Acuabio 1 nutritional supplement, as expresed by an increased size, weight and rate of growth. Similar results were obtained in goldfish fed carp recombinant GH [14] and in diploid and triploid salmon injected with porcine recombinant GH [15] or fed protein hydrolysates [16].

It has been suggested that the specific activity of CK and LDH indicate anaerobic capacity in fish [17]. The LDH is responsible for the catabolic reduction of pyruvate into lactate under anaerobic conditions, a pathway alternative to the aerobic oxidation of pyruvate into acetylcholine to enter the citric acid cycle and be finally oxidized into CO_2 and H_2O. The anaerobic oxidation of pyruvate brings an alternative pathway to generate ATP under stress conditions in muscle cells. On the other hand, CK catalyzes the direct phosphorilation of ADP

Table 4. Conditioning factor (k) and specific growth rate (SGR) obtained in the growth experiment with tilapia larvae (Oreochromis sp.)

Parameters	NC	Acuabio 1
Conditioning factor (k)	20.04 ± 0.47 (n = 51)	21.38 ± 0.75 (n = 162)
SGR (%/day)	6.862 ± 0.224 (n = 51)	8.327 ± 0.145* (n = 162)

Data are expressed as the mean ± standard error.
Asterisks (*) indicate significant differences between fish treated with Acuabio 1 or placebo (NC) for a value of $p < 0.05$ (Student's t test).
n: indicates the number of individually analyzed fish.
SGR: Specific growth rate.

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Table 5. Effect of the nutritional supplement Acuabio 1 on the biochemical parameters studied in tilapia (Oreochromis sp.) larvae

Parameters	NC	Acuabio 1
LDH (U/mg prot.)	308.8±67.23 (n = 6)	567.7 ± 82.66* (n = 7)
CK (U/mg prot.)	0.028 ± 0.005 (n = 8)	0.048 ± 0.008* (n = 7)
ASAT (U/mg prot.)	0.064 ± 0.010 (n = 10)	0.070 ± 0.009 (n = 9)
TAG (mmol/l)	0.753 ± 0.075 (n = 10)	0.698 ± 0.048 (n = 10)

Data are expressed as the mean ± standard error
Asterisks (*) indicate significant differences between fish treated with Acuabio 1 or placebo (NC) for a value of $p < 0.05$ (Student's t test).
n: indicates the number of goldfish larvae of approximately 250 mg analyzed in the trials.
U/mg prot.: Units per milligram of total proteins.
µg/g of tissue: Micrograms per gram of larvae tissue.

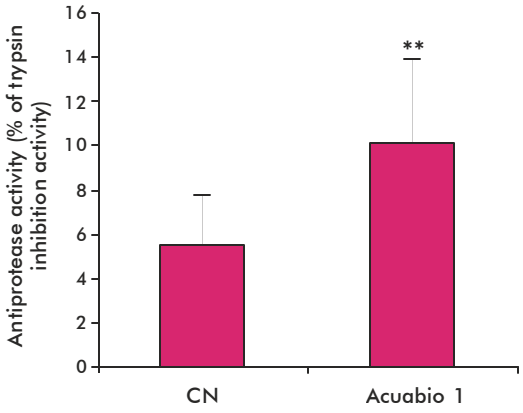

Figure 5. Anti-protease activity (trypsin activity percentage of inhibition) in homogenized extracts of tilapia larvae.
Legend:
Data are represented as the mean ± standard deviation. All the sampled were analyzed by duplicates (n=10). The group treated with Acuabio 1 showed the highest anti-protease activity compared to the negative control group (NC). Asterisks (*) indicate significant differences between animals treated with Acuabio 1 or placebo (NC); $p < 0.01$ (Student's t test); n: indicates the number of individually analyzed fish.

from phosphocreatine, bringing the ATP required during the first 30 s of fast contraction in the white muscle. Therefore, the significantly elevated levels of LDH in goldfish and tilapia larvae treated with Acuabio 1, compared to the control larvae, suggested an improved anaerobic metabolic capacity in these fish. Similar outcomes have been described in studies conducted in rainbow trout [18] and California halibut [19], with increased LDH activity leading to greater muscular mass and animal size.

Additionally, a significant increase in the CK activity of Acuabio 1-treated tilapia larvae was found and an increase, although non-significant, was observed in goldfish larvae in the present study. Similar results have been observed in rainbow trout, with relatively high pyruvate kinase or LDH activities [18]. Therefore, our results suggest that larvae treated with Acuabio 1 were improved with the energy coming from the anaerobic metabolism. The increased LDH activity is considered a potential mechanism for maintaining an optimal speed of displacement, irrespective of fish size (distance covered per second) [17,18].

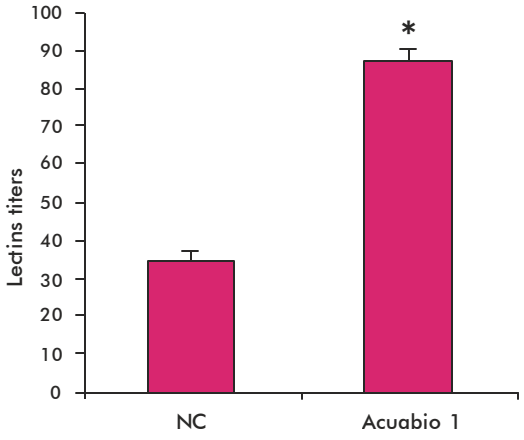

Figure 4. Lectins titers. The hemagglutination titer was determined in each sample, considering the highest sample dilution showing complete hemagglutination as the lectin titer.
Legend:
Data are expressed as the geometric mean ? confidence interval (n=10).
The group treated with Acuabio 1 showed the highest hemagglutination activity, compared to the negative control group (NC) Asterisks (*) indicate significant differences between animals treated with Acuabio 1 or placebo (NC); $p < 0.05$ (Student's t test);
n indicates the number of individually analyzed fish.

No significant differences were detected in the ASAT activity of animals treated with Acuabio 1, either of goldfish or tilapia, compared to control animals.

In adipose and hepatic tissues, lipolysis and fatty acid release are promoted as indirect effects of GH [19]. In our study, there were no significant differences in TAG concentrations in goldfish or tilapia larvae treated with Acuabio 1, indicating unaltered TAG metabolism in both species.

GH displays unrelated functions in immune activation and growth [20]. It is known that Acuabio 1 stimulates the release of the endogenous GH, and also the production of lysozyme in goldfish larvae. The stimulation of lysozyme release into the plasma, by administering GH homologueshas been also documented [21, 22], (*e.g.* salmon GH in freshwater-cultured rainbow trout [22]).

When brown trouts (*Salmo trutta*) are transferred from freshwater into saltwater, the GH rises and thyroid hormone traces decline in the plasma. These effects have been related to the augmented fagocytic activity of cephalic kidney lymphocytes and spiked concentrations of lysozyme in the plasma [23]. Yada *et al.* (2004) [24] reported that GH and prolactin increased plasma lysozyme in rainbow trout when it was administered in a dose-dependent regime. These experiments also showed that GH and prolactin stimulate the production of lysozyme in leukocytes without affecting their proliferation. Granular neutrophils are a major source of blood lysozyme in trout, as demonstrated [25].

Another parameter of innate immunity, lectins activity, was analyzed in tilapia. These plasma proteins are detected by hemagglutination assays,

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based on their bi- or polyvalent interaction with structural carbohydrate residues of glycolipids, which are also able to crosslink with diverse cellular types and lead to agglutination.

Suzuky and Otake (2000) [26] found in the skin of *Anguilla japonica* larvae an increased lectins activity, with cells that produce these proteins appearing at day 8 after hatching, a very early developmental stage. These proteins have also been identified in salmon [27] and *Paralichthys olivaceus* [28] eggs and *S. aurata* larvae [29]. Recently, it was demonstrated that the levels of mannose-binding lectins augmented after administering GH in humans, suggesting that the influence of Acuabio 1 on this parameter in fish could be mediated by the GH [30].

Additionally, the anti-protease activity in homogenized extracts of larvae treated also rose, compared to the negative control larvae. Several protease inhibitors have been described in fish plasma, mainly α1-antiprotease and α2-macroglobulin [31]. It has also been demonstrated that differences in the activity of α2-macroglobulin between two trout species are related to the infection of *A. salmonicida* [32]. Therefore, the improvement in this parameter with Acuabio 1 is beneficial to fish health.

Conclusions

The stimulating activity of the nutritional supplement Acuabio 1 on growth rate was corroborated in fish, by controlled growth trials in goldfish and tilapia larvae. Also, the activity of enzymes related to energy metabolism pathways and factors associated to innate immunity were studied. These explain the resistance observed in these fish species against pathogens after consuming this nutritional supplement in field trials.

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Received in september, 2006. Accepted for publication in november, 2006.