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Utilização de *Arthrospira platensis* em dietas para juvenis de tainha (*Mugil liza*)

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Utilização de *Arthrospira platensis* em dietas para juvenis de tainha (*Mugil liza*)

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Sumário

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Resumo

A utilização de ingredientes alternativos na aquicultura é um campo de estudo da nutrição, onde seus objetivos são a melhoria nos parâmetros zootécnicos, por estimulação dos sistemas imunológico e antioxidante de peixes. A cianobactéria *Spirulina* (= *Arthrospira platensis*) é reconhecida pelo seu alto teor proteico (60-70%) e também pelos amplos benefícios que proporciona à saúde, razão pela qual é utilizada na nutrição animal e humana. Para analizar seu uso na alimentação de juvenis de tainha (*Mugil liza*), três experimentos foram planejados; 1) cinco dietas experimentais foram elaboradas para se avaliar a substituição parcial e total da farinha de peixe por *Spirulina* (0, 30, 50, 70 e 100%). As dietas foram ofertadas por 80 dias, quando foi avaliado o efeito dessa inclusão no sistema imunológico inespecífico. 2) Baseado nos resultados do primeiro ensaio foi realizada a substituição concomitante parcial e total da farinha e óleo de peixe por *Spirulina* e óleo de linhaça (0, 30, 50, 70 e 100%). Além disso, foi incluída lisina para melhorar a qualidade proteica da *Spirulina*. O experimento teve duração de 75 dias. Os efeitos dessa substituição foram avaliados no sistema antioxidante e na qualidade do filé. 3) Foi comparado o efeito da inclusão de *Spirulina* (0, 5 e 10%) contra a inclusão de β-caroteno (50mg kg^{-1}). As dietas experimentais foram oferecidas por 75 dias e, após o período experimental foram avaliados os seus efeitos no sistema antioxidante e na qualidade do filé. 4) Neste último capítulo foi realizado uma revisão sobre a substituição e inclusão de *Spirulina* em dietas para a aquicultura, compilando grande parte da pesquisa nesta área e avaliando o porque o uso de *Spirulina* a nível comercial ainda não é uma realidade. Nossos primeiros resultados mostraram que até 50% da farinha de peixe pode ser substituída com benefícios no crescimento e na estimulação para a produção de linfócitos T e na estimulação para a apoptose. O segundo ensaio demonstrou que com dupla substituição e inclusão de lisina pode-se substituir até 70% da farinha de peixe sem prejudicar o crescimento da tainha. Também foi evidenciado que atributos como cor e carotenoides no músculo não aportam benefícios além dos 30% de inclusão em *Spirulina*, e que este suplemento aumenta as capacidades do sistema antioxidante. Por fim, neste expeirmento se concluiu que as tainhas acumulam ácidos graxos essenciais e que em grande parte refletem o perfil das dietas. No terceiro experimento verificou-se que a *Spirulina* e o β-caroteno trazem benefícios ao crescimento e na qualidade do filé. A *Spirulina* aporta mais benefícios do que o β-caroteno no sistema antioxidante da tainha.

Finalmente se avaliou que a produção da *Spirulina* tem custo elevado quando considerada a volatilidade do preço da farinha de peixe, assim mais alternativas são propostas no sentido de reduzir os custos em um futuro próximo. Como conclusão geral da tese acreditamos que a inclusão da *Spirulina* em dietas comerciais poderia se tornar uma realidade, pois ainda que em pouca quantidade, agrega beneficios à saúde dos organismos, o que de maneira geral, protege a produção de riscos inerentes à aquicultura.

P**alavras Chave**: Antioxidante, carotenoides, imuno estimulante, peixe, microalga, *Spirulina*.

Abstract

The use of alternative ingredients in aquaculture is a field of study of nutrition, the objectives of this research are beyond the search for improvements in growth, also are the stimulation of the fish immune system and the antioxidant system. The cyanobacterium *Spirulina* (*A. platensis*) has been recognized for its high protein content (60-70%) and also for its multiple health benefits, the reason for why it is so used in animal and human nutrition. To evaluate its benefits in the cultivation of mullet juveniles, three experiments were planned; 1) A total of 5 experimental diets were prepared in which the total and partial replacement of the fish meal by *Spirulina* meal 0, 30, 50, 70 and 100% were made, the diets were offered for 80 days, after the experimental period the effect of this inclusion was evaluated on growth and the immune system. 2) Based on the previous results, a total and partial replacement of fish meal and oil was made by *Spirulina* and linseed oil 0, 30, 50, 70 and 100%, lysine was also included to improve the protein quality of *Spirulina*. Experimental diets were offered for 75 days. The effects of this substitution were evaluated in the antioxidant system and in the quality of the fillet. 3) The effect of the inclusion of *Spirulina* (0, 5 and 10%) against the inclusion of β-carotene (50mg kg-1) was made. The experimental diets were offered for 75 days and, after the experimental period their effects were evaluated antioxidant system and fillet quality. 4) In this last chapter, a review on the replacement and inclusion of *Spirulina* in aquaculture diets was compiled, compiling much of the research in this area and evaluating why the commercial use of *Spirulina* is not yet a reality. Our first results showed that up to 50% of fishmeal can be replaced with *Spirulina* and have benefits in growth and stimulation for T-lymphocyte production and stimulation for apoptosis. The other results showed that with double substitution and addition of lysine we can substitute up to 70% of fish meal without prejudicing the growth of mullet. Also was found that attributes such as color and carotenoids in the muscle have no benefits beyond 30% inclusion of *Spirulina*, and that this suplement increas the capabilities of the antioxidant system. In our last experiment results was verify that *Spirulina* and β-carotene may have the same benefits in growth and fillet quality, but *Spirulina* overcomes the benefits of β-carotene in the antioxidant system of mullet. And finally assess that the production of *Spirulina* is high costs compared with even the volatility of the price of fishmeal, more alternatives are proposals that could reduce costs in the near future. As a

general conclusion of the thesis, we believe that the inclusion of *Spirulina* in commercial diets could become a reality since even in a small amount it adds many benefits to the health of the organisms, which in general could protect the production of many of inerent risks from aquaculture.

Key words: Antioxidant, carotenoids, immune stimulant, fish, microalgae, *Spirulina*.

1. Introdução Geral

A produção da aquicultura no ano 2014 atingiu 73 milhões de toneladas, o que representou 44.1% da produção anual de pescado no mundo. Mais de 580 espécies são produzidas globalmente o que inclui peixes, crustáceos e moluscos (FAO, 2016). Em um cenário onde os estoques pesqueiros estão sobre explorados a aquicultura pode atuar para suprir o aumento da demanda, uma vez que o consumo anual *per capita* alcançou 20 kg (FAO, 2016). O rápido crescimento da aquicultura tem sido apoiado pela indústria produtora de alimentos que teve crescimento anual estimado de 30%. Uma das limitantes no crescimento da aquicultura é o alto valor da ração de peixe, pois é o insumo na produção, podendo alcançar 60% dos custos de produção (Knapp, 2008), o que pode afetar a sustentabilidade econômica desta atividade.

A produção anual de farinha de peixe (FP) e de óleo de peixe (OP) tem atingido seu limite nos últimos anos, o que tem levado a um aumento no preço desses produtos especialmente no OP (Tacon e Metian, 2015). A aquicultura consome cerca de 75% da produção total de OP, enquanto que o consumo humano de OP utiliza 22% da produção total, sendo os 3% restantes utilizados nas industrias farmacêuticas. (FAO, 2016). O crescimento da aquicultura dependerá de práticas sustentáveis que permitam diminuir o uso desses produtos como os principais componentes das dietas aquícolas. A procura de ingredientes alternativos que possam substituir-los é uma prioridade de estudo segundo o National Reasearch Council (NRC, 2011), e uma das principais linhas de pesquisa da nutrição aquícola. A utilização de ingredientes de origem vegetal e de subprodutos de origem animal tem sido estudada, mas é limitada por fatores como:

1) Fatores anti-nutricionais - Os ingredientes de origem vegetal contém fatores anti-nutricionais tais como saponinas, lectinas ou inibidores de proteinases. As consequências de seu consumo podem promover redução da ingesta e da digestibilidade, imunossupressão e redução de crescimento (Francis *et al.*, 2001; Krogdahl *et al.*, 2010). Os subprodutos de origem de peixe também podem conter toxinas como a tetrodotoxina ou a dinogunellina que são lipoproteínas tóxicas (Halver e Hardy, 2002).

2) Desequilíbrio no conteúdo de aminoácidos essenciais (AAE). Os peixes marinhos têm em muitos casos hábitos alimentares carnívoros ou onívoros, a substituição de FP por farinhas vegetais tem implica em alteração doperfil de aminoácidos (AA), em especial por lisina, metionina e triptofano (Kaushik e Seiliez, 2010; Oliva-Teles *et al.*, 2015).

3) Desequilíbrio no conteúdo de ácidos graxos essenciais (AGE) nas dietas, em especial o ácido graxo eicosapentaenoico (EPA; 20:5n−3) e o docosahexaenoico (DHA; 22:6n−3) (Sargent, 2002; Tocher, 2015). Ácidos graxos poli-insaturados (PUFA) são essenciais em dietas de peixes, pois, os vertebrados não tem a capacidade de sintetizar estes compostos pela falta de enzimas elongases e desaturases. A substituição parcial de FP (25-50%) tem sido realizada para muitas espécies aquícolas como salmão, truta ou dourada, mas o crescimento da aquicultura impede uma diminuição na pressão pela utilização deste ingrediente (Hardy, 2010).

4) Gorduras oxidadas. O alto teor de lipídeos dos produtos de origem animal pode ser facilmente oxidado, em especial os produtos com alto teor de PUFAS, o consumo de produtos ranços (gordura oxidada) pode provocar mortalidade, degeneração do fígado e anemia em peixes (Halver e Hardy, 2002).

As diversas limitações que a aquicultura tem como o uso limitado de recursos como água e terra têm levado ao incremento na tecnificação dessa atividade, com o fim de aumentar a produção e o lucro com os mesmos recursos. Este fato provoca um incremento das densidades de produção o que teve consequências em diferentes setores da produção como o aumento da incidência de doenças (parasitos, vírus ou bactérias), toxicidade por acumulação de metabólitos do catabolismo dos organismos na água ou quedas no oxigênio em sistemas de criação. Por essas razões, outro desafio na nutrição aquícola surge; produzir alimento que além de cumprir as necessidades nutricionais do peixe, possam ser utilizados como uma ferramenta que incremente tanto a qualidade do produto final como sua saúde e resistência à fatores estressores, o que permitirá a sustentabilidade futura da aquicultura (Oliva-Telez, 2012; Pohlenz e Gatlin, 2014).

Dentre as alternativas existentes para resolver os desafios na nutrição aquícola, destaca-se o uso de microalgas, seja pelo seu potencial nutricional ou pela variedade de

espécies disponíveis. Dentre a variedade de microalgas existentes encontram-se especies com alto teor proteico, bom conteúdo de AGE e alta digestibilidade. Outro fator importante é que as microalgas podem ser produzidas de forma contínua e controlada. Além disso, as mesmas trazem benéficos para a saúde, uma vez que estes organismos são ricos em sustâncias antioxidantes, imuno-estimulantes ou probióticas entre muitas outras caraterísticas (Brown, 2002; Harun *et al.*, 2010).

1.1 Spirulina

A microalga *Arthrospira platensis* conhecida genericamente como *Spirulina* é uma microalga da classe *Cyanophyceae*, ordem Nostocales caracterizada por sua forma em hélice (Palmegiano *et al.*, 2008). Como a maioria das cianobactérias, a *Spirulina* é um organismo foto-autotrófico e não pode crescer na escuridão. São encontradas em diversos de ambientes, particularmente alcalinos e águas salobras ou salinas. A *spirulina* é considerada boa fonte de proteína além de conter componentes que apresentam efeitos positivos em diversas áreas da fisiologia animal (Ravi *et al.*, 2010). A Tabela 1 contém as características nutricionais da *Spirulina*.

Sua composição nutricional e a proporção de sustâncias ativas que contém podem ser modificadas pelo meio de cultivo onde é produzida. A produção de biomassa de *Spirulina* para tratar águas residuais resultantes da indústria, da agricultura ou até da aquicultura tem sido testado com sucesso como uma alternativa que transforma poluição em um produto alimentar de alta qualidade (Cheunbarn e Peerapornpisal, 2010; El-Kassas *et al.*, 2015; Wuang *et al.*, 2016). O seu cultivo pode ser realizado em tanques abertos ou em foto-biorreatores fechados onde precisará de alguns elementos traço como ferro, iodo, selênio, zinco, cobre ou manganésio. Os benefícios do cultivo da *spirulina* incluem uma menor utilização de área e água de cultivo, na comparação com outro alimento altamente proteico como a soja utiliza 20 vezes menos terra e um terço menos da água. As descargas de águas de cultivo não contém praticamente nenhum contaminante e ajuda a fixar dióxido de carbono do ambiente (Gershwin e Belay, 2007).

Tabela1. Componentes da *Spirulina* (*A. platensis*) e sua importância nutricional.

Nutriente	% Composição	Características	Referência
Proteína	55 – 70	9 aminoácidos essenciais: isoleucina, leucina, lisina, metionina, histidina, fenilalanina, treonina, triptófano e valina	1, 2.
Lipídeos	4 -6	Ácido Gamma-linolenico (GLA), ácido alpha-linolênico (ALA), ácido linoleico (LA), ácido estearidônico (SDA).	1, 2.
Carboidratos	17.8	Fibra. Ramnose (54%) Glicose (14%), ribose (10%) galactose, xilose e manose (menos de 5% dos açúcares totais)	3, 4.
Vitaminas	0.75	B1 tiamina, B2 riboflavina, B3 nicotinamida, B6 piridoxina, B9 ácido fólico, B12 cianocobalamina, vitamina C, vitamina D e vitamina E.	2, 3.
Minerais	8	Potássio, cálcio, cloro, cobre, ferro, magnésio, manganésio, fósforo, selênio, sódio e zinco.	2, 3.
Carotenoides	300-500 mg kg^{-1}	Alpha-caroteno, betacaroteno, xantofila, criptoxantina, equinenona, zeaxantina e luteína.	1, 2, 3.
Outros		Clorofila, ficocianina, porfirina, ficoeritrina.	1, 2, 3.
Umidade	3.4		1.

1) Ravi *et al.*, (2010);2) Habib *et al.*, (2008); 3), Gershwin e Belay (2007); 4) Chaiklahan *et al.*, 2013.

1.2 Efeitos da *Spirulina* em parâmetros zootécnicos

Essa microalga tem sido reconhecida mundialmente pela melhora nutricional que pode aportar na nutrição humana e animal. O seu uso na aquicultura tem sido amplamente estudado em muitas espécies. Alguns autores relatam efeitos benéficos como a melhora no crescimento

dos peixes pela estimulação na produção de enzimas ou pelo seu alto valor nutricional (Abdel-Tawwab e Ahmad, 2009; Adel *et al.*, 2016). A dose de *Spirulina* utilizada nas rações de organismos aquáticos difere amplamente, benefícios no crescimento e sobrevivência têm sido reportados para juvenis com doses de 0,5-1% de inclusão (Vonshak, 1997). Doses até 10% da inclusão também demonstraram benefícios no crescimento de peixe-gato gigante (*Pangasianodon gigas*) (Tongsiri *et al.*, 2010), peixe-papagaio (*Oplegnathus fasciatus*) (Kim *et al.*, 2013), truta-arco-íris (Teimouri *et al.*, 2013) e esturjão (Adel *et al.*, 2016). Com o objetivo de substituir grandes quantidades de FP, tem sido testadas doses que vão de 25 a 100% de substituição, com resultados variáveis. Nandeesha *et al.* (1998) observaram em carpa comum (*Cyprinus carpio* L.) que a inclusão de 75% de *Spirulina* na dieta não afetava os parâmetros zootécnicos, mas incrementou a digestibilidade proteica. Resultados similares foram encontrados por Nandeesha *et al.* (2001), que substituíram de maneira exitosa 100% da farinha de peixe por *Spirulina* em dietas para carpas *Catla catla* e *Labeo rohita*, observando ainda redução na deposição de lipídeo na carcaça. Para juvenis de tilapia *Oreochromis mossambicus*, o melhor nível de substituição encontrado foi de 40% da farinha de peixe (Olvera-Novoa *et al.*, 1998). Palmegiano *et al.* (2005) reportaram que o esturjão alimentado com dietas que incluem 50% *A. platensis* atingiram bons resultados no crescimento e parâmetros zootécnicos. A substituição total de FP por *Spirulina* tem alguns impedimentos, principalmente na composição proteica, pois ao ser a principal fonte proteica da ração pode ser deficiente em alguns dos AAE principalmente metionina, lisina e histidina (Nandeesha *et al.*, 1998; Olvera-Novoa *et al.*, 1998, Macias-Sancho *et al.*, 2014).

Além dos seus benefícios no crescimento outros benefícios têm sido achados no produto final do peixe como melhora na cor, sabor do filé cozido ou aumento no tempo de prateleira (Nandeesha, 1998; Teimouri *et al.*, 2013; Teimouri *et al.*, 2016). Os carotenoides que a *Spirulina* apresenta (principalmente zeaxantina e β-caroteno) são acumulados no músculo dos peixes e eles têm um efeito direto em sua coloração (Torrissen e Christiansen, 1995). A absorção dos carotenoides é influenciada por diferentes fatores, pois nem todos os peixes possuem as mesmas capacidades de catabolizar carotenoides, além disso, diferentes tecidos têm diferentes capacidades de absorção (Torrissen, 1989). A quantidade de lipídeos na

ração apresenta papel importante na quantidade de carotenoides a ser absorvidos sendo que os carotenoides são sustâncias lipofílicas (van het Hof *et al.*, 2000).

1.3 *Spirulina* no sistema imunológico

A principal função do sistema imunológico em animais é detectar e depois neutralizar ou destruir patógenos invasores, como vírus, bactérias, fungos e parasitas. E em segundo lugar a eliminação de células anormais. Para ter sucesso, os organismos vertebrados têm desenvolvido dois sistemas, o inato e o adaptativo (Bayne e Gerwick, 2001). Os peixes contam com os dois sistemas imunes adaptados para o ambiente aquático que funciona dentro dos limites da natureza poiquiliterma do peixe (Tort *et al.*, 2003). Dentro do meio aquático é normal a interação do peixe com organismos patógenos, onde as defesas inatas do peixe (não específicas) serão o primeiro meio de defesa e em caso de ser insuficientes ativarão a imunidade adaptativa (específica) para garantir sua sobrevivência (Magnadóttir, 2006). Essas respostas podem ser celulares (p.e. macrófagos) ou humorais (p.e. lisozima) (Bayne e Gerwick, 2001). A aquicultura tende a submeter aos peixes a grandes densidades de estocagens o que aumentará a pressão dos patógenos sobre os peixes, as constantes abrasões na pele do peixe podem diminuir a eficiência das principais defesas do peixe como o muco, um fator que pode dar origem a uma doença.

Os órgãos dedicados à manutenção da imunidade nos peixes são: o rim cefálico, como o órgão que mantém a memória imune, também é produtor de antígenos, anticorpos. O baço é um órgão secundário do sistema imune constituído de tecido linfoide onde amadurecem os linfócitos-T, e o timo como gerador de antígenos (Manning, 1994; Press e Evensen, 1999).

A utilização da ração como um ativador do sistema imune tem sido uma ferramenta muito utilizada na aquicultura (Pohlenz e Gatlin, 2014), dando lugar ao campo da nutrição que tem o objetivo de melhorar a função imunológica mediante a utilização de nutrientes específicos (Kirion, 2012). O caso específico da *Spirulina* como um produto ativador do sistema imune tem sido muito estudado na nutrição humana, pois além de ser imuno estimulante tem propriedades antivirais, antibacteriana e o seu uso como terapêutico está em avaliação (Habib *et al.*, 2008; Ravi *et al.*, 2010). Tem sido comprovado que a inclusão de *Spirulina* na dieta de peixes incrementrem a produção de macrófagos e a atividade deles

(Abdel-Tawwab e Ahmad, 2009; Ragap *et al.*, 2012), e inclusive o estrato de *Spirulina* (36% proteína, 10% polissacarídeos) tem estimulado a atividade dos macrófagos peritoniais em ratos (Hayashi *et al.*, 1994). A resposta humoral do sistema imune não específico tem mostrado incremento de atividade da lisozima, da interleucina e a citocina (Mao *et al.*, 2000; Ragap *et al.*, 2012; Ibrahem *et al.*, 2013) o que incrementa a eficiência da atividade bactericida do muco (Adel *et al.*, 2016). Além disso, tem sido documentado o incremento na produção de células sanguíneas brancas e vermelhas, assim como no hematócrito (Andrews *et al.*, 2011; Promya e Chitmanat 2011; Yeganeh *et al.*, 2015), e a diminuição no cortisol (Yeganeh *et al.*, 2015). Atua diretamente na resposta de apoptose em camarão (Macias-Sancho *et al.*, 2014) e na diminuição da expressão da proteína P53 (Ibrahem e Ibrahim 2014). Também já foi reportado que a *Spirulina* atua no incremento da produção de células `natural killers` (Abdel-Tawwab y Ahmad, 2009) e linfócitos T (Hayashi *et al.*, 1994).

O uso de *Spirulina* em dietas de peixes tem mostrado o incremento na resistência a bactérias patógenas (Tabela 2).

Tabela 2. Espécies criadas na aquicultura que ganharam resistência a bactérias patógenas mediante o consumo de *Spirulina*.

Espécie	Bactéria	Referencia
Catfish americano *Ictalurus punctatus*	*Edwardsiella ictalur*	Duncan e Klesius 1996
Carpa (Cyprinus carpio)	*Aeromonas hydrophila*	Watanuki *et al.*, 2006
Tilapia *(Oreochromis niloticus)*	*Aeromonas hydrophila*	Abdel-Tawwab e Ahmad, 2009
Tilapia *(Oreochromis niloticus)*	*Aeromonas hydrophila*	Ragap *et al.*, 2012
Tilapia *(Oreochromis niloticus)*	*Pseudomonas fluorescens*	Ibrahem *et al.*, 2013
Esturjão (*Huso huso*)	*Streptococcus iniae*, *Yersinia ruckeri*, *Aeromonas hydrophila* e *Lactococcus garviea*	Adle *et al.*, 2016

1.4 *Spirulina* no sistema antioxidante

Estresse oxidativo é o excesso na produção de espécies reativas de oxigênio (pela sigla em inglês ROS) durante o metabolismo normal, as quais em excesso não podem ser contidas pela ação de antioxidantes. As espécies reativas de oxigênio e nitrogênio (RONS) compreendem diversas espécies químicas, incluindo ânions superóxido, radicais hidroxilas e peróxidos de hidrogênio, e também peroxinitrito e óxido nítrico. ROS e RONS são moléculas com a capacidade de causar dano em biomoléculas, alterar sinalização de moléculas e reações bioquímicas (Pisoschi e Pop, 2015). Além disso, a peroxidação lipídica causada por ROS é uma causa importante de dano e destruição da membrana celular, pois os ácidos graxos podem gerar rapidamente peróxidos lipídicos (Gershwin e Belay, 2007).

A intensificação da aquicultura tem colocado os organismos em risco de estresse oxidativo por diversas razões, tais como a exposição às grandes quantidades de produtos nitrogenados (Maltez *et al.*, 2017), elevado consumo de ácidos graxos poli-insaturados de cadeia longa (LC-PUFAS) (Oliva-Teles, 2012), ou variações nos níveis de oxigênio na água (Lushchak e Bagnyukova, 2006).

Um antioxidante desde a perspectiva biológica refere-se a qualquer composto, que ao estar presente em baixas concentrações em comparação com o substrato oxidante é capaz de prevenir a oxidação (Halliwell e Gutteridge, 1999). Um sistema de defesa antioxidante é aquele que previne os danos gerados pelas ROS sequestrando radicais livres no citoplasma. Esse sistema pode ser enzimático ou não enzimático, o que tentará remover todas as biomoléculas danificadas antes que aconteçam alterações no metabolismo do organismo (Chesseman e Slater, 1993). Em anos recentes surgiu um grande interesse no uso de antioxidantes como suplementos alimentares (Habib *et al.*, 2008)

Dentre as melhorias no sistema antioxidante produzidas pela *Spirulina*, encontram-se a proteção da atividade das enzimas Glutationa S-tranferase (GST), aumento da glutationa total (GSH), Glutationa reductasse (GR) (Kim *et al.*, 2010), assim como o sequestro do ânion superóxido, e dos radicais hidroxila e peroxil (Estrada *et al.*, 2001; Chaiklahan *et al.*, 2013) e da inibição da peroxidação lipídica (Teimouri *et al.*, 2016) entre muitos outros benefícios. Abdelkhalek *et al.* (2015) mencionaram que a melhora no sistema antioxidante causada pela *Spirulina* pode ser indireta através do aprimoramento da atividade da glutatione peroxidase (GSH-Px), superóxido dismutase (SOD) e catalase (CAT), que são os eliminadores

enzimáticos de radicais livres nas células, ou diretamente por eliminação de radicais livres e inibição da peroxidação lipídica. As principais moléculas antioxidantes da *Spirulina* são: polissacarídeos (Chaiklahan *et al.* 2012), ficocianina (Chen *et al.* 2014) e carotenóides (Ahmed *et al.* 2014), também foi reportada a presença de compostos fenólicos (Kim *et al.*, 2013).

As ficobiliproteínas são um pequeno grupo de proteínas altamente conservadas que constituem o ficobilisómero, um complexo de proteína macromolecular cuja função principal é servir como um complexo de captação de luz para o aparelho fotossintético de cianobactérias (Gershwin e Belay, 2007). Diversos estudos das propriedades antioxidantes da biliproteina ficocianina tem demostrado a capacidade de quelar o ferro, e que seu efeito é dose dependente (Bermejo *et al.*, 2008). Alguns estudos *in vitro* demostraram que a ficocianina pode eliminar radicais hidroxil, alcoxil e diminuir a peroxidação lipídica (Romay *et al.*, 1998), e que é um antioxidante mais efetivo que astaxantina, α-tocoferol e até 20 vezes mais eficiente que o ácido ascórbico (Romay *et al.*, 2003). Por outro lado os carotenoides contidos na *Spirulina*, principalmente o β-caroteno, tem mostrado capacidades antioxidantes como sequestradores de ROS especialmente o óxido super ânion (Krinsky, 2001).

O uso da *Spirulina* tem demostrado um efeito hepato protetor e antioxidante em diversas espécies animais, inclusive em humanos (Belay *et al.*, 2010; Gad *et al.*, 2011; Kim *et al.*, 2010). Na aquicultura tem demostrado ter um bom efeito inibindo a peroxidação lipídica, e como consequência disso incrementando o tempo de prateleira do filé (Abdelkhalek *et al.*, 2015; Teimouri *et al.*, 2016).

1.5 Tainha (*Mugil liza*)

A família Mugilidae contém espécies de tainhas (Figura 1) de grande importância econômica em todo mundo. Inclui 12 gêneros e 80 espécies que habitam climas tropicais e subtropicais (Crosseti, 2016). No Brasil se encontram dentro das 20 espécies mais capturadas (Miranda e Carneiro, 2007). A maioria das espécies são eurialinas, habitam estuários, águas marinhas costeiras, lagoas de água salobra e podem entrar em água doce (Gonzáles-Castro, 2007).

A produção de Mugilídeos no mundo é oriunda de 80% da produção pesqueira e a aquicultura contribui com 20% da produção, o que representa 138,000 T (Crosseti, 2016).

Atualmente 15 países produzem tainhas de forma semi-intensiva sendo a África (84%) o continente de maior produção, seguido pela Ásia (14%) e Europa (2%). Cerca de 70% dessa produção é feita em águas salobras e o resto em água doce. No entanto, espera-se que a produção aumente gradualmente pelas facilidades alimentares que essa família apresenta (Crosetti, 2016).

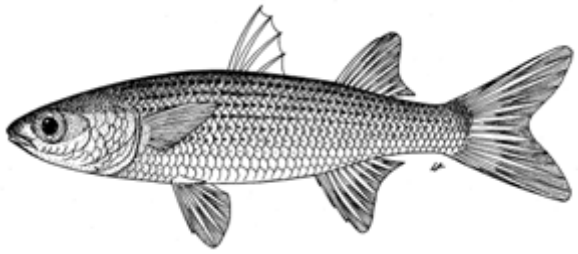

Figura1. *Mugil liza* (FAO, 2018)

A tainha *Mugil liza* (antes conhecida como *Mugil platanus*) é uma espécie que habita a costa Atlântica da América do Sul desde as costas do Caribe até a Argentina (Menezes *et al.*, 2010), atinge um peso máximo de 9 kg e 80 cm de comprimento (Cervigón *et al.*, 1993). Na década passada sua importância era principalmente artesanal, mais passou a ser uma espécie alvo das frotas industriais (Gasalla *et al.*, 2003). Atualmente a pressão de pesca sobre os estoques pesqueiros é severamente monitorada para evitar sua captura no período reprodutivo, pois a diminuição de reprodutores impede a oferta de juvenis o ano todo (Miranda *et al.*, 2011).

Na natureza as tainhas fêmeas maturam mais cedo que os machos e os superam em número. Na Baia de Sepetiba (RJ) a proporção entre machos e fêmeas de tainhas *M. liza* é de 1:1,7 (Albieri e Aráujo, 2010). A tainha é uma espécie migratória que intensifica sua maturação das gônadas nos meses de junho e julho (Esper *et al.*, 2001). Após o período de desova pela sua natureza semi-catádroma os juvenis se deslocam do mar aberto para áreas costeiras, baías, estuários e lagoas costeiras para alimentação e crescimento. Esses habitats desempenham um papel como áreas de viveiro e fornecem abrigo de predadores (Blaber, 1987). Vieira e Scalabrin (1991) analisaram o ciclo de vida de *Mugil platanus,* observando que é uma espécie que apresenta hábito alimentar zooplanctófago quando larva e passa a ter hábito

alimentar iliófago quando juvenil, após os 40 dias de vida, já possuindo capacidade de ingerir ração comercial (Galvão *et al.*, 1997).

A sua importância para a criação surge das características que fazem esta espécie apta para a aquicultura. As tainhas tem um filtro faríngeo-branquial complexo que permite alimentar-se de uma grande variedade de alimentos como micro-organismos e matéria orgânica em decomposição, algas e as fases larvares de insetos, crustáceos ou pequenos moluscos (Richard *et al.*, 2010). As tainhas exploram os sedimentos, pois seus mecanismos de alimentação lhes permite usar diferentes tamanhos de grãos e áreas de alimentação, pois tem um rápido desenvolvimento ontogénico (Galvão *et al.*, 1997). Nenhuma ração especial para esta espécie tem sido feita, mais a tainha tem uma fácil adaptação a ingredientes novos (Ramos *et al.*, 2015; Zamora-Sillero *et al.*, 2013). Antigamente os juvenis eram capturados em rios e canais em grandes quantidades e eram estocados em lagoas para sua engorda. No entanto, a disponibilidade natural de larvas e juvenis está sujeita a flutuações anuais e variações imprevisíveis de ocorrência e abundância que são difíceis de combinar com o planejamento e gestão da aquicultura (Godinho et al., 2013). Foi descrito por Aizen *et al.* (2005) que o uso do antagonista da dopamina (Domperidona) pode facilitar a indução à desova em tainhas.

2. Objetivo Geral

Avaliar a capacidade nutricional da *Spirulina* (*Arthrospira platensis*) em dietas para juvenis de tainha *Mugil liza*.

2.1 Objetivos Específicos

Analisar a possibilidade de se substituir completamente a farinha de peixe por *Spirulina* em dietas para juvenis de tainha e os seus efeitos no sistema imune não específico;

Avaliar a possibilidade de utilizar apenas fontes vegetais e *Spirulina* em dietas de tainha, e avaliar seu efeito no filé e na capacidade antioxidante;

Correlacionar a possível alteração de cor no músculo com a resposta antioxidante no músculo de tainha alimentada com diferentes níveis de inclusão de *Spirulina* e comparar-lhe com a inclusão de betacaroteno;

Avaliar a viabilidade econômica ao utilizar *Spirulina* em rações comerciais.

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CAPÍTULO 1.

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.

Resumo Expandido

A diminuição na produção da farinha de peixe (FP) e por consequência o incremento no valor econômico da mesma tem gerado a necessidade de se procurar novos ingredientes que possam substituir a farinha de peixe. A *Spirulina* é uma cianobactéria que tem sido reconhecida pelo alto teor proteico que possui (até 70%), sendo um possível candidato para substituir a FP. Além disso, este ingrediente apresenta propriedades imune estimulantes e antioxidantes. Os Mugilideos são peixes que têm incrementado sua importância econômica na aquicultura na última década, sendo a tainha *Mugil liza* um bom candidato para aquicultura pelos seus hábitos alimentares. Este trabalho teve como objetivo avaliar *A. platensis* como substituto à FP em dietas práticas de juvenis de tainhas, avaliando seus efeitos sobre o desempenho zootécnico e a resposta imune. O experimento foi aprovado pelo comitê de ética (FURG- CEUA Pq036/2014). Foram formuladas cinco dietas isoproteicas (38%) e isolipídicas (9%), onde a substituição da FP foi feita gradualmente 0% (D1), 30% (D2), 50% (D3), 70% (D4) e 100% (D5) com *A. platensis*. A composição das dietas experimentais assim como sua conformação de aminoácidos e ácidos graxos foram avaliados. O experimento foi realizado em um sistema de recirculação com 15 tanques, onde cada dieta foi testada em triplicata. Foram utilizadas 14 tainhas por tanque de 0,26 g. A quantidade de alimento oferecido foi de 8% da biomassa total de cada tanque. Os parâmetros da qualidade da água foram: oxigênio 6,1 ± 0,6 mg/L, temperatura 25,9 ± 0,6°C, pH 7,7 ± 0,1 e salinidade 29,2 ± 0,8 ppt. Após 80 dias foram avaliados os parâmetros zootécnicos, amostras de sangue foram coletadas para a contagem de células brancas. Os organismos foram eutanasiados com benzocaína (400 ppm) e foram coletados fígado e baço para os análises de imune histoquímica (apoptose e CD3 respectivamente). As análises das rações mostraram os níveis proteína e lipídeos próximos aos calculados. A análise de ácidos graxos mostrou níveis iguais de ácidos graxos altamente poli-

insaturados de cadeia longa (LC-PUFA). A análise de amino ácidos (AA) demostrou uma diminuição de lisina, histidina e metionina. Os resultados mostraram que a dieta 100% *Spirulina* teve menor desempenho nos parâmetros de crescimento, e maior mortalidade. Além disso, se observou que o tratamento 50% *Spirulina* teve o melhor desempenho. As análises do sistema imune (Tabela 1) mostraram diferenças entre os tratamentos, onde os tratamentos D2 e D3 tiveram uma menor produção de monócitos respeito aos outros tratamentos. O tratamento D3 demostrou ser mais eficiente na resposta de apoptose na comparação com os outros tratamentos.

Tabela 1. Contagem de glóbulos brancos, linfócitos (%), monócitos (%) e granulócitos (%), células reativas a CD3 e apoptose (mm^2).

	Tratamentos					ANOVA
	D1	D2	D3	D4	D5	P
Monócitos	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
Granulócitos	12.2 ± 3.5	7.25 ± 1.89	7.6 ± 4.92	15.4 ± 4.03	11.6 ± 7.37	> 0.05
Linfócitos	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 Baço	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

A alta mortalidade e o pobre desempenho de crescimento das tainhas do tratamento D5 explicam-se pela diminuição no conteúdo de amino ácidos essenciais, na comparação com a dieta controle a lisina teve uma diminuição de 21,01% e a histidina de 18,44%. Ambos AA apresentam papel importante na síntese de proteínas e no funcionamento do sistema imune. Baseado em nossos resultados a *A. platensis* pode ser um adequado substituto de FP (até 50%) em uma dieta prática de tainha. Além disso, a substituição parcial de FP por *A. platensis* afetou a proporção de leucócitos, melhorando a resposta imune celular inespecífica da tainha, aumentando a produção de células T e diminuindo a apoptose celular

CAPÍTULO 2.

Substituição total da farinha e do óleo de peixe em dietas de tainha TOTAL (*Mugil liza*) por Spirulina (*Arthrospira platensis*) e óleo de linhaça

Resumo expandido

A produção mundial de farinha de peixe (FP) e óleo de peixe (OP) tem diminuído na última década e, consequentemente, seus preços subindo. O uso de fontes alternativas que poderiam substituir a FP nas dietas de peixes marinhos enfrentam várias limitações, como as deficiências na quantidade de aminoácidos essenciais (EAA). Além disso, a substituição de OP por óleos vegetais diminui a quantidade de ácidos graxos essenciais (AGE), em particular os ácidos graxos poliinsaturados de cadeia longa ômega-3 (n-3) (LC-PUFA). Dois candidatos adequados para substituir FM e FO são *Spirulina* e óleo de linhaça, respectivamente. Em primeiro lugar, *Spirulina* é um produto alimentar com alto teor de proteína, também é uma rica fonte de compostos bioativos, como carotenoides, ficocianinas e ácido gama-linoleico que provaram ter muitos efeitos positivos durante a piscicultura. O efeito de substituir o OP tem mostrado efeitos na composição de AGE do filé do peixe, mas também têm sido reportado ser possível a substituição em dietas de peixes. A inclusão nas dietas de peixes ingredientes ricos em compostos bioativos tais como AG ou carotenoides pode resultar em uma melhora na qualidade do filé e na resposta antioxidante dos organismos.O objetivo desta pesquisa é a substituição total de FP e OP com produtos alternativos que possam levar a um bom crescimento e resposta antioxidante, além de melhorar a qualidade do produto final em termos de cor de filé, carotenoides e teor de FA. Esta pesquisa avaliou o efeito da substituição simultânea de FP e OP em diferentes proporções 0, 30, 50, 70 e 100% (tratamentos denominados SP0, SP30, SP50, SP70 e SP100) por *Spirulina* (*Arthrospira platensis*) e óleo de linhaça. As dietas experimentais foram ofertadas por 75 dias para juvenis de tainhas, o alimento oferecido foi o 8% da biomassa total de cada tanque. Foram estocadas 10 tainhas por tanque com um peso médio de 0,48 g. O experimento foi realizado em um sistema de recirculação onde os parâmetros da água foram os seguintes: oxigênio 6,38 ± 0,27 mg/L, temperatura 27,6 ± 1,06°C, pH 7,73 ± 0,18. e salinidade 32,6 ± 0,6 ppt. Cada tratamento foi testado em triplicata. Os parâmetros avaliados foram o crescimento, a cor do filé

(Luminosidade L, vermelho a*, amarelo b*e Matiz H), o teor total de carotenoides (TCC) do músculo e a capacidade antioxidante do fígado e do músculo mediante os testes de capacidade antioxidante contra radical peroxil (ACAP) e peroxidação lipídica (TBARS). A substituição total não foi possível, resultando em uma diminuição do peso final (P <0,05). As análises colorimétricas mostraram melhora na coloração do filé e no teor de carotenoides (P <0,05), também encontramos uma saturação de carotenoides no músculo a 30% de substituição (Tabela 1). O teor de ácidos graxos essenciais (DHA, EPA e ARA) diminui à medida que o nível de substituição aumenta e também o aumento do ácido linolênico (18-3n: 6) também foi proporcional ao nível de substituição. Encontramos um aumento na capacidade antioxidante até o tratamento SP70, o tratamento SP100 mostrou uma diminuição na capacidade antioxidante.

Tabela 1 Resultados colorimétricos no filé das tainhas alimentadas com os diferentes tratamentos experimentais.

	Tratamentos					ANOVA
	SP0	SP30	SP50	SP70	SP100	p-Value
L	46.0 ± 1.83	42.8 ± 1.4	44.2 ± 0.4	44.6 ± 2.2	44.3 ± 0.86	> 0.05
a*	7.2 ± 1.34 b	11.3 ± 0.49 a	11 ± 1.9 a	9.9 ± 1.7 ab	10.5 ± 1.02 ab	0.03
b*	8.7 ± 1.01	9.6 ± 0.53	9.48 ± 1.0	9.5 ± 1.3	9.9 ± 0.26	> 0.05
C	11.3 ± 1.61	14.9 ± 0.03	14.5 ± 2.1	13.7 ± 2.1	14.5 ± 0.87	> 0.05
H	39.4 ± 2.47 b	49.4 ± 2.76 a	49.1 ± 2.8 a	46.0 ± 2.4 ab	46.5 ± 2.38 a	0.005
TCC μg^{-1}	0.001 ± 0 b	0.012 ± 0 a	0.013 ± 0 a	0.014 ± 0 a	0.015 ± 0.00 a	0.006

Acreditamos que a inclusão parcial de *Spirulina* e óleo de linhaça (50%) pode ser alcançada e pode melhorar a qualidade do filé, também o excesso de seus antioxidantes pode diminuir a resposta antioxidante.

CAPÍTULO 3.

Comparação sobre a inclusão de β-CAROTENE ou *SPIRULINA* (*Arthrospira platensis*) em rações para tainha (*Mugil liza*) e seus efeitos sobre o crescimento e a capacidade antioxidante

Resumo expandido

A adição de carotenos puros é uma prática comum nas produções aquícolas. Seu uso em alimentos para peixes tem o objetivo de melhorar a qualidade da carcaça e indiretamente melhorar capacidade antioxidante. A cianobactéria *A. platensis* é uma fonte rica em carotenoides que apresentam benefícios para a saúde e bem-estar dos peixes. A sua utilização na aquicultura poderia ser um possível substituto para a inclusão de carotenoides puros. Dentro dos benefícios dados pelo consumo de *Spirulina* em peixes estão: o aumento no crescimento, melhora na qualidade do filé, a melhora na resposta antioxidante e incremento na capacidade imune estimulante. Com o fim de estudar possíveis substitutos dos carotenoides comerciais, comparamos a inclusão de *Spirulina* e β-carotenos em dietas de juvenis de tainha. Foram avaliamos seus efeitos na qualidade do filé e na resposta antioxidante contra danos oxidativos. Uma vez aprovado pelo comitê de ética (FURG- CEUA Pq036/2014). O experimento foi o seguinte, foram formuladas quatro dietas isoproteicas (38%) isolipídicas (9%), incluindo uma dieta de controle (livre de β-caroteno e *Spirulina*, SP0), dieta de β-caroteno (50 mg kg^{-1}, B0), 5% (SP5), e 10% (SP10) de inclusão de *Spirulina* (*A. platensis*) (Tabela 1). As dietas experimentais foram ofertadas por 75 dias para juvenis de tainhas, o alimento oferecido foi o 8% da biomassa total de cada tanque. Foram estocadas 10 tainhas por tanque com um peso médio de 0,48 g. O experimento foi realizado em um sistema de recirculação onde os parâmetros da água foram os seguintes: oxigênio 6,38 ± 0,27 mg/L, temperatura 27,6 ± 1,06°C, pH 7,73 ± 0,18. e salinidade 32,6 ± 0,6 ppt. Cada tratamento foi testado em triplicata. Os parâmetros avaliados foram o crescimento, a cor do filé (Luminosidade L, vermelho a*, amarelo b*e Matiz H), o teor total de carotenoides do músculo e a capacidade antioxidante do fígado e do músculo mediante os testes de capacidade antioxidante contra radical peroxil (ACAP) e peroxidação lipídica (TBARS). Encontramos

diferenças significativas (p <0,05) nos parâmetros de crescimento entre o tratamento SP0 e os tratamentos β0, SP5 e SP10. A análise colorimétrica mostrou que a tonalidade vermelha e a deposição de caroteno no músculo da tainha é a mesma entre β0 e SP10. As tainhas alimentadas com *Spirulina* (tratamentos SP5 e SP10) apresentaram maior capacidade antioxidante contra radicais peroxil no fígado e músculo do que os tratamentos β0 e SP0. Os tratamentos com *Spirulina* apresentaram menor peroxidação lipídica (TBARS) no fígado (Tabela 1).

Tabela 1. Capacidade de antioxidante total contra os radicais peroxil (ACAP) (área relativa) e teor de substâncias reativas do ácido tiobarbitúrico (TBARS) (nmol TMP mg tecido úmido^{-1}) no fígado e músculo de tainha (*Mugil liza*) (n = 5).

	Tratamentos				**ANOVA**
ACAP	SP0	β0	SP5	SP10	p-value
Fígado	3.33 ± 0.99b	3.11 ± 0.58b	1.48 ± 0.44 a	1.61 ± 0.64 a	<0.001
Músculo	4.22 ± 1.34b	3.46 ± 1.14b	1.71 ± 0.80 a	1.80 ± 0.50 a	<0.001
TBARS	(nmol TMP mg teçido úmido^{-1})				
Fígado	0.019 ± 0.001 b	0.015 ± 0.001 ab	0.013 ± 0.002 a	0.014 ± 0.002 a	0.005
Músculo	0.003 ± 0.000	0.006 ± 0.005	0.003 ± 0.000	0.007 ± 0.000	> 0.05

As análises do ACAP demonstrou que o músculo e fígado dos peixes com dietas contendo *Spirulina* (SP5 e SP10) apresentava uma capacidade antioxidante aumentada para neutralizar os radicais peroxil sobre os outros tratamentos. A *Spirulina* pode ser um substituto adequado para evitar a adição pura de β-caroteno em dietas de peixe, melhorando o crescimento, a qualidade do filé e incluso brindou melhor resposta antioxidante do que as dietas com inclusão de β-caroteno.

CAPÍTULO 4.

Possibilidade do uso de Spirulina em rações na aquacultura

Resumo expandido

O uso de *Spirulina* na alimentação humana e animal têm sido amplamente estudada demonstrando, na maioria dos casos, resultados favoráveis quando incluídos na dieta. A aquicultura aumentou o seu interesse por este ingrediente na última década, provando os seus efeitos como estimulador do crescimento e da saúde em múltiplas espécies de importância para a aquicultura. Seu uso como substituto da farinha de peixe tem sido amplamente avaliado, pois as caraterísticas nutricionais que contem a *Spirulina* incluem um alto teor proteico e diversas substancias bioativas que melhoram diversas áreas as saúde dos organismos. Durante a última década tem se incrementado consideravelmente os estudos de inclusão de *Spirulina* em dietas de peixes. Duas vertentes surgem dos estudos de *Spirulina* na nutrição aquícola, o primeiro tenta fazer a substituição parcial ou total da FP por *Spirulina*, o segundo tenta demostrar que pequenas quantidades de *Spirulina* em dietas de peixes podem melhorar o desempenho zootécnico, a qualidade do produto final, o sistema imune e a capacidade antioxidante dos organismos que a consomem (Tabela 1). De maneira geral a substituição total de *Spirulina* tem resultado ineficiente principalmente pela deficiência de aminoácidos essenciais que *Spirulina*. No entanto, a inclusão desta microalga seja em grandes quantidades ou pequenas tem provado aportar muitos benefícios nutricionais nos peixes que são traduzidos en melhora da saúde geral do organismo. Na aquicultura não é uma realidade o uso da *Spirulina.* O problema é atribuído ao seu alto valor econômico. Mesmo assim, é importante mencionar que, mesmo em pequenas quantidades, a *Spirulina* pode melhorar consideravelmente a saúde dos organismos, o que, em longo prazo, poderia compensar seu alto valor econômico, reduzindo os custos em outras áreas de produção. Algumas das alternativas que poderiam reduzir os custos na produção de *Spirulina* são: a utilização de novas cepas geneticamente selecionadas, a implementação de novas tecnologias que permitam o incremento da produção, a utilização de novas fontes nutricionais ou a utilização de águas

residuais como meio de cultivo de *Spirulina*. A diferença entre os preços da FP e da *Spirulina* é grande, portanto, uma substituição desse produto atualmente seria economicamente inviável. Por outro lado, uma pequena inclusão de *Spirulina* (1% ou menos) poderia ser uma alternativa viável para aumentar o valor nutricional de dietas para aquicultura, o que aumentará a saúde geral dos organismos, o que se traduz em menos estresse e maior ganho econômico.

Tabela 1. Exemplos de diferentes trabalhos de substituição e inclusão

% de Substituição	Espécie	Resumo de resultados	Referencia
25, 50, 75, 100	Dourada *(Rhabdosargus sarba)*	O 50% de substituição foi possível. 100% de Substituição não foi possível provavelmente por uma deficiência de AA.	El-Sayed 1994
20, 40, 60, 80, 100	Tilapia (*O. mossambicus)*	O 40% de Substituição foi possível. Diminuição em lisina e metionina foi reportado.	Olvera-Novoa *et al.*, 1998
25, 50, 75, 100	Carpa común (*Cyprinus carpio*)	O 100% de Substituição é possível, melhora a atividade de protease, lipase y amilase.	Nandeesha *et al.*, 1998
5, 10	Cocineiro *(Caranx vinctus)*	As duas inclusões melhoram o cor do tegumento.	Okada *et al.*, 1991
2.7	Bagre *Ictalurus punctatus*	*Spirulina* estimula a reposta imune celular não específica, aumenta a resposta de anticorpos timo-dependentes. Aumenta a resistença contra *Edwardsiella ictalur*	Duncan e Klesius 1996
2.5, 5	Larvas de *Litopenaeus schmitti*	A inclusão de *Spirulina* afetó o índice de desenvolvimento e diminuí o crescimento.	Jaime-Ceballos *et al.*, 2005

3. Discussão Gral

Em geral, nossa pesquisa demostrou que a inclusão de *Spirulina* desde 5 a 50% produz efeitos positivos significativos no desempenho zootécnico e na sobrevivência de juvenis de tainha, parâmetros como crescimento e taxa de crescimento específico mostraram um incremento na eficiência das dietas onde a *Spirulina* foi inclusa. O uso da *Spirulina* nas dietas de aquicultura tem sido avaliado de muitos jeitos, pois tem sido reportado que até o extrato da *Spirulina* (dose de 6 até 20 µg L^{-1}) pode melhorar o crescimento dos camarões (Tayag *et al.*, 2010). Muitos autores tem testado a substituição de farinha de peixe (FP) por *Spirulina* em dietas de peixes, e tem recomendado utilizar doses entre 40 e 60% (El-Sayed 1994; Olvera-Novoa *et al.*, 1998; Palmegiano *et al.*, 2005, Velasques *et al.*, 2016). Diferentes fatores devem ser considerados ao escolher a melhor dose de *Spirulina* a ser utilizada, no caso do Capítulo 1, a substituição de 50% e 70% de substituição não demostraram ter diferenças significativas no crescimento, mas no tratamento 70% à resposta imune diminui ao incrementar-se a dose de *Spirulina*, uma observação similar acontece no Capítulo 2 onde uma dose 100% *Spirulina* diminui a resposta antioxidante das tainhas. A substituição total de farinha de peixe (FP) por *Spirulina* não teve sucesso. No Capítulo 1 foi mencionado que uma das possíveis razões de isso acontecer pode ser a diminuição na quantidade de lisina e histidina provocada pelo aumento da inclusão de *Spirulina* resultando em alta mortalidade e baixo crescimento. A deficiência em lisina e histidina também tem sido reportada em dietas feitas com *Spirulina maxima* (Olvera-Novoa *et al.* 1998). A lisina é um dos aminoácidos (AA) mais limitantes nas dietas de peixes e, sua deficiência pode causar altas mortalidades, diminuição da síntese proteica, diminuição na produção de anticorpos e erosão na nadadeira caudal entre muitos outros efeitos (Li *et al.*, 2007; Li *et al.*, 2009). A histidina é um AA altamente importantes para o sistema imune, já que tem papel na síntese proteica, é precursora da histamina e tem um papel tamponador do pH (Tanaka e Ichikawa, 2006; Li *et al.*, 2009). Nossa segunda tentativa (Capítulo 2) a substituição total de *Spirulina* foi com a suplementação de lisina, a dieta SP100 conseguiu aumentar a sobrevivência dos organismos comparativamente ao primeiro experimento (provavelmente efeito da inclusão de lisina) mas não conseguiu bom desempenho nos parâmetros de crescimento. Os dois Capítulos onde a substituição de 100% foi feita o

nível de histidina foi similar (0.57 % do total da dieta no Capítulo 1 contra 0,56 % do total da dieta no Capítulo 2). Pelo que é provável que a deficiência de histidina nas dietas tivesse um papel importante na síntese proteica.

Além da redução na concentração de histidina, houve também diminuição dos ácidos graxos essenciais (AGE). Desta forma, a ausência tanto da histidina quanto dos AGE podem explicar o porquê do insucesso na substituição total da farinha e do óleo de peixe. Contrariamente aos nossos experimentos, alguns autores afirmam que a substituição total de *Spirulina* é possível em carpas e bagre gigante (Nandeesha *et al.*, 1998; Nandeesha *et al.* 2001; Tongsiri *et al.* 2010), espécies cujos hábitos alimentares são omnívoros. Nesse aspecto, precisa-se mais pesquisa sobre a inclusão de aminoácidos para a melhora da qualidade nutricional da *Spirulina*.

O alimento considerado imune estimulante pode ser aquele que atua aprimorando o sistema imune (Galindo-Villegas e Hosokawa, 2004). O funcionamento do sistema imune está intimamente ligado ao sistema antioxidante, pois este apresenta papel na prevenção de doenças (Huang *et al.*, 2005). Os pigmentos contidos na *Spirulina* (carotenoides e biliproteínas) têm sido considerados ambos antioxidantes e imunes estimulantes. As funções imunes protetoras dos carotenoides, por exemplo, dependem muito do tipo e concentração do carotenoide (Kirion, 2012). As funções no sistema imune vão desde o aumento da atividade da lisozima, do complemento, fagócitos e cito toxicidade não específica (Amar *et al.*, 2001) até a proliferação de linfócitos (Kirion, 2012). No Capítulo 1, a tainha teve importante resposta de melhora no sistema imune não específico ao ser alimentada com *Spirulina* até 50% de inclusão, o que acarretou em maior produção de linfócitos-T e melhorou a resposta apoptótica. A estimulação do sistema imune não especifica tem sido relatada em peixes pela ingesta de carotenoides (Amar *et al.*, 2004). O Capítulo 3 mostrou que inclusões pequenas de *Spirulina* podem aportar uma grande quantidade de carotenoides (maioritariamente β-caroteno), o caso dos tratamentos SP5 e SP10 tinham até 77,19 e 169,44 mg kg^{-1}, respectivamente, essas quantidades não excedem o limite recomendado pela European Food Safety Authority, que no caso de dietas para salmonídeos marca o limite em 908 mg kg^{-1} (EFSA, 2005), além disso as tainhas mostraram uma tendência a acumular os carotenoides no músculo que desde a dieta SP5 ate a dieta SP100 não excedem 0,021 μ g^{-1}. A tendência a acumular carotenoides significa

que eles são um ingrediente importante no metabolismo dos organismos, esses carotenoides serão utilizados como antioxidantes ou no caso do β-caroteno será precursor na via metabólica da síntese de vitamina A que também tem função na diferenciação dos linfócitos T e na regularização da resposta imune em intestino (Ross, 2012).

Têm sido reportados efeitos positivos da inclusão da *Spirulina* na qualidade do filé em peixes (Nandeesha 1998; Teimouri *et al.*, 2013a). O efeito de incluir carotenoides na ração para peixes tem um efeito direto na coloração do filé, esta prática é feita com o fim de produzir um produto que seja aceitável pelo consumidor (Christiansen, 1995). A *Spirulina* é uma fonte rica em carotenoides que pode atingir ate os 500 mg cada 100 g (Belay, 2008). No Capítulo 3 foi observado que a inclusão do β-caroteno puro (50 mg kg^{-1}) teve um incremento no tom avermelhado (a*) do filé e um incremento na retenção de carotenoides totais no músculo na comparação com o controle. Sabe-se que ofertar carotenoides puros em dietas de peixe tem maior bioeficiência e biodisponibilidade do que aquele mesmo carotenoide poderia ter incluso numa fonte alimentícia (ex. *Spirulina*) (van het Hof *et al.*, 2000; Priyadarshani, 2017), ainda assim, as inclusões de 5% e 10% de *Spirulina* na ração incrementaram a qualidade do filé por meio da coloração do músculo e do seu conteúdo de carotenoides totais. As tainhas que se alimentaram da dieta contendo 10% de inclusão de *Spirulina* igualou a qualidade de filé encontrada nos peixes alimentadas com a dieta contendo β-caroteno puro. Já que a tendência era aumentar a cor e a retenção de carotenoides na comparação com os resultados do Capítulo 2, podemos ver que todos os tratamentos (SP30, SP50, SP70 e SP100) mantiveram a qualidade do filé observada no Capítulo 3, mas não incrementaram a mesma. Foi verificado também que a acumulação dos carotenoides no músculo tem um limite que esta entre 10% e 30% da inclusão de *Spirulina* na ração, provavelmente pela diminuição na biodisponibilidade dos carotenoides (Teimouri *et al.*, 2013b) e a saturação dos lipídeos que são o meio de transporte dos carotenoides dentro do organismo (Nickell e Bromage, 1998).

A variabilidade entre os tons vermelhos (a*) e amarelos (b*) no filé tem a ver com o tipo de carotenoide ingerido ou a conversão dele nas vias metabólicas (Torrissen e Christiansen, 1995), pois o β-caroteno, por exemplo, pode ser oxidado e convertido em cantaxantina, que pode alterar a tonalidade a*, b* e o matiz (H) do produto final (Torrisen, 1989), já que o matiz (H) é uma medida em graus que vai de 0º a 90º, onde 0 indica amarelo e

90 vermelho todos os tratamentos dos Capítulos 2 e 3 mostraram uma tendência a balancear no meio desta medida (45º) o que é explicado pela grande variabilidade de pigmentos que *Spirulina* possui (Careri *et al.*, 2001; Ravi *et al.*, 2010). Ainda, observando a tendência de incremento da tonalidade amarela (b*) no filé do Capítulo 3 esse incremento não é proporcional ao incremento da dose de *Spirulina* (ex. 30%) observado no Capítulo 2. Contrario a isso, Teimouri *et al.* (2013a) observaram que a dose de *Spirulina* tinha um efeito no cor de filé, e que essa dose de carotenoides tinha mais importância na cor do filé do que o tempo que é ofertada.

A qualidade nutricional da *Spirulina* pode variar muito entre safras e técnicas de cultivo (Morais *et al.*, 2009). O conteúdo de lipídeos que *Spirulina* tem em geral é pequeno, mas é reconhecido pelo seu conteúdo do ácido graxo (AG) gama-linolênico (Belay, 2008), nossa pesquisa não detectou este AG em nenhuma análise de cromatografia feito no Capítulo 1 e 2, pelo que não podemos afirmar nenhuma propriedade benéfica ao seu conteúdo de AG. Referente à qualidade lipídica da tainha no filé observa-se que ela consegue refletir o conteúdo de AG das dietas com a tendência a acumular os AGE (EPA 20:5n−3, ARA 20:4n-6 e DHA 22:6n−3). Teimouri *et al.* (2016) mencionaram que a *Spirulina* consegue aumentar o tempo de prateleira do filé em fresco e congelado pois o acúmulo de carotenoides no musculo consegue proteger os AGE diminuindo a peroxidação ao longo do tempo.

A natureza autotrófica da *Spirulina* a obriga a produzir compostos que permitam ela coletar energia, em especial biliproteinas (ficocianina) e carotenoides, compostos que conferem uma grande capacidade antioxidante dando proteção contra espécies reativas de oxigênio (Gershwin e Belay, 2007). Como resultado da redução parcial de oxigênio na mitocôndria celular, diferentes espécies reativas de oxigênio (ROS) são produzidas (Pisoschi e Pop, 2015). Nossa avaliação da capacidade antioxidante da tainha pelo efeito da *Spirulina* atingiu dois dessas ROS, o radical peroxil e a peroxidação lipídica. Na análise de capacidade antioxidante contra radicais de peroxil (ACAPE) do Capítulo 3 foi observado que os tratamentos com *Spirulina* (SP5 e SP10) conferem uma atividade antioxidante superior no fígado e músculo a aquela dada pelo β-caroteno puro (uma redução dos radicais peroxil de quase 50% em ambos os órgãos), pois o β-caroteno tem capacidade de controlar principalmente o aníon superóxido (Stahl e Sies, 2003). O efeito superior conferido pela

Spirulina em relação ao β-caroteno puro pode ser explicado pela grande variedade de antioxidantes que possui o conteúdo de ficocianina da *Spirulina* por si só tem sido comprovado eficiente para sequestrar radicais alkoxil, hidroxil, peroxil e inibir peroxidação lipídica (Estrada *et al.*, 2001; Romay *et al.*, 2003, Bermejo *et al.*, 2008). Além disso, quando a dose de *Spirulina* é incrementada (desde 30 a 100% de inclusão) (Capítulo 2) a resposta ACAPE para fígado é incrementada (até 41% para o tratamento SP50) provavelmente pelas seguintes causas; 1) O fígado tem um papel importante na detoxificação e eliminação de compostos indesejados pelo que alta atividade antioxidante acontece ai, 2) Sua participação na beta oxidação lipídica faz ele um alvo na mobilização dos lipídeos (Si-Tayeb *et al.*, 2010), e os lipídeos são acarretadores de antioxidantes lipofílicos (Yi *et al.*, 2014). 3) A ficocianina é dose dependente (Bermejo *et al.*, 2008), mas a partir de 30% de inclusão não apresenta maior atividade pelo que poderia ter chegado a um ponto de saturação como foi reportado para os carotenoides no músculo. A resposta contra ACAPE no músculo se mantém constante entre os tratamentos SP5 e SP10 do Capítulo 3 em relação aos tratamentos SP30, SP50 e SP70 do Capítulo 2, no entanto, a inclusão de 100% *Spirulina* perde capacidade antioxidante o que poderia nos indicar evidencias de atividade pro-oxidante como tem sido reportado em outras espécies (Macari *et al.*, 2011, Dal Bosco *et al.*, 2014) provavelmente pela grande quantidade de antioxidante ofertado através da *Spirulina*. A resposta de TBARS não teve diferenças significativas para nenhum experimento e a produção de MDA no fígado e músculo foi similar para todos os tratamentos que ingeriram *Spirulina* (SP5, SP10, SP30, SP50, SP70 e SP100), sendo que o aumento da atividade antioxidante da *Spirulina* contra a peroxidação lipídica tem sido amplamente documentado (Kim *et al.*, 2010 Ponce-Canchihuamán *et al.*, 2010; Teimouri *et al.*, 2016). A explicação do porque não foi observado em nossos experimentos pode ser devido à diferentes motivos; a) a saturação dos órgãos pode causar possível efeito pro-oxidante (Dal Bosco *et al.*, 2014), b) a quantidade de antioxidantes ingeridos diminui a biodisponibilidade, somado à alta atividade metabólica dos peixes de diferentes tamanhos (Teimouri *et al.*, 2013b), c) o conteúdo de AGE nas dietas do Capítulo 2 diminui a medida que a inclusão do óleo de peixe diminui, pelo que existe menos quantidade de substrato disponíveis para gerar MDA pela peroxidação dos lipídeos (Teimouri *et al.*, 2016).

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4. Conclusões gerais

Nosso trabalho conclui que a *Spirulina* pode ter aplicações na nutrição de peixes, no caso da tainha *M. liza* um 50% da substituição da farinha de peixe por *Spirulina* pode ser feita melhorando o desempenho zootécnico. Além disso, a *Spirulina* se mostrou como um eficiente antioxidante e estimulante do sistema imune não específico. Também pode melhorar a qualidade do filé mais os seus benefícios só são atingidos ate um 30% de inclusão. A *Spirulina* pode atingir e sobre passar qualidade dada pelos carotenoides puros ainda suministrada em pequenas quantidades, pois ainda o seu uso em grandes quantidades não seja uma opção viável economicamente os benefícios que as suas sustâncias bioativas aportam incrementam o valor nutricional, e assim melhoram a saúde dos organismos que a consomem.

ANEXO 1

FISHMEAL SUBSTITUTION FOR SPIRULINA (*Arthrospira platensis)* IN JUVENILE MULLET, *Mugil liza* AND ITS EFFECTS ON GROWTH AND NON-SPECIFIC IMMUNE PARAMETERS.

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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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RESUMEN

La cianobactéria *Arthrospira platensis* (*Spirulina*) es un substituto potencial de la harina de pescado (HP) por su alto contenido de proteína, sus antioxidantes y sus propiedades inmune-estimulantes. Fueron analizados los efectos de la substitución parcial y total de HP por *A. platensis* (0, 30, 50, 70 y 100% substitución) para juveniles de lisa, *Mugil liza*. La substitución total resultó en un deficiente crecimiento y baja sobrevivencia. Analizando el sistema inmune, encontramos que el remplazo de HP produce cambios en las proporciones de macrófagos y linfocitos. También, la substitución de hasta un 50% HP aumenta la expresión de receptores CD3 en linfocitos T del bazo. Por otro lado, la substitución mayor a 50% HP disminuye la expresión de receptores CD3. También encontramos que la substitución parcial de HP disminuye el proceso de apoptosis. Basado en nuestros hallazgos, proponemos una substitución de HP del 50% por *A. platensis*, lo que ayudará a mantener la salud general de lisas.

Palabras Llave: Apoptosis, CD3, pez, inmune estimulante, microalga, *Spirulina*.

RESUMO

A cyanobacteria *Arthrospira platensis* (*Spirulina*) é um potencial substituto da farinha de peixe (FP) pelo seu alto conteúdo de proteína, antioxidantes e características imuno estimulantes. 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*. A troca total de FP resulta em déficits de crescimento e baixa sobrevivência. A avaliação do sistema imune demostrou que a substituição da FP produz mudaças 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. Baseado em nossos descobrimentos, se propõe até 50% de substituição da FP por *A. platensis* o que manterá a saúde geral das tainhas.

Palavras Chave: Apoptose, CD3, peixe, imune estimulante, microalga, *Spirulina*.

ABSTRACT

The cyanobacterium *Arthrospira platensis* (*Spirulina*) is a potential fishmeal (FM) substitute because of its high protein content and antioxidant and immune stimulant properties. Here we evaluated the effects of total and partial substitution of FM with *A. platensis* (0, 30, 50, 70 and 100% substitution) for juvenile mullet, *Mugil liza*. A full replacement of FM resulted in growth deficits and low survival. By evaluation of the immune system, we found that FM replacement induced changes in the proportion of macrophages and lymphocytes. We found that up to 50% FM replacement increased the expression of CD3 receptors in spleen T lymphocytes (T-Cells). Whereas FM replacement >50% decreased the expression of CD3 receptors. We also found that partial FM substitution diminishes the apoptotic process. Based on our findings, we propose that up to 50% FM substitution with *A. platensis* would help to maintain overall health in mullets.

Keywords: Apoptosis, CD3, fish, immune stimulant, microalgae, *Spirulina*.

1. Introduction

Identifying alternative protein sources that could substitute the use of fishmeal (FM) in aquaculture diets is a major challenge (Oliva-Teles, 2012). Because of their excellent lipid and protein profilés, as well as their high biomass production capacity, microalgae are candidate FM substitutes (Palmegiano *et al.*, 2008). Among the microalgae, *Arthrospira platensis* has been widely used in animal and human nutrition because of its high protein content (60–70%) and might be useful as a feedstuff in fish diets (Belay, 2002). Indeed, *A. platensis* has already been used as a FM replacement in aquaculture diets, improving the 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). Moreover, *A. platensis* bioactive components provide a wide variety of characteristics desirable in aquaculture production, such as improved carcass quality (carotenoids) (Nandeesha *et al.*, 1998), immune stimulants (polysaccharides) (Abdel-Tawwab and Ahmad, 2009), antioxidant activity (β-carotene and C-phycocyanin) (Ravi *et al.*, 2010), and polyunsaturated fatty acids (γ-linolenic acid – GLA) (Jafari *et al.*, 2014).

As well as the improvement of growth parameters, enhancement of the immune system is a critical factor in aquaculture production. This has been achieved with the use of a wide variety of macro and micronutrients (Ringø *et al.*, 2010). Oral administration of *A. platensis* trough the diet might benefit some specific and non-specific cellular immune parameters in fish, as already shown in macrophage and natural killers cell (NK) activities in tilapia (*O. niloticus*) (Abdel-Tawwab and Ahmad, 2009), increasing mucus protease production in sturgeon (Adel *et al.*, 2016), as well as in the increase of interleukin gene expression in leucocytes of *C. carpio* (Watanuki *et al.*, 2006), and the increased number of granular hemocytes in 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 ingredients (Ramos *et al.*, 2015; Zamora-Sillero *et al.*, 2013). Here we aimed to evaluate *A. platensis* as a FM substitute in practical diets of juvenile mullets, evaluating its effects on growth performance and immune response.

2. Materials and methods

2.1 Fish source and experimental design

Once approved by the Etic Comity (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 the Laboratory of Nutrition of Aquatic Animals (LANOA) at the Federal University of Rio Grande (FURG). 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). The photoperiod manteined was 12:12 h (light: dark). To maintain water quality conditions, a full water renewal was carried out every day.

After the acclimatization period, an 80-days experiment was conducted in 15 50-L fiberglass tanks, all of which were connected by a recirculation system, which consisted of a biological filter, an UV light filter (18 w Philips®) and a protein skimmer. During this period, the 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. The 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) and maintained at 6.1 ± 0.6 mg/L, 25.9 ± 0.6°C. 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). The ammonium and nitrite concentrations were determined according to the methods presented by Benderschneider and Robinson (1983) and Strickland and Parsons (1972), respectively. The 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 was then adjusted to 7% of the total biomass for the remainder of the experiment. The biomass was calculated daily assuming a feed conversion ratio of 2:1.

2.2 Diet formulation

The microalgae *A. platensis* was obtained from a commercial brand (Prilabsa®). The composition of all feed ingredients was analyzed at the Laboratorio de Nutrição de Organismos Aquaticos-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 this study.

Table1. Proximal composition of fishmeal and *A. platensis* in dry basis (g kg^{-1}).

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 100− (crude protein + lipids + ash+ moisture+ fiber).

Diets were formulated to contain 35% CP (Carvalho *et al.*, 2010) and 9% lipids. The pre-weighed ingredients were mixed mechanically (Marconi, MA200) then mixed with oil and water to produce stiff dough; the mixtures were pelleted using a meat grinder (Metalúrgica 9000, PC-22). Pellets were air dried at 60°C for 24 h in an oven (Marconi, MA035). The size of the resulting pellets was adjusted gradually to the fish growth; diets were stored in plastic bags at -18°C until use. Diet formulations and proximate compositions are shown in Table 2.

Table 2. Diets formulation and final composition. Proximate analyses values expressed in g kg^{-1}.

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 Ul kg^{-1}), Vit. D3 (250.000 Ul kg^{-1}), Vit. E (5.000 mg kg^{-1}), Vit. K3 (500 mg kg^{-1}), Vit. B1 (1.000 mg kg^{-1}), Vit. B2 (1.000 mg kg^{-1}), Vit. B6 (1.000 mg kg^{-1}), Vit. B12 (2.000 mg kg^{-1}), Niacin (2.500 mg kg^{-1}), Calcium pantothenate (4.000 mg kg^{-1}), Folic acid (500 mg kg^{-1}), Biotin (10 mg kg^{-1}), Vit C (10.000 mg kg^{-1}), Choline (100.000 mg kg^{-1}), Inositol (1.000 mg kg^{-1}). Trace elements: Selenium (30 mg kg^{-1}), Iron (5.000 mg kg^{-1}), Copper (1.000 mg kg^{-1}), Manganese (5.000 mg kg^{-1}), Zinc (9.000 mg kg^{-1}), Cobalt (50 mg kg^{-1}), Iodine (200 mg kg^{-1})).

2.3 Diet fatty acid identification

The fatty acid (FA) analyses were made at the Facultad de Ciencias, UDeLaR (Montevideo, Uruguay). Lipids were extracted according to Folch *et al.* (1957) and transesterified using 1 mL of 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 49ºC 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 -20ºC until chromatography reading.

FA was quantified using gas chromatography (Hewlett Packard 5890, GMI, USA) provided with a capillary column of melted silica Supelco wax as stationary phase (30 m × 0.32 mm D.I., Supelco, USA). Nitrogen was used as carrier gas and split mode for the injection. The injector and detector temperatures were both 250°C. Initially, the 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^{-1}) 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.

2.4 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 at 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

2.5 Evaluation of growth parameters and sampling

At the end of the 80-day experimental trial, each organism was anesthetized (benzocaine 50 ppm) and individual weights were obtained to determinate growth parameters for each dietary treatment. The evaluated 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;

Afterward, 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 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.

2.6 White blood cell count

Smears of obtained blood were fixed in methanol and stained with Wright-Giemsa stain for determination of the differential White Blood Cells (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.

2.7 Immunohistochemistry (IHC)

For IHC, analyses were elaborated at the Laboratório de Imunologia e Patologia de Organismos Aquáticos (FURG). Five spleens for each tank were fixed in 20% buffered formalin, embedded in paraplast, and stained using the ABC peroxidase method (Vectastain Elite ABC Kit, Canada), 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 for each tank were examined under an optical microscope.

CD3 receptor expression was evaluated by quantitative analysis of the phenotypic percentage by 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).

2.8 Apoptosis evaluation

For the apoptosis evaluation, five livers from fish of each tank were fixed in 20% buffered formalin and embedded in paraplast; the evaluation was carried out using the TUNEL method for ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore) according

to Charriaut-Marlangue and Ben-Ari (1995). The apoptotic cell expression was evaluated by a quantitative analysis of the expression percentage cells by square millimeter (mm^2) of tissue. The apoptotic cells in the liver were also quantified using Bioscan OPTIMAS 6.1 software.

2.9 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 5% significance level.

3. *Results*

A summary of the growth parameters recorded here is given in Table 4. Except for FCR, all parameters increased up to 50% FM substitution (D3 treatment), followed by a gradual decrease until D5 treatment. When fed D5, almost all growth parameters were significantly different from all other diets, final weight (FW) and weight gain (WG) shown no significant difference between D1 and D5 (Table 5). Mullet survival ranged from 100% to 47.62% and was significantly 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. Mullet growth performance with different levels of *A. platensis* substitution.

Parameters	Treatments					ANOVA
	D1	D2	D3	D4	D5	P
IW (g)	0.26 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	> 0.05
FW (g)	3.93 ± 0.69^{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 ± 0.68^{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 ± 0.28^{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 ± 0.16^{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 ± 0.11^{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 ± 4.12^{a}	97.62 ± 4.12^{a}	100 ± 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 in 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 proximal composition analysis of *M. liza* (wet weight basis, g kg^{-1}) fed with different levels of *A. platensis* substitution.

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 in each row show significant differences in Tukey test at p ≤0.05.

The proportions of WBCs varied among the treatments (Table 7). The proportion of monocytes was different between the D2 and D3 treatments compared to the D4 treatment. The proportion of lymphocytes was different between the D3 and the D4 treatments. However,

we detected no statistically significant differences among treatments for the granulocyte or T-Cell CD3 receptor expression. Apoptotic expression was significantly different between treatments without *A. platensis* (1.34 ± 0.12 mm^2) and (D2 treatment (0.54 ± 0.10 mm^2) (P= 0.015).

Table 7. White blood cell count, lymphocytes (%), monocyte (%) and granulocytes (%), CD3 and apoptosis reactive cells (mm^2) of mullet fed with diets containing different levels of *A. platensis.*

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	$1.34 \pm 0{,}12^{a}$	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 in each row show significant difference.

4. *Discussion*

Spirulina use has been widely recommended by the Food and Agriculture Organization of the United Nations (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) showed that 50% of *Spirulina* inclusion resulted 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 for *Oreochromis sp*. and results in the best FCR. Velasquez *et al.* (2016) found that the best FCR and PER could be achieved at 30% of *Spirulina* substitution in tilapia (*O. 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 resulted in

best weight gain, FCR, and PER. However, the total substitution of FM (D5) resulted in reduced performance indexes, growth, and survival.

The poor growth and survival rates observed at full FM substitution (D5) might be related to an imbalance in FA and AA. Since the needs of AA 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 results observed in survival and growth. 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 growth and survival of fish (Hauler and Carter, 2001). Borlongan and Coloso (1993) observed that a deficiency in histidine could result in elevated mortality and small differences in growth in Milkfish (*Chanos chanos*). Waagbø et al. (2010) found a promotion in the growth of salmons (*Salmo salar*) in 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 the deficiency of these AAs in *A. platensis* might affect mullet performance (survival and growth).

Enhancement of the immune system through diet has been a widely used tool to improve the health and growth of cultured fish (Pohlenz and Gatlin, 2014). *Spirulina* has been tested for its immune stimulant properties in fish feed at low inclusions (up to 10% inclusion). Its effects on the immune system of fish species are many; as enhancer of phagocyte, serum and complement activity in carp (*Cyprinus carpio*) and trout (*Onchorincus mykiss*) (Amar et al., 2004; Watanuki et al., 2006), increases the resistance against pathogens in shrimp (*Litopenaeus vannamei*) and tilapia (*O. niloticus*) (Tayag et al., 2010; Ragap et al., 2012), as an improver of hematocrit and lysozyme activity in tilapia (*O. niloticus*) (Ibrahem et al., 2013), and as an activator of the leucocyte functions (Adel et al., 2016), among other effects. However, 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 the addition of *Spirulina* in their diet and modifies the proportions of these cells. In the present report, significant differences were found in the monocyte and lymphocyte count between the D2 and D3 treatments. In particular, the D3 treatment demonstrated the lowest value for monocytes proportion (1.4 ± 1.14%) and an elevated value for lymphocytes (91 ± 2.38%). This same trend was observed by Abdel-Tawwab and Ahmad (2009), where the lowest monocyte production and the highest lymphocyte production matched the diet that showed the best growth for tilapias fed with *Spirulina* inclusion (5%). Watanuki et al. (2006) observed an increase in the macrophage activity in diets supplemented with *Spirulina* in carp (*Cyprinus carpio*). This effect was also obtained by supplementing with 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 (monocytes 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). 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 the proliferation of T-Cells on lymphoid tissue in rats (Simsek et al., 2007), and increase the 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 cellular 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.*

5. *Conclusions*

Based on our data, *A. platensis* might be suitable as an 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 the non-specific cellular immune response of mullet by increasing the production of T-Cells and decreasing cell apoptosis.

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ANEXO 2

TOTAL REPLACEMENT OF FISH OIL AND MEAL IN MULLET (*Mugil liza*) DIET BY *SPIRULINA* (*Arthrospira platensis*) AND LINSEED OIL.

Fish oil and meal replacement in mullet (*Mugil liza*) diet with *Spirulina* (*Arthrospira platensis*) and linseed oil

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Abstract

This research evaluated the effect of the simultaneous substitution of fish meal and fish oil in different proportions 0, 30, 50, 70 and 100% (treatments named SP0, SP30, SP50, SP70 and SP100) for *Spirulina* (*Arthrospira platensis*) and linseed oil and its effect on growth, colorimetric analyses, fatty acid (FA) profilé and antioxidant system of mullet. The experimental diets were supplemented with lysine to supply the deficiency of this amino acid from *Spirulina*. Full substitution was not possible resulting in a diminution of final weight ($P < 0.05$). The colorimetric analyses shown an improvement in fillet coloration and carotenoid content ($P < 0.05$), also we found a saturation of carotenoids in muscle at a 30% of substitution. The content of essential fatty acids (DHA, EPA and ARA) diminishes as the substitution level increases and also an increase on the linolenic acid (18-3n:6) were also proportional to the substitution level. Also we found an increase in the antioxidant capacity up to the treatment SP70, the SP100 treatment shown a diminution in the antioxidant capacity. Finally, we believe that partial inclusion of *Spirulina* and linseed oil (50%) can be achieved and can improve fillet quality, also the excess of its antioxidants can diminish the antioxidant response.

Key words: Antioxidant, double substitution, fatty acid, fillet quality, *Spirulina*.

1. Introduction

Given the high and growing demand for fish meal (FM) and fish oil (FO) and its limited global production, the current trend in the formulation of fish diets is to replace these ingredients for alternative sources. The current production of FM and FO has been diminishing over the decades and consequently, its prices have been rising (Tacon and Metian, 2015). The use of alternative sources that could substitute FM in marine fish diets face several limitations, as the deficiencies on the amount of essential amino acids (EAA) especially for lysine, methionine and tryptophan (Oliva-Teles *et al.,* 2015). Also, the substitution of FO for vegetal oils diminish the amount of essential fatty acids (EFA) in particular the omega-3 (n−3) long-chain polyunsaturated fatty acids (LC-PUFA) of which FO are rich, as the eicosapentaenoic (EPA; 20:5n−3) and docosahexaenoic (DHA; 22:6n−3). The fatty acids (FA) have critical functions in the maintenance of homeostasis and the functionality of immune system (Tocher, 2015). The substitution of FO in marine fish diet can be possible when the FM inclusion in diets is elevated, this way all the LC-PUFAS will be contributed by the FM (Regost *et al.*, 2003; Bell *et al.*, 2004). So the usage of two simultaneous substitutes to the full amount fish products in marine fish diets can be a risky solution, some authors tried these double substitution for vegetable products as Drew *et al.* (2007) just achieve the full FO substitution in rainbow trout (*Oncorhynchus mykiss*) for linseed oil, at a low level of FM substitution (25%) results in a diminution of growth efficiency. Benedito-Palos *et al.*, 2007 successfully substituted up to 66% of the FM and FO in gilthead sea bream (*Spaurus aurata*), and blame the failure of the full substitution to the imbalance of AA content in the casein.

Two suitable candidates to substitute FM and FO are *Spirulina* and linseed oil respectively. First, *Spirulina* is a food product with high protein content (up to 700 g kg^{-1}), also is a rich fount of bioactive compounds such as carotenoids, phycocyanins and gamma-linoleic acid that have proven to have many positive effects during fish culture. Many studies have proven *Spirulina* effects as an immune stimulant (Abdel-Tawwab and Ahmad, 2009), antioxidant (Teimouri *et al.*, 2015) and as an improver of fillet quality (Teimouri *et al.*, 2013). Our previous research prove that 50% of FM substitution was possible in *Mugil liza* (Rosas *et al.* In Press), and also we believe that with the right amino acid (AA) supplementation the

level of substitution can be higher. Second, linseed oil is a rich fount of linolenic acid (18: 3n3) which is a good precursor of n-3 LC-PUFAs in organisms capable of elongating and unsaturating linolenic acid (Monroig *et al.*, 2011). It is necessary to evaluate the effect of the use of linseed oil in marine fish, since they have a limited capacity for synthesis of DHA from linolenic acid (Tocher, 2003). This is why, when replacing fish oil with alternative sources, not only the growth of the organisms should be considered, but also the quality of the final product (FAO, 2016). These changes can be observed in the amount of n-3 and n-6 fatty acids of the muscle (Bell *et al.*, 2001), in the susceptibility to oxidation (Kjær *et al.*, 2008) or the capability of facilitate bioaccessibility of carotenoids (Sotomayor-Gerding *et al.*, 2016).

Fish nutrition can improve growth, and also can be used as a tool to improve fish health and fillet quality. Good quality from fillet can be measure in many ways such as desirable tons of color (Cheng *et al.*, 2015), its content of carotenoids (Choubert, 2010) or the quality of its FA (Tocher, 2015). All these characteristics are desirable to the consumer, so the use from alternative ingredients that can improve growth and fillet quality should be analyzed.

Mullets are worldwide specie that is produced in at least 15 countries, which represent almost 20% from total world mullet consumption (FAO, 2016). Its production is steadily growing each year due their facility of been cultured (Crosetti, 2016), it is a specie at a low level in the trophic chain so its protein requirement is low (Carvalho *et al.*, 2010) and due its feeding habits it is easy to include novel ingredients for mullets (Ramos *et al.*, 2015). For these reasons we believe that *M. liza* could be a suitable candidate to consume diets low in FM and FO content.

The aim of this research it is to achieve a total FM and FO substitution with alternative products that could lead to a good growth and antioxidant response, and also improve the quality of the final product in terms of fillet color, carotenoids and FA content.

2. Materials and methods

2.1 Fish source and experimental design

Once approved by the Etic Comity (FURG- CEUA Pq036/2014). Juvenile mullets were capture in the Cassino beach/Southern Brazil (Latitude: -32.1833, Longitude: -52.1667)

and acclimated at the Laboratorio de Nutrición de Organismos Aquaticos (LANOA) at the Universidade Federal de Rio Grande-FURG. The fishes (n=150) were gradually acclimatized (one month) to the laboratory conditions in a recirculation system of a 500L tank, the water parameters were controlled: salinity (30ppt), temperature (26°C), photo period 12:12 h (light: dark). During this period mullets were hand feed four times per day (9:00, 12:00 14:00 and 16:00 h) with the SP0 diet. Renewal of 30% of the water was carried out every day. Once finished the acclimation period, a 75 days experiment was conducted in recirculation aquaculture system (RAS). The experimental system includes 15 experimental tanks of 80L, connected to a biological filter, a UV light sterilizer (18 w Philips®) and a protein skimmer. Each tank was stocked with 10 individuals (0.47 ± 0.01 g, initial weight). The water parameters were measured daily in all tanks and remained stable throughout the experimental period. During the experimental period the water parameters were: salinity 32.6 ± 0.6 ppt (measure with refractometer RTS 101, ATAGO), pH 7.73 ± 0.18 (digital pH meter Hanna Instruments, HI221), oxygen and temperature 6.38 ± 0.27 mg L^{-1}, 27.6 ± 1.1°C, respectively (multi-parameter electrode YSI, 550A, Yellow Springs, Ohio). The ammonium and nitrite concentrations were determined according to the methods presented by Benderschneider and Robinson (1983) and Strickland and Parsons (1972) respectively. The total ammonia and nitrite levels were 0.17 ± 0.15 mg $L^{-1}$and 0.21 ± 0.20 mg L^{-1} respectively. Alkalinity was maintained via the addition $CaCO_3$ to maintain 100 mg L^{-1} of $CaCO_3$ in the water to maintain the biofilter and was measured according to APHA (2005). All the tanks were fed initially with the 10% of the total tank biomass for the first 15 days, and then feeding was adjusted to the 8% of the total biomass per tank. The feed daily offered was calculated assuming a feed conversion ratio of 2:1. Feed was distributed in four times per day (9:00, 12:00 14:00 and 16:00 h).

2.2 Diet formulation and elaboration

Five experimental isoproteic (35%) and isolipidic (9%) diets were formulated (Carvalho *et al.*, 2010), in which the fish meal and fish oil were simultaneously substituted by *Spirulina* (*A. platensis*) and lineseed oil, the experimental diets were named according to its level of substitution: control (SP0), SP30, SP50, SP70 and SP100. Also, aminograms of the main diet ingredients were made, and then the levels of lysine from all experimental diets were

leveled by the addition of Biolys® L-lysine Sulfate (Evonik Industries, Germany). All the ingredients were pre-weighted and mixed in a blender (Marconi, MA200), then the oil was added and mixed, and finally, water was added to produce a stiff dough, the pellets were made mechanically in a grinder (Metalúrgica 9000, PC-22). The resulting pellets were air dried at 65 °C for 24 h in an oven (Marconi, MA035). The dried pellet was crushed to different sizes according to the fish needs, and stored at -18°C in plastic bags. Diets formulation and proximate composition are shown in Table 1.

Table 1. Diets formulation and final composition.

	Treatments				
Ingredients	SP0	SP30	SP50	SP70	SP100
Fish Meal [a]	45	30	22.5	15	0
A. platensis [b]	0	15	22.5	30	45
Soybean meal [c]	10	10	10	10	10
Wheat meal [d]	4	4	4	4	4
Fish oil [e]	6	4.2	3	1.8	0
Linseed Oil [f]	0	1.8	3	4.2	7
Starch [g]	19	19	19	19	19
Cellulose	11	11	11	11	10
Mineral/Vitamin Premix [h]	2	2	2	2	2
Gelatin	3	3	3	3	3
Lysine [i] (g kg^{-1})	0	4.83	7.24	9.66	14.5
TOTAL	100	100	100	100	100
Proximate analyzes (dry weight g kg^{-1})					
Protein	38.33	38.47	39.31	39.20	39.48
Lipids	9.75	8.63	8.43	8.34	8.23
Ash	11.18	9.40	7.39	7.28	4.97
N.N.E	40.74	43.50	44.87	45.19	47.33

[a]Leal Santos, Rio Grande, RS, Brazil.
[b]Prilabsa®, Brazil.
[c]Sulino RS, Brazil.
[d]Sulino , RS, Brazil.
[e]Campestre®, São Paulo. Brazil.
[f] Pazze ®, Brazil.
[g]Maizena®, Brazil
[h]Premix M. Cassab, São Paulo, Brazil (Vit. A (500.000 Ul kg^{-1}), Vit. D3 (250.000 Ul kg^{-1}), Vit. E (5.000 mg kg^{-1}), Vit. K3 (500 mg kg^{-1}), Vit. B1 (1.000 mg kg^{-1}), Vit. B2 (1.000 mg kg^{-1}), Vit. B6 (1.000 mg kg^{-1}), Vit. B12 (2.000 mg kg^{-1}), Niacin (2.500 mg kg^{-1}), Calcium pantothenate (4.000 mg kg^{-1}), Folic acid (500 mg kg^{-1}), Biotin (10 mg kg^{-1}), Vit C (10.000 mg

kg^{-1}), Choline (100.000 mg kg^{-1}), Inositol (1.000 mg kg^{-1}). Trace elements: Selenium (30 mg kg^{-1}), Iron (5.000 mg/kg), Copper (1.000 mg kg^{-1}), Manganese (5.000 mg kg^{-1}), Zinc (9.000 mg kg^{-1}), Cobalt (50 mg kg^{-1}), Iodine (200 mg kg^{-1})).
[i]Biolys® L-lysine Sulfate (Evonik Industries, Germany)

2.3 Amino acid evaluation in diets

With the aim of improve the *Spirulina* meal quality the amino acids (AA) of the main ingredients contained in the diets were obtained, *Spirulina* was analyzed by Evonik Industries AG (São Paulo, Brazil) by the AMINONIR® (Nitrogen infrared, NIR), the FM was analyzed by the Laboratorio de Análises Micotoxicológicos (LAMIC, Universidade Federal de Santa Maria, RS, Brazil) also by the NIR method, the remain ingredients data was gathered from the NRC (2011) (for soybean meal, wheat meal and gelatin). The first AA evaluation shown that the lysine was diminishing 31% from the treatment SP0 to the treatment SP100, then lysine supplement was added to the final formulation. The final AA values were calculated according to the inclusion of each ingredient (Table 2).

Table 2. Essential and non-essential amino acids contained in the experimental diets, expressed at percentage (%) of the diet.

Essential Amino acid	Treatments				
	SP0	SP30	SP50	SP70	SP100
Methionine	0.8	0.77	0.75	0.73	0.70
Lysine	2.26	2.26	2.26	2.26	2.26
Threonine	1.23	1.32	1.36	1.4	1.49
Arginine	2.25	2.25	2.25	2.26	2.26
Isoleucine	1.32	1.46	1.53	1.6	1.75
Leucine	2.42	2.54	2.59	2.65	2.77
Valine	1.5	1.66	1.73	1.81	1.97
Histidine	0.79	0.71	0.68	0.64	0.56
Phenylalanine	2.21	2.12	2.01	1.9	1.79
Nonessential amino acid					
Cysteine	0.36	0.35	0.35	0.34	0.33
Tyrosine	1.16	1.25	1.29	1.33	1.42

2.4 Fatty acid identification

Lipids from samples were extracted according to Folch *et al.* (1957) and trans-esterificated using 1ml of sulfuric acid (1%) in methanol (Christie 1982). Butilhidroxitoluene (BHT) (50 mg L^{-1}) was used to prevent oxidation of the FA. Samples were incubated at 49 ºC

for 16 h in nitrogen atmosphere. Afterward, an hexane:ether (1:1 v/v) solution was used for FA extraction and KHCO3 (20g L^{-1}) was used to wash the hexane:ether solution. Finally, FA were dried for 24 h and a dilution of chloroform 30 mg ml^{-1} was made and kept under nitrogen atmosphere at -20 ºC until chromatography reading.

FA were quantified using gas chromatography (Hewlett Packard 5890) provided with a capillary column of melted silica Supelco wax as stationary phase of 30 m x 0.32 mm D.I (Supelco, 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 during 10 min. Afterwards, temperature raised at a rate of 2.5°C/min up to 212 °C, final temperature was kept 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^{-1}).

	Treatments				
FA	SP0	SP30	SP50	SP70	SP100
14:0	5.40	5.18	3.45	1.46	0.32
16:0	21.66	26.81	23.49	19.96	17.77
16:1n-7	8.07	6.18	4.84	2.84	1.50
16:2n4	1.08	0.65	0.52	0.32	0.22
16:3n-4	1.20	0.81	0.67	0.38	0.34
18:0	5.35	5.98	5.30	4.81	4.04
18:1n-9	15.02	13.93	14.77	16.61	16.21
18:1n-7	3.88	3.18	2.62	1.50	1.03
18:2n-6	9.90	11.11	14.27	18.03	21.39
19:0	0.22	1.09	1.74	2.56	3.81
18:3n-3	1.39	6.54	13.63	24.03	32.55
20:4n-6[a]	0.97	0.66	0.57	0.24	0.05
20:5n-3[b]	7.92	4.23	3.67	1.43	0.08
22:6n-3[c]	4.76	2.51	2.28	1.18	0.09
SAFA[d]	35.17	41.37	34.74	28.17	22.96
MUFA[e]	31.48	27.71	25.75	23.55	20.44
PUFA[f]	33.36	30.92	39.51	48.28	56.60
n-3 LC-PUFA[g]	14.23	7.91	6.38	3.44	0.61
n-6/n-3	0.65	0.78	0.75	0.68	0.66

[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.

2.5 Evaluation of performance and sampling

At the end of the experiment, all organisms were anesthetized (benzocaine 50 ppm) and weighted to determinate performance parameters for all treatments. The evaluated growth parameters were: weight gain (WG), Feed conversion ratio (FCR), Specific growth rate (SGR), Protein efficiency ratio (PER):

a) WG = (final weight (g) - initial weight (g)) / initial weight (g);
b) FCR = average dry feed intake / average weight gain;
c) SGR = 100 × [(ln (final weight) - ln (initial weight)) / days of feeding];
d) PER= weight gain (g) / protein intake (g);
e) Survival = (final number of fish / initial number of fish) * 100;

After weighing, all fish were euthanized with benzocaine overdose (300 ppm). Afterwards, organs of five fish per tank were collected and submersed on liquid nitrogen (muscle and liver), and then stored in ultrafreezer (-82ºC) for further analyses. The remaining carcasses were also stored on ultrafreezer for body composition analyses according to the A.O.A.C. (2000) methodology.

2.6 Determination of total carotenoid content (TCC)

The extraction of carotenoids in muscle was made according to the methodology described by Yanar *et al.* (2004). For each treatment nine samples of 0.5 g of muscle were used. Each sample was macerated, then equal amounts of anhydrous sodium sulfate was added. The mixture was washed twice with 5 ml of acetone, and transferred to a 10 ml amber glass bottle, preserved for 3 d at 25°C in the dark. After that, the samples were centrifuged for 5 min at 5000 rpm^{-1}. The supernatant was taken and scanned in a spectrophotometer (Biospectro,SP-22, Brazil), the TCC ($\mu g\ g^{-1}$) was calculated at the absorption value of 480 nm according to the formula:

$$TCC(\mu g\ g^{-1}) = A_{\lambda=480\ nm} * K * V/(E * G)$$

Where A is the absorbance value; K is a constant (104); V is the volume of the extracting solution (ml); E is the extinction coefficient (1900); and G is the sample mass (g).

2.7 Colorimetric analyses

For each treatment, nine fillets were assessed using the Minolta Chroma Meter analysis (Minolta Chroma Meter, CR-400 Minolta, Japan) that measures the reflectance of light from the fish flesh. The measured colour parameters were lightness (L*), red/green chromaticity (a*), and yellow/blue chromaticity (b*). The instrument was placed on the flesh, and triplicate measurements were taken at each fillet area in order to calculate a mean value. The a* value represented the redness and the b* value the yellowness of the flesh. From the a* and b* values, the chroma (C_{a^*b}) and hue (H_{a^*b}) were calculated (Hunt, 1977). The chroma (C) is an expression of the intensity and clarity of the colour, whereas the hue (H) is the relationship between the redness and the yellowness of the fillet, where 0° indicates a red hue and 90° denotes a yellow hue. The following expressions were employed to calculate these parameters:

$$C_{a^*b} = (a^{*2} + b^{*2})^{1/2} \qquad \text{Eq. (1)}$$

$$H_{a^*b} = \tan^{-1}(b^*/a^*) \qquad \text{Eq. (2)}$$

2.8 Antioxidant capacity against peroxyl radicals

Total antioxidant capacity against peroxyl radicals (ACAP) was determined according to the method described by Amado *et al.* (2009), which consist in the detection of reactive oxygen species (ROS) in biological samples treated or not with a peroxyl radical generator. All samples were diluted with homogenization buffer to 2.0 mg protein mL^{-1}, then exposed to peroxyl radicals generated by thermal (37°C) decomposition of 2,2′-azobis(2 methylpropionamidine) dihydrochloride (ABAP, 4 mM). Peroxyl radicals reacted with a fluorescent substrate (2′,7′ dichlorofluorescein diacetate–H2DCF-DA) and fluorometry (excitation 485 nm; emission 520 nm) were measured through a microplate reader (BioTek, Synergy HT) with readings every 5 min for 35 min, employing software Gen5 2.00 to program readings intervals properly. The results were expressed as a relative area (the difference between the ROS area with and without ABAP relative to that without ABAP). For result interpretation purposes, a higher relative area means a lower antioxidant capacity.

2.9 Lipid peroxidation

To determine the thiobarbituric acid reactive substances (TBARS) by the quantification of MDA (malondialdehyde) to mesure lipid peroxidation levels the method of Oakes and Van Der Kraak (2003) was used, 20 μL of BHT solution (67 μM), 150 μL 20% acetic acid solution, 150 μL 0.8% TBA solution, 50 μL Milli-Q H_2O and 20 μL of 8.1% SDS (sodium dodecyl sulfate) were added to samples (liver 50 μL; muscle 100 μL) before being heated at 95°C for 30 min. Thereafter, 100 μL of Milli-Q H_2O and 500 μL of n-butanol were added to the final solution.

The remaining supernatant after centrifugation (3000 × g, 10 min, 15°C) was used to determine the fluorescence (excitation 520 nm; emission 580 nm) through a microplate reader (BioTek, Synergy HT), and the results were expressed as nanomole TMP milligram wet tissue^{-1}, where TMP stands for tetramethoxypropane (ACROS Organics), employed as a standard.

2.10 Statistical analyses

All data were expressed as the mean ± SD. The normality and homogeneity from the data was tested according to Shapiro-Wilk and Levene tests, respectively. The one-way Analysis of variance (ANOVA) was used to identify possible differences among the treatments results. When significances between treatments was detected, a posterior means comparison were performed by Tukey's test at a significance level of 5%. The linear regression analysis between diets and FA was made by the Coefficient of determination (R^2).

3. Results

The analysis of the diets showed a tendency to diminish in the amount of LC-PUFA contained in the diets (especially in araquidonic acid (ARA), EPA and DHA), this diminution is highly correlated ($p<0.05$) to the amount of FM and FO substitution (ARA $R^2=0.97$, EPA $R^2=0.96$ and DHA $R^2=0.95$), also an increase in linoleic (18:2n-6) and linolenic (18:3n-3) acid in highly correlated ($p<0.05$) to the increase in the amount of linseed oil (18:2n-6 $R^2=0.95$ and 18:3n-3 $R^2=097$). The AA analysis showed a decrease in some of the essential AA ($p<0.05$). Histidine was the AA that diminish the most between the SP0 and the SP100 diets (30%, $R^2=0.98$), also the amount of phenylalanine and methionine diminish 20% and 13%

respectively and were highly correlated ($p<0.05$) to the level of FM substitution in diets ($R^2=0.99$ and $R^2=1$ respectively).

Growth parameters are presented in Table 4. The SP0 treatment showed significant differences ($p<0.05$) against all the *Spirulina* treatments having the lowest growth. The treatment SP100 had significant differences against the SP30 and SP50 treatments showing less growth gain. The most efficient FCR were those of the treatments SP30 and SP50 (1.62 ± 0.05 and 1.64 ± 0.03 respectively) presenting significant differences ($p<0.05$) with the treatments SP0 and SP100. Also, the SP0 treatment shown significant differences ($p<0.05$) for all the other performance parameters (SGR, PER, Survival). For the performance parameters in most of the case, mullet achieved the best performance at the level of 50% of FM and FO substitution, bigger substitutions results in a decrease in growth efficiency. The carcass proximal composition presented significant differences ($p<0.05$) only in the ashes content (Table 5).

Table 4. *Mugil liza* growth performance.

	Treatments					
	SP0	SP30	SP50	SP70	SP100	p-Value
IW	0.48 ± 0.00	0.48 ± 0.01	0.48 ± 0.00	0.48 ± 0.00	0.47 ± 0.00	> 0.05
FW	5.92 ± 0.29 c	11.56 ± 0.47 a	11.71 ± 0.59 a	10.61 ± 0.83 ab	8.59 ± 1.32 b	<0.001
FCR	2.38 ± 0.24 c	1.62 ± 0.05 a	1.64 ± 0.03 a	1.81 ± 0.20 ab	2.20 ± 0.31 bc	0.002
SGR	3.38 ± 0.07 c	4.30± 0.04 a	4.32 ± 0.08 a	4.19 ± 0.12 ab	3.89 ± 0.20 b	<0.001
PER	1.01 ± 0.10 c	1.54 ± 0.05 a	1.49 ± 0.03 a	1.38 ± 0.14 ab	1.10 ± 0.18 bc	<0.001
Survival	73.81 ± 2.06 b	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	<0.001

Values are expressed as means ± SD of three replicate groups. Similar letters in each row show absence of significant differences in Tukey test or global ANOVA test at $p \leq 0.05$. IW= initial weight; FW= final weight; SGR=Specific growth ratio; FCR=Feed conversion ratio; PER=Protein efficiency ratio.

Table 5. Carcass proximal composition analysis of *Mugil liza* (wet weight basis. g kg^{-1}).

Analyses	SP0	SP30	SP50	SP70	SP100	ANOVA p-Value
Moisture	66.77 ± 1.73	65.24 ± 1.59	66.37 ± 1.24	65.12 ± 0.45	64.63 ± 2.41	> 0.05
Protein	18.39 ± 2.46	18.28 ± 1.87	18.68 ± 3.69	18.01 ± 2.90	19.09 ± 2.92	> 0.05
E.E.*	9.68 ± 4.94	11.41 ± 1.88	10.32 ± 2.24	11.13 ± 1.20	10.83 ± 1.39	> 0.05
Ash	4.40 ± 0.51 a	3.62 ± 0.93 b	3.54 ± 0.65 b	3.90 ± 1.35 ab	3.30 ± 1.08 b	< 0.001

Values are expressed as means ± SD of three replicate groups. Similar letters in each row show absence of significant differences in Tukey test or global ANOVA test at p ≤0.05. * Ether extract.

As shown in Table 6, the fillets coloration were no affected by treatments in the luminosity (L), the yellowness (b*) and the chroma parameters. Also, *Spirulina* affected the redness (a*) of the fillet in the treatments SP30 and SP50, presenting a significant increase ($p<0.05$) in the red intensity of the fillet (11.3 and 11 respectively) in comparison to the treatment SP0. The treatments SP70 and SP100 showed no differences against the control group (SP0) in the expression of redness. Also the hue showed a tendency to yellowness from all the *Spirulina* treatments.

The ACAPE analyses show that the *Spirulina* treatments had an increase in its antioxidant response in liver ($p<0.05$). The response from muscle showed the best antioxidant capacity for the *Spirulina* treatments ($p<0.05$), no significant difference was found between the treatments SP0 and SP100. The TBARS response shows no difference in the production of TMP for liver and muscle between the treatments.

Table 6. *Mugil liza* fillet colorimetric analyses and total carotenoid content extracted from muscle.

	Treatments					ANOVA
	SP0	SP30	SP50	SP70	SP100	p-Value
L	46.0 ± 1.83	42.8 ± 1.4	44.2 ± 0.4	44.6 ± 2.2	44.3 ± 0.86	> 0.05
a*	7.2 ± 1.34 b	11.3 ± 0.49 a	11 ± 1.9 a	9.9 ± 1.7 ab	10.5 ± 1.02 ab	0.03
b*	8.7 ± 1.01	9.6 ± 0.53	9.48 ± 1.0	9.5 ± 1.3	9.9 ± 0.26	> 0.05
C	11.3 ± 1.61	14.9 ± 0.03	14.5 ± 2.1	13.7 ± 2.1	14.5 ± 0.87	> 0.05
H	39.4 ± 2.47 b	49.4 ± 2.76 a	49.1 ± 2.8 a	46.0 ± 2.4 ab	46.5 ± 2.38 a	0.005
TCC μg^{-1}	0.001 ± 0 b	0.012 ± 0 a	0.013 ± 0 a	0.014 ± 0 a	0.015 ± 0.00a	0.006

L= lightness; a*=red/green chromaticity; b*= yellow/blue chromaticity; C= chroma; H=hue; TCC= Total Carotenoid Content. Values are expressed as means ± SD of three replicate groups. Similar letters in each row show absence of significant differences in Tukey test or global ANOVA test at p ≤0.05

Table 7. Total antioxidant capacity against peroxyl radicals (ACAP) (relative area) and thiobarbituric acid reactive substances (TBARS) content (nmol TMP mg wet tissue^{-1}) in liver and muscle of juvenile mullet (*Mugil liza*) (n=5).

	Treatments					ANOVA
ACAPE	SP0	SP30	SP50	SP75	SP100	p-Value
Liver	3.33 ± 0.99 b	0.64 ± 0.28 a	0.61 ± 0.23 a	0.72 ± 0.31 a	0.66 ± 0.21 a	<0.001
Muscle	4.22 ± 1.34 b	1.87 ± 0.34 a	1.97 ± 0.66 a	1.54 ± 0.32 a	2.32 ± 0.14 ab	<0.001
TBARS						
Liver	0.022 ± 0.003	0.021 ± 0.002	0.023 ± 0.003	0.019 ± 0.004	0.020 ± 0.003	> 0.05
Muscle	0.019 ± 0.019	0.022 ± 0.009	0.018 ± 0.006	0.016 ± 0.002	0.016 ± 0.006	> 0.05

Values are expressed as means ± SD of five replicate groups. Different letters in each row show significant differences in Tukey test at $p \leq 0.05$

4. Discussion

The finite resources referring to FM and FO have increased aquaculture attention to search alternative sources that could substitute these ingredients. The *Spirulina* production can be feasible due its high biomass production (Morais *et al.*, 2009). *Spirulina* has proven to be a good source of protein that achieved full FM replacement in fish diets according to some authors (Nandeesha *et al.* 2001; Tongsiri *et al.* 2010). The positive effects in fish growth given by the addition of *Spirulina* are many, such as a prebiotic fount or as an improver of enzymatic activity (Teimouri *et al.*, 2013; Adel *et al.*, 2016). In contrast, not all authors recommend this full substitution (El-Sayed, 1994; Olvera-Novoa *et al.*, 1998; Rosas el al., In Press).

Our results indicate that the mullets of the treatments SP30 and SP50 had the best performance in terms of growth, SGR, PER and survival. The full FM and FO substitution treatment had a significant effect in terms of growth; two possible explanations arise from such results. One can be an imbalance in some of the AA contained in the 100% *Spirulina* fed, the AA analyses show a diminution in three of the EAA been the histidine one the most significant decrease. In our previous work (Rosas *et al.*, In Press) we found that full FM substitution for *A. platensis* meal results in elevated mortality and poor mullet growth, probably by the low levels of lysine and histidine found, this diminution in these EAA also was found in diets with *Spirulina maxima* (Olvera-Novoa *et al.*, 1998). The fact that no mortality was found in the treatment SP100 could lead us to think that the lysine

supplementation in diets have a positive role in this parameter. On the other hand, the diminution of growth might be associated to histidine. According to Li *et al.* (2009) since its participation in one-carbon unit metabolism, which affects protein synthesis, also histidine is a precursor of histamine which is essential in the modulation of immune system (Tanaka and Ichikawa, 2006). The second reason is the diminution in amount EFA especially ARA, EPA and DHA, which are essential to marine fish due their limited capacity of synthetize them. Palmegiano *et al.* (2005) saw that *Spirulina* inclusion in diets diminish the levels of LC-PUFA and that partial substitution (up to 60%) did not affect growth or EFA accumulation in muscle of white sturgeon. Moreover, Palmegiano *et al.* (2008) elaborate diets were partially replace FM (66%) for *Spirulina* and totally replace FO for soybean oil or corn oil, both diets had no adverse effects on growth or survival of white sturgeon, also they notice that EFA were accumulated by fish.

Regarding the FO substitution, many authors have found that full FO substitution for linseed oil is possible in some species as in salmon (Menoyo *et al.*, 2005), white bass (*Atractoscion nobilis*) (Lewis *et al.*, 2011) or gray mullet (*Mugil cephalus*) (Argyropoulou *et al.*, 1992). In mullets, the necessities of EFA might be low as was found by Argyropoulou *et al.* (1992), also mullets might have variations according to its size, because mullets have a variation of the amount of lipases on digestive organs that is dependent of weight, meaning that smaller fish have more lipases on stomach and intestine and bigger fish have more lipase activity on liver (Nayak *et al.*, 2003).

It is difficult to evaluate the degree of FO substitution due several reasons; external factors such as temperature or salinity play a role in the amount of EFA that the fish would need (Turchini *et al.*, 2009), also the FM can contribute with several of the EFA due the composition of its lipid content, in this sense Bowyer *et al.*, (2012) mentioned that if the fish diet match the minimal requirement of EFA (i.e.LC-PUFAS) full FO substitution will not affect growth. The diets SP70 and SP100 did not have the contribution of EFA that FM provides (3.44% and 0.61% LC-PUFAS from total FA respectively). Their values were under the minimal amount of LC-PUFAS (from 5% to 11% of total FA) that is recommended (Websterand and Lovell, 1990) therefore resulting in poor growth. The LC-PUFAS are considered poor substrate for the mitochondrial β-oxidation (Sargent *et al.*, 2002), also fish have the tendency to accumulate them.

There is few evidence of the effect of full inclusion of *Spirulina* in the colour of fish fillet and the carotenoid retention in muscle tissue. It is known that carotenoids have a direct effect in the colour expression of the muscle fillet (Teimouri *et al.*, 2013) and in this context make it a more acceptable product for consumption (Christiansen *et al.*, 1995). The carotenoid retention in muscle tissue did not increases as the *Spirulina* inclusion increase in the experimental diets, so no significant difference was found in the retention of carotenoids between the *Spirulina* treatments. Nickell and Bromage (1998) suggested that there is a limit in the deposition of carotenoids in muscle; it is provable that the amount of *Spirulina* that saturates the muscle with carotenoids goes under the 30% of inclusion. Even so, the redness (a*) of the muscle show higher values for the treatments SP30 and SP50, and the treatments SP70 and SP100 showed no significant difference compared to the SP0, also carotenoids tend to mask the yellow coloration in muscle fillet (Nickell and Bromage, 1998), this can be appreciated in the Hue, it is also a reason that explain lack of differences found in the yellowness coloration (b*). There are many factors that play a part in the carotenoid retention of muscle, as the type of fish (Ytrestøyl and Bjerkeng, 2007) or the type of carotenoid that ingest (Torrissen and Christiansen, 1995). The coloration of muscle given by diets might not depend exclusively from carotenoids but from other pigments. *Spirulina* is a rich fount of carotenoids such as zeaxanthin, β-cryptoxanthin and β-carotene (Careri *et al.*, 2001) and from other pigments as Chlorophyll, phycocyanin and porphyrin (Ravi *et al.*, 2010), but the limit of saturation of these pigments probably was reached at an inclusion of SP30. There is evidence that the amount of lipids have a direct impact in muscle coloration due the fact that these macromolecules are used to transport carotenoids (Yi *et al.*, 2014). All experimental diets tested in the experiment had the same amount of lipids and may have not interfered on carotenoid transport. However, there is no evidence for if the type of FA can affect the fillet colorations deserving further investigations.

The limited resources to the aquaculture production such as inland water or fish derivate ingredients (FO and FM) have led to the search of alternatives that could help intensify this commercial activity with the same resources. The increase in fish culture density is a common practice that can result in risky situations such as the diminution of oxygen or the increase of ammonia production the water (Lushchak *et al.*, 2005, Hegazi *et al.*, 2010). These situations plus the normal metabolism can cause the increase in the amount of reactive oxygen

species (ROS); uncontrolled ROS causes several cellular damage and malfunction of normal metabolism (Pisoschi and Pop, 2015). *Spirulina* is known by being a rich fount of antioxidants (Ahmed *et al.*, 2014; Chen *et al.*, 2014) that can diminish ROS damage as shown at the ACAPE results. All treatments containing *Spirulina* improve fish capacity to neutralize peroxyl radical in liver. By the other hand, the ACAPE response for muscle increase at levels of 30%, 50% and 70% after that it decrease when the *Spirulina* inclusion reaches 100%. The capacity of *Spirulina* to neutralize peroxyl radicals is attributed mainly to the biliprotein phycocyanin (Bhat and Madyastha, 2000), and has been proved to have scavenging properties *in vitro* (Huang *et al.*, 2007) and *in vivo* from rats (Estrada, *et al.*, 2001). We believe that the biomolecule responsible for the protection against peroxyl radicals should be phycocyanin due the next reason; the mainly constituent of *Spirulina* carotenoids its β-carotene mainly known for its scavenger activity against singlet oxygen ($^1O_2$) (Stahl and Sies, 2003), but phycocyanin comprise scavenger activity on superoxide radical, hydrogen peroxide, peroxyl radical and inhibitory activity of lipid peroxidation (Romay *et al.*, 2003; Bermejo *et al.*, 2008). We saw that the protection against peroxyl radical diminish in mullet after receiving a high *Spirulina* dose in the treatment SP100, Bermejo *et al.* (2008) report that the scavenging capacity of phycocyanin (against hydroxyl a peroxyl radicals) is dose dependent, also there are some suggestions that indicate in certain situations *Spirulina* could have a pro-oxidant effect (Macari el al., 2011, Dal Bosco *et al.*, 2014).

The TBARS technique determine lipid peroxidation. Two factors in the experimental diets play a high importance role in this response from fish; the levels of LC-PUFAS and the quantity of antioxidants ingested. There is evidence that elevated amounts of LC-PUFAS increase the production of MDA in fish muscle (Kjær *et al.*, 2008). The experimental result show a decrease in the values of LC-PUFAS as the inclusion of linseed oil increases, even so there were no significant differences in the MDA values of liver and muscle among the treatments. *Spirulina* is highly efficient inhibitor of lipid peroxidation because of its biomolecules (Bermejo *et al.*, 2008). Teimouri *et al.*, (2016) also found no differences in the lipid peroxidation value on day 0 of storage between the fish feed with the control diet and those feed with *Spirulina*, also mention that lipid peroxidation increases over time even at freeze storage.

In summary, our data strongly support that combined replacement at a medium level of substitution (50%) from FM and FO for *Spirulina* and linseed oil its possible in juvenile mullets diets and results in an efficient growth. Full FM and FO substitution is not a feasible due the negative effect that has on growth for the lack of some EAA and EFA. *Spirulina* improves the redness on fillet but the carotenoids saturate the muscle at a level under 30% of inclusion. Finally, the antioxidant capacity can be improved until the 100% of *Spirulina* inclusion; at that level might cause a pro-oxidant effect. Further studies are recommended to improve the *Spirulina* meal quality so a full fish meal substitution could be achieved with success.

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ANEXO 3

COMPARISON OF β-CAROTENE AND *SPIRULINA* (*Arthrospira platensis*) IN MULLET (*Mugil liza*) DIETS AND EFFECTS ON ANTIOXIDANT PERFORMANCE AND FILLET COLOURATION.

Submetido para Aquaculture

Comparison of β-carotene and *Spirulina* (*Arthrospira platensis*) in mullet (*Mugil liza*) diets and effects on antioxidant performance and fillet colouration

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Abstract

The addition of pure carotenes is a common practice in aquaculture production since its use in fish feed is known to improve carcass quality and antioxidant capacity. *Spirulina* cyanobacteria are a rich source of carotenoids that has proven health benefits for fish welfare. In the current experiment, four isonitrogenous (38%) isolipidic (9%) diets were made, including a control diet (free of β-carotene and *Spirulina*, SP0), β-carotene diet (β0), 5% (SP5), and 10% (SP10) *Spirulina* (*A. platensis*) inclusion. The experimental diets were given to juvenile mullets in a controlled recirculation system for 75 days. The parameters evaluated were growth, colour, total carotene content of muscle, and antioxidant capacity of liver and muscle. We found significant differences ($p < 0.05$) in the growth parameters having the SP0 treatment less growth than the β0, SP5, and SP10 treatments. The colorimetric analysis showed that the redness and carotene deposition in muscle of mullet is the same between β0 and SP10. Mullets fed with *Spirulina* (SP5 and SP10 treatments) had a higher antioxidant capacity against radical peroxyls in liver tissue, showing lower lipid peroxidation (TBARS). In conclusion, *Spirulina* can be a suitable substitute at 10% of inclusion for pure β-carotene addition in fish diets, improving some of the health benefits from this carotene.

Keywords: Antioxidant capacity, colorimetric analysis, fish, TBARS

1. Introduction

Carotenoids are one of the most important pigment groups in living organisms (Britton, 1995). The adding of carotenoids to the diet is a common practice in aquaculture productions (EFSA, 2005), since the presence of colour on skin and fillet is a desirable attribute for the consumer and a quality indicator (Torrissen and Christiansen, 1995). Also, the content of carotenoids in animal feed gives many valuable health benefits. They are precursors of vitamin A (Nagao, 2011), enhance the immune system (Chew and Jean, 2004) and have antioxidant properties that neutralise free radicals resulting from oxidative stress (Ahmed *et al.*, 2014; Biswal, 2014).

As aquaculture intensification grows, fish susceptibility to oxidative stress also increases, leading to an overproduction of reactive oxygen-nitrogen species (RONS) that trigger mitochondrial dysfunction (Halliwell, 2007). Fish are potentially at risk of oxidative attack for some of the following reasons: elevated highly unsaturated fatty acids (HUFA) content in feed (Oliva-Teles, 2012), exposure to metabolic subproducts of protein catabolism (i.e., ammonia) (Maltez *et al.*, 2017). Adequate nutrition can be a tool to maintain health, improve growth, and increase the quality of the produced organisms (Pohlenz and Gatlin, 2014).

Spirulina has great varieties of bioactive compounds that have immune stimulant and antioxidant properties (Belay, 2002; Habib *et al.*, 2008; Adel *et al.*, 2016). Among these bioactive compounds are carotenoids, phycocyanin, gamma-linolenic acid (GLA), vitamins, and minerals (Ravi *et al.*, 2010). The quantity of carotenoids extracted from *Spirulina* can vary according to the production methods, but can reach up to 500 mg 100 g^{-1} (Belay, 2008). Moreover, the proportion of the many carotenoids that *Spirulina* has might vary; the most abundant are β-carotene and zeaxanthin (Habib *et al.*, 2008). Many fractions of the *Spirulina* have proven antioxidant activity as the polysaccharides in its cell membrane (Chaiklahan *et al.*, 2012), the phycocyanin biliprotein (Chen *et al.*, 2014) and carotenoids (Ahmed *et al.*, 2014). For these reasons, we hypothesised that the inclusion of small amounts of *Spirulina* in fish feed would be a suitable substitute for carotenoids employed in the aquaculture industry, giving more benefits than the traditional practice.

Mullets have high economic importance worldwide, for fisheries and aquaculture production (Crosetti, 2016). From the 740,000 t produced in 2014, almost 20% were produced

in aquaculture systems in 15 different countries (FAO, 2016). The mullet *Mugil liza* is a species with fast ontogenic development (Galvão *et al.*, 1997), easy adaptation to consume novel ingredients (Zamora-Sillero *et al.*, 2013; Ramos *et al.*, 2015). All of these characteristics make mullets a desirable species for aquaculture production.

In order to study alternative substitutes for commercial carotenes, we compared small inclusion of *Spirulina* vs. β-carotene in mullet diets and measured resulting fillet quality and fish antioxidant and oxidative damage responses.

2. Materials and methods

2.1 Fish and experimental design

Once approved by the Etic Comity (FURG- CEUA Pq036/2014). Juvenile mullets (n = 120) were captured using a 3 mm beach seine net at Cassino beach/southern Brazil (Latitude: -32.1833, Longitude: -52.1667) and taken to the Laboratory of Nutrition of Aquatic Animals (LANOA) at the Federal University of Rio Grande (FURG). The fish were acclimated to laboratory conditions for one month in a 500 L tank under controlled temperature (26°C), salinity (30 ppt), and a photoperiod of 12:12 (light:dark). During the acclimation period, mullets were hand fed the control diet (SP0) four times per day (9:00, 12:00, 14:00, and 16:00 h). Tank water was replaced every day in order to keep high water quality conditions.

Afterwards, a 75-day experiment was conducted in 12 80-L plastic tanks connected by a recirculation aquaculture system (RAS), which consisted of a biological filter, a UV light sterilizer (18W Philips®), and a protein skimmer. During this period, the 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 10 individuals (0.47 ± 0.01 g, initial mean weight). Water quality parameters were measured daily in all tanks and remained stable throughout the experimental period. Dissolved oxygen concentrations and temperature were measured with a multi-parameter equipment (YSI, 550A, Yellow Springs, Ohio) and maintained at 6.38 ± 0.27 mg L^{-1} and 27.6 ± 1.1°C, respectively. Water pH was measured with a digital pH meter (Hanna Instruments, HI221) and presented a mean value of 7.73 ± 0.18. Salinity was kept at a constant 32.6 ± 0.6 ppt and was measured with an optical refractometer (RTS 101, ATAGO). 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 ± 0.15 mg L^{-1} and 0.21 ± 0.20 mg L^{-1}, respectively. We added $CaCO_3$ to maintain an alkalinity of 105 ± 0.5 mg L^{-1} of $CaCO_3$; we measured alkalinity according to APHA (2005).

Four diets were prepared, including a control diet (SP0) and three experimental diets: control+β-carotene (β0) and 5% (SP5) and 10% (SP10) of *Spirulina* (*Arthrospira platensis)* inclusion. Each diet was tested in triplicate in a randomised design. Fish were fed 10% of the total weight biomass per tank for the first 15 days. We then made adjustments, according to the feed intake, to 8% of the total biomass for the rest of the experiment, four times per day (9:00, 12:00, 14:00, and 16:00 h). The feed offered daily was adjusted assuming a feed conversion ratio of 2:1 (Zamora-Sillero et as., 2016).

2.2 Diet formulation and elaboration

Diets were formulated to contain 35% crude protein (Carvalho *et al.*, 2010) and 9% lipids. The dry microalgae *A. platensis* was obtained from a commercial brand (Prilabsa®). The pre-weighed ingredients were mixed mechanically (Marconi, MA200) and then mixed with oil and water to produce a stiff dough; the mixtures were pelleted using a meat grinder (Metalúrgica 9000, PC-22). Pellets were air dried in an oven at 65°C for 24 h (Marconi, MA035). The resulting pellet size was adjusted gradually to the fish growth starting from 0.3 to 500 mm, and pellets were stored in plastic bags at -18°C until use. Diet formulation and proximal composition are shown in Table 1.

Table 1. Diet formulation and final composition (g/100g). (SP0 = Control Diet, β0 = Control+ β-carotene, SP5 = *Spirulina* inclusion 5%, SP10 = *Spirulina* inclusion 10%).

Ingredients	**SP0**	**β0**	**SP5**	**SP10**
Fish meal [a]	45	45	42.975	40.5
A. platensis [b]	0	0	2.025	4.5
Soybean meal [c]	10	10	10	10
Wheat meal [d]	4	4	4	4
Fish oil [e]	6	6	6	7
Starch [f]	19	19	19	19
Cellulose	11	11	11	10
Mineral/Vitamin				
Premix [g]	2	2	2	2
Gelatin	3	3	3	3
TOTAL	100	100	100	100
β-carotene [h] (mg kg^{-1})	0	50	0	0
Proximate analyses (dry weight g/ 100g)				
Protein	38.33	38.98	38.73	38.45
Lipids	9.75	9.88	9.43	9.82
Ash	11.18	12.36	10.91	10.82
N.N.E	40.74	38.77	40.93	40.91
TCC[i] (mg kg^{-1})	4.13	60.98	77.19	169.44

[a]Leal Santos, Rio Grande, RS, Brazil.
[b]Prilabsa®, Brazil.
[c]Sulino RS, Brazil.
[d]Sulino , RS, Brazil.
[e]Campestre®, São Paulo. Brazil.
[f] Pazze ®, Brazil.
[g]Maizena®, Brazil
[h]Premix M. Cassab, São Paulo, Brazil (Vit. A (500.000 Ul kg^{-1}), Vit. D3 (250.000 Ul kg^{-1}), Vit. E (5.000 mg kg^{-1}), Vit. K3 (500 mg kg^{-1}), Vit. B1 (1.000 mg kg^{-1}), Vit. B2 (1.000 mg kg^{-1}), Vit. B6 (1.000 mg kg^{-1}), Vit. B12 (2.000 mg kg^{-1}), Niacin (2.500 mg kg^{-1}), Calcium pantothenate (4.000 mg kg^{-1}), Folic acid (500 mg kg^{-1}), Biotin (10 mg kg^{-1}), Vit C (10.000 mg kg^{-1}), Choline (100.000 mg kg^{-1}), Inositol (1.000 mg kg^{-1}). Trace elements: Selenium (30 mg kg^{-1}), Iron (5.000 mg/kg), Copper (1.000 mg kg^{-1}), Manganese (5.000 mg kg^{-1}), Zinc (9.000 mg kg^{-1}), Cobalt (50 mg kg^{-1}), Iodine (200 mg kg^{-1})).
[i]Biolys® L-lysine Sulfate (Evonik Industries, Germany)
[i] Total Carotenoid Content.

2.3 Evaluation of growth parameters and sampling

At the end of the 75-day experiment, each organism was anesthetised (benzocaine 50 ppm), and individual weights were obtained to determinate growth parameters for each dietary treatment. The evaluated 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 weight gain (g) / average protein intake (g); and survival = (final number of fish / initial number of fish) * 100.

All fish were euthanised with an overdose of benzocaine (300 ppm). We collected muscles and livers tissue of six fish per tank; the tissues were submerged in liquid nitrogen and stored in an ultrafreezer (-82ºC). The carcasses of 12 fish per treatment were frozen for body composition analyses.

All proximal analyses were conducted according the methodologies described by the AOAC (2000). The protein content analyses were made according to the micro-Kjendahl method (Ma and Zuazago, 1942), and the total ether extract analyses according to the Soxhlet method (AOAC, 2000). Ashes were obtained by oven incineration at 600ºC and the non-nitrogenized extract was quantified by the difference between all other constituents and 100% of the diet (AOAC, 2000).

2.4 Colorimetric analyses

For each treatment, nine fillets were assessed using the Minolta Chroma Meter analysis (Minolta Chroma Meter, CR-400 Minolta, Japan) that measures the reflectance of light from the fish flesh. The measured colour parameters were lightness (L*), red/green chromaticity (a*), and yellow/blue chromaticity (b*). The instrument was placed on the flesh, and triplicate measurements were taken at each fillet area in order to calculate a mean value. The a* value represented the redness and the b* value the yellowness of the flesh. From the a* and b* values, the chroma (C_{a^*b}) and hue (H_{a^*b}) were calculated (Hunt, 1977). The chroma is an expression of the intensity and clarity of the colour, whereas the hue is the relationship between the redness and the yellowness of the fillet. The following expressions were employed to calculate these parameters:

$$C_{a^*b} = (a^{*2} + b^{*2})^{1/2} \quad \text{Eq. (1)}$$

$$H_{a^*b} = \tan^{-1} (b^*/a^*) \quad \text{Eq. (2)}$$

2.5 Total Carotenoids Content determination (TCC)

The TCC analyses were elaborated according to the method proposed by Yanar *et al.* (2004). Nine samples from each treatment of 0.4 – 0.5 g of muscle tissue per sample were collected and macerated. Equal amounts of anhydrous sodium sulfate and 5 ml of acetone were added and mixed with the muscle sample. Acetone (5 ml) was used to wash the homogeniser twice, which was transferred to a 10 ml amber glass bottle, preserved for 3 days at 25°C in the dark, and then centrifuged for 5 min at 5000 r min^{-1}. The supernatant was scanned in a spectrophotometer (Biospectro, SP-22, Brazil) from 400 to 700 nm. Finally, TCC (µg g^{-1}) was calculated at the absorption value of 480 nm by the formula:

$$\text{TCC } (\mu g\ g^{-1}) = A_{\lambda=480\ nm} * K * V / (E * G), \quad \text{Eq. (3)}$$

where $A_{\lambda=480\ nm}$ is the absorbance value at λ = 480 nm; K is a constant (104); V is the volume of the extracting solution (ml); E is the extinction coefficient (1900); and G is the sample mass (g).

2.6 Antioxidant capacity against peroxyl radicals

Total antioxidant capacity against peroxyl radicals (ACAP) was determined through detection of reactive oxygen species (ROS) in samples treated and not treated with a peroxyl radical generator, according to Amado *et al.* (2009). All samples were diluted with homogenisation buffer to 2.0 mg protein mL^{-1}, then exposed to peroxyl radicals generated by thermal (37°C) decomposition of 2,2′-azobis(2 methylpropionamidine) dihydrochloride (ABAP, 4 mM). Peroxyl radicals reacted with a fluorescent substrate (2′,7′ dichlorofluorescein diacetate–H2DCF-DA), and fluorometry (excitation 485 nm; emission 520 nm) was measured with a microplate reader (BioTek, Synergy HT) with readings every 5 min for 35 min, employing Gen5 2.00 software to program reading intervals properly. The results were expressed as a relative area (the difference between the ROS area with and without ABAP

relative to that without ABAP). For result interpretation purposes, a higher relative area means a lower antioxidant capacity.

2.7 Lipid peroxidation

To determine the thiobarbituric acid reactive substances (TBARS) by the quantification of MDA (malondialdehyde) to measure lipid peroxidation levels, we used the method of Oakes and Van Der Kraak (2003): 20 μL of BHT solution (67 μM), 150 μL 20% acetic acid solution, 150 μL 0.8% TBA solution, 50 μL Milli-Q H_2O and 20 μL of 8.1% SDS (sodium dodecyl sulfate) were added to samples (liver 50 μL; muscle 100 μL) before being heated at 95°C for 30 min. Thereafter, 100 μL of Milli-Q H_2O and 500 μL of n-butanol were added to the final solution. The remaining supernatant after centrifugation (3000 × g, 10 min, 15°C) was used to determine the fluorescence (excitation 520 nm; emission 580 nm) with a microplate reader (BioTek, Synergy HT), and the results were expressed as nanomole TMP milligram wet tissue^{-1}, where TMP (tetramethoxypropane, ACROS Organics) employed as a standard.

2.8 Statistical analyses

A one-way analysis of variance (ANOVA) was used to identify possible differences among the treatments results. Normality and homogeneity of variance were tested according to Shapiro-Wilk and Levene tests, respectively. For each case, when significance between treatments was detected, a posterior means comparison was performed using Tukey's test. All tests were conducted at the 5% significance level.

3. Results

Table 2 summarises the growth performance of mullets fed on the experimental diets. Significant differences ($p < 0.05$) for FW, SGR, PER, FCR and survival between the control group and all other treatments were observed. No significant difference was observed in growth performance parameters between the β0 treatment and *Spirulina* treatments. Carcass proximal composition (Table 3) showed no significant differences ($p > 0.05$) in the muscle and lipids composition on all the experimental treatments. However, the carcass ash content presented significant differences ($p<0.05$) between the treatment SP0 and the SP5 treatment.

The fish muscle carotenoids concentrations are presented in Table 4. The highest carotenoid concentrations were observed in β0 (0.027 ± 0.08 μg g^{-1}) and SP10 (0.021 ± 0.06 μg g^{-1}) treatments, which were both significantly different ($p<0.05$) with respect to the SP0 treatment.

Table 2. Mullet *Mugil liza* growth performance. (SP0 = Control Diet, β0 = Control+ β-carotene, SP5 = *Spirulina* inclusion 5%, SP10 = *Spirulina* inclusion 10%).

	Treatments				**ANOVA**
	SP0	β0	SP5	SP10	p-value
IW (g)	0.48 ± 0.00	0.48 ± 0.01	0.48 ± 0.01	0.48 ± 0.01	>0.05
FW (g)	5.92 ± 0.29 b	10.97 ± 0.77 a	10.40 ± 1.08 a	10.86 ± 1.18 a	<0.001
FCR	2.38 ± 0.24 b	1.70 ± 0.11 a	1.82 ± 0.20 a	1.75 ± 0.22 a	0.009
SGR	3.38 ± 0.07 b	4.24 ± 0.08 a	4.15 ± 0.16 a	4.21 ± 0.17 a	<0.001
PER	1.01 ± 0.01 b	1.46 ± 0.09 a	1.37 ± 0.17 ab	1.42 ± 0.18 a	0.01
Survival(%)	73.81 ± 2.06 b	96.7 ± 5.77 a	100 ± 0 a	95.2 ± 8.25 a	<0.001

Values are expressed as means ± SD of three replicate groups. Similar letters in each row show absence of significant differences in Tukey's test or global ANOVA test at $p \leq 0.05$. IW = initial weight; FW = final weight; SGR = specific growth ratio; FCR = feed conversion ratio; PER = protein efficiency ratio.

Table 3. Carcass proximal composition analysis of mullet *Mugil liza* (wet weight basis, g Kg^{-1}) (SP0 = Control Diet, β0 = Control+ β-carotene, SP5 = *Spirulina* inclusion 5%, SP10 = *Spirulina* inclusion 10%).

Analyses	**Treatments**				**ANOVA**
	SP0	β0	SP5	SP10	p-value
Moisture	66.77 ± 1.73 a	65.30 ± 0.39 a	66.06 ± 2.43 a	66.04 ± 2.05 a	>0.05
Protein	18.39 ± 2.46 a	19.30 ± 2.06 a	18.65 ± 2.11 a	18.53 ± 1.69 a	> 0.05
Ether Extract	9.68 ± 4.94 a	11.03 ± 1.60 a	10.44 ± 2.06 a	10.23 ± 2.99 a	>0.05
Ash	4.40 ± 0.51 a	4.51 ± 0.64 ab	3.58 ± 0.46 b	4.64 ± 0.78 ab	0.001

Values are expressed as means ± SD of three replicate groups. Similar letters in each row show absence of significant differences in Tukey's test or global ANOVA test at $p \leq 0.05$.

Some colour measurement parameters of the fillets were affected by dietary treatment (Table 4). The relative yellowness (b*) and the lightness (L) parameters showed no statistical

differences among the treatments. The a* values, which indicate the relative redness of a sample, showed less redness for the SP0 treatment in comparison to the treatments β0 and SP10 treatments ($p<0.05$), muscles from fish in the treatments β0 and SP10 showed more redness than the other treatments. Also, the chroma parameter was significantly higher ($p<0.05$) in the SP10 treatment (14.38 ± 0.33) than the SP0 treatment. The hue parameter was significantly higher (50.39 ± 2.82; $p < 0.05$) in fish from the β0 treatment than in the SP0 treatment, while hue values in fish from the *Spirulina* treatments showed no significant differences compared to the pure β-carotene treatment.

Table 4. Mullet *Mugil liza* fillet colorimetric analyses and total carotenoid content (TCC) extracted from muscle. (SP0 = Control Diet, β0 = Control+ β-carotene, SP5 = *Spirulina* inclusion 5%, SP10 = *Spirulina* inclusion 10%).

Colour	**Treatments**				**ANOVA**
	SP0	β0	SP5	SP10	p-value
L	46.01 ± 1.83	43.18 ± 1.62	45.22 ± 3.01	43.36 ± 1.15	>0.05
a*	7.23 ± 1.34 b	10.36 ± 0.48 a	9.26 ± 1.02 ab	10.27 ± 0.06 a	0.007
b*	8.73 ± 1.01	8.58 ± 0.57	9.38 ± 1.01	10.06 ± 0.52	>0.05
C	11.34 ± 1.61 b	13.46 ± 0.35 ab	13.20 ± 0.98 ab	14.38 ± 0.33 a	0.02
H	39.42 ± 2.47 b	50.39 ± 2.82 a	44.65 ± 4.55 ab	45.62 ± 1.63 ab	0.01
TCC μ g^{-1}	0.001 ± 0.01 b	0.027 ± 0.06 a	0.018 ± 0.03 ab	0.021 ± 0.08 a	0.012

L = lightness; a* = red/green chromaticity; b* = yellow/blue chromaticity; C = chroma; H = hue; TCC = Total Carotenoid Content. Values are expressed as means ± SD of three replicate groups. Similar letters in each row show absence of significant differences in Tukey's test or global ANOVA test at $p \leq 0.05$.

The ACAP analyses showed significant differences ($p<0.05$) from those diets supplemented with *Spirulina* (SP5 and SP10) and the ones without it (SP0 and β0) (Table 5), showing for all the organs analysed a smaller relative area, that means a higher antioxidant capacity against hydroxyl radicals. The antioxidant capacity of livers from the *Spirulina* treatments was 52% and 48% (SP5 and SP10, respectively) witch were higher to the β0

treatment. For muscle, the increase was 50% in the SP5 and 47% in the SP10 diets, with respect to the β0 treatment.

The TBARS analyses (Table 5) showed significant differences in liver tissue ($p < 0.05$) between the SP0 treatment and the SP5 and SP10 treatments. The TBARS values of livers from the β0 treatment showed no significant difference ($p > 0.05$) from the control treatment. The TBARS levels from muscle did not present significant differences ($p > 0.05$) for any treatment.

Table 5. Total antioxidant capacity against peroxyl radicals (ACAP) (relative area) and thiobarbituric acid reactive substances (TBARS) content (nmol TMP mg wet tissue−1) in liver and muscle of juvenile mullet (*Mugil liza*) (n=5). (SP0=Control Diet, β 0= Control+ β-carotene, SP5 = *Spirulina* inclusion 5%, SP10 = *Spirulina* inclusion 10%)

	Treatments				**ANOVA**
ACAP	SP0	β0	SP5	SP10	p-value
Liver	3.33 ± 0.99 b	3.11 ± 0.58 b	1.48 ± 0.44 a	1.61 ± 0.64 a	<0.001
Muscle	4.22 ± 1.34 b	3.46 ± 1.14 b	1.71 ± 0.80 a	1.80 ± 0.50 a	<0.001
TBARS	(nmol TMP mg wet tissue^{-1})				
Liver	0.019 ± 0.001b	0.015 ± 0.001 ab	0.013 ± 0.002 a	0.014 ± 0.002 a	0.005
Muscle	0.003 ± 0.000	0.006 ± 0.005	0.003 ± 0.000	0.007 ± 0.000	> 0.05

Values are expressed as means ± SD of five replicate groups. Different letters in each row show significant differences in Tukey's test at $p \leq 0.05$

4. Discussion

Our results indicate that *Spirulina* as a feed additive showeds no difference in comparison to the β-carotene diet in terms of growth performance or carcass proximal analyses. Also the β0, SP5, and SP10 treatments showed superior growth and survival when compared to the SP0 treatment. These reasons for these differences vary. The addition of pure β-carotene have already shown positive effects in growth in tilapia hybrids (*Oreochromis niloticus* × *O. aureus*) (Hu *et al.*, 2006), although other authors found no improvement of growth by the addition of β-carotene in other aquaculture species, such as *Penaeus monodon* (Niu *et al.*, 2014) and rainbow trout (*Oncorhynchus mykiss*) (Amar *et al.*, 2001). The

improvements in growth given by β-carotene can be explained due that this carotenoid is a precursor of vitamin A in the organisms. This conversion is known to be catalysed by enzymes in mammals (Olson, 1989) and fish (Kaisuyama and Matsuno, 1988). On the other hand, the improvement in fish growth performance as a result of the addition of *Spirulina* in diets has been widely reported in fish species like sturgeon (Adel *et al.*, 2016), parrot fish (*Oplegnathus fasciatus)* (Kim *et al.*, 2013), giant catfish (Tongsiri *et al.*, 2010), and rainbow trout (Teimouri *et al.*, 2013b). The benefits of *Spirulina* have many explanations, such as the increase in lipase and protease activity (Adel *et al.*, 2016), the improvement in feed efficiency by the increase of gut bacterial colonization (Teimouri *et al.*, 2013a), or the high vitamin content (Abdel-Tawwab and Ahmad, 2009). Also, *Spirulina* is a rich source of natural carotenoids that can also be converted to vitamin A. This fact could be seen by the higher total carotenoids content presented in SP5 and SP10 (77.19 and 169.54 mg kg^{-1}, respectively) diets compared to the control SP0. The internal transport of carotenoids in the organism is strictly linked to lipid availability (Erdman *et al.*, 1993). In the carcass analyses, no difference in lipid content was found, so all the treatments had, *a priori*, the same availability of lipids for internal carotenoid transport.

We observed an increase in the a* (redness) coloration of mullet fillet in both β0 and SP10 treatments. Also, the TCC of muscle showed the same tendency, meaning that higher accumulation of carotenoids happened with pure β-carotene or 10% *Spirulina* inclusion. Similar results have been presented by Teimouri *et al.* (2013a) who showed that 5% *Spirulina* inclusion reached the a* and b* coloration of astaxanthin in salmon, and that 10% inclusion could obtain even better results. Teimouri *et al.* (2013b) also found the highest deposition of carotenoids in trout muscle at 10% *Spirulina* inclusion. Promya and Chitmanat (2011) also observed that 10% *Spirulina* inclusion resulted in higher carotenoid retention in the muscle of catfish. Moreover, Tongsiri *et al.* (2010) recommend a dose of *Spirulina* higher than 10% to obtain a more efficient carotenoid retention in muscle. Although some carotenoids, such as astaxanthin, have proven to take priority in the fillet deposition (Amar *et al.*, 2001; Niu *et al.*, 2014) for some species, it is also known that the carotene uptake is selective among different species intestines, causing differences in the accumulation of carotenoids in muscle (Nagao, 2011). *Spirulina* can contain a numerous variety of carotenoids, such as lutein, zeaxanthin, β-cryptoxanthin, and β-carotene (Miki *et al.*, 1986; Careri *et al.*, 2001), which also play a role in

fillet coloration and that might be responsible for the increase in carotenoids deposition in fillets from fish in the SP10 treatment, leading to the increase in chroma and hue parameters in fish from the *Spirulina* diets. In fact, some authors have indicated that, parallel to the increases in carotenoid retention in muscle, there also is an increase in some colorimetric parameters, such as a*, b*, and chroma (Christiansen *et al.*, 1995; Nickell and Bromage, 1998). Some other factors, such as metabolism of carotenoids, participate in the coloration of muscle (Torrissen and Christiansen, 1995). For example, the zeaxanthin is in the metabolic conversion route of astaxanthin (Kaisuyama and Matsuno, 1988), and the latter is one of the most preferred carotenoids to be absorbed by fish intestinal cells. There are numerous varieties of carotenoids in *Spirulina*, and each one of them has different bioavailability behaviour. Some of the factors that modify carotenoids availability are their lipophilicity and the amount of fat included in the diet (van het Hof *et al.*, 2000). These reasons could explain why the SP5 diet, despite having a high amount of carotenoids, showed no significant difference with the SP0 treatment for any parameter. Our results indicate that, in mullet fillet, 10% *Spirulina* inclusion can match the colorimetric quality of the pure β-carotene inclusion in most of the colour parameters (a*, b*, hue, and chrome). Even β-carotene has more bioavailability when provided pure than when it is naturally present in food (van het Hof *et al.*, 2000), and different forms of the same carotenoid (synthetic or natural) differ in the absorption mechanisms and bioefficacy (Priyadarshani, 2017).

Production of ROS comprises diverse chemical species, including superoxide anions, hydroxyl radicals, and hydrogen peroxides, formed during normal metabolism processes, and they can trigger lipid peroxidation (Pisoschi and Pop, 2015). Moreover, these processes can happen under normal freezer storage of fish fillet (-20°C) and increase over time (Teimouri *et al.*, 2016), which results in lipid oxidation (lipid rancidity) that is responsible for the fillet colour change and off-odour of the final product (Sohn *et al.*, 2005). The ACAP analyses demonstrated that muscle from fish with diets containing *Spirulina* (SP5 and SP10) had a significant increased antioxidant capacity to neutralize peroxyl radicals over the other treatments. It is known that β-carotene has antioxidant capacity against singlet oxygen ($^1O_2$) related to its conjugated double bond structure (Stahl and Sies, 2003). On the other hand, *Spirulina* has been considered a source of many antioxidants, as reported by Estrada *et al.* (2001) who showed the phycocyanin fragment of *Spirulina* increases the antioxidant activity

against hydroxyl radicals. Also, Bermejo *et al.* (2008) found that *Spirulina* protean extract scavenged hydroxyl and peroxyl radicals and presented inhibitory activity against lipid peroxidation. Due to the fact that *Spirulina* has an antioxidant capacity against a wide variety of ROS sub-products, it could be assumed that *Spirulina* is more efficient in protecting against oxidative stress than β-carotene.

The reduction in TBARS detected in livers from fish in *Spirulina* treatments is in accordance with the antioxidant protection in livers found in many species of rats (Gad *et al.*, 2011) and rabbits (Kim *et al.*, 2010). The liver is one of the main converter organs of carotenoids to vitamin A (Khachik *et al.*, 2002) and, for this reason, a high amount of carotenoids are stored in this organ, a feature that should explain the improvement in terms of antioxidant capacity and reduction in lipid peroxidation found in the present study. The muscle, however, showed no significant difference between the treatments. Teimouri *et al.* (2016) found no effect, in terms of TBARS levels, at the end of the feed trial from trout fed with *Spirulina*, but they also reported an antioxidant effect in the muscle in frozen storage over time.

In conclusion, both β-carotene and *Spirulina* diets have been proven to promote more benefits in health and growth in fish than the control diet (β0). Benefits from both additive products showed the same improvement in terms of growth and colorimetric parameters, but it should be noted that *Spirulina* surpasses the benefits in antioxidant activity and has a greater capacity to inhibit lipid peroxidation. For this reason, *Spirulina* is a more suitable additive for mullet diet than pure β-carotene.

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ANEXO 4

FACTIBILIDAD DEL USO DE *SPIRULINA* EN LA ACUICULTURA.

Factibilidad del uso de *Spirulina* en la acuicultura.

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Resumen

El uso de la *Spirulina* en la nutrición humana y animal ha sido ampliamente evaluado en la nutrición demostrando, en la mayoría de los casos resultados favorables al incluirla en la alimentación. La acuicultura ha incrementado su interés en este ingrediente en la última década, comprobando sus efectos como estimulador del crecimiento y de salud en múltiples especies de importancia para la acuicultura. El hecho de que su implementación en la acuicultura no sea una realidad es atribuido a su valor económico elevado. Aun así, es importante mencionar que inclusive en pequeñas cantidades la *Spirulina* puede mejorar considerablemente la salud de los organismos, lo que a largo plazo podría compensar su elevado valor económico disminuyendo costos en otras áreas de producción.

1. Introducción

La harina de pescado (HP) es un ítem muy valorado en la industria de la acuicultura ya que es uno de los ingredientes más nutritivos y completos en la formulación del pienso para peces (FAO, 2016). Pues esta década llego a su límite de producción pues las pesquerías han venido a ser sobreexplotadas en las últimas décadas, lo que en consecuencia incremento su valor económico (Tacon y Metian, 2015). Hay evidencias de que el uso de la harina de pescado está disminuyendo siendo substituido por fuentes alternativas, aun así su precio es muy volátil, lo que puede implicar riesgos en la sustentabilidad de la acuicultura (Asche *et al.*, 2013), pues los costos de producción referentes a la alimentación pueden llegar a ocupar hasta el 60% de los costos totales en un sistema de producción (Knapp, 2008). La *Spirulina* ha sido

evaluada extensamente por sus excelentes propiedades nutricionales como un posible suplemento de la nutrición humana y animal (Belay *et al.*, 1996; Ravi *et al.*, 2010).

En el área de la acuicultura la investigación de las propiedades nutricionales de la *Spirulina* ha tomado dos caminos: el de la substitución y el de la inclusión. Para definir estos caminos hemos de diferenciar los términos substitución e inclusión; reconociendo que los trabajos que realizan la substitución de HP u otras fuentes nutricionales proteicas (p.ej. soya) se refieren al hecho de quitar un ingrediente de la formulación y substituirlo por otro en la misma proporción. Para fines de esta investigación se consideró substitución el intercambio de un ingrediente que alcanzaba nivel(es) superior(es) al 20% de su inclusión. En el caso de la inclusión, consideramos dos tipos de trabajos, los que realizan substituciones menores al 20% de inclusión o aquellos que establecían una dieta control y suplementaban con pequeñas cantidades de *Spirulina* independientes de la formulación original. Fueron desconsiderados trabajos que utilizaran *Spirulina* en dietas de organismos acuáticos pero que no evaluaran sus efectos en crecimiento. Una vez definidos estos términos se puede apreciar que la inclusión y la substitución han sido ampliamente estudiadas, principalmente en la última década donde ha aumentado considerablemente su investigación.

2. Substitución de fuentes proteicas por *Spirulina* en acuicultura.

Los estudios de substitución de fuentes proteicas para balanceados en acuicultura (Tabla 1) abarcan principalmente estudios de piscicultura y en segundo lugar carcinocultura y otros. Estos tienen como objetivo principal la disminución en el uso de la HP; donde la *Spirulina* se destaca como un posible substituto por tener un alto valor proteico (hasta 70%), y por inferir resistencia en los cultivos debido las propiedades de sus nutrientes tales como vitaminas, minerales, ácidos grasos y pigmentos (carotenoides y biliproteinas) (Gershwin y Belay, 2007; Habib *et al.*, 2008; Ravi *et al.*, 2010). Los porcentajes a ser substituidos varían mucho entre especies, pues los hábitos alimenticios de los organismos estudiados influencian fuertemente la digestibilidad, retención y absorción de nutrientes (Hansen *et al.*, 2008; Santigosa *et al.*, 2008). Los únicos reportes donde la substitución total fue realizada con éxito sin perjudicar el rendimiento en los parámetros zootécnicos son los hechos con carpas (Nandeesha *et al.*, 1998; Nandeesha *et al.*, 2001). De forma más detallada, la investigación referente a la substitución total de HP por *Spirulina* ha reportado deficiencias en la

composición de amino ácidos (AA) de las diferentes variedades de *Spirulina*, principalmente en las proporciones de lisina y también en metionina, histidina, arginina y treonina (Olvera-Novoa *et al.*, 1998; Jaime-Ceballos *et al.*, 2006; Macías-Sancho *et al.*, 2014; Rosas *et al.*, In Press). Como resultado de estas deficiencias se puede observar un menor crecimiento de los animales y en casos mas severos mortalidades. Además de las carpas, solamente el camarón, *Litopenaeus vannamei* aceptó un alto nivel de substitución (75%) sin afectar al crecimiento (Macías-Sancho *et al.*, 2014). La mayoría de los experimentos realizados en la piscicultura aceptan un nivel de substitución que oscila entre los valores de 40-60%, en estos valores es donde ocurre el mejor rendimiento de los parámetros zootécnicos (El-Sayed 1994; Olvera-Novoa *et al.*, 1998; Palmegiano *et al.*, 2005; Palmegiano *et al.*, 2008; Dernekbasi *et al.*, 2010, Ahmadzade-Nia *et al.*, 2011; Rosas *et al.*, In Press). Estas mejorías en el rendimiento causadas por la presencia de *Spirulina* tienen diferentes explicaciones entre ellas está su efecto prebiótico (Ramakrishnan *et al.*, 2008), el incremento en la actividad enzimática (Nandeesha *et al.*, 1998; Adle *et al.*, 2016), su alto contenido vitamínico (Abdel-Tawwab y Ahmad, 2009), y su actividad antioxidante e inmunoestimulante (Belay *et al.*, 1996; Ravi *et al.*, 2010). Aún con todos estos beneficios reportados es importante considerar que en exceso la *Spirulina* puede provocar indicios de actividad pro oxidante (Macari *el al.*, 2011, Dal Bosco *et al.*, 2014).

Tabla 1. Estudios efectuados con substitución de *Spirulina* en la acuicultura.

Substitución %	Especie	Resumen de resultados	Referencia
25, 50, 75, 100	Silver seabream *(Rhabdosargus sarba)*	El 50% de substitución fue posible. 100% de substitución no fue posible probablemente por una deficiencia de AA.	El-Sayed 1994
20, 40, 60, 80, 100	Tilapia (*O. mossambicus)*	El 40% de substitución fue posible. Disminución en lisina y metionina fue reportado.	Olvera-Novoa *et al.*, 1998
25, 50, 75, 100	Carpa común (*Cyprinus carpio*)	El 100% de substitución es posible, mejora actividad de Proteasa, lipasa y amilasa.	Nandeesha *et al.*, 1998
25, 50, 75, 100	Carpas (*Catla catla* y *Labeo rohita*)	Ambas especies aceptaron el 100% de substitución.	Nandeesha *et al.*, 2001
100	Tilapia *(Oreochromis Niloticus)*	La substitución disminuye el crecimiento y el contenido de ácidos grasos esenciales, por otro lado aumenta la sobrevivencia	Takeuchi *et al.*, 2002
40, 50, 60	Esturión (*Acipenser baeri*)	El 50% de substitución da el mayor rendimiento.	Palmegiano *et al.*, 2005
25, 50, 75, 100	Larvas de *Litopenaeus schmitti*	*Spirulina* substituyó *Chaetoceros muelleri*, 25%, de substitución resulto en los mejores índices de desarrollo. La dieta de 100% de inclusión de *Spirulina* fue deficiente en lisina e histidina y disminuyó el crecimiento.	Jaime-Ceballos *et al.*, 2006
45	Esturión (*Acipenser transmontanus)*	La substitución de 45% *Spirulina* fue exitosa pero los niveles de LC-PUFAS disminuyeron en dietas y por consecuencia en el musculo.	Palmegiano *et al.*, 2008
10, 20, 30, 40	Guppy (*Poecilia reticulata*)	Puede realizarse 40% de inclusión con éxito.	Dernekbasi *et al.*, 2010
5, 10, 100	Bagre gigante *Pangasianodon gigas*	Recomienda una substitución del 10%. El 100% de substitución incrementa la pigmentación de piel.	Tongsiri *et al.* 2010
20, 40, 60, 80	Trucha arco-íris	La *Spirulina* substituyó harina de soya, 60% de la substitución mostró el mejor rendimiento.	Ahmadzade-Nia *et al.*, 2011
25, 50, 75, 100	Trucha (*Oncorhynchus mykiss albaum*)	La dieta con 75% *Spirulina* y 25% soya dio el mejor rendimiento, pero no superó el crecimiento del tratamiento control.	Hernández *et al.*, 2012
25, 50, 75, 100	*Litopenaeus vannamei*	La substitución fue exitosa hasta el nivel de 75%, la dieta de 100% de inclusión de *Spirulina* fue deficiente en arginina, treonina y lisina. La *Spirulina* aumenta la inmunidad.	Macias-Sancho *et al.*, 2014
25, 50, 75, 100	Tenca (*Tinca tinca*)	25% de substitución fue posible.	Vasconi *et al.*, 2014
25, 50, 75, 100	Pacific geoduck clam (*Panopea generosa*)	Se intentó substituir el uso de las microalgas *Chaetoceros muelleri* y *Tisochrysis lutea*, por *Spirulina* seca en spray sin éxito en ningún nivel de substitución.	Arney *et al.*, 2015
30, 45, 60, 75	Tilapia *(Oreochromis Niloticus)*	30% de substitución da el mejor desempeño La presencia de *Spirulina* disminuye los triglicéridos en sangre.	Velasques *et al.*, 2016

30, 50, 70, 100	Lisa (*Mugil liza*)	La substitución del 50% dio el mejor rendimiento en crecimiento e incrementó la producción de linfocitos T y mejoró la respuesta apoptotica.	Rosas *et al.*, In Press

3. Inclusión de *Spirulina* en acuicultura.

Se puede apreciar que en la última década el incremento en la cantidad de experimentos referentes a *Spirulina* como suplemento en el área de acuicultura, y que las diferentes áreas de la acuicultura que utilizan este ingrediente son diversas, pues engloban producción de peces de engorda, producción de peces ornamentales, carcinocultura y otras.

Los estudios de inclusión de *Spirulina* (Tabla 2) muestran que aún con pequeñas cantidades de este ingrediente se consigue causar efectos benéficos en la salud general del organismo objetivo, pues incluso dosis de 10 mg diario por pez en la dieta consiguió estimular la inmunidad y la resistencia de la tilapia (Ragap *et al.*, 2012). Las áreas principales de acción de la *Spirulina* dentro del organismo son cuatro: 1) Mejora el crecimiento (CR), ya evaluado en la sección anterior; 2) Mejora la calidad del producto final (PF), esta área de acción de *Spirulina* varía según el fin zootécnico de la especie, pues en las especies que tienen como objetivo productivo el filete incrementa los tonos rojos características que es atractiva para el mercado. Por otro lado también puede mejorar los índices reproductivas de los reproductores como fecundidad y fertilidad (Kohal *et al.*, 2017) y finalmente infiere color en la piel, característica deseada en la creación de peces ornamentales (James *et al.*, 2006; Karadal *et al.*, 2017); 3) Mejora el sistema inmune (SI), que puede englobar el sistema inmune específico (Duncan y Klesius, 1996), sistema inmune no-específico (Ibrahem e Ibrahim, 2013), resistencia bacteriana (Adle *et al.*, 2016) o memoria inmune (Ragap *et al.*, 2012); 4) Mejora de la respuesta antioxidante (RA) contra radicales libres y peroxidación lipídica (Kim *et al.*, 2013; Teimouri *et al.*, 2015).

Las ventajas del uso de *Spirulina* pueden ser variadas y en la mayor parte de los casos están estrictamente relacionadas. Un ejemplo, es el caso de los carotenoides. El consumo de carotenoides a través de la inclusión de *Spirulina* en las dietas de peces hace que sean absorbidos y transportados mediante los lípidos de baja densidad a diferentes órganos/células del organismo donde serán acumulados (van het Hof *et al.*, 2000). Su acumulación en el músculo infiere color (Promya y Chitmanat, 2011), lo que en general resulta en un incremento

de valor como producto en el mercado (Christiansen *et al.*, 1995). De acuerdo a las características del carotenoide que fue ofrecido, puede ser metabolizado a un tipo de carotenoide específico (Torrisen, 1989). En estas células incrementará la resistencia a la peroxidación lipídica, lo que implica en un mayor tiempo de estante en fresco y congelado (Teimouri *et al.*, 2015). En el torrente sanguíneo la concentración de carotenoides adquirida a través de la *Spirulina* es proporcional a la cantidad de esta en la dieta (Teimouri *et al.*, 2013b), aquí los carotenoides pueden ser fácilmente distribuidos al organismo y sirven como protección contra radicales libres (Fiedor y Burda, 2014). Los beta-carotenos estimulan la citotoxicidad de los linfocitos y aumentan la producción de superóxido de los macrófagos, y también protegen a los macrófagos de ser inhibidos por otras sustancias (Chew *et al.*, 1997).

Otro caso a considerar es el aporte de biliproteinas que las cianobacterias verde-azules pueden agregar a las dietas de organismos acuáticos, siendo la *Spirulina* una de las mayores productores de ficocianina para consumo humano y animal. Esta biliproteina consiste de dos sub unidades α- y β-ficocianina (Saxena, 1988), cada una con diferentes capacidades inmunoestimulantes (Wang *et al.*, 2007). Chen *et al.* (2014) mostraron que la cantidad de ficocianina producida por la *Spirulina* podía ser modulada por la cantidad de sulfato de amonio en el agua, 40% de este reactivo incrementaba significativamente la producción de esta proteína. Los beneficios que esta proteína agrega a la dieta de organismos acuáticos son muchas, sirve como modulador selectivo de la ciclooxigenasa-2, enzima catalizadora de prostaglandinas (Reddy *et al.*, 2000) de esta forma tiene un papel importante en la respuesta inflamatoria. Posiblemente el papel importante de esta biliproteina es el de antioxidante, pues tienen un papel importante secuestrando radicales peroxyl, hidroxilo, alcoxilo y superóxido, e inducen enzimas que participan en la defensa contra el daño oxidativo (Romay et al., 1998; Romay et al., 2003). En estudios de acuicultura, pocas veces fueron comprobados los beneficios de la ficocianina en la salud del pez. Por lo que esta área de estudio aún cuenta con gran potencial de exploración.

Tabla 2. Estudios efectuados con inclusión de *Spirulina* en la acuicultura.

% inclusión	Especie	Resumen de resultados	Área	Referencia
5, 10	Striped Jack *(Caranx vinctus)*	Todas las dosis mejoran el color del tegumento.	PF	Okada *et al.*, 1991
2.7	channel catfish *Ictalurus punctatus*	*Spirulina* estimula la respuesta inmune celular no específica, aumenta la respuesta de anticuerpos timo-dependientes. Aumenta la resistencia contra *Edwardsiella ictalur*	SI	Duncan y Klesius 1996
2.5, 5	Larvas de *Litopenaeus schmitti*	La inclusión de *Spirulina* afectó el índice de desenvolvimiento y disminuyó el crecimiento.	CR	Jaime-Ceballos *et al.*, 2005
1, 3, 5, 8	*Xiphophorus helleri*	*Spirulina* aumenta la coloración en piel, la retención de carotenoides en musculo, y mejora la inmunidad no específica.	PF, SI	James *et al.*, 2006
1, 10, 25 g diario	Carpa *(Cyprinus carpio)*	Aumenta la fagocitosis, la expresión de los genes IL-1β and TNF-α en hígado y leucocitos, también aumentó la resistencia a infección bacteriana.	SI	Watanuki *et al.*, 2006
0.125, 0.25, 0.5, 0.75, 1	Tilapia *(Oreochromis Niloticus)*	*Spirulina* incremento el crecimiento, también aumento la respuesta de macrófagos y células `natural killers` así como la resistencia a infección por *Aeromonas hydrophila.*	SI	Abdel-Tawwab y Ahmad, 2009
1, 2, 3	Carpa (*Cyprinus carpio*)	*Spirulina* incrementó el crecimiento y la carga microbiana en intestino.	CR	Ramakrishnan *et al.*, 2008
1, 2, 4	*Labeo rohita*	Todos los niveles de inclusión de *Spirulina* aumentaron los parámetros del suero, haciendo más eficiente la inmunidad no especifica.	SI	Andrews *et al.*, 2011
3, 5	Bagre (*Clarias gariepinus*)	El 5% de inclusión incrementa la producción de células blancas y rojas, por consecuencia mejora la inmunidad.	SI	Promya y Chitmanat 2011
1, 10 mg diario	Tilapia *(Oreochromis Niloticus)*	La dosis 10mg aumentó la actividad bactericida, la actividad de fagocitos y lisozima, la producción de anticuerpos y la resistencia a infección bacteriana.	SI	Ragap *et al.*, 2012
2.5, 5, 10	Ciclido cola amarilla (*Pseudotropheus acei)*	La *Spirulina* mejoró el crecimiento, la coloración y el desempeño reproductivo.	CR, PF	Guroy *et al.*, 2012
2.5, 5, 7.5, 10	Trucha arcoíris	*Spirulina* mejoró la coloración del filete y piel, esta mejora en el color es proporcional al aumento en la inclusión de *Spirulina*.	PF	Teimouri *et al.*, 2013a
2.5, 5, 7.5, 10	Trucha arcoíris	*Spirulina* aumenta la concentración de carotenoides en sangre proporcional a la inclusión. Mejora la coloración del filete y mantiene la calidad de filete congelado hasta por seis meses.	PF	Teimouri *et al.*, 2013b
0.5, 0.75, 1, 1.5, 2	Tilapia *(Oreochromis Niloticus)*	La dosis 2% aumento el crecimiento, la inmunidad no especifica, la actividad de la lisozima y la sobrevivencia a infección bacteriana.	SI	Ibrahem *et al.*, 2013
0.5, 0.75, 1, 1.5, 2	Tilapia *(Oreochromis Niloticus)*	*Spirulina* disminuye la expresión de la proteína P53 que es relacionada con la activación de la apoptosis. De esta forma mejorando el sistema inmune no específico.	SI	Ibrahem y Ibrahim 2013
5, 10, 15	Parrot Fish (*Oplegnathus*	La presencia de *Spirulina* en dietas aumenta proporcionalmente los compuestos fenólicos y	RA	Kim *et al.*, 2013

	fasciatus)	por consecuencia aumenta la respuesta antioxidante.		
0.1, 0.3, 0.5	Carpa común	La inclusión de *Spirulina* aumenta el crecimiento y el rendimiento.	CR	Abdulrahman *et al.*, 2014
0.5, 1	Tilapia *(Oreochromis Niloticus)*	*Spirulina* incrementa las defensas en suero y disminuye la peroxidación lipídica.	RA, SI	Abdelkhalek *et al.*, 2015
2.5, 5, 7.5, 10	Trucha arcoíris	A partir de 7.5% de inclusión disminuye el colesterol, e incrementa la producción de células blancas y rojas, por consecuencia mejora la inmunidad.	SI	Yeganeh *et al.*, 2015
2.5, 5, 7.5, 10	Trucha arcoíris	La inclusión de *Spirulina* mejora la calidad del filete, disminuye la peroxidación lipídica y aumenta la durabilidad del filete fresco y congelado.	RA,PF	Teimouri *et al.*, 2015
2.5, 5, 10	Esturión (*Huso huso*)	El 10% de inclusión mejora el crecimiento, la actividad de enzimas digestivas (lipasa y proteasa), aumenta la respuesta inmune e incrementa resistencia bacteriana.	SI	Adle *et al.*, 2016
2,5. 5, 10, 20	Gourami (*Trichopodus trichopterus*)	Organismos alimentados con *Spirulina* tuvieron un incremento significativo en los parámetros reproductivos y de crecimiento.	PF, CR	Khanzadeh *et al.*, 2016
1, 3, 5, 8, 10	Camarón (*Neocaridina davidi*)	Los niveles de 8%y 10% mejoraron el crecimiento y los índices reproductivos.	PF, CR	Kohal *et al.*, 2017
20	kenyi cichlids (M*aylandia lombardoi*)	Organismos alimentados con *Spirulina* tuvieron un incremento en crecimiento y coloración.	PF, CR	Karadal *et al.*, 2017

Áreas de investigación; CR= Crecimiento; PF= Producto final; RA= Respuesta antioxidante; SI= Sistema inmune;

5. Viabilidad en el uso de *Spirulina* en la acuicultura.

Siendo llamativo el número de investigaciones que indican las ventajas del uso de *Spirulina* en la acuicultura, se hace necesario analizar la viabilidad económica del uso de este ingrediente en acuicultura. Una posible explicación pueden ser los costos de producción del micro alga. La producción de kilo de micro alga ha sido estimada en 310US$ kg^{-1}(Wijffels *et al*., 2010), más específicamente el valor de la *Spirulina* se estimó en 44US$ kg^{-1} (Brennan y Owende, 2009), si comparamos su precio con el máximo alcanzado por la HP en 2013 que fue de 1.74 US$ kg^{-1} (FAO, 2016) la diferencia de precios hace inviable la substitución de HP por *Spirulina*, por lo que se requiere más investigación en el área de producción para conseguir disminuir los costos productivos y hacerla competitiva como ingrediente de ración. También, algunas de sus substancias bioactivas son reconocidas por su alto valor económico, produciendo sub productos de alto valor económico, como es el caso de las ficocianina que tiene un precio entre 3 - 25 US$ mg^{-1} (Spolaore *et al.*, 2006) o los β-carotenos que varía entre 30 - 3000 US$ kg^{-1} (Ben-Amotz, 2004).

Los métodos de producción de *Spirulina* son ampliamente conocidos, y fueron descritos al detalle por Soni *et al.* (2017), incluyendo cultivos en estanques abiertos, fotobioreactores o sistemas mixtos. Diferentes áreas que envuelven la producción de *Spirulina* son investigadas con el fin de convertirla en una actividad económica más eficientes. Algunas alternativas que pueden disminuir los costos de producción son el uso de sistemas de producción con mayor tecnificación (Gao *et al.*, 2015); el uso de aguas residuales para el cultivo de *Spirulina* puede ser una alternativa sostenible, siempre y cuando los residuos del agua no sean tóxicos para animales o humanos. Como es el caso de Cheunbarn y Peerapornpisal (2010) que diluyeron (10%) aguas residuales de porcicultura para cultivo de *Spirulina* obteniendo mayor producción de micro alga, El-Kassas *et al.* (2015) utilizaron aguas residuales industriales de una industria confitera para la producción de *Spirulina*, el perfil nutricional de la misma no se vio afectado y posteriormente se utilizó como enriquecedor de copépodos mejorando la calidad nutricional de los mismos. Otra opción que puede reducir los costos de producción es el uso de fuentes nutricionales alternativas que puedan disminuir los costos para la producción de *Spirulina* es un área importante de investigación, pues variaciones en el medio de cultivo de las microalgas puede definir su composición química y la producción de biomasa de su cultivo (Mosterte y Grobbelaar, 1987), la utilización de urea para su producción, por ejemplo disminuye la cantidad de proteína contenida en la *Spirulina* lo que afectara su valor económico en el mercado (Madkour *et al.*, 2012). Otro ejemplo es el uso de orina humana como fuente de amonio en la producción de *Spirulina*, donde se concluyó que es posible cultivarla sin afectar su rendimiento o calidad nutricional siendo necesario agregar fuentes de carbono (Chang *et al.*, 2013). Raoof *et al.* (2006) produjeron un medio de cultivo con fertilizantes de uso común en la agricultura (súper fosfato, nitrato de sodio, muriato de potasio, cloruro de sodio, sulfato de magnesio, cloruro de calcio y bicarbonato de sodio) para remplazar el medio de cultivo tradicional (Zarrouk´s) de la *Spirulina*, donde la calidad de la misma no se vio afectada, consiguiendo así disminuir el valor del medio de cultivo de 80 US$ a 16 US$ lo que significa 1/5 del valor. Y por último, el uso de cepas mejoradas para la producción de esta micro alga en diferentes condiciones ambientales (Raoof y Kaushik, 2002; Morais *et al.*, 2009).

Podemos concluir que la diferencia entre los precios de la HP y la *Spirulina* es grande por lo que una substitución de ese producto en la actualidad sería inviable económicamente.

Por otra parte, una pequeña inclusión de *Spirulina* (1% o menos) podría ser una alternativa viable para incrementar el valor nutritivo de dietas para acuicultura, que incrementará la salud general de los organismos, que se traduce en menos estrés y mayor gano económico.

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ANEXO 5

COMISSÃO DE ÉTICA EM USO ANIMAL
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CEUA

AUTORIZAÇÃO PARA USO DE MAIS ANIMAIS Nº 003/2016

PROCESSO Nº	23116.009160/2014-26
CEUA Nº	Pq036/2014
UNIDADE	Instituto de Oceanografia
TÍTULO DO PROJETO	Utilização de *Arthrospira platensis* (Spirulina) em dietas para juvenis de tainha, *Mugil liza*
NÚMERO DE ANIMAIS E VIGÊNCIA	465 (*Mugil liza*) – 01/03/2018
ENVIO DO RELATÓRIO FINAL	Março de 2018
PROFESSOR RESPONSÁVEL	Marcelo Borges Tesser

PARECER DA CEUA:

Após a análise de sua solicitação de utilização de mais 225 animais para a realização dos protocolos experimentais descritos no processo, realizada em 21 de outubro de 2016, o seu pedido foi considerado **APROVADO**, sendo autorizado o uso total de **690** animais, pois cumpre o disposto na Lei no 11.794, nas demais normas aplicáveis e nas Resoluções Normativas e Diretrizes do Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Rio Grande, 27 de outubro de 2016.

Med. Vet. Márcio de Azevedo Figueiredo
Coordenador da CEUA-FURG

Controle da CEUA - Parecer(es) prévio(s) relacionado(s) ao processo: P003/2015; P014/2015. Página 1/1