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Original research article

Fishmeal substitution for *Arthrospira platensis* in juvenile mullet (*Mugil liza)* and its effects on growth and non-specific immune parameters¤

Substitución de harina de pescado por Arthrospira platensis en juveniles de lisa (Mugil liza) y sus efectos en el crecimiento y parámetros del sistema inmune no-específico

Substituição da farinha de peixe por Arthrospira platensis em juvenis de tainha (Mugil liza) e seus efeitos no crescimento e nos parâmetros do sistema imune não-especifico

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Abstract

Background: Cyanobacterium *Athrospira platensis* (*Spirulina*) is a potential fishmeal (FM) substitute in fish diets because of its high protein content, antioxidant and immunostimulant properties.

Objective: To evaluate the effects of total and partial substitution of FM with *A. platensis* (0, 30, 50, 70 and 100% substitution) in juvenile mullet (*Mugil liza).*

Methods: Juvenile mullets (n=210) were maintained in a recirculation system under optimal water parameters for the species. Mullets were fed five experimental diets for 80 days. Each diet was tested in triplicate tanks. At the end of the experimental period growth parameters were measured and samples of blood, liver and spleen were taken to evaluate the immune system.

Results: Full replacement (100%) of FM resulted in growth deficits and low survival. The FM replacement induced changes in the proportion of macrophages and lymphocytes. Up to 50% FM replacement increased the

expression of CD3 receptors in spleen T lymphocytes (T-Cells), whereas >50% FM replacement decreased the expression of CD3 receptors. We also found that partial FM substitution diminished the apoptotic process.
Conclusions: Up to 50% FM substitution with *A. platensis* can improve performance of non-specific immune system of mullets.

Keywords: *apoptosis, CD3, fish, immunostimulant, microalgae, Spirulina.*

Resumen
Antecedentes: La cianobacteria *Arthrospira platensis* (*Spirulina*) puede usarse como substituto potencial de la harina de pescado (HP) por su alto contenido de proteína, sus antioxidantes y sus propiedades inmunoestimulantes.
Objetivo: Analizar el efecto de la substitución parcial y total de HP por *A. platensis* (0, 30, 50, 70 y 100% de substitución) en juveniles de lisa (*Mugil liza)*.
Métodos: Juveniles de lisa (n=210) se mantuvieron en un sistema de recirculación con parámetros de calidad de agua en niveles óptimos para la especie. Las lisas se alimentaron con las dietas experimentales durante 80 días. Cada dieta fue evaluada en triplicado. Al final del periodo experimental se midieron los parámetros de crecimiento y se colectaron muestras de sangre, hígado y bazo para evaluación del sistema inmune.
Resultados: La substitución total (100%) resultó en deficiente crecimiento y baja sobrevivencia. El remplazo de HP produjo cambios en las proporciones de macrófagos y linfocitos. La substitución de hasta un 50% HP aumentó la expresión de receptores CD3 en linfocitos T del bazo. Por otro lado, la substitución mayor a 50% HP disminuyó la expresión de receptores CD3. La substitución parcial de HP disminuyó el proceso de apoptosis.
Conclusiones: Proponemos una substitución de HP del 50% por *A. platensis,* lo cual mejora el desempeño del sistema inmune no especifico de las lisas.

Palabras clave: *apoptosis, CD3, inmunoestimulante, microalga, pez, Spirulina.*

Resumo
Antecedentes: A cianobactéria *Athrospira platensis* (*Spirulina*) é um potencial substituto da farinha de peixe (FP) pelo seu alto conteúdo de proteína, antioxidantes e características imune estimulantes.
Objetivo: Foram avaliados os efeitos da substituição parcial e total da FP por *A. platensis* (0, 30, 50, 70 e 100% substituição) em juvenis de tainha (*Mugil liza*).
Métodos: Juvenis de tainha (n=210) foram mantidos em um sistema de recirculação com os parâmetros da água sendo mantidos em níveis ótimos para a espécie. As tainhas foram alimentadas com as dietas experimentais por 80 dias, cada dieta foi testada em triplicata, ao final do período experimental foram avaliados os parâmetros de crescimento e amostras de sangue, fígado e baço foram coletadas para a avaliação do sistema imune.
Resultados: A substituição total de FP resultou em redução do crescimento e baixa sobrevivência. A avaliação do sistema imune demostrou que a substituição da FP produz alterações nas proporções de macrófagos e linfócitos. Provou-se que até 50% de substituição da FP incrementa a expressão de receptores CD3. Além disso, a substituição parcial da FP diminui o processo de apoptose.
Conclusão: Baseado em nossos descobrimentos, se propõe a substituição de até 50% da FP por *A. platensis* que melhorará o desempenho do sistema imunológico não específico das tainhas.
Palvras-chave: *apoptosis, CD3, estimulante, imune, microalga, peixe, Spirulina.*

Introduction

Identifying alternative protein sources that could substitute fishmeal (FM) in aquaculture diets is a major challenge (Oliva-Teles, 2012). Because of its excellent lipid and protein profiles, as well as high biomass production, microalgae are potential substitutes for FM (Palmegiano *et al.*, 2008). Among microalgae, *Athrospira platensis* has been widely used in animal and human nutrition because of its high protein content (60–70%), and can be used as a feedstuff in fish diets (Belay, 2002). Indeed, *A. platensis* has already been used as a FM replacement in aquaculture diets, improving growth performance of tilapia *Oreochromis niloticus* (Abdel-Tawwab and Ahmad, 2009), common carp

(*Cyprinus carpio* L.) (Teimouri *et al.,* 2015), and sturgeon (*Huso huso*) (Adel *et al.,* 2016). Due to its bioactive components, *A. platensis* improves carcass quality (carotenoids) (Nandeesha *et al.,* 1998), and provides immune stimulants (polysaccharides) (Abdel- Tawwab and Ahmad, 2009), antioxidant activity (β-carotene and C-phycocyanin) (Ravi *et al.,* 2010), and polyunsaturated fatty acids (□-linolenic acid – GLA) (Jafari *et al.,* 2014). Besides improving growth parameters, it enhances the immune system -critical in aquaculture production- due to a wide variety of macro and micronutrients (Ringø *et al.,* 2010). Dietary administration of *A. platensis* might benefit specific and non-specific cellular immune parameters in fish, as already shown in macrophage and natural killers cell (NK) activities in tilapia (*Oreochromis niloticus*) (Abdel-Tawwab and Ahmad, 2009), increasing mucus protease production in sturgeon (Adel *et al.,* 2016), interleukin gene expression in *C. carpio* leucocytes (Watanuki *et al.,* 2006), and granular hemocytes in white shrimp (*Litopeneaus vannamei)* (Macias-Sancho *et al.,* 2014).

The mullet (*Mugil liza)* has desirable characteristics for aquaculture, including its iliophagus habits (Vieira, 1991), fast ontogenic development (Galvão *et al.,* 1997), and easy adaptation to consuming novel dietary ingredients (Ramos *et al.,* 2015; Zamora-Sillero *et al.,* 2013). Therefore, we evaluated the effects of *A. platensis* as a FM substitute in practical diets on growth performance and non-specific immune cellular response of juvenile mullets

Materials and Methods

Fish source and experimental design

Once approved by the Ethics Committee (FURG- CEUA Pq036/2014), juvenile mullets (n=210) were captured using a 3 mm beach seine net at Cassino Beach/ Brazil (Latitude, -32.1833; Longitude, -52.1667) and taken to Laboratório de Nutrição de Organismos Aquáticos-FURG at the Universidade Federal do Rio Grande (FURG), Brazil. The fish were acclimated to the laboratory conditions for one month in a 500-L tank under controlled temperature (26 °C) and salinity (30 ppt). During the acclimation period, the mullets were hand-fed the control diet (D1) four times per day (at 9:00, 12:00, 14:00, and 16:00). To maintain water quality conditions, a full water renewal was carried out every day.

After the acclimatization period, an 80-day experiment was conducted in 15 50-L fiberglass tanks connected by a recirculation system, consisting in a biological filter, a UV light filter (18 w Philips®, São Paulo, Brazil) and a protein skimmer. During this period, water flow rate was 3 L/min, and a daily water exchange corresponding to 10% of the total tank volume was carried out. Each tank was stocked with 14 individuals (0.26 ± 0.01 g initial weight). Water quality parameters remained stable throughout the experimental period. Water parameters were measured daily in all tanks. Dissolved oxygen concentrations and temperature were measured using a multi-parameter electrode (YSI, 550A, Yellow Springs, Ohio, USA) and maintained at 6.1 ± 0.6 mg/L, 25.9 ± 0.6 °C, respectively. The pH was measured with a digital pH meter (Hanna Instruments, HI221) and presented a mean value of 7.7 ± 0.1. Salinity was kept constant at 29.2 ± 0.8 ppt and was measured with an optical refractometer (RTS 101, ATAGO, Tokyo, Japan). A photoperiod of 12:12 h (light: dark) was maintained. The ammonium and nitrite concentrations were determined according to the methods presented by Benderschneider and Robinson (1983), and Strickland and Parsons (1972), respectively. Total ammonia and nitrite levels were 0.17 ± 0.09 mg/L and 0.31 ± 0.34 mg/L, respectively. Alkalinity was maintained by the addition $CaCO_3$ (to maintain 100 mg/L of $CaCO_3$ in the water), thereby maintaining the biofilter. Alkalinity was measured according to APHA (2005).

Five experimental diets were prepared by replacing 0 (D1), 30 (D2), 50 (D3), 70 (D4) and 100% (D5) of FM with *A. platensis*. Each diet was tested in triplicate tanks, which were randomly distributed. Fish were fed four times per day (9:00, 12:00, 14:00, and 16:00). At each feeding, fish were fed 10% of the total weight biomass per tank for the first 15 experimental days, and adjusted to 7% of the total biomass for the remaining days. Biomass was calculated daily assuming a feed conversion ratio of 2:1.

Diet formulation

A commercial *A. platensis* microalgae was used (Prilabsa®, Natal, Brazil). Composition of all feed ingredients was analyzed at Laboratório de Nutrição de Organismos Aquáticos-FURG according to the Association of the

Official Analytical Chemists (A.O.A.C., 2000) methodology. Table 1 presents the proximal composition of *A. platensis* and the FM used in the study.

Table 1. Proximal composition of fishmeal and *A. platensis* in dry basis (g/kg).

Ingredient	Moisture	Crude Protein	Ether Extract	Fiber	Ash	NFE [a]
Fish Meal	5.57	61.54	9.82	7.46	11.80	11.27
A. platensis	2.22	61.61	0.50	2.51	3.12	32.55

[a] Calculated value (Merrill and Watt, 1973). NFE= Nitrogen Free Extract, calculated as 100 − (crude protein + lipids + ash + moisture + fiber).

Table 2. Diet formulation and final composition. Proximate analyses values expressed in g/kg.

Ingredients	Treatments				
	D1	D2	D3	D4	D5
Fish Meal [a]	39	27	19.5	12	0
A. platensis [b]	0	12	19.5	27	39
Soybean meal [c]	10	10	10	10	10
Wheat meal [d]	10	10	10	10	10
Fish oil [e]	4	5	6	7	8
Starch [f]	19	19	19	19	19
Cellulose	13	12	11	10	9
Mineral/Vitamin Premix [g]	2	2	2	2	2
Gelatin	3	3	3	3	3
TOTAL	100	100	100	100	100
Proximate Analyses (dry base)					
Crude Protein	36.89	37.74	37.27	37.63	37.30
Ether Extract	9.42	9.30	9.13	9.23	8.74
Fiber	5.95	5.70	6.21	6.12	3.50
Ash	7.39	6.66	7.19	5.98	4.19
Moisture	3.08	2.58	2.99	2.17	4.24

[a] Leal Santos, Rio Grande, RS, Brazil; [b] Prilabsa® , Brazil; [c] Sulino RS, Brazil; [d] Sulino , RS, Brazil; [e] Campestre®, São Paulo. Brazil; [f] Maizena, Brazil ®; [g] Premix M. Cassab, São Paulo, Brazil ((Vitamin A (500,000 lU/kg), Vit. D3 (250,000 lU/kg), Vit. E (5,000 mg/kg), Vit. K3 (500 mg/kg), Vit. B1 (1,000 mg/kg), Vit. B2 (1,000 mg/kg), Vit. B6 (1,000 mg/kg), Vit. B12 (2,000 mg/kg), Niacin (2,500 mg/ kg), Calcium pantothenate (4,000 mg/kg), Folic acid (500 mg/kg), Biotin (10 mg/kg), Vit C (10,000 mg/kg), Choline (100,000 mg/kg), Inositol (1,000 mg/kg). Trace elements: Selenium (30 mg/kg), Iron (5,000 mg/kg), Copper (1,000 mg/kg), Manganese (5,000 mg/kg), Zinc (9,000 mg/kg), Cobalt (50 mg/kg), Iodine (200 mg/kg)).

Diets were formulated to contain 35% CP (Carvalho *et al.*, 2010) and 9% lipids. The pre-weighed ingredients were mechanically mixed (Marconi, MA200, São Paulo. Brazil), and then mixed with oil and water to produce a stiff dough. Then, mixtures were pelleted using a meat grinder (Metalúrgica 9000, PC-22, São Paulo, Brazil). Pellets were air dried at 60 °C for 24 h in an oven (Marconi, MA035). The size of the resulting pellets was gradually adjusted to fish growth. Diets were stored in plastic bags at -18 °C until use. Diet formulation and proximate

composition is shown in Table 2.

Diet fatty acid identification

Fatty acid (FA) analyses were made at the Facultad de Ciencias, Universidad de la republica (Montevideo, Uruguay). Lipids were extracted according to Folch *et al*. (1957) and transesterified using 1 mL sulfuric acid (1%) in methanol (Christie, 1982). The antioxidant butylhydroxytoluene (BHT) (0.5 mL, 50 mg/L) was used to prevent FA oxidation. Samples were incubated at 49 ºC for 16 h in a nitrogen atmosphere. Next, hexane:ether (1:1 v/v) solution was used for FA extraction and $KHCO_3$ (20g/L) was used to wash the hexane:ether solution. Finally, FA was dried for 24 h, and a dilution of chloroform 30 mg/mL was made and kept under a nitrogen atmosphere at -20 ºC until chromatography was performed.

The FA was quantified using gas chromatography (Hewlett Packard 5890, GMI, Minneapolis, MN, USA), with a capillary column of melted silica Supelco wax as stationary phase (30 m × 0.32 mm D.I., Supelco, Pennsylvania, USA). Nitrogen was used as carrier gas and split mode for the injection. Injector and detector temperatures were both 250 °C. Initially, temperature was 180 °C for 10 min, then increased at a rate of 2.5 °C/min up to 212 °C. Final temperature was maintained for 13 min. Chromatography Station for Windows (CSW Data Apex 1.7) was used for data processing of chromatograms. All FA were identified (Table 3) by comparing its retention time with cod fish oil standard (Supelco), according to Salhi and Bessonart (2013).

Table 3. Fatty acid composition of diets (g/kg of lipids).

FA	Treatments				
	D1	D2	D3	D4	D5
14:0	5.16	4.72	5.05	5.30	5.58
16:0	18.98	22.79	24.76	26.84	29.44
16:1n-7	5.98	6.10	6.04	5.98	6.44
16:2n-4	0.88	0.77	0.77	0.74	0.71
16:3n-4	0.70	0.65	0.64	0.61	0.60
18:0	4.77	4.51	4.35	4.27	3.54
18:1n-9	15.08	13.22	11.79	10.86	8.42
18:1n-7	3.09	3.10	2.96	2.67	3.27
18:2n-6	10.65	8.71	8.77	9.30	10.01
18:3n-3	1.96	1.94	1.89	1.86	1.67
18:4n-3	1.55	1.79	1.91	1.92	1.99
20:1n-9	1.57	1.25	1.18	1.11	1.11
20:4n-6(ARA)[a]	1.08	1.38	1.24	1.13	1.02
20:5n-3(EPA)[b]	7.54	7.37	7.60	7.46	7.06
22:5n-3	2.13	1.57	1.51	1.32	1.06
22:6n-3(DHA)[c]	10.16	10.92	10.66	9.95	8.56
SAFA[d]	31.37	35.03	36.98	39.30	41.55
MUFA[e]	28.10	26.51	24.96	23.60	22.89
PUFA[f]	40.53	38.46	38.06	37.10	35.56
n-3HUFA[g]	20.68	20.57	20.41	19.36	17.29
n-6HUFA[h]	1.88	2.62	2.30	2.11	2.02
n-3/n-6	1.85	2.07	2.12	1.98	1.70
DHA/EPA	1.35	1.48	1.40	1.33	1.21
DHA/ARA	9.39	7.90	8.58	8.82	8.40
EPA/ARA	6.97	5.34	6.11	6.61	6.93

[a] arachidonic acid, [b] eicosapentaenoic acid, [c] docosahexaenoic acid, [d] saturated fatty acids, [e] monounsaturated fatty acids, [f]poliunsaturated fatty acids, [g] highly unsaturated fatty acids n-3, [h] highly unsaturated fatty acids n-6.

Amino acid evaluation in diets

The amino acids (AA) contained in the diets were calculated according to the AA profile of the principal protein sources (FM and *Spirulina*) analyzed by Evonik Industries AG (São Paulo, Brazil) by the AMINONIR® (Nitrogen infrared, NIR) technique and data gathered from the NRC (2011) (for soybean meal, wheat meal and gelatin), then calculated according to the inclusion of each ingredient (Table 4).

Table 4. Essential amino acids contained in the experimental diets, expressed as percentage (%) of the diet.

	Treatments				
	D1	**D2**	**D3**	**D4**	**D5**
Essential Amino acid					
Methionine	0.74	0.72	0.72	0.71	0.69
Lysine	1.95	1.82	1.75	1.68	1.54
Threonine	1.25	1.33	1.37	1.41	1.50
Arginine	2.09	2.15	2.18	2.21	2.27
Isoleucine	1.22	1.40	1.49	1.58	1.75
Leucine	2.09	2.32	2.43	2.55	2.78
Valine	1.53	1.68	1.75	1.83	1.98
Histidine	0.69	0.65	0.63	0.61	0.57
Tryptophan	0.34	0.40	0.42	0.45	0.51
Phenylalanine	1.29	1.37	1.41	1.45	1.53
Nonessential amino acid					
Cysteine	0.28	0.30	0.31	0.31	0.33
Tyrosine	1.24	1.30	1.33	1.36	1.43

Evaluation of growth parameters and sampling

At the end of the 80-day experiment, each organism was anesthetized (benzocaine 50 ppm) and individual weights were obtained to determine growth parameters. The performance parameters were:

Weight gain (WG) = individual final weight (g) - individual initial weight (g)/individual initial weight (g);

Feed conversion ratio (FCR) = average individual dry feed intake/average individual weight gain;

Specific growth rate (SGR) = 100% × [ln (final weight) - ln (initial weight)]/days of feeding;

Protein efficiency ratio (PER) = average individual weight gain (g)/average individual protein intake (g);

Survival = (final number of fish/initial number of fish) * 100;

Afterwards, blood samples were collected from the caudal vein of six fish per treatment and a drop of blood was smeared onto a clean glass slide and dried. Next, all fish were euthanized with a benzocaine overdose (300 ppm). Nine samples of liver and spleen from each treatment were collected and fixed in 20% formalin solution for subsequent analyses. The carcasses of 12 fish per treatment were frozen for body composition analyses. All

proximal analyses were conducted according to the AOAC (2000) methodology.

White blood cell count

Smears of blood were fixed in methanol and stained with Wright-Giemsa stain for determination of the differential White Blood Cell (WBC) count. At least 100 WBCs for each smear were counted for differential WBC determinations under an optical microscope. Six smears for each tank were counted.

Immunohistochemistry (IHC)

For IHC, analyses were conducted at Laboratório de Imunologia e Patologia de Organismos Aquáticos (FURG). Five spleens per each tank were fixed in 20% buffered formalin, embedded in paraplast and stained using the ABC peroxidase method (Vectastain Elite ABC Kit, California, USA), as described by Hsu *et al*. (1981). The sections were incubated with monoclonal anti-CD3 antibodies (Sigma®, USA), as previously tested by Romano *et al*. (2004) and Führ *et al*. (2016). Subsequently, the sections were washed (0.1% diaminobenzidine solution) and dehydrated; six slides per tank were examined under optical microscope.

The CD3 receptor expression was evaluated by quantitative analysis of phenotypic percentage per square millimeter (mm^2) of tissue. The expression of these receptors in the spleen was quantified using Bioscan OPTIMAS 6.1 software according to the method proposed by Weibel (1981) and Romano *et al*. (1996).

Apoptosis evaluation

Five livers per tank were fixed in 20% buffered formalin and embedded in paraplast; the evaluation was conducted using the TUNEL method for ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore), according to Charriaut-Marlangue and Ben-Ari (1995). Apoptotic cell expression was evaluated using quantitative analysis of the expression percentage cells per square millimeter (mm^2) of tissue. The apoptotic cells in the liver were also quantified using Bioscan OPTIMAS 6.1 software.

Statistics

To test possible differences among treatments, we used one-way Analysis of variance (ANOVA). Normality and heterogeneity were tested according to Shapiro-Wilk and Levene tests, respectively. Percentage data were transformed into arcsine values before statistical significance tests. For each case, when significance between treatments was detected, a posterior means comparison was performed by Tukey's test. All tests were conducted at the 5% significance level.

Results

A summary of growth parameters is given in Table 5. Except for FCR, all parameters increased for up to 50% FM substitution (D3 treatment), followed by gradual decrease until D5 treatment. When fed D5, almost all growth parameters were different from the other diets. Final weight (FW) and weight gain (WG) showed no significant difference between D1 and D5 (Table 5). Mullet survival ranged from 100 to 47.62%, and was different for full FM substitution compared to all other treatments.

For proximal analyses of the carcass, we detected no statistically significant differences among treatments (Table 6).

Table 5. Growth performance of mullets fed different *Arthrospira platensis* levels in substitution of fish meal.

Parameters	Treatments					ANOVA
	D1	D2	D3	D4	D5	p
IW (g)	0.26 ± 0.0	0.26 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	> 0.05
FW (g)	3.93 ± 0.6^{ab}	4.34 ± 0.44^{a}	5.28 ± 0.39^{a}	4.17 ± 0.77^{ab}	2.32 ± 0.36^{b}	0.008*
WG (g)	3.67 ± 0.6^{ab}	4.07 ± 0.43^{a}	5.02 ± 0.38^{a}	3.90 ± 0.76^{ab}	2.06 ± 0.36^{b}	0.008*
SGR (%.day^{-1})	3.36 ± 0.2^{a}	3.50 ± 0.17^{a}	3.75 ± 0.12^{a}	3.44 ± 0.31^{a}	2.70 ± 0.25^{b}	0.003*
FCR	2.04 ± 0.1^{a}	2.00 ± 0.12^{a}	1.89 ± 0.12^{a}	2.12 ± 0.22^{a}	3.20 ± 0.22^{b}	0.000*
PER	1.23 ± 0.1^{a}	1.25 ± 0.09^{a}	1.35 ± 0.09^{a}	1.18 ± 0.14^{a}	0.75 ± 0.07^{b}	0.000*
Survival (%)	95.24 ± 4.1^{a}	97.62 ± 4.12^{a}	100.00 ± 0^{a}	97.62 ± 4.12^{a}	47.62 ± 8.25^{b}	0.000*

Values are expressed as means ± SD of three replicate groups. Different letters within ([a, b]) each row show significant differences in Tukey test at p≤0.05. IW= initial weight; FW = final weight; WG = weight gain; SGR = specific growth ratio; FCR = feed conversion ratio; PER = protein efficiency ratio.

Table 6. Whole-body proximate composition of mullets (wet weight basis, g/kg) fed different *Arthrospira platensis* levels in substitution of fish meal.

Analyses	Treatments					ANOVA
	D1	D2	D3	D4	D5	p
Dry Matter	35.29 ± 1.52	34.66 ± 1.13	35.23 ± 1.18	33.97 ± 0.95	34.07 ± 1.70	> 0.05
Ash	5.20 ± 0.46	5.04 ± 0.51	4.41 ± 0.50	4.83 ± 0.68	4.99 ± 0.55	> 0.05
Protein	15.68 ± 2.06	16.08 ± 0.71	16.81 ± 0.03	15.92 ± 0.29	15.75 ± 0.37	> 0.05
Ether extract	10.89 ± 0.13	11.23 ± 0.69	11.95 ± 0.26	11.13 ± 0.60	10.36 ± 0.84	> 0.05

Values are expressed as means ± SD of three replicate groups. Different letters within each row show significant differences in Tukey test at p≤0.05.

Table 7. White blood cell count, lymphocytes (%), monocyte (%) and granulocytes (%), CD3 and apoptosis reactive cells (mm^2) of mullets fed different *Arthrospira platensis* levels in substitution of fish meal.

Cell	Treatments					ANOVA
	D1	D2	D3	D4	D5	p
Monocyte	3.4 ± 1.34^{ab}	1.2 ± 0.97^{b}	1.4 ± 1.14^{b}	4.4 ± 1.94^{a}	2.3 ± 1.52^{ab}	*0.016
Granulocyte	12.2 ± 3.5	7.25 ± 1.89	7.6 ± 4.92	15.4 ± 4.03	11.6 ± 7.37	> 0.05
Lymphocyte	84.4 ± 4.39^{ab}	91.5 ± 5.52^{ab}	91 ± 2.38^{a}	80.2 ± 5.54^{b}	86 ± 8.88^{ab}	*0.028
CD3 Spleen	3.1 ± 1.57	7.5 ± 4.51	10.8 ± 5.95	6.78 ± 4.48	4.3 ± 2.25	> 0.05
Apoptosis	1.34 ± 0,12^{a}	1.10 ± 0.54^{ab}	0.54 ± 0.10^{b}	0.94 ± 0.23^{ab}	1.28 ± 0.52^{ab}	*0.015

Values are expressed as means ± SD of three replicate groups. Different letters within ([a, b]) each row show significant difference.

The proportions of WBCs varied among treatments (Table 7). Proportion of monocytes was different between D2 and D3 treatments compared to D4 treatment. Proportion of lymphocytes was different between D3 and D4 treatments. However, no statistically significant differences among treatments for the granulocyte or T-Cell CD3 receptor expression were detected. Apoptotic expression was different between treatments without *A. platensis* (1.34 ± 0.12 mm^2), and D2 treatment (0.54 ± 0.10 mm^2) (p=0.015).

Discussion

Spirulina has been widely recommended by the FAO as a food supplement in humans and as a high-quality feed ingredient in animal nutrition (Habib *et al.*, 2008). The use of these microalgae as a FM substitute has been tested in many fish species and at various levels of FM substitution. El-sayed (1994) found that 50% of the FM substitution results in the best growth for silver seabream (*Rhabdos argussarba*). Palmegiano *et al*. (2005) show that 50% of *Spirulina* inclusion results in the best growth, FCR, and PER performance for sturgeon (*Acipenser baeri*). Rincón *et al*. (2012) observed that 30% of FM substitution for *Spirulina* results in the best growth and FCR for *Oreochromis sp*. Velasquez *et al*. (2016) found that the best FCR and PER could be achieved at 30% of *Spirulina* substitution in tilapia (*Oreochromis niloticus*). In the present study, we demonstrated that up to 50% of FM might be substituted with *A. platensis* in diets for juvenile mullets, showing that this level of inclusion produced the best weight gain, FCR, and PER results. However, total substitution of FM (D5) resulted in reduced growth performance and survival (47.62%).

The poor growth and survival rates observed at full FM substitution (D5) might be related to an imbalance in FA and AA. Since AA requirements are species-specific, the quality of dietary AA may affect growth, survival, or

both (Li *et al.,* 2009). For this reason, FA and AA compositions were evaluated for all diets. Lipid analyses demonstrated no variation in the quantity of essential fatty acids (EFA) among the tested diets. Neither the content of total n-3 HUFA nor the content of DHA or DHA/EPA ratio presented differences between diets that might have accounted for the survival and growth results. However, the AA calculation table showed a decrease in histidine (-18.44%) and lysine (-21.01%), when comparing the full FM diet (D1) and the 100% *A. platensis* diet (D5). Each of these AA plays an important role in fish growth and survival (Hauler and Carter, 2001).

Borlongan and Coloso (1993) observed that a histidine deficiency could result in elevated mortality and small differences in Milkfish (*Chanos chanos*) growth. Waagbø *et al*. (2010) found a promotion in salmon (*Salmo salar*) growth for diets supplemented with histidine, while lysine deficiency had no effect on survival but could strongly affect growth. The effects of lysine deficiency are observed in many species, including red sea bream (*Pagrus major*) (Forster *et al.,* 1998), striped bass (*Morone saxatilis)* (Small and Soares, 2000), and black sea bream (*Sparus macrocephalus*) (Zhou *et al.,* 2010). As such, it is clear that deficiency of these AAs in *A. platensis* might affect mullet survival and growth.

Immune system enhancement through the diet has been widely used to improve health and growth of cultured fish (Pohlenz and Gatlin, 2014). *Spirulina* has been tested for its immune-stimulant properties in fish feed at low inclusion levels (up to 10% inclusion). It has several effects on the immune system of fish species, by enhancing phagocyte, serum and complement activity in carp (*Cyprinus carpio*) and trout (*Onchorincus mykiss*) (Amar *et al.,* 2004; Watanuki *et al.,* 2006), increasing resistance against pathogens in shrimp (*Litopenaeus vannamei*) and tilapia (*Oreochromis niloticus*) (Tayag *et al.,* 2010; Ragap *et al.,* 2012), improving hematocrit and lysozyme activity in tilapia (*Oreochromis niloticus*) (Ibrahem *et al.,* 2013), and activating leucocyte functions (Adel *et al.,* 2016), among other effects. However, to the best of our knowledge, there are no reports in the literature regarding the influence of full substitution of FM for *Spirulina* in fish diets with respect to immune stimulation.

According to Simsek *et al*. (2007), WBC production in rats is increased by dietary addition of *Spirulina*, with changes in the proportions of these cells. In the present report, significant differences were found in monocyte and lymphocyte counts between D2 and D3 treatments. In particular, the D3 treatment resulted in the lowest monocyte proportion (1.4 ± 1.14%) and elevated lymphocytes (91 ± 2.38%). This same trend was observed by Abdel-Tawwab and Ahmad (2009), where the lowest monocyte and the highest lymphocyte production matched the diet that showed the best growth for tilapias fed *Spirulina* inclusion (5%). Watanuki *et al.* (2006) observed an increase in macrophage activity in diets supplemented with *Spirulina* in carp (*Cyprinus carpio*). This effect was also obtained by supplementing β-carotene in trout (*Oncorhinchus mykis*), which increased the protection of macrophage receptors against oxidative stress (Amar *et al.,* 2000); *Spirulina* is known to be a rich source of carotenoids, such as β-carotene (Leema *et al.,* 2010). These findings suggest a more efficient response to stress (monocyte activity) when β-carotene is present, which might account for the decrease in the production of this cell type observed here.

The spleen is a secondary immune organ consisting of lymphoid tissue, in which maturation of T-Cells occurs (Manning, 1994). The T-Cell co- receptor expression has been linked to the speed of development of lymphoid tissue (Miceli and Parnes, 1993). The CD3 complex technique has been recently used in fishes to detect the CD3 co-receptors on the T-Cells (Øvergard *et al.*, 2009). The CD3 co-receptor analyses of spleen cells did not show any significant difference between treatments. Although fish from the 50% treatment showed the highest quantity of CD3 co-receptors (10.8 ± 5.95 mm^2), and those of the control treatment had the lowest reaction (3.1 ± 1.57 mm^2), this did not reach statistical significance. In some monogastric animals, β-carotene can be metabolized into various vitamin A metabolites, including Retinoic acid (RA) (Wang, 1994). RA can enhance T-Cell proliferation and stimulate a more accurate immune response (Ertesvag *et al.,* 2002, Ross, 2012). In this context, *Spirulina* has been shown to stimulate proliferation of T-Cells on lymphoid tissue in rats (Simsek *et al.,* 2007), increase lymphocyte proliferation in the spleen of parrotfish (*Oplegnathus punctatus*) (Tachibana *et al.,* 1997), and trout (Amar *et al.*, 2000). Our results suggest that *Spirulina* might have stimulated the development of

lymphoid tissue, which results in a major detection of CD3 co-receptors. Moreover, Führ *et al*. (2016) suggested that a greater quantity of expressed CD3 co-receptors might represent the most immune stimulated physiological situation in fish.

Cell deletion is an important mechanism for health and disease maintenance (Elmore, 2007). Apoptosis might occur as a controlled alternative to infected cells (Valentim-Neto *et al.*, 2014), but also via the incapacity of cells to resist ambient stressors (Tabas and Ron, 2011). Ibrahem *et al*. (2013) found that S*pirulina* might promote expression of the P53 protein, which plays an important role in cell maintenance and repair in tilapia. Here we detected significant differences in apoptotic expression between the treatment without *A. platensis* (D1) and the treatment with 50% substitution (D3). In this case, the decrease in the apoptotic process in organisms not exposed to pathogen agents is probably due to a more efficient cell response to oxidant damage and repair. The same response was observed by Chu *et al*. (2010) with *Spirulina* extract in fibroblast cells, and Macias-Sancho *et al*. (2014), wherein less apoptotic cells were observed in white shrimp feed with *Spirulina.*

In conclusion, *A. platensis* might be suitable as a FM substitute (up to 50%) in a practical fish diet for mullet. Also, partial substitution of FM for *A. platensis* affected the proportion of WBCs, improving non-specific cellular immune response of mullets by increasing the production of T-Cells and decreasing cell apoptosis.

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

References

Abdel-Tawwab M, Ahmad M. Live *Spirulina* (*Arthrospira platensis*) as a growth and immunity promoter for Nile tilapia, (*Oreochromis niloticus L.*), challenged with pathogenic *Aeromonas hydrophila*. Aquac Res 2009; 40: 1037–1046.

Adel M, Yeganeh S, Dadar M, Sakai M, Dawood MAO. Effects of dietary *Spirulina platensis* on growth performance, humoral and mucosal immune responses and disease resistance in juvenile great sturgeon (*Huso huso* Linnaeus, 1754). Fish Shellfish Immunol 2016; 56: 436–444.

Amar EC, Kiron V, Satoh S, Okamoto N, Watanabe T. Effects of dietary β-carotene on the immune response of rainbow trout *Oncorhynchus mykiss*. Fisheries Sci 2000; 66: 1068–1075.

Amar EC, Kiron V, Satoh S, Watanabe T. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss*) associated with dietary intake of carotenoids from natural products. Fish Shellfish Immunol 2004; 16: 527–537.

American Public Health Association (APHA). Standard methods for the examination of water and wastewater, 21 st ed. APHA, Washington, 2005 pp.1193.

Association of Official Analytical Chemists. Official methods of analysis of the AOAC. Washington: AOAC International. 16 ed. 2000; pp 1141.

Belay A. The Potential Application of *Spirulina* (*Arthrospira*) as a Nutritional and Therapeutic Supplement in Health Management. JANA 2002; 5, 26–48.

Benderschneider K, Robinson RJ. A new spectrophotometric method for determination of nitrate in seawater. J Mar Res 1952; 1: 69–87.

Borlongan IG, Coloso RM. Requirements of juvenile milkfish (*Chanos chanos Forsskal*) for essential amino

acids. J Nutri 1993; 123: 125–132.

Carvalho C, Bianchini A, Tesser MB, Sampaio LA. The effect of protein levels on growth, postprandial excretion and tryptic activity of juvenile mullet *Mugil platanus* (Günther). Aquac Res 2010; 41: 511–518.

Charriaut-Marlangue C, Ben-Ari Y. A cautionary note on the use of the TUNEL stain to determine apoptosis. Neuroreport 1995; 7: 61–64.

Christie GW. Lipid analysis. Pergamon Press.1982; 207.

Chu WL, Lim YW, Radhakrishnan AK, Lim PE. Protective effect of aqueous extract from *Spirulina platensis* against cell death induced by free radicals. B.M.C. Complem Alterna Med 2010; 10: 53.

Elmore S. Apoptosis: a review of programmed cell death. Toxicol Path 2007; 35: 495–516.

El-Sayed AFM. Evaluation of soybean meal, *Spirulina* meal and chicken offal meal as protein sources for silver seabream (*Rhabdos argussarba*) fingerlings. Aquac 1994; 127: 169–176.

Ertesvag A, Engedal N, Naderi S, Blomhoff HK. Retinoic acid stimulates the cell cycle machinery in normal T cells: involvement of retinoic acid receptor-mediated IL-2 secretion. J Immunol 2002; 169: 5555–5563.

Folch J, Lees M, Stanley G. A simple method for isolation and purification of total lipids from animal tissues. J Bio Chem 1957; 226: 497–509.

Forster I, Ogata HY. Lysine requirement of juvenile Japanese flounder *Paralichthys olivaceus* and juvenile red sea bream *Pagrus major*. Aquac 1998; 161: 131–142.

Führ F, Tesser MB, Rodrigues RV, Pedron J, Romano LA. Artemia enriched with hydrolyzed yeast improves growth and stress resistance of marine pejerrey *Odontesthes argentinensis* larvae. Aquac 2016; 450: 173–181.

Galvão MSN, Fenerich-Verani N, Yamanaka N, Oliveira IR. Histologia do sistema digestivo da tainha *Mugil platanus* Günther, 1880 (*Osteichthyes, Mugilidae*) durante as fases larval e juvenil. B Inst Pesca 1997; 24: 91–100.

Habib MAB, Parvin M, Huntington TC, Hasan MR. A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals and fish. Food and Agriculture Organization of the United Nations. 2008.

Hauler RC, Carter CG. Reevaluation of the quantitative dietary lysine requirements of fish. Reviews in Fish Sci 2001; 9: 133–163.

Hsu SM, Raine L, Fanger H. Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC an unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29: 577–585.

Ibrahem MD, Mohamed MF, Ibrahim MA. The role of *Spirulina platensis* (*Arthrospira platensis*) in growth and immunity of Nile tilapia (*Oreochromis niloticus*) and its resistance to bacterial infection. J Agric Sci 2013; 5*:* 109.

Jafari SMA, Rabbani M, Emtyazjoo M, Piryaei F. Effect of dietary *Spirulina platensis* on fatty acid composition of rainbow trout (*Oncorhynchus mykiss*) fillet. Aquac Int 2014; 22: 1307–1315.

Leema JM, Kirubagaran R, Vinithkumar NV, Dheenan PS, Karthikayulu S. High value pigment production from Arthrospira (*Spirulina*) platensis cultured in seawater. Biores Techno 2010; 101: 9221–9227.

Li P, Mai K, Trushenski J, Wu G. New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids 2009; 37*:* 43–53.

Macias-Sancho J, Poersch LH, Bauer W, Romano LA, Wasielesky W, Tesser MB. Fishmeal substitution with *Arthrospira* (*Spirulina platensis*) in a practical diet for *Litopenaeus vannamei*: Effects on growth and immunological parameters Aquac 2014; 426–427, 120–125.

Manning MJ, Turner RJ. Immunology: a comparative approach. John Wiley, New York, 1994; pp 69–100.

Merrill AL, Watt BK. Energy value of foods: basis and derivation. United States Department of Agriculture (U.S.D.A.), Handbook 1973; pp 74.

Miceli MC, Parnes JR. Role of CD4 and CD8 in T cell activation and differentiation. Advances Immuno 1993; 53: 59–122.

Nandeesha M, Gangadhar C, Varghese T, Keshavanath P. Effect of feeding *Spirulina platensis* on growth proximal composition and organoleptic quality of common carp, *Cyprinus carpio* L. Aquac Res 1998; 29: 305–313.

NRC (National Research Council). Nutrient Requirement of Fish and Shrimps. National Academy Press, Washington. 2011.

Oliva-Teles A. Nutrition and health of aquaculture fish. J Fish Disea 2012; 35: 83–108.

Øvergard AC, Hordvik I, Nerland AH, Eikeland G, Patel S. Cloning and expression analysis of Atlantic halibut (*Hippoglossus hippoglossus*) CD3 genes. Fish Shellfish Immunol 2009; 27: 707–713.

Palmegiano GB, Agradi E, Forneris G, Gai F, Gasco L, Rigamonti E, Zoccarato I. *Spirulina* as a nutrient source in diets for growing sturgeon (*Acipenser baeri*). Aquac Res 2005; 36: 188–195.

Palmegiano GB, Gai F, DapraØ F, Gasco L, Pazzaglia M, Peiretti PG. Effects of *Spirulina* and plant oil on the growth and lipid traits of white sturgeon (*Acipenser transmontanus*) fingerlings. Aquac Res 2008; 39: 587–595.

Pohlenz C, Gatlin DM. Interrelationships between fish nutrition and health. Aquac 2014; 431: 111–117.

Ragap HM, Khalil RH, Mutawie HH. Immunostimulant effects of dietary *Spirulina* platensis on tilapia *Oreochromis niloticus*. J Appl Pharma Sci 2012; 2: 26.

Ramos LRV, Romano LA, Monserrat JM, Abreu PC, Verde PE, Tesser MB. Biological responses in mullet *Mugil liza* juveniles fed with guar gum supplemented diets. Animal Feed Sci and Tech 2015; 205: 98–106.

Ravi M, Lata S, Azharuddin DS, Paul S. The beneficial effects of *Spirulina* focusing on its immunomodulatory and antioxidant properties. Nutri Dietary 2010; 2: 73–83.

Rincón DD, Velásquez, HA, Dávila MJ, Semprun AM, Morales ED, Hernández JL. Substitution levels of fish meal by Arthrospira (= Spirulina) maxima meal in experimental diets for red tilapia fingerlings (*Oreochromis sp*.). Revista Colombiana de Ciencias Pecuarias 2012; 25: 430–437.

Ringø E, Olsen RE, Gifstad TØ, Dalmo RA, Amlund H, Hemre GI, Bakke AM. Prebiotics in aquaculture: a review. Aquac Nutri 2010; 16: 117–136.

Romano LA, Ferder MD, Stella IY, Inserra F, Ferder LF. High correlation in renal tissue between computed image analysis and classical morphometric analysis. J Histotechnol 1996; 19: 121–123.

Romano LA, Marozzi V, Zenobi C. Utilización de anticuerpos humanos en la marcación de receptores CD3 y CD4 de linfocitos en *Xiphophorus helleri*. Na Soc Cient Argent 2004; 43: 123–127.

Ross AC. Vitamin A and retinoic acid in T cell–related immunity. American J Clin Nutri. 2012; 96: 1166S–1172S.

Salhi M, Bessonart M. Growth, survival and fatty acid composition of *Rhamdia quelen* (Quoy and Gaimard, 1824) larvae fed on artificial diet alone or in combination with Artemia nauplii. Aquac Res 2013; 44: 41–79.

Simsek N, Karadeniz A, Karaca T. Effects of the *Spirulina platensis* and *Panax ginseng* oral supplementation on peripheral blood cells in rats. Revue Méd Vét 2007; 158: 483–488.

Small BC, Soares JR. Quantitative dietary lysine requirement of juvenile striped bass *Morone saxatilis*. Aquac Nutri 2000; 6: 207–212.

Strickland JL, Parsons TR. A practical handbook of seawater analysis. Fish. Res. Board Can. Bull, Ottawa. 1972.

Tachibana K, Yagi M, Hara K, Mishima T, Tsuchimoto M. Effects of feeding of β-carotene-supplemented rotifers on survival and lymphocyte proliferation reaction of fish larvae (Japanese parrotfish (*Oplegnathus fasciatus*) and Spotted parrotfish (*Oplegnathus punctatus*)): preliminary trials. In. Live Food in Aquac 1997; 313–316.

Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 2011; 13: 184–190.

Tayag CM; Lin YC, Li CC, Liou CH, Chen JC. Administration of the hot-water extract of *Spirulina platensis* enhanced the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. Fish Shellfish Immunol 2010; 28: 764–773.

Teimouri M, Yeganeh S, Amirkolaie AK. The effects of *Spirulina platensis* meal on proximate composition, fatty acid profile and lipid peroxidation of rainbow trout (*Oncorhynchu s mykiss*) muscle. Aquac Nutri 2015; 22: 559–566.

Valentim-Neto PA, Fraga AP, Marques MR. 2014. Differential expression of proteins in the gills of *Litopenaeus vannamei* infected with white spot syndrome virus. Aquac Inter 22, 1605–1620.

Vieira JP. Juvenile *Mullets* (Pisces: *Mugilidae*) in estuary of lagoa dos Patos, RS, Brazil. Copeia 1991; 2: 409–418.

Velasquez SF, Chan MA, Abisado RG, Traifalgar RFM, Tayamen MM, Maliwat GCF, Ragaza JA. Dietary Spirulina (Arthrospira platensis) replacement enhances performance of juvenile Nile tilapia (*Oreochromis niloticus*). J Appl Phyco 2016; 28: 1023–1030.

Waagbø R, Tröße C, Koppe W, Fontanillas R, Breck O. Dietary histidine supplementation prevents cataract development in adult Atlantic salmon, *Salmo salar* L., in seawater. Brit J Nutri 2010; 104: 1460–1470.

Wang XD. Review: absorption and metabolism of beta-carotene. J Ame Col Nutri 1994; 13: 314–325.

Watanuki H, Ota K, Malina AC, Tassakka AR, Kata T, Sakai M. Immunostimulant effect of dietary *Spirulina platensis* on carp, *Cyprinus carpio*. Aquac 2006; 258: 157–163.

Weibel ER. Stereological methods. Vol. 1. Practical methods for biological morphometry. J Microsc 1981; 121: 131–132.

Zamora-Sillero J, Ramos LRV, Romano LA, Monserrat JM, Tesser MB. Effect of dietary dextrin levels on the growth performance, blood chemistry, body composition, hepatic triglicerides and glycogen of Lebranche mullet juveniles (*Mugil liza* Valenciennes 1836, *Mugilidae*). J Appl Ichthyol 2013; 29: 1342–1347.

Zhou F, Shao J, Xu R, Ma J, Xu Z. Quantitative L-lysine requirement of juvenile black sea bream (*Sparus macrocephalus*). Aquac Nutri 2010; 16: 194–204.