UNIVERSIDADE FEDERAL DO RIO GRANDE
INTITUTO DE OCEANOGRAFIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

USO DE POLISSACARÍDEOS NÃO-AMILÁCEOS POR JUVENIS DE TAINHA *MUGIL LIZA* (VALENCIENNES, 1836)

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RIO GRANDE
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Tese apresentada como parte dos requisitos para a obtenção do grau de doutor em Aquicultura no Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande

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1 **DEDICATÓRIA**

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24 **Dedido esta tese à minha família, em especial ao meu avô**

25 **José, meu exemplo de força, perseverança, paciência**

26 **e mais do que tudo, resiliência.**

27

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1 **RESUMO GERAL**

2 Com a crescente expansão da atividade aquícola mundial, torna-se necessário a redução
3 da dependência sobre a farinha de peixe aliado ao estudo de alimentos alternativos
4 sustentáveis e ambientalmente amigáveis. Porém, a presença de fatores antinutricionais
5 e componentes fibrosos nesses produtos vegetais fazem com que sua utilização na
6 alimentação de organismos aquáticos seja limitada, principalmente pela falta de
7 conhecimento das ações destes alimentos sobre o organismo alvo. Os objetivos do
8 presente estudo foram avaliar o efeito da goma guar (GG – experimento 1) e da pectina
9 cítrica (PC - experimento 2), dois polissacarídeos não-amiláceos solúveis (PNAs),
10 sobre o crescimento, composição proximal, morfologia do trato intestinal, comunidade
11 microbiana e parâmetros hepáticos de juvenis de tainha *Mugil liza*. Ainda, avaliar a
12 inclusão de enzimas exógenas (experimento 3) numa ração base de farelo de soja e
13 verificar a performance, composição proximal muscular, histologia intestinal,
14 comunidade microbiana do trato e deposição de Ca e P nos ossos. Para o primeiro e
15 segundo experimento, foi formulada uma ração purificada basal sem níveis
16 significativos de fibra e a ela foram suplementados três níveis de polissacarídeos não-
17 amiláceos (4, 8 e 12%), goma guar (experimento 1) e pectina cítrica (experimento 2) ao
18 longo de 60 dias. Os resultados do experimento 1 indicam que a goma guar atuou como
19 um fator antinutricional com a inclusão de 8 e 12%, reduzindo o desempenho dos
20 animais. A adição de GG alterou a composição corporal, o glicogênio e o colesterol
21 hepático além da comunidade microbiana em diferentes secções do trato, contudo não
22 foram observadas alterações na morfologia do trato. No experimento 2, a inclusão de
23 diferentes níveis PC não alterou a performance, mas alterou a composição corporal e o
24 glicogênio hepático. Não foi observado efeito modulador na comunidade microbiana e
25 os peixes alimentados com PC apresentaram lesões intestinais semelhantes à enterite.
26 No experimento 3, uma ração basal composta por farelo de soja como a principal fonte
27 protéica (controle) foi suplementada com quatro níveis de coquetel enzimático exógeno
28 (50, 100, 150 e 200 g t^{-1}) e fornecida aos peixes durante 75 dias. Não foi observado
29 melhoria no desempenho, alterações na composição do músculo e comunidade
30 microbiana. Foi constatada maior retenção de Ca nos ossos dos peixes alimentados com
31 a inclusão de enzima. Peixes alimentados com a ração controle apresentaram alterações
32 morfológicas de grau leve à severa, com necrose e alterações das vilosidades intestinais,
33 que a longo prazo, podem comprometer a performance. Animais alimentados com

supplementação de enzima nas dietas não desenvolveram nenhuma patologia intestinal, indicando que as enzymas exógenas podem ter eliminado ou neutralizado os fatores antinutricionais presentes no farelo de soja. Como conclusão, recomenda-se o uso de GG como aglutinante em dietas para tainhas somente até o nível de 4%; o uso de PC em dietas como aglutinantes para essa espécie deve ser cauteloso quando realizado por períodos longos; e adição de enzimas exógenas apresentou potencial para mitigar as lesões intestinais induzidas pela inclusão do farelo de soja em dietas para *Mugil liza*.

Palavras-chave: Aglutinantes; enterite; enzimas exógenas; goma guar; microorganismos; pectina cítrica.

GENERAL ABSTRACT

With the increasing of world aquaculture activities, it is necessary to reduce the dependence upon the fishmeal coupled to the evaluation of sustainable plant resources and environmental friendly ingredients. However, the antinutritional factors and fibrous material in vegetable ingredients make their use as aquafeeds unsuitable, mainly for the lack of knowledge of the actions of that feedstuffs on the organisms. The purpose of this study were evaluates the effects of guar gum (GG – experiment 1) and citrus pectin (CP – experiment 2), both soluble non-starch polysaccharides (NSP), over the growth, body composition, gastrointestinal tract histology, microbial community and liver parameters of *Mugil liza* juvenile. Moreover, it was also evaluated the effects of an enzyme cocktail inclusion in a soybean meal-based diet and the assessment of the animal performance, muscle proximal composition, intestinal tract histology, tract microbial community and Ca and P bone deposition. In the first and second experiments, a purified diet was formulated without significantly levels of crude fiber, and at it was supplemented three levels of non-starch polysaccharides (4, 8 and 12%), guar gum (experiment 1) and citrus pectin (experiment 2), during 60 days. The experiment 1 results indicates that guar gum acts like an antinutritional factor in the 8 and 12% inclusions, lowering the performance of the animals. The GG inclusion has altered the body composition, liver glycogen and cholesterol and microbial community in the different tract sections, however, it was not observed alterations in digestive tract morphology. In the experiment 2, the pectin inclusion did not alter the fish performance, but has changed the body composition and liver glycogen. It was not observed a modulatory effect in the microbial community from the tract sections, but fishes fed on CP supplemented diets have shown intestinal lesions enteritis-like. In the experiment 3, a soybean meal-based diet (control) was supplemented with four levels of an enzyme cocktail (50, 100, 150 and 200 g t^{-1}) and fed the fishes for 75 days. No performance improvements were observed in the animals, muscle proximate composition and in the gastrointestinal microbial community. Was detected higher calcium retention in the bones of the fishes fed with enzyme-supplemented diets. Fishes feed in the control diet exhibited from light to serious morphological alterations, with necrosis and alterations in the villus morphology, which for longer periods could impair the animal performance. Animals fed on enzyme-supplemented rations dit not display any intestinal pathology, indicating that exogenous enzyme could eliminate or neutralize the antinutritional factor that induce those disease observed in the animals fed on soybean meal. As conclusion, is recommended the GG

as binder in diets for mullet only at 4% inclusion level; the uses of PC as binder in this specie feed should be cautious when performed for longer periods; the enzyme supplemented diets shown potential for mitigates intestinal lesions soybean meal-induced in diets for *Mugil liza.*

Key-words: binder; citrus pectin; enteritis; exogenous enzymes; guar gum; microorganisms.

INTRODUÇÃO GERAL

Produção aquícola

A contribuição de pescados provenientes da aquicultura chegou a 42,2%, do total de 158 milhões t provenientes da pesca e aquicultura, sendo um resultado crescente em relação aos dados de 1990 e 2000, com respectivamente, 13,4 e 20,7% de participação. Somente no período entre 2000 e 2012, a produção global de pescado proveniente da aquicultura para alimentação mais que dobrou, passando de 32,4 para 66,6 milhões t (FAO 2014).

Atualmente existe uma grande tendência a intensificação da atividade aquícola, com o aumento da produção de espécies onde há uma dependência maior de alimento inerte e redução na produção de espécies filtradoras, não-dependentes de rações como as carpas e moluscos bivalves. Em 2012, a produção total de espécies filtradoras foi de 20,5 milhões t, isso representa uma redução de 33,5% em 2010 para 30,8% em 2012, ainda, refletindo um grande crescimento na produção de espécies dependente de dietas formuladas (FAO 2014).

Parte dessa produção aquícola também é direcionada para outros usos que não a alimentação humana. Desde 1990, houve um crescente aumento no destino dos pescados para a alimentação humana, sendo que no ano de 2012, esse valor chegou ao patamar de 136 milhões de toneladas, representando 86% da produção total de pescados provenientes da pesca e aquicultura. Os 14% restantes foram direcionados para fins não alimentícios, e destes, 75%, o equivalente a 16.3 milhões de t, foi destinado à obtenção da farinha e óleo de peixe (FAO 2014). Ainda, em relação ao total produzido, esse valor continua sendo uma parcela significativa, contudo, segue em declínio. Sobre a farinha de peixe, duas características principais justificavam seu uso na alimentação de organismos aquáticos: o baixo preço e bom perfil protéico. Contudo, hoje os preços estão cada vez mais crescentes pelo aumento da demanda por razão da intensificação da atividade; 35% da farinha de peixe em 2012 foram proveniente de subprodutos de pescado, apresentando elevado teor de cinzas e reduzido valor biológico da proteína, além da qualidade ser variável (FAO 2014; Olsen e Hasan, 2012). Esses fatores têm feito aumentar recentemente o número de estudos voltados para a redução da dependência da farinha de peixe, muitos dos quais contribuíram para reduzir a quantidade desse ingrediente nas rações de espécies de alto valor comercial e de algumas espécies carnívoras marinhas (Olsen e Hasan, 2012).

Há cerca de 10 anos, avanços foram obtidos na substituição da farinha de peixe por fontes alternativas de proteínas, reduzindo a porcentagem em até 50% dessa fonte nas dietas para peixes carnívoros de alto valor comercial como salmão, truta, sea bream e sea bass, e também rações para peixes onívoros, principalmente na fase de engorda (Hardy, 2010). Mas para se conseguir a total substituição da farinha de peixe, são necessárias fontes protéicas alternativas aliadas a aditivos e técnicas que permitam às espécies manterem o rápido crescimento e a boa eficiência alimentar obtida com a farinha de peixe.

Substitutos à farinha de peixe

Enquanto existir limitação no fornecimento da farinha de peixe, alternativas sustentáveis devem ser estudadas e a indústria da aquicultura percebeu há muito a importância do uso de produtos vegetais na produção de alimentos formulados ao setor, sendo um gargalo importante para o futuro da atividade (Gatlin *et al.*, 2007; Merrifield *et al.* 2007). E junto a essa perspectiva, a produção mundial de grãos tem aumentado nas últimas duas décadas como resultado da alta produtividade obtida nos campos tanto por técnicas de fertilização como por melhorias no sistema de irrigação (Hardy, 2010).

Proteínas de origem vegetal são, e provavelmente serão, a principal opção de substituição à farinha de peixe na aquicultura (Olsen e Hasan, 2012). Contudo, a escolha do produto vegetal deve ser baseada em características que o façam comparáveis a farinha de peixe, como um bom nível protéico, e consequentemente, de aminoácidos, boa palatabilidade, além da disponibilidade na região e a ausência de fatores antinutricionais (Sinha *et al.* 2011). Entre os candidatos, podem ser citados a cevada, canola, milho, algodão, ervilha, tremoço, soja e trigo, além de seus produtos derivados como o isolado protéico de soja e o glúten de trigo e milho. No entanto, em relação à farinha de peixe esses produtos apresentam geralmente mais carboidratos insolúveis e fibras, que aumentam a excreção, e redução da absorção de minerais (Naylor *et al.*, 2009). Melhorias têm sido feitas na qualidade genética desses vegetais, transgenia e técnicas de processamento pós-colheita que aumentam o valor nutricional dos concentrados protéicos, além de manipulações dietéticas que podem ser usadas para permitir o seu melhor aproveitamento, como a adição de alimentos complementares para obter o perfil de aminoácidos adequado; suplementação com aminoácidos sintéticos; adição de enzimas exógenas para compensar alguns fatores antinutricionais e probioticos (Naylor *et al.*, 2009; Barrows, 2008).

1 Proteínas vegetais já representam a principal fonte protéica na alimentação de
2 espécies como tilápias, carpas e bagres, representantes de níveis tróficos inferiores, e
3 dentre essas fontes, o farelo de soja é o destaque, sendo a proteína de origem vegetal
4 mais comum em dietas tanto para peixes herbívoros como onívoros e crustáceos (Tacon
5 *et al.*, 2011).
6 O alto conteúdo protéico e o perfil de aminoácidos favorável justificam o uso do
7 da soja como alimento na aquicultura e, além disso, é a principal oleaginosa produzida
8 em escala global, sendo seus produtos (farelo, floco, concentrado protéico e isolado
9 protéico) já avaliados em dietas para peixes, com o farelo de soja sendo o mais
10 amplamente utilizado (Gatlin *et al.*, 2007; Merrifield *et al.*, 2007). Contudo, o grande
11 gargalo de sua plena utilização são algumas qualidades nutricionais desfavoráveis; o
12 farelo de soja, em relação à farinha de peixe, apresenta concentração geralmente menor
13 de 10 aminoácidos essenciais, a presença de fatores antinutricionais como
14 oligossacarídeos e polissacarídeos não-amiláceos indigestíveis, fitato, presença de fator
15 anti-tríptico entre outros (Gatlin *et al.* 2007; Dersjant-Li, 2002).
16 Dentro da perspectiva de produção e disponibilidade da soja, os dados da
17 projeção agrícola do MAPA (2013) para a produção dessa *commodity* são otimistas. De
18 acordo com o documento, no ano de 2022/23 o Brasil ocupará a primeira posição na
19 produção mundial desse grão, com o montante de 63,8 milhões de t, com participação
20 de 44,2%. Para o farelo, a projeção aponta que o país ocupará a segunda posição com
21 16,9 milhões de toneladas com participação de 22,9%.
22 **Fatores antinutricionais**
23 Fatores antinutricionais (FAT) são definidos como metabólitos secundários
24 produzidos por vegetais, e além de contribuírem para a composição de odores
25 específicos, sabor e cor, agem também como sistema de defesa contra predadores e
26 patógenos (Bennett and Wallsgrove, 1994). Alguns FAT podem ter efeitos benéficos
27 como antioxidantes, imunoestimulantes ou prebióticos dependendo da quantidade
28 ingerida (Krogdahl *et al.*, 2010). Os possíveis efeitos deletérios incluem redução da
29 palatabilidade, menor eficiência na utilização de nutrientes para o crescimento, alteração
30 do balanço de nutrientes das dietas, disfunções intestinais, alteração na comunidade
31 microbiana, modulação imunológica entre outros, e dependendo da espécie animal, sua
32 idade, tamanho, gênero, estado nutricional e saúde e qualquer fator de estresse podem
33 modificar estas respostas.

1 Francis e*t al.* (2001) fizeram uma extensa revisão sobre o efeito de FAT
2 presentes em dietas e seus efeitos em peixes, e os dividiram em quatro grupos distintos:
3 1) fatores que afetam a digestão e uso de proteínas; 2) fatores que afetam o uso de
4 minerais; 3) antivitamínicos; 4) compostos diversos como polissacarídeos não-
5 amiláceos, micotoxinas, cianogênicos, nitratos, alcaloides, saponinas entre outros.
6 Alguns FATs podem ser facilmente eliminados por processamento, enquanto que outros
7 são mais difíceis de serem neutralizados, mas para a grande maioria, fermentação ou
8 tratamento enzimático pode reduzir o conteúdo ou sua atividade no alimento (Krogdahl
9 *et al.*, 2010).
10 De acordo com Choct (1997), mundialmente por ano são produzidas
11 aproximadamente 2,0 bilhões de toneladas de cereais e 140 milhões de toneladas de
12 leguminosas e sementes, e destes, são produzidas 230 milhões de toneladas de co-
13 produtos que poderiam ser usados em dietas de peixes como uma fonte barata de
14 ingrediente vegetal. Contudo, grande parte desse material é composta por
15 polissacarídeos não-amiláceos, que naturalmente são parte da parede celular vegetal e
16 também material de reserva energética em leguminosas. Polissacarídeos não-amiláceos
17 (PNAs) são constituídos por celulose, hemicelulose, pectinas, gomas e mucilagens que
18 não são passíveis de digestão por enzimas animal e humana, contudo, podem ser
19 fermentados por microorganismos presente no trato gastrointestinal do hospedeiro
20 (Bach Knudsen, 2001; Choct, 1997; Asp, 1996; McDougall *et al.*, 1996).
21 A capacidade de digerir carboidratos estruturais vegetais, que são geralmente
22 impassíveis de digestão por enzimas endógenas, é definida como característica de
23 herbívoros, ao menos no ambiente terrestre (Choat & Clements, 1998). A utilização de
24 carboidratos fibrosos por peixes devem incluir uma série de aparatos digestivos, que
25 incluem dentição e outros mecanismos para trituração (estômago), uma complexa
26 arquitetura alimentar e a presença de micro-organismos simbióticos necessários para a
27 fermentação de tais carboidratos (Choat & Clements, 1998).
28 Em peixes herbívoros marinhos, a capacidade de fermentação foi demonstrada e
29 quantificada para *Kyphosus sydneyanus, Odax pullus* e *Aplodactylus arctidens*
30 (Mountfort *et al.*, 2002). A capacidade de quebra de paredes celulares de algas pelo
31 baixo pH estomacal e a lise enzimática realizada por microorganismos mostrou-se
32 efetiva na alimentação do *Holacanthus passer*, outro herbívoro marinho (Martínez-
33 Díaz & Pérez-España, 1999). Kihara *et al.* (2002) demonstraram, em ensaios *in vitro*,
34 que oligossacarídeos indigestíveis provenientes da soja tem grande potencial

fermentativo pela microbiota da carpa onívora *Cyprinus carpio*, sendo produzidos os ácidos acético, propiônico e butírico. Leenhouwers *et al.* (2008) demonstraram a capacidade de utilização de diferentes fontes de carboidratos (incluindo PNAs) *in vito* pela microbiota intestinal da tilápia *Oreochromis niloticus* e do carnívoro sea bass *Dicentrarchus labrax.*

Polissacarídeos não-amiláceos

PNAs possuem uma série de propriedades físico-químicas de importância nutricional: capacidade de troca de cátions, hidratação, viscosidade e absorção de compostos orgânicos (Bach Knudsen, 2001). De acordo com Lunn & Buttriss (2007), PNAs insolúveis têm propriedade de atrair e reter água passiva e lentamente, que auxilia a aumentar o volume, amolecer fezes e encurtar o tempo de trânsito no trato intestinal. Além disso, também é resistente à fermentação intestinal. Para o conceito de PNAs solúveis, ponderam que esse tipo de fibra imediatamente retém água, formando uma solução viscosa enquanto passa ao longo do trato gastrointestinal (TGI), e é fermentada no intestino.

Os principais polissacarídeos dos PNAs são celulose, pectinas, β-glucanos, pentosanas e xilanas, e apresentam a característica de não serem hidrolisados por quaisquer enzimas animais (Montagne *et al.*, 2003). Após a passagem pelo trato, parte dos PNAs chegam ao intestino grosso praticamente intactos, são hidrolisados e fermentados por microorganismos desse compartimento, gerando ácidos graxos voláteis (AGVs) butirato, que tem ação específica como fonte de energia do epitélio do colón; propionato e acetato, que são absorvidos e exercem efeitos no metabolismo de carboidratos e lipídeos, respectivamente (McDougall *et al.*, 1996; Alles *et al.*, 1999; Asp, 1996; Wenk, 2001; Lunn & Buttriss, 2007) entre outros. Além dos AGVs, são produzidos ainda água, gases (CO_2, H_2, CH_4), biomassa de células bacterianas (Montagne *et al.*, 2003), lactato e etanol (Alles *et al.*, 1999).

Existem diversos efeitos que os PNAs podem causar ao organismo animal e humano. No campo da fisiologia, em termos gerais, há o consenso de que as fibras solúveis aumentam o tempo do trânsito intestinal, atrasam o esvaziamento gástrico, retarda a absorção de glicose, aumentam as secreções pancreáticas e torna a absorção mais lenta, enquanto que as fibras insolúveis diminuem o trânsito intestinal, aumentam a capacidade de reter água e auxiliam no volume fecal em animais não-ruminantes (Montagne *et al.*, 2003). Além disso, os PNAs melhoram o fluxo de matéria seca e as perdas endógenas tanto de fontes endógenas como exógenas, levando a redução na

1 digestibilidade da energia e dos nutrientes no íleo e nas fezes, incluindo amido,
2 proteínas e lipídeos (Souffrant, 2001). Também exercem efeito sobre o metabolismo do
3 colesterol, reduzindo seus níveis na corrente sanguínea e inibindo sua síntese no fígado
4 (Hara *et al.*, 1999; Lunn & Buttriss, 2007).

5 Interações entre os PNAs e a modificação da morfologia do TGI estão bem
6 documentadas na literatura, porém, o mecanismo responsável ainda não é muito bem
7 definido. Alguns tipos de fibras, particularmente, têm a propriedade de aumentar a
8 viscosidade da digesta, e a presença desta no lúmen intestinal eleva a taxa de perda
9 celular nas vilosidades, o que causa atrofia da mesma, sendo um fenômeno associado
10 com a proliferação de células das criptas intestinais e geralmente é acompanhada pelo
11 aumento da profundidade das mesmas (Montagne *et al.*, 2003). Scheppach (1994)
12 afirma que os AGVs possuem efeito trófico sobre as células da cripta intestinal,
13 causando sua proliferação. Esse efeito trófico já foi descrito para diversas espécies de
14 animais, incluindo ratos (Sakata, 1987) frangos de corte (Iji *et al.*, 2001), suínos
15 (Mcdonald *et al.*, 2001) e coelhos (Chao & Li, 2008).

16 Na nutrição de peixes, os efeitos dos PNA foi revisado por Sinha *et al.* (2011).
17 Além de presentes nos vegetais comumente usados nas formulações de dietas, alguns
18 PNA solúveis são usados na estabilização dos pellets (aglutinantes), como por exemplo,
19 a goma guar. Os efeitos adversos do uso desses compostos são dependentes da espécie
20 de peixe, hábito alimentar, da idade, do tipo de produto vegetal empregado e quantidade
21 adicionada à dieta, e devido a esses fatores torna-se difícil fazer comparações entre os
22 dados disponíveis na literatura.

23 Storebakken (1985) trabalhou com PNAs solúveis (Goma guar e alginato) e
24 observaram redução da digestibilidade da proteína e gordura, além de redução da
25 ingestão alimentar e o crescimento reduzido na truta arco-íris com a adição de goma
26 guar. Hossain *et al.* (2001) e Hossain *et al.* (2003) demonstraram que o endosperma da
27 semente de *Sesbania aculeata*, rica em galactomananos, um PNA solúvel, reduziu o
28 crescimento e a utilização dos nutrientes pela carpa comum e pela tilápia,
29 respectivamente. A constatação de que a adição de PNA solúveis provoca alterações na
30 viscosidade da dieta em peixes foi comprovada numa série de estudos. Com o bagre
31 africano *Clarias gariepinus* foi demonstrado que a adição de goma guar aumentou a
32 viscosidade da dieta e consequentemente reduziu a digestibilidade dos nutrientes, além
33 de causar aumento do peso relativo dos órgãos digestivos; a adição de cereais (centeio,
34 trigo, milho, cevada) às dietas promoveu o aumento da viscosidade e redução da

1 digestibilidade dos nutrientes, além de afetar a fermentação intestinal; com a tilápia do
2 Nilo *Oreochromis niloticus*, dietas com a inclusão dos mesmos cereais aumentaram
3 também a viscosidade da digesta com reflexos negativos na absorção de sódio, redução
4 da digestibilidade da matéria seca além de efeitos negativos no balanço de água
5 intestinal (Leenhouwers *et al*. 2006, 2007a, 2007b).
6 Peixes e outros monogástricos não possuem enzimas intestinais para degradação
7 dos PNAs, e a melhoria no aproveito das dietas ricas nesse FAT pode ser obtida pela
8 inclusão de enzimas degradadoras de PNAs, ou carboidrases, nas dietas (Sinha *et al*.,
9 2011). Na indústria de frangos e suínos, carboidrases e fitases são comuns nas
10 formulações de dietas, contudo, pouca atenção é dada aos efeitos dessas enzimas nas
11 dietas para peixes (Bedford e Cowieson, 2012; Ai *et al.*, 2007).
12 Diferentemente dos alimentos formulados para animais domésticos, alimentos
13 voltados para a aquicultura necessitam manter sua estabilidade em água para reduzir a
14 perda dos nutrientes por lixiviação (Paolucci *et al.* 2012). Logo, a utilização de
15 aglutinantes nas dietas para peixes torna-se imprencidível para se assegurar o
16 fornecimento de nutrientes sem perdas siginificativas, além disso, também para
17 aumentar a firmeza das fezes quando eliminadas na água reduzindo a poluição (Brinker
18 *et al.* 2009; Amirkolaie *et al.* 2005). Contudo, um mesmo aglutinante não é adequado
19 para todas as espécies, e mesmo dentro da mesma espécie o hábito alimentar é alterado
20 pela idade, logo estudos devem ser feitos para determinar o tipo, o nível de inclusão e os
21 efeitos dos aglutinantes na fisiologia das diferentes espécies usadas na aquicultura
22 (Paolucci *et al.* 2012).
23 Os aglutinantes naturais provenientes de PNAs (biopolímeros) são os mais
24 estudados e os derivados de vegetais os mais comuns, tanto os insolúveis, como a
25 celulose, como os solúveis, como a goma guar, carragena, ágar e pectina (Paolucci *et al.*
26 2012).
27 **Goma guar**
28 Gomas têm sido descritas como exudatos vegetais solúveis e dispersíveis
29 contendo polissacarídeos de cadeias longas, e sua adição em alimentos aumentam sua
30 densidade sem adicionar calorias, melhorando o seu valor (Chawla e Patil 2010). Goma
31 guar é um polissacarídeo linear (galactomana) derivado do endosperma do feijão
32 indiano (*Cyamopsis tetragonolobus*), baseado quimicamente numa raiz de β(1,4)-D-
33 manose ligados com cadeias laterais α(1,6)-D-galactose, as galactomananas (Paolucci *et*
34 *al.* 2012; Brinker *et al.* 2007). A propriedade mais importante da goma guar é sua

1 capacidade de se hidratar e manter uma alta viscosidade e adesão mesmo em baixas
2 concentrações, o que o torna amplamente utilizado na indústria de alimentos, como
3 espessante e aglutinante em saladas, sorvetes, macarrões instântenos, rações para
4 animais domésticos, carnes processadas e bebidas (Paolucci *et al.* 2012; Butt *et al.*
5 2007).
6 Na nutrição humana, é recomendado seu consumo pelos seus efeitos
7 hipocolesterolêmico e hipoglicêmico, redução de doenças cardiovasculares, obesidade e
8 diabetes (Chawla e Patil 2010; Butt *et al.* 2007); em suínos, já foi descrito a relação
9 negativa com a distibilidade da energia e proteína e redução da performance
10 (Mcdonalds *et al.* 2001; Owusu-Asiedu *et al.* 2006); em frangos, já foram reportados o
11 aumento do peso intestinal e alteração da morfologia do trato, redução do crescimento e
12 ingestão alimentar e o aumento da viscosidade da digesta (Lee *et al.* 2003a, 2003b). Na
13 nutrição de peixes, efeitos sobre o crescimento, digestibilidade, estabilidade fecal,
14 viscosidade da digesta, metabolismo da glicose e lipídeos e efeitos sobre o estresse
15 oxidativo do trato já foram descritos (Enes *et al.* 2013; Brinker e Reiter 2012; Enes *et*
16 *al.* 2012; Brinker *et al.* 2009, 2007; Leenhouwers *et al.* 2006; Amirkolaie *et al.* 2005;
17 Storebakken 1985). Contudo, apesar de se saber que PNAs solúveis tem efeitos sobre a
18 microbiota do trato gastrointestinal e pelos dados na literatura ainda serem contraditório
19 sobre efeitos antinutricionais da goma guar, novos estudos devem ser realizados com o
20 intuito exclarescer melhor seus efeitos na biologia de peixes.

21 **Pectina Cítrica**

22 Pectinas estão presente como material "cimentante" da parede celular vegetal,
23 interligando moléculas de celulose, hemicelulose e lignina (Chawla e Patil 2010).
24 Quimicamente, são polissacarídeos com a raiz formada de α-(1,4)-D-ácido
25 galacturônico, os ácidos urônicos, ligados com uma variedade de acúcares nas cadeias
26 laterais (arabinose, galactose, xilose, raminose) (Farris *et al.* 2009). Na indústria de
27 alimentos, a principal aplicação da pectina é como aditivo alimentar gelificante e
28 expessante, e sua utilização vai além das aplicações alimentícias, visto que possuem
29 efeitos benéficos à saúde (Tungland *et al.* 2002); na indústria farmacêutica, é indicadoo
30 como um promissor veículo para o fornecimento de medicamentos protegidos,
31 encapsulados, "escoltando" a droga até seu ponto de ação (Liu *et al.* 2003).
32 Redução do colesterol, do risco cardiovascular e do câncer colorretal são ações
33 benéficas à saúde humana atribuídas ao consumo da pectina (Chawla e Patil 2010; Lunn
34 e Buttriss, 2007; Tungland *et al.* 2002). Até o momento, não existe informações sobre o

uso de pectina como aglutinante em dietas para peixes. Contudo, já foi testada como aglutinantes em dietas para a lagosta *Cherax albidus* onde se observou resultados promissores tanto para a qualidade da ração como para o desempenho animal (Volpe *et al.* 2008, 2011).

A pectina cítrica é obtida a partir da extração ácida do bagaço da laranja, um co-produto da produção do suco de laranja, que conta com 50% da massa total do fruto (Oreoupolou e Tzia 2007). O Brasil é o maior produtor de laranja do mundo, com 25% da parcela mundial dessa produção, aproximadamente 18 milhões de toneladas, e desse total, cerca de dois milhões de toneladas (11%) são processados na forma de suco (MAPA, 2007; Neves *et al.* 2010). Logo, o país possui um grande potencial para a produção desse aglutinante e visto o grande potencial na indústria de alimentos e seus efeitos sobre a saúde humana, seu uso poderia ser estimulado e explorado em dietas voltadas para organismos aquáticos.

Complexos enzimáticos

Na década de 1950, pesquisadores iniciaram os estudos com a adição de enzimas na dieta de vários animais domésticos, como proteases e amilases, e observaram melhorias na produtividade, sendo que atualmente, o uso de enzimas exógenas na ciência animal é uma das áreas mais estudadas e promissoras nas áreas de nutrição, alimentação e biotecnologia (Adeola e Cowieson, 2011). A suplementação de rações com enzimas melhora o valor nutricional dos ingredientes, reduz a variação na qualidade dos nutrientes e auxiliam na quebra de FAT, que interferem com a fisiologia do trato gastrointestinal. (Bedford, 2000; Barletta, 2010).

Segundo Adeola e Cowieson (2011), o mercado global de enzimas alimentares, que em 2011 ultrapassou os 550 milhões de dólares, é dividido nos segmentos das enzimas fitase e das não-fitase (carboidrases e proteases). Essas enzimas são secretadas naturalmente por fungos e bactérias com o objetivo de suprir suas necessidades metabólicas, e a partir disso, sistemas de fermentação foram desenvolvidos baseados em organismos geneticamente modificados para superproduzirem a enzima de interesse.

Fitase

O fósforo é um elemento crítico para peixes e outros animais domésticos, participando da estrutura e função celular através dos fosfolipídeos, ácidos nucléicos, proteínas, coenzimas e como componente integral do trifosfato de adenosina (ATP), compostos estes que atuam no metabolismo energético, divisão e crescimento celular, transporte e metabolismo de gorduras, absorção e utilização de carboidratos, ácidos

graxos e proteínas (Kumar *et al.*, 2011). Esse elemento está presente nos ingredientes de origem vegetal na forma do fitato, e nas plantas, este composto forma complexos com minerais (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+}, Fe^{3+}), proteínas e amido que se tornam indisponíveis para monogástricos, que não possuem ou não sintetizam quantidade suficiente de enzimas que o hidrolisem (Singht e Satyanarayana, 2014; Barletta, 2010; Vats *et al.*, 2009; Francis *et al.*, 2001). Como consequência, muitas rações são formuladas com excesso de fósforo inorgânico para suprir as exigências dos animais, e esse elemento acaba sendo excretado para o ambiente causando poluição. A introdução de enzimas que degradam o fitato nas dietas pode reduzir significativamente a poluição causada pela excreção do fósforo (Bedford, 2000).

As enzimas degradadoras de fitato, ou fitase, é um grupo de enzimas de diferentes tamanhos, estruturas e atividades catalíticas que quebram a molécula de fitato, liberando os seis grupos fosfatos que a compõem e que podem se quelar a cátions (Kumar *et al.*, 2011; Greiner e Konietzny, 2010). Dessa forma, a hidrólise desse composto pode aumentar a disponibilidade não só do fósforo inorgânico (ortofosfato), mas também de outros minerais nas dietas dos animais (Singht e Satyanarayana, 2014).

Apesar dos peixes conseguirem absorver o P pelas brânquias e pelo trato digestório, sua concentração é muito baixa na água, fazendo com que a dependência do P seja quase exclusivamente proveniente da dieta (Kumar *et al.*, 2011). Com o aumento do uso de proteínas de origem vegetal na aquicultura, 50 a 80% do P vegetal estão armazenados na forma de fitato, e sendo indisponível, muito do P acaba excretado, poluindo o meio aquático e provocando a ocorrência da eutrofização e floração de algas/microalgas potencialmente tóxicas (Baruah *et al.*, 2004; Kumar *et al.*, 2011).

Estudos avaliando a aplicação de fitase nas dietas de peixes comprovaram a melhora da disponibilidade de minerais além do P, como Ca, Mg e Zn (Ai *et al.*, 2007; Sajjadi *et al.* 2004; Yan e Reigh., 2002; Storebakken *et al.*, 1998), melhora na digestibilidade de proteínas (Storebakken *et al.*, 1998; Cheng e Hardy., 2004), aumento do ganho de peso (Jackson *et al.*, 1996; Storebakken *et al.*, 1998) e redução da excreção de P no meio aquático (Ai *et al.*, 2007; Sajjadi *et al.*, 2004; Vielma *et al.*, 2000)

Carboidrases

Todos os produtos vegetais utilizados na alimentação possuem fibra em sua constituição. Ela é formada por polissacarídeos não-amiláceos solúveis e insolúveis (Barletta, 2010). Não-ruminantes não possuem enzimas digestivas próprias capazes de hidrolisar essa fração dos alimentos, logo a suplementação de carboidrases exógenas é

1 necessária para a degradação dos PNA complexos existentes nas dietas atuais, que são
2 responsáveis pela redução da utilização do alimento pelos animais causado pelo
3 aumento da viscosidade da digesta, pela disponibilização de nutrientes aprisionados pela
4 teia de carboidratos insolúveis e pela liberação de oligossacarídeos para a microbiota
5 intestinal benéfica, agindo como prebióticos (Castillo e Gatlin, 2014; Barletta, 2010).
6 Todas as enzimas que são capazes de degradar os polímeros de carboidratos de
7 alto peso molecular são denominadas carboidrases, e mais de 80% do mercado global
8 destas enzimas são representados pelas glucanase e xilanase, que respectivamente
9 degradam a celulose e PNAs, e além dessas são incluídas ainda α-amilases, β-
10 mananases, α-galactosidase e pectinases (Adeola e Cowieson, 2011; Jackson, 2011;
11 Paloheimo *et al.*, 2011).
12 O uso de alimentos suplementados com enzimas exógenas na aquicultura é uma
13 área relativamente nova e com crescente aumento no número de trabalhos envolvendo
14 as diversas espécies, contudo, ainda existem muitos dados contraditórios, com o
15 resultado dependente das espécies envolvidas, idade, fonte alimentar utilizada e a
16 mistura ou não das enzimas suplementares (Castillo e Gatlin, 2014). Resultados
17 positivos já foram observados no desempenho zootécnico do salmão (*Salmo trutta*
18 *caspius*), carpa capim, esturjão (*Huso huso*), bagre africano (*Clarias gariepinus*), sea
19 bass japonês (*Lateolabrax japonicus*), da tilápia híbrida (*Oreochromis niloticus* x *O.*
20 *aureus*) e truta arco-íris (Zamini *et al.*, 2014; Zhou *et al.*, 2013; Ghomi *et al.*, 2012;
21 Yildirim e Turan, 2010; *Ai et al.*, 2007; Lin *et al.*, 2007; Carter *et al.*, 1994). Por outro
22 lado, outros estudos não apontam resultados significativos para os parâmetros de
23 crescimento avaliados, como os reportados para a truta arco-íris e perca prateada
24 (*Bidyanus bidyanus*) (Dalsgaard *et al.*, 2012; Fahangi and Carter, 2007; Ongukoya *et*
25 *al.*, 2006; Stone *et al.*, 2003).

26 **Tainha *Mugil liza***

27 A tainha *Mugil liza* é um importante recurso pesqueiro na região Sul do Brasil,
28 com o último boletim pesqueiro apontando a captura de cerca de 18 mil toneladas
29 (MPA, 2011). Segundo Reis & D'Incao (2000), no Estuário da Lagoa dos Patos, a
30 tainha é uma espécie com potencial econômico na região. Contudo, afirmam sua pesca
31 não ocorre como atividade econômica, e as capturas são feitas apenas no nível de
32 subsistência.

33 A tainha *Mugil liza* atinge cerca de 60 cm de comprimento, podendo atingir
34 entre 6 e 8 kg de peso vivo (Vieira & Scalabrin, 1991). Benetti & Fagundes Netto

(1991) afirmam que essa espécie é adequada tanto para sistemas de monocultivo como em policultivo com outras espécies de peixes e crustáceos. Para a criação dessa espécie, foram desenvolvidas pesquisas voltadas para os aspectos nutricionais (Ito & Barbosa, 1997; Carvalho *et al.* 2010; Zamora-Sillero *et al*. 2013), ambientais (Fonseca Netto & Sparch, 1999; Okamoto *et al.*, 2006, Poersch *et al.*, 2007), densidades de estocagem (Scorvo Filho *et al.*, 1992; Sampaio *et al.*, 2001), biologia reprodutiva (Viera e Scalabrin 1991; Esper *et al.*, 2001) e do desenvolvimento ontogênico do sistema digestório (Galvão *et al.*, 1997a; Galvão *et al.*, 1997b).

De acordo com Vieira & Scalabrin (1991), *Mugil liza* é uma espécie que apresenta hábito alimentar zooplanctófago quando larva e passa a ter hábito alimentar iliófago quando juvenil. Tanto juvenis quanto adultos apresentam como principais itens alimentares as bacilariofíceas e detritos, e de acordo com a época do ano, esses itens sofrem variações para ambas as idades de desenvolvimento (Oliveira & Soares, 1996). Galvão *et al.* (1997a) analisou a histologia do sistema digestivo da tainha durante as fases larval e juvenil e verificaram que, aos 60 dias, a região pilórica é caracterizada como uma moela de aves, capaz de triturar itens alimentares de diversas naturezas. Ainda de acordo com os autores, essa região não contribuí com enzimas digestivas ou HCl para a digestão, mas pode auxiliar a digestão por meios mecânicos. A presença dessa estrutura indica hábito alimentar micrófago, detritívoro ou herbívoro.

Recentemente, Menezes *et al*. (2010) fizeram a revisão taxonômica do gênero *Mugil sp.* e a partir de dados de análises morfométricas e merísticas, ponderaram que todas as espécies de tainhas encontradas da região do Caribe e até a costa Atlântica da América do Sul representam apenas uma espécie de tainha, e *Mugil liza* deve ser o nome mais adequado para tal.

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32

1 **OBJETIVOS**

2 **Objetivo Geral**

3 Avaliar os efeitos nutricionais de polissacarídeos não-amiláceos solúveis inertes,
4 goma guar e pectina cítrica, e de rações com farelo de soja, uma fonte de proteína
5 vegetal, suplementada com enzimas exógenas em juvenis de *Mugil liza*.

6 **Objetivos Específicos**

7 **1.** – Identificar qual nível máximo de goma guar e pectina, PNAs solúveis, que
8 podem ser suplementados à dieta sem comprometer o desempenho zootécnico
9 da tainha;
10 **2.** – Avaliar se a inclusão de diferentes níveis ou se as diferentes qualidades de
11 PNA na dieta causam modificações na morfologia do trato gastrointestinal;
12 **3.** – Observar possíveis alterações na comunidade microbiana, quantitativa e
13 qualitativamente, do trato gastrointestinal com a adição de PNAs à dieta;
14 **4.** – Investigar alterações biológicas e proximais nos tecidos dos peixes
15 alimentados com diferentes níveis e tipos de PNAs;
16 **5.** – Identificar se a utilização de diferentes níveis de coquetel enzimático
17 suplementar numa ração base de farelo de soja pode melhorar o aproveitamento
18 da fração fibrosa da mesma, com possível aumento do desempenho dos peixes;
19 **6.** – Avaliar a composição proximal de peixes alimentados com suplementação
20 enzimática à ração;
21 **7.** – Observar a presença de lesões intestinais causadas pela inclusão do farelo de
22 soja, e se a suplementação de enzimas exógenas pode mitigar as lesões;
23 **8.** – Investigar a capacidade de retenção de Ca e P nos ossos de peixes alimentados
24 com coquetel enzimático contendo fitase numa ração base de farelo de soja.
25 **9.** – Quantificar e qualificar a microbiota do trato gastrointestinal dos peixes
26 alimentados com ração base de farelo de soja e suplementadas com coquetel
27 enzimático.
28

CAPÍTULO 1

(artigo aceito para publicação pela Animal Feed Science and Technology)

Biological responses in mullet *Mugil liza* juveniles fed with guar gum supplemented diets

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Abstract

This study aimed to evaluate the effects of adding guar gum, a non-starch polysaccharide, to the diet of *Mugil liza* juveniles. The juveniles (mean weight = 0.38 ± 0.01 g) were fed one of three diets with increasing supplement levels (4, 8, and 12%) and a control diet without additional gum for 60 days, in order to evaluate the effects on zootechnical performance, proximate composition, liver parameters, morphological alterations to the intestinal tract, and modulation of gastrointestinal microbiota. The animals fed 8 and 12% gum presented a significantly lower mean final weight, weight gain, specific growth rate, food intake rate, and protein intake rate than the control. Adding gum to the diets also reduced the dry matter, crude protein, and carcass fat levels. All treatments with added gum resulted in increased liver glycogen, and the cholesterol levels were significantly reduced in fish fed 4 and 8% supplement levels. No intestinal morphological alterations were observed in the animals. However, a modulating effect was noted on the microbial community, altering the bacterial quantity and composition throughout the tract segments. The use of guar gum is not recommended in *Mugil liza* diets, at least above a 4%.

1 ***Keywords:*** Binder, dietary fibers, microorganisms, non-starch polysaccharide.

1 1. Introduction
2 Unlike formulated food for terrestrial animals, aquaculture diets require
3 adequate processing to ensure sufficient stability in the water for the animals to
4 consume them without loss to the environment (Paolucci *et al.,* 2012). Therefore, "inert"
5 additives, termed binders, of either organic or inorganic origin are typically included in
6 diet formulas. Organic binders consist of complex carbohydrate polymer chains called
7 non-starch polysaccharides (NSPs) and include pectin, laminarin, guar gum, agar,
8 carrageenan, alginate, and chitosan (Paolucci *et al.,* 2012).
9 However, NSPs can be considered antinutritional factors when present in a fish
10 diet (Francis *et al.,* 2001). Recently, Sinha *et al.* (2011) reviewed the antinutritional
11 effects of these compounds, many of which are associated with changes in the diet's
12 viscosity resulting in slowed gastric emptying and reduced gastrointestinal transit time,
13 altered morphology and physiology of the digestive tract, changes in intestinal
14 microbial community and additionally altered the levels of glucose and cholesterol.
15 Moreover, the increased tendency to substitute fishmeal with plant-based protein
16 ingredients leads to an increased of the presence of NSPs in fish diets. In that sense,
17 studies dealing with the usage of purified NSPs are important to simulate the effects of
18 higher levels of plant feedstuffs, which have been widely used in fish diets
19 Guar gum (galactomannan) is an NSP derived from *Cyamopsis tetragonolobus*
20 (Indian bean endosperm) and acts as an excellent thickener because it is a water-soluble
21 polymer (Storebakken, 1985). Some studies have evaluated the addition of guar gum to
22 fish diets, yet most have only observed the physical attributes of the feces (Amirkolaie
23 *et al.,* 2005; Brinker 2007, 2009; Brinker & Reiter, 2012), nutrient digestibility
24 (Leenhouwers *et al.,* 2006), oxidative status, gastrointestinal tract morphology (Enes *et*
25 *al.,* 2012), and the effects on glucose and lipid metabolism (Enes *et al.,* 2013).
26 However, the mechanisms by which guar gum acts on body composition and modulates
27 the microbial community in the tract remain uncertain.
28 The mullet *Mugil liza* reach approximately 60 cm in length and may weigh
29 between 6 and 8 kg (Vieira & Scalabrin, 1991). They are consumers of the low trophic
30 layers, being therefore suitable for both monoculture and polyculture with other fish and
31 shellfish (Benetti & Fagundes Netto, 1991). Studies on the farming of this species have
32 focused on nutritional aspects (Ito & Barbosa, 1997; Carvalho *et al.,* 2010; Zamora-
33 Sillero *et al.,* 2013), rearing environment (Fonseca Neto & Sparch, 1999; Okamoto *et*
34 *al.*, 2006, Poersch *et al.*, 2007), stocking densities (Scorvo Filho *et al.,* 1992; Sampaio

et al., 2001), ontogenetic development of the digestive system (Galvão *et al.*, 1997a, 1997b), and reproductive biology (Viera & Scalabrin 1991; Esper *et al.*, 2001). Many organisms like tilapia, carp, mullet and shrimp have been recognized as more suitable for aquaculture in tropical countries as they forage primarily on detritus (Moriarty & Pullin, 1987). By definition, detritus consists of dead organic matter primarily formed by plant material (Bowen, 1987). Ecologically, in the Lagoa dos Patos estuary (Southern Brazil), mullet primarily forage on detritus and microalgae that are undergoing microbial decomposition (Seeliger *et al.,* 1997). However, it is unknown if the mullet is able to use any fibrous fraction of the detritus or if ingesting this material is a strategy for consuming the microbial matter that decomposes it.

This study aimed to evaluate the effects of adding guar gum to the diets of *Mugil liza* mullet by comparing performance, proximate composition, digestive tract morphology, modulation of the tract microbial community, and changes in liver cholesterol, triglyceride, and glycogen levels.

2. Materials and Methods

2.1. Fish conditioning

Mullet (*Mugil liza*) juveniles were caught by trawl (2.5 m x 1.5 m; mesh size 5.0 mm) at Cassino beach (Rio Grande, RS, Brazil), transferred to the Laboratório de Piscicultura Marinha e Estuarina of the Universidade Federal do Rio Grande - FURG, and stocked in one 300-L tanks (two fishes per liter) for feed training (hand-fed four times per day). After one week, the fish were weighed (0.38 g ± 0.01) and randomly distributed throughout a static system consisting of 12 rectangular tanks (50 L) at a density of 15 fish per tank. After the first daily feeding, the tanks were siphoned and filled with seawater previously filtered through bag filters (5 µm) and treated with chlorine. Sodium thiosulfate was used to neutralize the chlorine before the utilization. Submerged heaters maintained the temperature at 25°C, the salinity was held near 30, and a photoperiod of 14L:10D was maintained.

The fish were hand-fed four times daily (8:00 AM, 11:00 AM, 12:00 PM, 3:00 PM) until apparent satiation. At the end of each day, the diets were weighed in a precision analytical scale (± 0.01 g, BL-3200H, Marte, São Paulo, Brazil) to record daily intake.

2.2. Water parameters

1 The water parameters were monitored daily. Dissolved oxygen and temperature
2 were measured using an oximeter (YSI 50A, Ohio, USA), pH was measured using a
3 digital pH meter (±0.01, YSI®-pH100, Ohio, USA), and salinity was measured with a
4 handheld Atago® refractometer (model 103, Tokyo, Japan). Ammonia content was
5 measured every other day, and alkalinity was measured weekly via the UNESCO (1983)
6 method.
7
8 *2.3. Diet formulas*
9 The experiment was randomized, with four treatments performed in triplicate,
10 consisting of reference diet (control) (350 g kg^{-1}% crude protein; 16.45 MJ g^{-1}) and
11 three other diets with increasing guar gum (GG) supplement levels (Farmaquímica S.A.,
12 São Paulo, Brazil) (4%: GG4; 8%: GG8, 12%: GG12) (Tables 1 and 2). Care was taken
13 to ensure that no ingredients contained significant crude fiber levels. The dry
14 ingredients were homogenized with oil and distilled water at 60°C until a consistent
15 texture was obtained that could be pelleted in a meat grinder with a 2-mm-diameter
16 opening. Next, the pellets were dried in a forced-circulation oven for 5 h at 60°C, and
17 after drying, they were maintained in a freezer at -20°C until used.
18 Diet and carcasses proximate analyses at the onset and in the end of the
19 experiment were conducted according to the methodology described by the AOAC
20 (1999): Dry matter (#934.01) was obtained after drying in an oven for 5 h at 102°C; for
21 ash (#942.05), the samples were burned in a muffle for 5 h at 600ºC. The Kjeldahl
22 method was utilized to determine the crude protein (#984.13) level after sample
23 digestion and nitrogen distillation; the results were multiplied by 6.25. To obtain an
24 ether extract (#920.39), a Soxhlet extraction was conducted for 6 h with petroleum ether
25 as the solvent. The following methodology described by Silva & Queiroz (2009) was
26 utilized for the crude fiber analysis: Acid and base digestions of the samples were
27 conducted for 30 minutes each; then, the residues were burned in a muffle at 500°C, and
28 the crude fiber value was obtained from the weight difference. The non-nitrogenous
29 extract was calculated from the difference between the total crude protein, the ether
30 extract, the crude fiber, and the ash values. Viscosity was measured according to the
31 adapted methodology by Refstie *et al.* (1999): a 50-g diet was added to 450 mL distilled
32 water and incubated for 30 minutes at 25°C under agitation (80 rpm). Next, the rations
33 were centrifuged (10.000 g, 10 minutes), and the supernatant was collected and

1 analyzed using a rheometer (Brookfield, DV – III Ultra, Massachusetts, USA) rotating
2 at 250 rpm.
3 Table 1. Feed ingredients and proximal composition of reference diet

	Dry matter (g kg^{-1})
Feed Ingredients	
Fishmeal	60.0
Casein[1]	250.0
Gelatin[1]	100.0
Maize	550.0
Fish oil	30.0
Premix[2]	10.0
Proximal composition	
Dry matter	890.0
Crude protein	338.4
Ether extract	27.2
Ashes	17.3
Crude fiber	0.43
Metabolizable energy (MJ g^{-1})	18.45
Viscosity (cP)	18.4

4 [1]Rhoster (São Paulo, Brazil); [2] Premix M. Cassab, SP, Brazil (Vit. A (500,000 UI kg^{-1}), Vit. D3 (250,000
5 UI 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}),
6 Vit. B6 (1,000 mg kg^{-1}) Vit. B12 (2,000 mcg kg^{-1}), Niacin (2,500 mg kg^{-1}), Calcium pantotenate (4,000
7 mg kg^{-1}), folic acid (500 mg kg^{-1}), biotin (10 mg kg^{-1}), vit. C (10,000 mg kg^{-1}). Colin (100,000mg kg^{-1}),
8 Inositol (1,000 mg kg^{-1}). Trace elements: selenium (30 mg kg^{-1}), iron (5,000 mg kg^{-1}), copper (5,000 mg
9 kg^{-1}), manganese (5,000 mg kg^{-1}), zinc (9,000 mg kg^{-1}), cobalt (50 mg kg^{-1}), iodine (200 mg kg^{-1}). [3]
10 Calculated from the physiological standard values, where 1 kg of carbohydrate (N-free extract), protein
11 and lipid yields 16.7, 16.7 and 37.6 MJ, respectively (Garling and Wilson, 1976).
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1 Table 2. Formulation and proximal composition of experimental diets. GG4, GG8 and
2 GG12: means the % of inclusion of guar gum in the reference diets

	Diets		
	GG4	GG8	GG12
Diet formulation (g kg^{-1})			
Reference diet	960	920	880
Guar Gum	40	80	120
Dietary component (g kg^{-1} dry matter)			
Dry matter	890.4	892.4	897.0
Crude protein	324.0	323.7	301.2
Ether extract	29.2	28.1	3.02
Ashes	17.1	17.1	16.6
Crude fiber	0.55	0.58	0.84
Viscosity (cP)	41.1	101.0	135.8

3
4 *2.4. Growth trial*
5 The experiment lasted 60 days, after which all fish (15 per tank) were weighed
6 and measured to obtain the following zootechnical and biometric indexes:
7 1. Weight gain (g): final weight – initial weight
8 2. Apparent feed conversion: diet supplied/weight gain
9 3. Specific growth rate (% day^{-1}): [(ln final weight – ln initial weight)/days farming] ×
10 100
11 4. Protein efficiency rate: weight gain (g)/protein intake (g)
12 5. Condition factor: 100 × body weight (g)/body length (cm)^3
13 6. Hepatosomatic index: ($weight_{liver}/weight_{body}$) × 100
14 7. Viscerosomatic index: ($weight_{viscera}/weight_{body}$) × 100
15 8. Intestinal quotient relative to length: $length_{intestine}/length_{body}$
16 9. Intestinal quotient relative to weight: $length_{intestine}/weight_{body}$
17 Afterwards, all the fishes were euthanized with an overdose of Benzocaine (300
18 ppm) and the liver and gastrointestinal tract were collected and weighted; intestines
19 were also measured for intestinal quotient quantifications. All livers were separated and
20 frozen at −80°C for subsequent analyses; the stomach and intestines (three fishes per

1 tank) were fixed in 20% formalin for histological analysis. For microbial quantification,
2 prior to removing the tract from the animals (three fishes per tank), the outer surfaces
3 were sterilized with povidone iodine. Then, the tract was collected and fixed in 4%
4 formalin solution. At the beginning nine fish were subjected to the same procedure for
5 initial microbial quantification. The carcasses of all the animals were used for body
6 composition analysis at the conclusion of the experiment, 50 other fish were euthanized
7 with overdose of Benzocaine (300 ppm) for initial whole body composition.
8
9 *2.5. Glycogen, cholesterol, and liver triglyceride levels*
10 The frozen liver samples were mixed for 40 minutes in a sonicator with
11 perchloric acid (6%) in a volume 7.5 times the sample weight according to Zamora-
12 Sillero *et al.* (2013). After sonication, the homogenates were neutralized with the same
13 volume of potassium bicarbonate (1 M). Then, the homogenates were centrifuged
14 (13.000 g × 30 minutes), and the supernatants were used for the analyses. The total
15 triglyceride and cholesterol levels were estimated using commercial kits (Triglicérides
16 Enzimático Líquido, Colesterol Enzimático Líquido, Doles, Goiânia, GO, Brazil).
17 The liver glycogen content was estimated in duplicate according to the method
18 of Carr & Neff (1984), later modified by Nery & Santos (1993). The glycogen content
19 was obtained via enzymatic breakdown (amyloglucosidase, Sigma) into glucose. The
20 product was measured using a commercial kit (Glicose enzimática, Doles, Goiania, GO,
21 Brazil). All measurements were obtained on a spectrophotometer with a microplate
22 reader at a wavelength of 490 nm (ELx808, Biotek Instruments Inc., Winooski,
23 Vermont).
24
25 *2.6. Histological analysis*
26 The fixed material was processed in a LUPE PT 05 automatic processor
27 embedded in Paraplast® and cut into 5-μm-thick sections in a LUPETEC MRPO3
28 microtome. The sections were stained with hematoxylin-eosin (HE).
29
30 *2.7. Microbial enumeration*
31 The fixed samples were taken to the Laboratório de Fitoplâncton e
32 Microorganismos Marinhos/IO – FURG for bacterial count; for this, the intestines and
33 stomach were carefully removed from the solution and sectioned into pieces in
34 previously autoclaved petri dishes. After being opened, the stomach and intestines were

1 washed with 10 mL distilled water. The solution was transferred to 40-mL glass jars and
2 sonicated (Cole-Parmer Instrument Co., Chicago, Illinois, USA) in three 10-second
3 increments with 10-second intervals between them. One 0.5-mL aliquot was removed
4 and filtered through polycarbonate membrane filters (Nucleopore, Kent, UK, 0.2-µm
5 porosity) that were previously darkened with 12% Irgalan black. The filtrate was then
6 stained with acridine orange (1 µg mL^{-1}) (Hobbie *et al.,* 1977). The bacteria were
7 counted in 30 random fields using a Zeiss Axioplan epifluorescence microscope
8 (Oberkochen, Germany) equipped with a blue filter (487709 – BP 450-490, FT 510, LT
9 520) and a Watec CCD (Watec Co., Yagamata, Japan) (0.0003 Lux).
10 The results of performance, body composition and liver parameters were
11 subjected to analysis of variance (ANOVA). Since that bacteria count were performed
12 along the digestive tract (stomach, proximal, mid and distal intestine) and these
13 measurements were clustered within each animal, a Poisson Generalized Linear Mixed
14 Effects model was used to identify the influence of the levels of inclusion of guar gum
15 in the multivariate microbial counts. The treatments groups were used as fixed effects
16 and the animal as random effect. The contrasts of interest analyzed were the control
17 group vs. 4, 8 and 12% of gum guar supplemented diets. Interactions between
18 treatments and the different localizations in the digestive tract were also analyzed by a
19 two-way Poisson Generalized Linear Mixed Effects method. Statistical significant
20 differences were declared for p-values less than 0.05. The corresponding statistical test
21 for group comparison used in the analysis was a likelihood ratio test. Statistical
22 computations were performed with the statistical software R version 3.1.0 (R Core
23 Team, 2014) and using the package lme4 (Bates et al., 2014).
24 Data normality (Shapiro-Wilks) and variance homogeneity (Cochran test) were
25 previously checked. The transformation $\log_{10}$ (microbial counts + 1) was applied in
26 order to satisfy analysis assumptions. A Tukey test was applied to identify any
27 significant difference from experimental groups. All statistical tests were performed
28 using a 5% of significance level (Zar, 1984).
29
30 3. Results
31 *3.1. Water quality*
32 Throughout the experiment, the mean temperature was 24.9°C ± 0.03, the
33 dissolved oxygen content was 6.82 ± 0.04 mg L^{-1}, the pH was 8.12 ± 0.05, and the mean
34 alkalinity was 147 ± 12.93 mg L^{-1} $CaCO_3$. The mean values for ammonia in the control,

GG4, GG8, and GG12 treatments were 0.69 ± 0.38, 0.62 ± 0.34, 0.42 ± 0.23, and 0.39 ± 0.22 mg L^{-1}, respectively.

3.2. Zootechnical performance

The zootechnical performance results are provided in Table 3. No mortality occurred during the experimental period. Final weight, weight gain, feed intake rate, and protein intake rate were significantly higher ($P < 0.05$) in the control group than in the 8 and 12% added guar gum treatment groups. No significant differences ($P > 0.05$) were found in apparent feed conversion, protein efficiency rate, and biometric indexes.

Table 3. Growth performance and biometric index of juvenile mullets *Mugil liza* fed with increasing levels of guar gum*. GG4, GG8 and GG12: means the % of inclusion of guar gum in the ration

Parameters	Control	GG4	GG8	GG12	PSE	*P*
$AW_{initial}$	0.38	0.38	0.38	0.38	0.01	-
AW_{final}	3.67^{a}	2.63^{ab}	2.52^{ab}	2.42^{b}	0.20	0.031
WG	3.29^{a}	2.26^{ab}	1.94^{ab}	1.86^{b}	0.24	0.032
SGR	3.78	3.47	3.16	3.09	0.11	0.090
FI	6.90^{a}	4.63^{b}	4.32^{b}	4.00^{b}	0.44	0.007
FCR	1.94	2.01	2.29	2.12	0.08	0.466
PI	2.41^{a}	1.62^{b}	1.51^{b}	1.40^{b}	0.15	0.007
PER	1.47	1.42	1.28	1.35	0.04	0.501
		Biometric indexes				
K	1.29	1.31	1.26	1.29	0.02	0.827
HSI	1.62	1.68	1.49	1.62	0.04	0.545
VSI	10.57	10.96	10.16	11.32	0.22	0.316
QIR_{CL}	2.49	2.6	2.42	2.52	0.05	0.678
QIR_{BW}	4.51	5.35	5.63	5.97	0.25	0.209

AW: average weight; WG: weight gain; SGR: specific growth rate; FI: feed intake; FCR: feed conversion rate; PI: protein intake; PER: protein efficiency rate; *K*: condition factor; HSI: hepatic somatic index; IVS: viscera somatic index; QIR: Quotient intestinal relative (CL: corporal length; BW: body weight). *Mean values of triplicates groups. Mean with different subscript letters in the same column differ e significantly ($P < 0.05$). PSE: Pooled standard error

3.3. Body composition

Increased guar gum supplementation in the fish diets resulted in reduced dry matter, crude protein, and carcass fat levels (Table 4). The control treatment exhibited

1 significantly higher ($P < 0.05$) dry matter and ether extract levels than the treatments
2 with added guar gum; the crude protein was significantly higher ($P < 0.05$) in the
3 control and GG4 treatments, with the lowest value found for the 8% guar gum
4 treatment. The ashes were significantly reduced ($P < 0.05$) by the addition of 8% guar
5 gum (Table 4).
6 Table 4. Proximal body composition of juvenile mullet *Mugil liza* fed with increasing
7 levels of guar gum in diets* GG4, GG8 and GG12: means the % of inclusion of guar
8 gum in the ration

Body composition	Initial	Final				PSE	*P*
		Control	GG4	GG8	GG12		
Dry matter	22.25	28.59^{a}	27.47^{b}	24.66^{c}	16.36^{d}	1.28	0.000
Crude protein	15.70	17.60^{a}	17.51^{ab}	16.26^{c}	16.59^{bc}	0.18	0.030
Ether extract	0.89	7.53^{a}	7.02^{b}	6.06^{c}	6.24^{c}	0.16	0.000
Ashes	5.34	3.25^{a}	3.26^{a}	3.05^{b}	3.19^{ab}	0.03	0.006

9 *Values are means of triplicate groups. Means with different superscript letters in the same column differ
10 significantly ($P < 0.05$). PSE: Pooled standard error.
11
12 *3.4. Liver parameters*
13 All guar gum treatments caused significantly higher ($P < 0.05$) glycogen values
14 compared with the control group. Liver cholesterol content for the GG8 and GG4 were
15 below those for the GG12 and control treatments ($P < 0.05$). No significant difference
16 was noted in the triglyceride levels between the treatment groups (Table 5).
17
18 Table 5. Levels of hepatic glycogen, triglycerides and cholesterol (mg g^{-1}) of juvenile
19 mullets *Mugil liza* fed with increasing levels of guar gum in diets* GG4, GG8 and
20 GG12: means the % of inclusion of guar gum in the ration

Treatment	Glycogen	Triglycerides	Cholesterol
Control	2.95^{b}	5.50	0.058^{ab}
GG4	6.25^{a}	4.43	0.039^{b}
GG8	5.87^{a}	4.88	0.034^{b}
GG12	5.09^{a}	4.69	0.144^{a}
PSE	0.34	0.43	0.01
P	0.000	0.924	0.022

21 *Values are means of triplicate groups. Means with different superscript letters in the same column differ
22 significantly ($P < 0.05$). PSE: Pooled standard error.

3.5. Digestive tract histological analysis

No morphological or pathological alterations associated with guar gum supplementation to the diets were observed.

3.6. Digestive tract bacterial count

Significant differences were detected ($P < 0.05$) between the guar gum inclusion and the control in the total microbial counts. In the stomach, control treatment exhibited higher counts than GG4 and GG8 treatments; in the mid intestine, control was higher than GG4, but lower than GG8 treatment; in the distal intestine, stomach had more total bacteria than GG4 treatment (Table 6).

Regarding the bacterial morphotypes, three bacterial groups were identified: cocci, bacilli and filamentous. Significant differences ($P < 0.05$) were observed between the counts in the different tract sections. Cocci bacteria showed higher counts in the control when compared with GG4 treatment in the distal intestine section. Bacilli count in the proximal intestine had lower results in the control than GG4, while in the mid and distal intestine sections the control showed elevated counts when compared with this same treatment. Finally, filamentous count in the stomach was higher in the control treatment than the treatments with guar gum inclusion; the proximal intestine had more filamentous bacteria in the control than GG4 treatment; mid intestine had less filamentous bacteria in the control than the GG8 treatment; and in the distal intestine GG4 and GG8 had more filamentous counts than control (Table 6).

Interactions between guar gum inclusion and tract section in the bacterial count tract were identified. The inclusion of 4% guar gum showed higher ($P < 0.05$) total bacteria, cocci, bacilli and filamentous counts in the stomach and proximal intestine than distal intestine. The inclusion of 8% guar gum exhibited higher ($P < 0.05$) for filamentous counts in the stomach and proximal in comparison to distal intestine. Also, filamentous counts were significantly higher ($P < 0.05$) in the stomach section than distal intestine in the treatment with 12% of guar gum (Data not shown).

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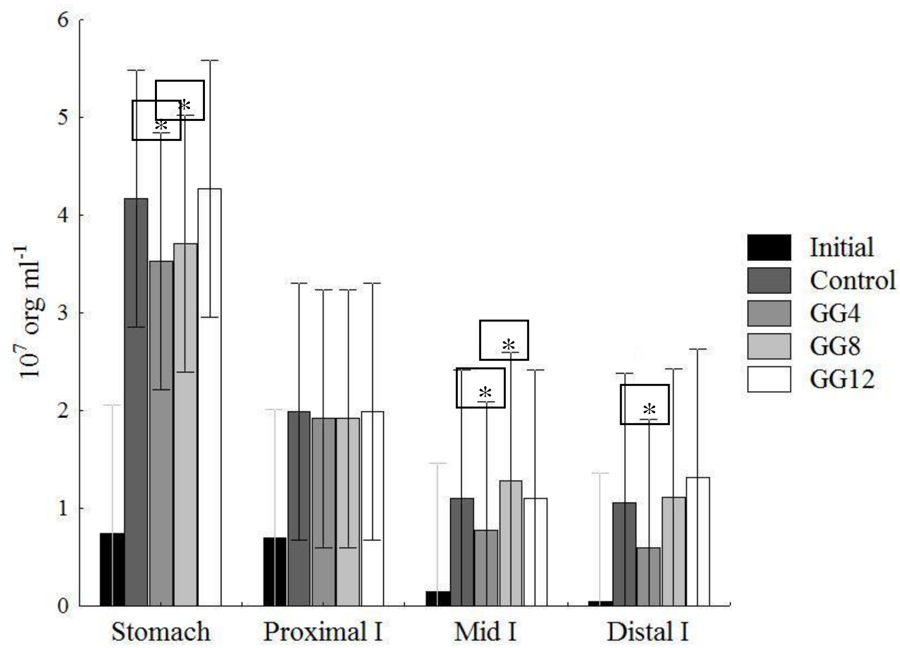

13 Figure 1. Total bacteria count in the different tract sections of juvenile mullet *Mugil liza*
14 fed with increasing levels of guar gum. Asterisk (*) denotes difference statistic ($P <$
15 0.05) from control group. GG4, GG8 and GG12: means the % of inclusion of guar gum
16 in the ration. I.: intestine.
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Table 6. Summary of results from bacterial morphotypes count (total bacteria: 10^7 org ml^{-1}; Cocci: 10^7 org ml^{-1}; Bacilli: 10^6 org ml^{-1}; Filamentous: 10^5 org ml^{-1}) in the different tract sections of juvenile mullets *Mugil liza* fed with increasing levels of guar gum*. GG4, GG8 and GG12: means the % of inclusion of guar gum in the ration. I.: intestine

Total bacteria	Treatments				P-Values		
	Control (a)	*GG4 (b)*	*GG8 (c)*	*GG12 (d)*	*(a vs b)*	*(a vs c)*	*(a vs d)*
Stomach	4.17	**3.52**	**3.71**	4.27	0.020	0.006	
Proximal I.	1.99	1.91	1.91	1.99			
Mid I.	1.10	**0.77**	**1.27**	1.10	>0.001	0.016	
Terminal I.	1.06	**0.60**	**1.27**	1.32	0.0563	>0.001	
PSE	0.462	0.500	0.406	0.505			
Cocci							
Stomach	24.24	27.09	28.01	31.24			
Proximal I.	18.08	17.08	17.01	16.77			
Mid I.	7.61	6.38	9.46	9.08			
Terminal I.	12.31	**5.23**	9.62	11.31		>0.001	
PSE	2.802	14.261	3.092	3.652			
Bacilli							
Stomach	6.69	5.15	6.31	7.46			
Proximal I.	0.84	**1.85**	1.07	2.00		0.022	
Mid I.	1.92	**0.54**	1.84	1.23		0.024	
Terminal I.	1.30	**0.38**	0.76	1.15		0.027	
PSE	0.774	0.718	0.873	1.090			
Filamentous							
Stomach	8.77	**3.00**	**2.77**	**4.00**	>0.001	>0.001	>0.001
Proximal	1.61	**0.23**	1.07	1.15	>0.001		
Mid	0.61	0.84	**1.46**	0.69		>0.001	
Terminal	0.23	0.38	**0.76**	0.69		0.033	
PSE	1.301	0.354	0.403	0.584			

*Values are mean of triplicate groups. PSE: Pooled standard error

4. Discussion

Although the mullet *Mugil liza* in its natural environment forages for foods such as algae and detritus that contain soluble and insoluble polysaccharide (Vieira, 1991; Oliveira & Soares, 1996), the addition of soluble guar gum polysaccharide to its diet caused a negative growth response. Despite their presence in a wide variety of plant-based ingredients, soluble NSPs are related to depressed growth in some monogastric species (Sinha *et al.*, 2011). The antinutritional effects of guar gum are due to its physical properties. According to Paolucci *et al.* (2012), the main property of guar gum is its ability to rapidly hydrate, creating a highly viscous material even at relatively low levels, a response that was observed in this study (Table 2). Leenhouwer *et al.* (2006) demonstrated that among the adverse physiological effects associated with viscosity, soluble NSPs (guar gum) induce increased digesta viscosity in fish. Delayed gastric emptying is among the factors responsible for reduced food intake (Sinha *et al.*, 2011), which affects animal performance, and could explain the reduced food intake and consequent reduced zootechnical performance in the fish fed with guar gum.

Furthermore, adding guar gum to the diets markedly affected the carcass proximate composition. As the gum supplement increased, water quantity increased, with a concurrent reduction in carcass lipid, protein, and ash levels. The same result was observed in studies using soluble polysaccharide-rich plant sources, including those by Hossain *et al.* (2001, 2003), Siddhuraju and Becker (2001), Krogdahl *et al.* (2003) and Kumar *et al*. (2011). Some physiological effects of high viscosity induced by guar gum may be related to reduce carcass lipid and protein levels. Sinha *et al.* (2011) explain that the addition of NSP supplements to diets reduces protein digestibility and consequently interferes with amino acid absorption, which in turn, influences body protein formation. Pasquier *et al.* (1996) used *in vitro* assays to demonstrate that soluble polysaccharides (guar gum, pectin, and arabic gum) reduce fat emulsification and triglyceride lipolysis, hindering digestion and absorption, while Vahouny *et al.* (1980) demonstrated how soluble fibers can bind to bile salts, hindering their intestinal absorption. This latter mechanism is described by Potter *et al*. (1995), and according to the authors, increased bile salt excretion creates an environment in which cholesterol is removed from the body, making the liver to provide them for bile acid re-synthesis.

Surprisingly, adding 12% guar gum increased the liver cholesterol levels. Several studies of fish have reported that soluble NSP supplements reduce dietary lipid use (Sinha *et al.,* 2011), and they have subsequently been indicated for the human diet

1 (Tungland *et al.,* 2002). However, Enes *et al.* (2013) observed increased plasma
2 cholesterol levels when 8 and 12% guar gum was added to the diets of *Diplodus sargus*
3 (sea bream). The increased liver cholesterol levels and reduced carcass fat observed in
4 this study indicate that muscular fat reserves are more mobile than liver fat reserves, as
5 noted previously by Potter *et al.* (1995).
6 Enes *et al.* (2013) evaluated the effect of guar gum on liver glycogen in
7 *Diplodus sargus*; however, the authors found no alterations, concluding that guar gum
8 aids in reducing endogenous glucose production in this fish species. In our study, fish
9 fed diets containing guar gum supplement exhibited significantly higher liver glycogen
10 values than the control group, regardless of supplement level. Non-ruminant animals
11 derive an additional source of energy from the fermentation products that are not
12 digestible by endogenous enzymes, some of which are absorbed and used as a source of
13 glucose in the liver (Montagne *et al.,* 2003); this process may have occurred in this
14 study.
15 Typically, direct microbial counts in the fish digestive tract only evaluate the
16 intestine; the stomach is often neglected. In this study, the highest bacterial counts were
17 observed in this organ and decreased further along the tract toward the distal intestinal
18 segment regardless of diet. Conversely, studies conducted on marine herbivorous fish
19 caught in their natural environment found increased bacterial density toward the distal
20 intestinal segment (Rimmer, 1986; Clements 1991; Fidopiastis *et al.,* 2006). According
21 to Clements (1991), the absence of microorganisms in the anterior portion of the
22 gastrointestinal tract and their abundance in the terminal portions indicate that the
23 organisms present are not only consumed together with the food particles but also form
24 an endosymbiotic bacterial community profile that assists with polysaccharide
25 digestion.
26 However, histological analysis of the mullet stomach (Galvão *et al.,* 1997)
27 demonstrated that the pyloric region – with deep folds, highly developed muscles, and
28 no digestive glands – has the primary function of grinding food, comparable to the
29 gizzard in birds. The absence of secretory glands for both enzymes and hydrochloric
30 acid in the pyloric region creates a proper environment for microbial colonization,
31 which could explain the high bacterial density in the stomachs of these animals. Thus, it
32 is very likely that *M. liza* utilizes the bacterial biomass produced after the
33 decomposition of consumed food. If this is the case, mullet fish should have a ruminant-
34 like feeding behavior, with the incorporation of bacteria into its biomass.

In ruminant animals, microorganisms are the main source of high-quality protein and are subsequently digested in the abomasum (Allison, 1993). The hypothesis that the stomach microbial community can be used as a protein supplement may also apply to mullet because the microbial density follows a trend wherein gradually decreases throughout the intestinal tract, which could denote absorption of digested bacteria throughout the intestines. Chun-Fang *et al.* (2012) also evaluated the effect of purified NSPs (raffinose and stachyose) on the microbial profile of the silver crucian carp digestive tract and verified that the bacterial community remained unchanged.

To our knowledge, this is the first study that has evaluated the effect of guar gum in microbial population in the fish tract. In this work, guar gum modulated the bacterial community of the mullet tract, and markedly the inclusion 4% guar gum had impacted not only in the total counts, but in all bacteria morphotype composition, mainly in the bacilli group, which are the major group of bacteria that present some role as a probiotic (Balcázar *et al*. 2006). However, this manipulation does not seem to affect the animal's performance, since the worst results were observed in the inclusion of 12% of guar gum. In grower pigs, guar gum causes the increasing of intestinal bacteria populations, mainly bacteria of bacilli morphotypes (lactobacilli, clostridia, enterobacteria and bifidobacteria), and this effect is related with changes in the physiology and ecosystem caused by the increased viscosity in the gut (Owusu-Asiedu *et al.* 2006).

5. Conclusion

Supplementing the diets of *Mugil liza* mullet with guar gum caused an antinutritional effect, reducing growth and feed intake. Furthermore, the supplementation altered the body composition and increased cholesterol and glycogen levels in the liver. Guar gum modulated the bacterial profile and the bacteria densities in the different tract sections. This research indicates the use of guar gum is not recommended in *Mugil liza* diets at levels exceeding 4%.

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CAPÍTULO 2

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Effects of supplementing the diets of *Mugil liza* juveniles with citrus pectin

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Summary

The aim of this study was to evaluate whether increasing the levels of citrus pectin has anti-nutritional effects when included in the diets of *Mugil liza* juveniles, including its effects on hepatic metabolism and modulation of the microbial community. Fish (mean weight 0.38 g ± 0.01) were stocked at density of 15 fishes per tank and fed for 60 days with either a control diet or one of three diets containing different levels of pectin (4, 8 and 12%), in triplicates. The temperature, dissolved oxygen, pH, salinity and alkalinity during the trial were, respectively, 25.0 ºC ± 0.1, 6.82 ± 0.02 mg L^{-1}, 8.10 ± 0.06 and 147 mg ± 12.93 $CaCO_3$. The TAN in PC4, PC8 and PC12 treatments were, respectively, 0.69 ± 0.38; 0.57 ± 0.35; 0.64 ± 0.39 and 0.45 ± 0.23 mg L^{-1}. The increasing diet

viscosity with pectin inclusion did not cause significant differences in growth. The fish fed with pectin demonstrated a reduction in their percentage body dry matter, crude protein and ashes. Hepatic glycogen levels were elevated in the group fed with 12% of pectin, while there were no effects in the cholesterol and triglycerides levels. Citrus pectin did not exert modulatory effect on the microbial community. Although the pectin supplemented fish showed enteritis during the experimental period, this did not impair animal performance. The use of this polysaccharide as a binder in diets of mullet for longer periods should be considered with caution.

***Keywords*: Binder, dietary fibers, enteritis, microorganisms, mullet, non-starch polysaccharides.**

1. Introduction

Approximately two billion tons of grain and 140 million tons of vegetables and seeds are produced worldwide per year, and approximately 230 million tons of fibrous materials are produced as by-products primarily comprising non-starch polysaccharides (NSP) (Choct, 1997). NSPs are constituents of dietary fiber made by the soluble and insoluble polysaccharides present in vegetable cell walls, which are resistant to enzyme attack in the animal and human digestive tract. However, NSPs are susceptible to fermentation by microorganisms present in the gastrointestinal tract of the host organism (Choct, 1997; McDougall *et al.* 1996; Sinha *et al.* 2011).

In animal and human nutrition, the role of pectin, a soluble NSP, in health maintenance is well known. Pectin is responsible for lowering plasma glucose and serum cholesterol levels, thus reducing the risks of cardiovascular disease (Lunn and Buttriss. 2007; Tungland *et al*. 2002) and exerting a preventive effect against colorectal cancer in humans (Lunn and Buttriss. 2007) and antioxidant effects in mice (Kohen *et*

1 *al.* 1993). Beyond forages, pectin is present in a wide range of feedstuffs from citrus by-
2 products in the diets of ruminants and non-ruminants, such as in fresh citrus pulp, dried
3 citrus pulp, and citrus meal, which are used as high energy feeds (Bampidis and
4 Robinson 2006). However, although recommended as a suitable binder in the diets of
5 aquatic organisms (Paolucci *et al.* 2012), to date, only two studies have tested their use
6 in formulating diets for crayfish diets (Volpe *et al.* 2008, 2012).
7 Mullets have several attributes that make them suitable for aquaculture, such as
8 hardiness and easy feed handling, because they easily accept rations and exhibit a wide
9 tolerance to salinity and temperature variations (Miranda-Filho *et al.* 2010). However,
10 there is not yet commercial production of this species in Brazil and some studies of *M.*
11 *liza* have focused on the nutritional requirements (Carvalho *et al.* 2010; Ito and Barbosa
12 1997; Zamora-Sillero *et al.* 2013), in order to create a technological rearing package.
13 However, to our knowledge, there is no information regarding the use of NSP in the
14 diets of Mugilids.
15 Thus, the purpose of this study was to evaluate the effect of the inclusion of
16 citrus pectin in the diets of mullet *Mugil liza* by observing their effects on performance,
17 body composition, the gastrointestinal bacterial community, the morphology of the
18 gastrointestinal tract and the levels of hepatic cholesterol, triglycerides and glycogen.
19
20 2. Materials and Methods
21 *2.1. Fish conditioning*
22 Mullet (*Mugil liza*) were caught by trawl (2.5 m x 1.5 m; mesh size 5.0 mm) at
23 Cassino beach (Rio Grande – RS, Brazil; 32º17'S-52º10'W) and transferred to the
24 Laboratório de Piscicultura Marinha e Estuarina of the Universidade Federal do Rio
25 Grande – FURG. The fish were stocked in one 300-L tank (25ºC, static system, 2 fishes

per liter) for feed training – hand-fed, four times per day (see below). After one week, 180 fishes were weighed (0.38 g ± 0.01) and randomly distributed throughout a static system consisting of 12 rectangular tanks (50 L) at a density of 15 fish/tank. After the first daily feeding, the tanks were siphoned and filled with seawater previously filtered through bag filters (5 μm) and chlorinated. The water was daily exchanged at the rate of 90% of the tank volume. Before utilization, the chlorinated water was treated with sodium thiosulfate for chlorine neutralization. Submerged heaters maintained the temperature at 25ºC, the salinity was held at 30, and the photoperiod was fixed in 14L:10D. The static system was employed in order to avoid water mixture among treatments, which could affect the colonization of the intestinal microbiota (Roeselers *et al.* 2011). In that sense, the water quality parameters were carefully evaluated to ensure conditions to fish development (.

The fish were hand-fed four times per day until apparent satiation (8:00 h, 11:00 h, 14:00 h, 17:00 h) according to the NRC (2011) recommendations. At the end of each day, the rations were weighed in a precision analytical scale (± 0.01 g, BL-3200H, Marte, São Paulo, Brazil), and the feed consumption was recorded.

2.2. Water parameters

The dissolved oxygen and temperature were measured daily using an oxygen meter (YSI 50A, Ohio, USA), the pH was recorded with a digital pH meter (± 0.01, YSI®-pH100, Ohio, USA), and the salinity was measured with a handheld Atago® refractometer (model 103, Tokyo, Japan). The total ammonia-nitrogen (TAN) content was measured every other day, and the alkalinity was measured weekly both *via* the UNESCO (1983) method. All water quality parameters were measured for all tanks.

2.3. Diet composition and proximate analysis

1 The experimental design was randomized, consisting of four treatments
2 performed in triplicate, which comprised a reference diet (control) (350 g kg^{-1} crude
3 protein; 16.45 MJ g^{-1}) and three other diets with increasing levels of citrus pectin (CP)
4 (Farmaquímica S.A., São Paulo, Brazil) supplemented to the reference diet (4% - CP4;
5 8% - CP8; 12% - CP12) (Tables 1 and 2). Care was taken to ensure that no ingredients
6 contained significant crude fiber levels. The dry ingredients were homogenized, and
7 subsequently oil and distilled water at 60ºC were added until achieving a consistent
8 texture that enabled pelleting in a meat grinder with a die 2 mm in diameter. Next, the
9 pellets were dried in a forced-circulation oven for 5 h at 60 ºC. After drying, the diets
10 were stored in hermetically sealed plastic bags in a freezer (-20 ºC) until use.
11 Proximate analysis of the diets and carcasses at the onset and end of the
12 experiment was performed according to the AOAC (1999) methodology: dry matter was
13 obtained after drying in an oven at 102 ºC for 5 hours; for ash, the samples were burned
14 in the muffle at 600 ºC for 5 h; crude protein measurement followed the Kjeldhal
15 method after sample digestion and nitrogen distillation, multiplying the result by 6.25;
16 and the lipid content was determined by ether extraction using a Soxhlet. For crude
17 fiber, the employed methodology was described by Silva & Queiroz (2009): both acid
18 and base digestions of the sample were conducted for 30 min each, followed by burning
19 the residue in a muffle at 500 ºC, and the value of the crude fiber was obtained by
20 weight difference. The non-nitrogenous extract was calculated from the difference of
21 the summed values of crude protein, lipid, ashes and crude fiber. The viscosity was
22 measured according to the adapted methodology by Refstie *et al*. (1999): a 50 g ration
23 was added to 450 ml distilled water and incubated for 30 minutes at 25 ºC under
24 agitation (80 rpm). Next, the rations were centrifuged (10.000 x g, 30 minutes), and the

1 supernatant was collected and analyzed using a rheometer (Brookfield, DV – III Ultra,

2 Massachusetts, USA) rotating at 250 rpm.

3 Table 1. Feed ingredients and proximal composition of reference diet

	Dry matter (g kg^{-1})
Feed ingredients	
Fishmeal	60.0
Casein[1]	250.0
Gelatin[1]	100.0
Corn starch	550.0
Fish oil	30.0
Premix[2]	10.0
Proximal composition	
Dry matter	890.0
Crude protein	338.4
Crude lipid	27.2
Ashes	17.3
Crude Fiber	0.43
Metabolizable energy[3] (MJ g^{-1})	18.45
Viscosity (cP)	18.4

4 [1]Rhoster (São Paulo, Brazil); [2] Premix M. Cassab, SP - Brazil (Vit. A (500,000 UI kg^{-1}),

5 Vit. D3 (250,000 UI kg^{-1}), Vit. E (5,000 mg kg^{-1}), Vit. K3 (500 mg kg^{-1}), Vit. B1 (1,000

6 mg kg^{-1}), Vit. B2 (1,000 mg kg^{-1}), Vit. B6 (1,000 mg kg^{-1}) Vit. B12 (2,000 mcg kg^{-1}),

7 Niacin (2,500 mg kg^{-1}), Calcium pantothenate (4,000 mg kg^{-1}), folic acid (500 mg kg^{-1}),

8 biotin (10 mg kg^{-1}), vit. C (10,000 mg kg^{-1}). Colin (100,000 mg kg^{-1}), Inositol (1,000

1 mg kg^{-1}). Trace elements: selenium (30 mg kg^{-1}), iron (5,000 mg kg^{-1}), copper (5,000
2 mg kg^{-1}), manganese (5,000 mg kg^{-1}), zinc (9,000 mg kg^{-1}), cobalt (50 mg kg^{-1}), iodine
3 (200 mg kg^{-1}). [3] Calculated from the physiological standard values, where 1 kg of
4 carbohydrate (N-free extract), protein and lipid yields 16.7, 16.7 and 37.6 MJ,
5 respectively (Garling and Wilson, 1976).
6 Table 2. Formulations and proximal compositions of the experimental diets

	Diets		
	CP4	CP8	CP12
Diet formulation (g kg^{-1})			
Reference diet	960	920	880
Citrus pectin	40	80	120
Dietary component (g kg^{-1} dry matter)			
Dry matter	895.5	894.8	900.5
Crude protein	328.2	326.8	306.3
Crude lipid	26.6	22.1	16.7
Ashes	17.9	16.1	17.1
Crude fiber	0.49	0.42	0.43
Viscosity (cP)	25.0	36.2	101.0

7
8 *2.4. Growth trial*
9 The experiment lasted 60 days, after which all fish were weighed and measured
10 to obtain the following growth and biometrical indexes:
11 1. Weight gain (g): final weight – initial weight
12 2. Apparent feed conversion ratio: diet supplied/weight gain

3. Specific growth rate (% day^{-1}): [(ln final weight – ln initial weight)/days farming] × 100

4. Protein efficiency ratio: weight gain (g)/protein intake (g)

5. Condition factor: 100 × live weight (g) / corporal length $(cm)^3$

6. Hepatosomatic index: $(weight_{liver}/weight_{body}) \times 100$

7. Viscerosomatic index: $(weight_{viscera}/weight_{body}) \times 100$

8. Intestinal quotient relative to length: $length_{intestine}/length_{body}$

9. Intestinal quotient relative to weight: $length_{intestine}/weight_{body}$

Afterwards, the fish were euthanized with an overdose of Benzocaine (500 ppm) to collect the liver and gastrointestinal tract to obtain the biometrical indexes and perform the subsequent analysis. The livers (18 fish per treatment) were separated and frozen at -80 ºC for subsequent analysis; the stomach and intestine (nine fish per treatment) were fixed in buffered formalin (20%) for histological analysis. For microbial quantification, prior to removing the tract from the remaining animals (nine fish per treatment), the outer surfaces were sterilized with povidone iodine. Then, the tract was sampled and fixed in 4% formalin. Before the beginning of the experiment, nine random fish were subjected to the same procedure for initial microbial quantification.

2.5. Glycogen, cholesterol and liver triglyceride levels

The frozen liver samples were homogenized for 40 min in a sonicator with perchloric acid (6%) in a volume 7.5 times the sample weight according to Zamora-Sillero *et al.* (2013). After sonication, the homogenates were neutralized with the same volume of potassium bicarbonate (1 M). Then, the homogenates were centrifuged

(13.000 x g during 30 min), and the supernatants were used for the analyses. Total cholesterol and triglyceride levels were estimated using commercial kits (Triglicérides Enzimático Líquido, Colesterol Enzimático Líquido, Doles, Goiânia, GO, Brazil).

The liver glycogen content was estimated in duplicate according Carr & Neff (1984), later modified by Nery & Santos (1993). The glycogen content was obtained *via* the enzymatic breakdown (amiloglicosidase, Sigma) of glucose. The resulting product was measured with a commercial kit (Glicose enzimática, Doles, Goiânia, GO, Brazil). All measurements were performed on a spectrophotometer with a microplate reader at a wavelength of 490 nm (ELx800, Biotek Instruments Inc., Winooski, Vermont).

2.6. Histological analysis

The fixed material was processed in a LUPE PT 05 automatic processor, embedded in Paraplast® and cut into 5-µm-thick sections in a LUPETEC MRPO3 microtome. The sections were stained with hematoxylin-eosin (HE).

2.7. Microbial count

The fixed samples were taken to Laboratório de Fitoplâncton e Microorganismos Marinhos/IO – FURG for bacterial count; for this, the intestine and stomach were carefully removed from the solution and sectioned into pieces in previously autoclaved petri dishes. After being opened, the stomach and intestines were washed with 10 mL of distilled water. The solution was transferred to 40-mL glass jars and sonicated (Cole-Parmer Instrument Co., Chigado, Illinois, USA) in three 10-second increments with 10-second intervals between them. One 0.5-mL aliquot was removed and filtered through polycarbonate membrane filters (Nucleopore, Kent, UK, 0.2 µm porosity) that were previously darkened with 12% Irgalan black. The filtrate was then stained with acridine

1 orange (1 µg mL^{-1}) (Hobbie *et al.,* 1977). The bacteria were counted in 30 random fields
2 using a Zeiss Axioplan epifluorescent microscope (Oberkochen, Germany) equipped
3 with a blue filter (487709 – BP 450-490, FT 510, LT 520) and a Watec CCD (Watec
4 Co., Yagamata, Japão) (0,0003 Lux).

5 The results of the performance, body composition and liver parameters were
6 subjected to analysis of variance (ANOVA). The ANOVA assumptions (normality by
7 Shapiro-Wilks, and variance homogeneity by Levene) were previously evaluated. The
8 microbial quantification was subjected to two-way ANOVA to identify interactions
9 between the citrus pectin level inclusion and the bacterial population in the different
10 digestive tract segments. The Tukey test was applied to identify any significant variance
11 from the mean (Zar, 1984). In all cases, the significance level was fixed at 5%.

12

13 3. Results

14 *3.1. Water parameters*

15 The water quality parameters evaluated did not present significant differences (*P*
16 > 0.05) among the treatment groups (Table 3).

17 Table 3. Water quality parameters (mean ± S.D) during the evaluation of juvenile
18 mullets fed with increasing levels of citrus pectin.

Parameters	Control	CP4	CP8	CP12
Temperature (ºC)	24.9 ± 0.70	24.9 ± 0.74	24.9 ± 0.72	24.9 ± 0.74
Dissolved oxygen (mg L^{-1})	6.81 ± 0.37	6.83 ± 0.40	6.80 ± 0.39	6.82 ± 0.38
Salinity	29 ± 1.13	29 ± 1.13	29 ± 1.13	29 ± 1.13
pH	8.11 ± 0.06	8.14 ± 0.06	8.15 ± 0.08	8.14 ± 0.09
Alkalinity (mg $CaCO_3$ mL^{-1})	147 ± 12.93	147 ± 12.93	147 ± 12.93	147 ± 12.93
TAN (mg L^{-1})	0.68 ± 0.45	0.57 ± 0.35	0.64 ± 0.39	0.45 ± 0.24

19

20

1 *3.2. Growth performance*

2 The results of growth performance are provided in Table 4. No significant
3 differences ($P > 0.05$) among the treatments were recorded for any of the tested
4 parameters. No mortality was registered during the experimental period in any
5 treatment.

6 Table 4. Growth performance and biometric index of juvenile mullets fed with
7 increasing levels of citrus pectin*

Parameter	Control	CP4	CP8	CP12
$AW_{Initial}$	0.38 ± 0.06	0.36 ± 0.01	0.38 ± 0.01	0.37 ± 0.02
AW_{Final}	3.67 ± 0.50	3.33 ± 0.42	3.51 ± 0.26	3.20 ± 0.67
WG	3.29 ± 0.50	2.96 ± 0.42	3.13 ± 0.26	2.82 ± 0.66
SGR	3.78 ± 0.24	3.65 ± 0.17	3.70 ± 0.14	3.56 ± 0.29
FI	6.51 ± 0.96	5.24 ± 0.37	6.25 ± 0.30	5.23 ± 1.14
FCR	1.94 ± 0.01	1.79 ± 0.14	2.00 ± 0.12	1.86 ± 0.19
PI	2.23 ± 0.33	1.84 0.14	2.19 ± 0.10	1.83 ± 0.40
PER	1.47 ± 0.01	1.61 ± 0.13	1.43 ± 0.09	1.55 ± 0.15
		Biometric índex		
K	1.29 ± 0.08	1.30 ± 0.02	1.30 ± 0.01	1.32 ± 0.08
HSI	1.62 ± 0.11	1.77 ± 0.23	1.65 ± 0.12	1.63 ± 0.01
VSI	10.57 ± 0.48	10.25 ± 0.37	10.74 ± 1.07	10.69 ± 0.72
QIR_{CL}	2.49 ± 0.16	2.45 ± 0.18	2.55 ± 0.21	2.62 ± 0.07
QIR_{BW}	4.51 ± 0.66	4.71 ± 0.50	4.7 ± 0.41	5.16 ± 0.87

8 AW: average weight; WG: weight gain; SGR: specific growth rate; FI: feed intake;
9 FCR: feed conversion rate; PI: protein intake; PER: protein efficiency rate; *K*: condition
10 factor; HSI: hepatic somatic index; IVS: viscera somatic index; QIR: Quotient intestinal

1 relative (CL: corporal length; BW: body weight). * Mean values ± S.D. of triplicates
2 groups; n = 15
3
4 *3.3. Body composition*
5 The inclusion of citrus pectin in the diets resulted in significant (P < 0.05)
6 alterations in body composition. The control group showed higher dry matter and crude
7 protein content. On the other hand, the lipid levels in the control were not significantly
8 different (P > 0.05) from those of treatments CP8 and CP12, and the ashes content was
9 not significantly (P > 0.05) different from that of treatment CP4, while was significantly
10 higher (P < 0.05) than CP8 and CP12 treatments (Table 5).
11 Table 5. Proximal body composition of juvenile mullets fed diets with increasing levels
12 of citrus pectin*

Body composition	Initial	Final			
		Control	CP4	CP8	CP12
Dry matter	22.25 ± 0.10	28.59 ± 0.04[a]	27.05 ± 0.03[b]	25.53 ± 0.04[d]	26.55 ± 0.19[c]
Crude protein	15.70 ± 0.39	17.60 ± 0.30[a]	16.48 ± 0.19[b]	15.64 ± 0.29[c]	16.58 ± 0.22[b]
Crude lipid	0.89 ± 0.03	7.53 ± 0.10[ab]	7.12 ± 0.03[b]	7.25 ± 0.40[ab]	7.65 ± 0.07[a]
Ash	5.34 ± 0.03	3.25 ± 0.06[a]	3.04 ± 0.02[ab]	2.71 ± 0.14[c]	2.86 ± 0.11[bc]

13 *Values are means ± S.D. of triplicates groups. Means with different superscript letters
14 in the same column differs significantly (P < 0.05); n = 15
15
16 *3.4. Liver parameters*
17 The liver glycogen concentrations at CP12 treatment were significantly higher
18 (P < 0.05) than control. No significant differences (P > 0.05) in triglycerides or in the
19 cholesterol concentrations among the treatments were observed (Table 6).

1 Table 6. Levels of hepatic glycogen, triglycerides and cholesterol (mg g^{-1}) in juvenile

2 mullets fed diets with increased levels of pectin*

Treatment	Glycogen	Triglycerides	Cholesterol
Control	2.95 ± 0.63^{c}	5.50 ± 2.02^{a}	0.058 ± 0.02^{a}
CP4	4.45 ± 0.91^{ab}	4.69 ± 1.64^{a}	0.070 ± 0.24^{a}
CP8	3.61 ± 1.13^{bc}	4.43 ± 3.27^{a}	0.045 ± 0.02^{a}
CP12	5.07 ± 1.10^{a}	4.88 ± 2.63^{a}	0.046 ± 0.02^{a}

3 *Values are means ± S.D. of triplicate groups. Means with different superscript letters

4 in the same column differ significantly ($P < 0.05$); n = 6

5

6 *3.5. Digestive tract histological analysis*

7 Intestinal pathologies were identified in all of the fish fed with pectin; however,

8 the degree of severity was independent of the level of pectin inclusion. Chronic

9 inflammations with a predominance of lymphocytes and some macrophage infiltrates in

10 the mucosa, as well the release of epithelium and intestinal villus shortening, were

11 observed (Figure 1).

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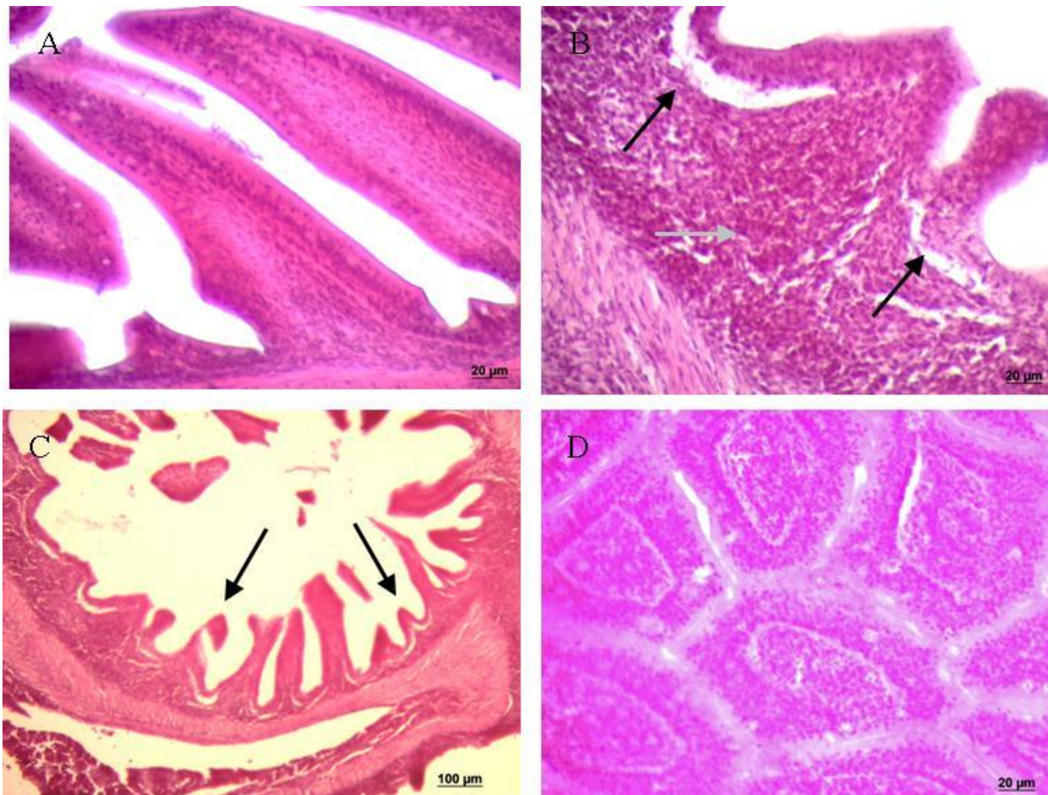

Figure 1. Morphological changes in the intestines of juvenile mullets fed diets with increased levels of citrus pectin. A) Longitudinal section of the normal villus in the Control; B) epithelium release in CP8 (black arrow) and infiltrates in the mucosa (gray arrow); C) villus disruption and shortening in CP8 (black arrow); and D) cross-section of the villus, showing inflammations in the CP12 treatment.

3.6. Digestive tract bacterial count

No significant differences ($P > 0.05$) were identified among the pectin treatments in the modulation of the microbial community, and no interaction between the level of pectin inclusion and the tract section in the microbial community was observed. However, individual effects were observed when the comparisons considered the bacterial morphotypes in the tract sections isolated in each treatment.

Three main bacteria classes were identified: cocci, bacilli and filamentous. These three bacteria morphotypes exhibited in the stomach bacterial densities

1 significantly higher ($P < 0.05$) when compared to the intestinal segments, mainly in the

2 distal section. This was noted in the CP4 and PC8 treatments for the cocci; in the CP8

3 treatment for the bacilli group; and in the Control, CP4 and CP8 treatments for the

4 filamentous morphotypes (Figure 2).

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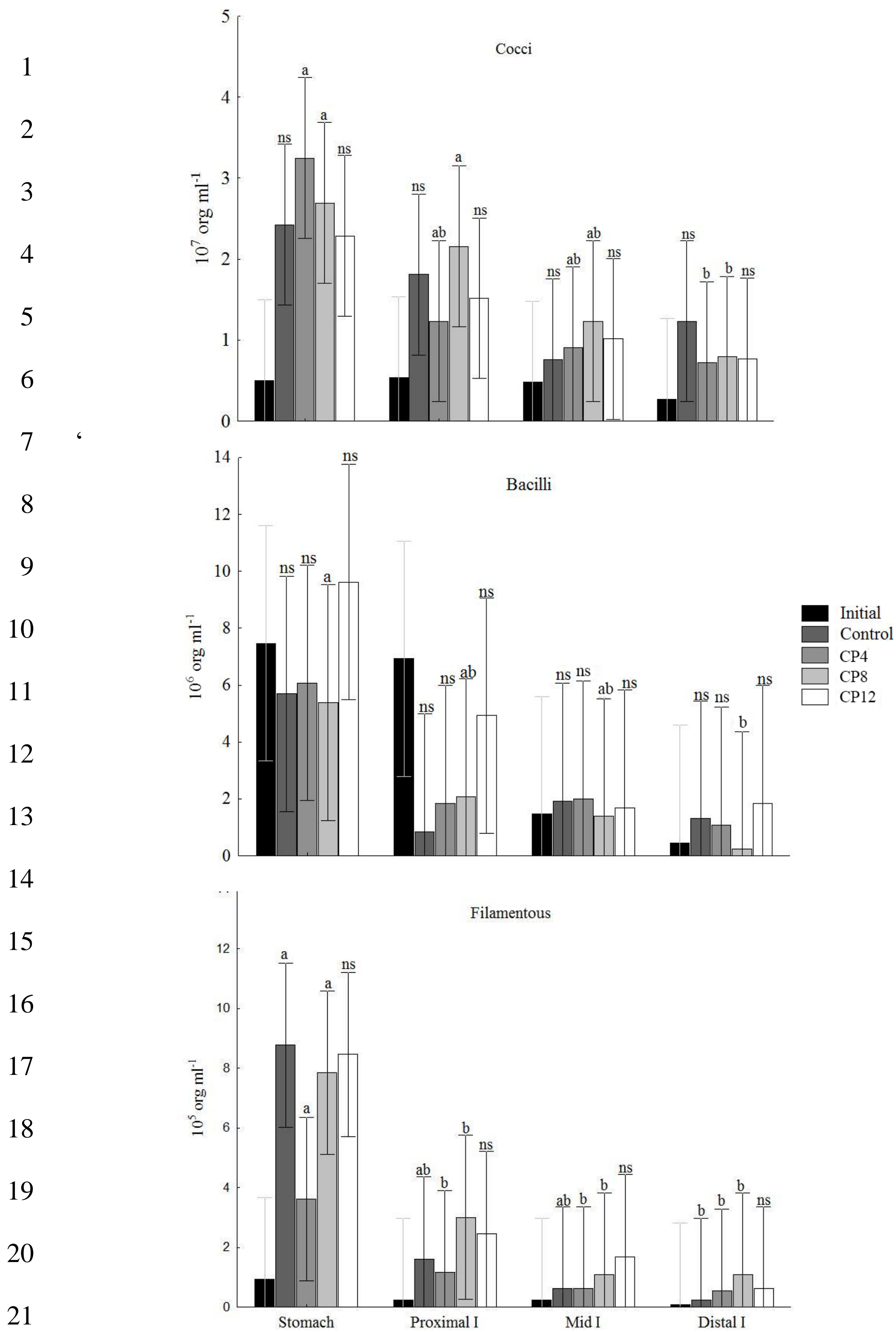

22 Figure 2. Coccoid, rods and filamentous bacteria densities in different tract sections of
23 *Mugil liza* juvenile mullets fed with increasing levels of citrus pectin. Different
24 lowercase letters indicate significant differences ($P < 0.05$) between sections of the
25 same treatment (ns = non-significant).

1 4. Discussion

2 According to the Brazilian Ministry of Agriculture - MAPA (2007), the
3 Brazilian projection of orange production in 2013/2014 is about 20 millions tons, which
4 represents 50% of the world orange production. Moreover, 98% of this production is
5 designed to the orange juice industry. The production of orange juice generated 50% of
6 the dry weight in by-products (processed fruit), which can be used for the production of
7 citrus pulp, molasses and pectin, which is obtained by acid extraction of peel
8 (Oreopoulou and Tzia 2007). Considering the local availability of orange juice by-
9 products and its low inclusion level in fish diets, citrus pectin may represent a good
10 candidate to be included as binder in fish diets.

11 In the present study, it was observed that the inclusion of pectin in the diets of
12 juvenile mullets did not cause growth reduction. To the best of our knowledge, there are
13 no studies considering the use of pectin as a fish diet binder. Nevertheless, it is widely
14 used as an ingredient in the food and pharmaceutical industries due to its gelling and
15 thickening activity (Liu *et al.* 2003), and according to Farris *et al.* (2009), among
16 polysaccharides, the use of pectin is particularly promising for a wide number of
17 applications in the food industry.

18 Volpe *et al*. (2008) evaluated the growth response as well as the pellet stability
19 in water of diets containing three different binders (agar, alginate and pectin; all
20 included at 2.5% of the diet) used in feed for lobster *Cherax albidus*. The inclusion of
21 pectin was found to provide higher pellet stability in the water, and the animals also
22 showed better performance with this binder. Similarly, Volpe *et al.* (2012) evaluated the
23 effect of three binders (pectin, alginate and chitosan, all included at 5%) on the growth
24 of juvenile *Cherax albidus* and on the stability of the pellets after the process of cold

1 extrusion. Again, pectin proved to be the best option for cold extrusion and animal
2 performance.

3 Liver glycogen content of the fish fed with pectin was higher than that of the
4 control treatment, and according to Panserat *et al.* (2000), glycogen in the liver is
5 related to the intake of digestible carbohydrates in the diet. However, pectin is
6 indigestible by fish or any other vertebrate due to the lack of an enzyme that breaks
7 down this molecule, but it can be easily fermented by microorganisms in the digestive
8 tract to produce primarily short chain fatty acids used as a source of both glucose and
9 fat, which can be stored in extra intestinal tissues, such as in the liver and muscle, by the
10 fish (Montagne *et al.* 2003; Willmott *et al.* 2005). In addition, Semova *et al.* (2012)
11 showed, for zebrafish, the diet-dependent role of the microbiota in stimulating fatty acid
12 absorption in the intestine. Those mechanisms could explain the increase in the liver
13 glycogen in the fish fed with pectin.

14 Despite the presence of intestinal pathologies in pectin fed fish, their growth
15 performances were not affected. Non-starch polysaccharides are present in a wide
16 variety of ingredients of plant origin. In salmonids, the use of soybean meal is limited
17 because it causes intestinal inflammatory responses (enteritis) characterized by the
18 presence of inflammatory cells, the shortening of villi, the disruption of microvilli and
19 the widening of lamina propria caused by the infiltration of inflammatory cells
20 (Merrifield *et al*. 2011) and some of these pathologies were also observed in the present
21 study. It is known that the anti-nutritional effect of soluble NSP arises from its viscous
22 nature (Sinha *et al.* 2011), yet soybean meal contains high concentrations of
23 carbohydrates, mainly formed by NSP and oligosaccharides, which are the pectic
24 polysaccharides responsible for approximately 50% of the NSP fraction (Choct 1997).

In that sense, it can be inferred that the presence of enteritis in the mullets fed pectin may be associated with the same effect observed in salmonids fed with soybean meal.

In poultry, the use of pectin was investigated, and negative effects were observed in the feed intake, weight gain and feed conversion upon the addition of 3% pectin to the diet (Shakouri *et al*. 2006). The same study showed a pectin modulatory effect on the microflora, with an increase in the total count of anaerobic bacteria in the anterior intestine section. In swine, negative effects from the use of pectin have also been reported, such as depression of the growth performance of weaning pigs (Choct *et al*. 2010).

Some studies have indicated the importance of this nutrient in the diets of non-ruminant animals and humans, primarily due to its modulatory role on the microbial community (Tungland *et al.* 2002; Montagne *et al.* 2003). While individual effects of the diet over the density of bacterial morphotypes, which was increased in the stomach and was lower in the distal intestine, were observed on the course of the experiment, there were no significant changes between the inclusion of citrus pectin and the control diet. Usually, in studies that perform direct bacteria counting in fish tracts, higher levels of bacteria are observed in the intestine compared to the stomach (Clements 1991; Fidopiastis *et al.* 2006; Navarrete *et al.* 2009; Rimmer 1986). Unexpectedly, the juvenile mullets showed the opposite behavior: the microbial population was abundant in the stomach and decreased as it approached the distal portion of the intestine. This may indicate a ruminant-like feeding behavior, where there is bacterial uptake along the intestine and use of this microbial biomass as an additional source of protein.

5. Conclusion

The inclusion of citrus pectin in the diets of *Mugil liza* caused no adverse effects on growth. There were changes in the body composition, liver glycogen and microbial community of animals. The occurrence of enteritis in animals fed with the pectin levels tested was not sufficient to reduce the performance of the animals. Nevertheless, because of the occurrence of enteritis, it is recommended to use caution in applying this binder in fish diets.

6. Acknowledgments

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16

CAPÍTULO 3

Exogenous enzyme cocktail prevents intestinal soybean meal-induced enteritis in *Mugil liza* (Valenciennes, 1836) juvenile.

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1. Resume

The use of plant proteins in aquafeeds to replace fish meal are increasing, being the soybean meal the more suitable and widely protein source in aquaculture feed industry. Despite the numerous antinutritional factors, new technology has arisen to improve their utilization by the fish, such as the supplementation of exogenous enzyme cocktails. The purpose of the present work was to evaluate the enzyme inclusion in fed for *Mugil liza* juvenile, evaluating the growth parameters, muscle composition, intestinal histology and the microbial counts from the gastrointestinal tract (stomach and proximal, mid and distal intestine). The animals were fed in the soybean meal-based diet (Control – E0) and others four diets with increasing levels of an enzyme cocktail (E50, E100, E150 and E200, respectively, 50, 100, 150 and 200 g ton^{-1}) during 75 days. There were not significant differences (P>0.05) in the growth and muscle composition. The bacterial counts were not affected by the enzyme inclusion, however isolated effects between tract sections could be observed inside the treatments. The histological screening shows that fishes fed in control diet exhibited serious from moderate infiltrate of inflammatory cells, modification in villus morphology and necrosis in some cases, whilst the fishes

from all enzyme-treated diets show only light infiltrates. The use lower levels of exogenous enzyme are recommended in diets for *Mugil liza* when soybean meal are used as main source of protein.

***Key-words*: Antinutritional factors, growth, microorganisms, muscle composition.**

2. Introduction

The feed industry has recognized for many years that viable utilization of plant feedstuffs formulated in aquafeeds for the production of aquatic species is an essential requirement for future development of aquaculture (Gatlin *et al.* 2007). However, most of the potential alternative plant-derived nutrient sources are known to contain a wide variety of antinutritional substances (Francis *et al*. 2001, Gatlin *et al.* 2007; Hardy, 2010). Also there are various forms of nutrient that are unavailable to animals because they require specific digestive enzymes to access them (NRC, 2011). Since a combination of plant-derived feed ingredients are necessary to replace the fish meal supplements such as amino acids, flavourings and exogenous enzymes will be needed to produce aquafeeds without fish meal that support growth rates similar to fish meal based diets (Gatlin *et al.* 2007).

The antinutrients are secondary metabolites found in many plants having a role in defense against herbivores, pest and pathogens and in plant feedstuffs, including also structural components such non starch-polysaccharides (NSP), nutrients and energy storing components such phosphorous-rich phytic acid, allergens and others chemicals defenses (Bennett & Wallsgrove, 1994; Krogdahl *et al*, 2010). In fish nutrition, these compounds may reduce feed intake, growth, nutrient digestibility and utilization, affects the function of internal organs and alter disease resistance (Krogdahl *et al.* 2010). According to Francis *et al.* (2001), the processing techniques like dry and wet heating, extracting with water and addition of feed supplements have been widely used to reduce the concentration of antinutrients in plant feeds.

The feed is the most important cost in any animal production system and in on-farm profitability, but if the animal underutilizes the feed, there is a cost to both to producer and the environment (Barletta 2010). The feed supplementation with specific exogenous enzymes can improve the nutritional value of the raw material and reduce the variation in the nutritional quality of ingredients, also helps in the breakdown of antinutritional factors (Bedford 2000; Barletta 2010). The global feed enzyme market is

1 worth in excess of $550 million US dollars and saves the global feed market an
2 estimated $3 to 5 billion per year, in mainly two enzymes segments, phytase
3 (approximately 60%) and nonphytase (40%), while most of them are obtained from
4 fermentation systems based in genetically modified bacteria and/or fungi as *Aspergillus*
5 *niger*, *Bacillus licheniformis*, *Escherichia coli* and *Trichoderma reesei* (Adeola and
6 Cowieson, 2011).
7 As reviewed by Barletta (2010), the enzymes usage arose in 1980s in poultry
8 nutrition in Europe, which wheat and barley are the main feeds used in rations
9 formulation. The introduction of fiber degrading enzymes improved bird production and
10 lowered feed costs. However, the bigger progress has come with the phytase feed
11 enzyme introduction, in 1990s, evolving benefits to animal production and environment
12 by the reduction of phosphorus excretion by poultry and swine. Now, the feed enzymes
13 are widely used in poultry and swine production, in a combination of phytase and
14 carbohydrases that targeting different antinutrients in the diet releasing even more
15 nutrients comparing with the use of the isolated enzymes. Recently, a review of enzyme
16 supplementation in aquaculture points that the research in this field is not extensive, and
17 based in results of non-ruminant animals, more attention should be given to application
18 of this additive in the fish nutrition (Castillo & Gatlin, 2014).
19 The mullet *Mugil liza* is a important marine resource in the southern of Brazil,
20 with the catches counting about 18,000.00 ton in the last fisheries bulletin (MPA, 2011).
21 It is recommended specie for aquaculture (Miranda-Filho *et al.* 2010) and studies have
22 been made for this purpose in the last years focusing on the nutritional requirements.
23 Thereby, Carvalho *et al.* (2010) determined that 35 g kg^{-1} is the crude protein level for
24 that specie; Zamora-Sillero *et al.* (2013) investigate the inclusion of dextrin in their
25 diets and pointing that inclusions levels above 35 g kg^{-1} do not causes reduction in the
26 growth; Ramos *et al.* (2015) included citrus pectin, a soluble non-starch polysaccharide,
27 in the fish diets and do not observe any alteration in the growth.
28 The aim of this study is evaluate the optimum exogenous enzyme cocktail level
29 in diets for *Mugil liza*, evaluating the growth performance, carcass composition, hepatic
30 metabolites, gastrointestinal tract histology, gastrointestinal microbial profile and
31 calcium and phosphorus bone retention.
32
33 3. Material and Methods.
34 *3.1. Fish capture and conditioning*

This work agrees with ethic norms of animal experimentation from Conselho Nacional de Controle de Experimentação Animal (CONCEA) in process number 23116.0014242014-01.

Juvenile mullet were caught in Casino beach (Rio Grande – RS, Brazil; 32º17'S-52º10'W) by a trawl net (2.5 m x 1.5 m; mesh size 5.0 mm) and conduced to the Laboratório de Piscicultura Marinha e Estuarina of the Universidade Federal do Rio Grande – FURG. Then the animals were stocked in one circular 300-L tanks at the density of two fishes per litter before the beginning of the experiment for prophylactic treatment (formaldehyde, 100 ppm, 30 min) and feed training (hand-fed four times per day) for one week.

3.2 Water parameters

Daily, the water parameters were monitored; dissolved oxygen and temperature were measured with an oximeter (YSI 50A, Ohio, USA); pH with a digital ph meter (±0.01, YSI®-pH100, Ohio, USA); salinity using a handheld refractometer (Atago®, model 103, Tokyo, Japan). Ammonia and alkalinity ware measured every other day via UNESCO (1983) method.

3.3 Experimental diets

Experimental diets were composed for one basal control diet (Table 1), formed by a soybean-based diet without inclusion of enzyme cocktail (E0), and other four diets, composed by different levels of enzyme cocktail (E50: 50 g ton^{-1}; E100: 100 g ton^{-1}, E150: 150 g ton^{-1} and E200: 200 g ton^{-1}) supplementing the soybean-basal diet. The diets enzymatic activity were measured at Centre d'Analyse, de Recherche et d'Appui Technique (Adisseo, Commentry, France) (Table 1). The enzymatic cocktail are composed by Xilannases (endo-1,4 β-xylanase, α-arabinofuranosidase, β-xylosidase, feruloyl esterase, endo-1,5 α-arabinase), β-glucanases (endo-1,3(4) β-glucanase, β-1,3 glucanase [laminarinase] endo-1,4 β-glucanase [cellulase], cellobiohydrolase, β-glucosidase), pectinases (pectinase, polygalacturonase, pectinesterase, rhamnogalacturonase) mannanases (endo-1,4 β-mannanase), phytase (6-phytase) and others (α-galactosidase, aspartate protease, metalloprotease). For diet manufacture, dry ingredients were homogenized and subsequently added oil and distilled water at 50ºC, and in the diets with enzyme inclusion, the last was added to the water and mixed with the mixture, until acquisition of a consistent texture that allowed to be pelleted in a meat grinder with die of 2-mm-diameter. Care was taken to do not overheat (over 60ºC) the water and denature the enzymes. After, pellets were dried in a forced-circulation oven

1 for 6 h at 55 ºC. Finally, the diets were stored in sealed plastic bags in a freezer at -20ºC
2 until used.
3 Table 1. Feed ingredients and proximate composition of basal diet, and feed enzyme
4 activity of the experimental diets (E0: Control – 0 g ton^{-1}; E50: 50 g ton^{-1}, E100: 100 g
5 ton^{-1}, E150: 150 g ton^{-1}, E200: 200 g ton^{-1})

Feed ingredients				*Dry matter (g kg^{-1})*
Fish meal				2.0
Soybean meal				46.0
Wheat gluten				8.0
Rice meal				20.0
Wheat meal				20.0
Fish oil				3.0
Premix				1.0
Proximal composition				
Dry matter				975.3
Crude protein				360.7
Ether extract				85.3
Ashes				58.5
Crude fiber				52.1
Non-nitrogenous extract				443.4
Metabolizable energy (MJ g^{-1})[2]				16.45
Enzymatic activity				
	Phytase activity		Xylanase activity	
Experimental Diets	Target	FTU kg^{-1}	Target	Visco units kg^{-1}
E50	500	814	1100	904
E100	1000	1256	2200	2198
E150	1500	1402	3300	2821
E200	2000	1696	4400	3927

6 [1]Premix M. Cassab, SP, Brazil: Vit. A (500000 UI kg^{-1}), Vit. D3 (250000 UI kg^{-1}), Vit. E (5000 mg kg^{-1}),
7 Vit. K3 (500 mg kg^{-1}), Vit. B1 (1000 mg kg^{-1}), Vit. B2 (1000 mg kg^{-1}), Vit. B6 (1000 mg kg^{-1}) Vit. B12
8 (2000 mcg kg^{-1}), Niacin (2500 mg kg^{-1}), Calcium pantotenate (4000 mg kg^{-1}), folic acid (500 mg kg^{-1}),
9 biotin (10 mg kg^{-1}), vit. C (10000 mg kg^{-1}). Colin (100000mg kg^{-1}), Inositol (1000 mg kg^{-1}). Trace
10 elements: selenium (30 mg kg^{-1}), iron (5000 mg kg^{-1}), copper (5000 mg kg^{-1}), manganese (5000 mg kg^{-1}),
11 zinc (9000 mg kg^{-1}), cobalt (50 mg kg^{-1}), iodine (200 mg kg^{-1}). [2]Calculated from the physiological
12 standard values, where 1 kg of carbohydrate (N-free extract), protein and lipid yields 16.7, 16.7 and 37.6
13 MJ, respectively (Garling and Wilson, 1976).
14
15 *3.4 Growth trial*
16 A design with five treatments with three replicates each was set in this study.
17 After the conditioning period, fishes were subject to an initial biometry weighing (0.18

1 ± 0.005 g) and distributed in a static system formed by 15 50-L rectangular tanks at a
2 density of 20 fishes per tank. The fishes were hand-fed four times per day (9:00 AM,
3 12:00 AM, 15:00 PM, 18:00 PM) until apparent satiation. At the end of the each day,
4 the diets were weighted in a precision analytical scale (± 0.01 g, BL-3200H, Marte, São
5 Paulo, Brazil) to record daily intake. Daily, after the first feeding, the tanks were
6 cleaned and filled with treated seawater (chlorinated, filtered in bag filter 5 µm and pass
7 through ultraviolet light). Sodium thiosulfate was used to neutralize the chlorine before
8 utilization. Environmental conditions were maintained in 25ºC for temperature with a
9 room air conditioning, 30 for salinity and photoperiod of 14L: 10D until the end of the
10 experimental period, 75 days.
11 At the end of experiment, all fishes were weighted and measured to obtain the
12 growth parameters and biometrical index that follow: weight gain, apparent feed
13 conversion rate and protein efficiency ratio. For hepatosomatic index, viscerosomatic
14 index, carcass composition, histological analysis and bacterial counts, all fishes were
15 euthanized with an overdose benzocaine (300 ppm). Liver and gastrointestinal tract
16 were removed from all fishes and weighted separated, carcass backbone were separated
17 to access the calcium and phosphorus bone retention and then were frozen at -20ºC. The
18 gastrointestinal tract of three fishes per tank (nine per treatment) were sectioned in
19 stomach and intestine and fixed in buffered formaline 4% for bacterial count and at the
20 beginning of the experiment, ten fishes were subjected to the same procedure for the
21 initial counts. The viscera of others three fishes per tank (nine per treatment) were
22 removed and fixed for 24h in Bouin's fluid and after in ethanol 70% for histological
23 analysis.
24
25 *3.5 Analytical methods*
26 Proximal analysis of ration and muscle were conduced according AOAC (1999)
27 methods: dry matter was assessed after drying of samples in a oven for 5h at 102ºC;
28 ashes by the burning in a muffle for 5h at 600ºC; Kjeldhal method was used for crude
29 protein determination, which the samples were digested followed by nitrogen
30 distillation then the results were multiplied for 6.25. Soxhlet extractor was utilized for
31 lipid determination, using petroleum ether as solvent for 6h. Calcium, phosphorus and
32 crude fiber followed the methodology described by Silva & Queiroz (2009). Non-
33 nitrogen extract was calculated from the difference between the total crude protein,
34 ashes, ether extract and crude fiber values.

1 Ethanol 70% maintened material was taken to Laboratório de Imunologia e
2 Patologia de Organismos Aquáticos – FURG and processed in a LUPE PT 05 automatic
3 processor embedded in Paraplast® and cut into 5-μm-thick sections in a LUPETEC
4 MRPO3 microtome. The sections were stained with hematoxylin-eosin (HE). To
5 measure the intestinal tissue damage, was used a modified quantitative scoring adapted
6 from Bakke-McKellep *et al.* (2007), according with the pathological description of
7 Baeverfjord and Krogdhal (1996) that follows: I: light lymphocyte infiltrate; II:
8 Moderate lymphocyte infiltrate with alterations in villus; III: Serious lymphocyte
9 infiltrate with alterations in villus structure; IV: Serious lymphocyte infiltrate with
10 alterations in villus structure and necrosis on the mucosa tissue. The peritoneal lipid
11 accumulation was measured in the software AxioVision 4.8, using one histological
12 section per fish.
13 Formaline 4% fixed samples of stomach and intestine was taken to the
14 Laboratório de Fitoplâncton a Microoganismos Marinhos/IO – FURG for bacterial
15 count. The samples were carefully removed from solution, sectioned (stomach, intestine
16 proximal, mid and distal) and opened in previously autoclaved Petri dishes and washed
17 with 10 mL of milliQ water. Then the solution was transferred to 40-mL glass jars and
18 sonicated (Cole-Parmer Instrument Co., Chicago, Illinois, USA) in three 10-second
19 increments with 10-seconds interval between them. One 1.0-mL aliquot was taken and
20 filtered through polycarbonate membrane filters (Nucleopore, Kent, UK, 0.2 μm
21 porosity) that were previously darkened with 12% Irgalan black. The filtrate was then
22 stained with acridine orange (1 μg mL^{-1}) (Hobbie *et al.,* 1977). Bacteria were count in
23 30 random fields using a Zeiss Axioplan epifluorescence microscope (Oberkochen,
24 Germany) equipped with a blue filter (487709 – BP 450-490, FT 510, LT 520) and a
25 Watec CCD (Watec Co., Yagamata, Japan) (0.0003 Lux). The software Image Tool UT
26 3.0 was utilized to perform the counts.
27 For phosphorus and calcium analysis, the backbones were dried at 105ºC for 5
28 hour in an oven; macerate, thereafter defatted in a soxhlet extractor using petroleum
29 ether as a solvent for 6 hours. Then, the samples were taken to Laboratório de
30 Hidroquímica/IO – FURG, suffer an acid digestion with nitric and perchloric acids and
31 the resultant solutions were diluted in distilled water, filtered in paper filters and
32 afterward, the analysis follow Silva & Queiroz (2009) methodology. The samples were
33 read in an atomic absorption spectrophotometer flame at the wave length of 422.7 nm

1 for calcium analysis, and in a digital spectrophotometer at the wave length of 725 nm
2 for phosphorus analysis.
3 *3.6 Statistical analysis*
4 Water quality, growth performance, body composition and liver parameters
5 results were subjected to analysis of variance (ANOVA), with previous check of
6 assumptions (normality by Shapiro-Wilks and variance homogeneity by Levene)
7 evaluated. Mathematical transformation were applied when the premises was not
8 accessed in order to satisfy the analysis of variance assumptions. ANOVA two-way was
9 applied for microbial counts in order to indentify interactions between the enzyme level
10 inclusion and the bacteria populations in the different tract segments. The Tukey test
11 (5% of significance) was applied to identify significant variances from the means (Zar,
12 1984).
13
14 4. Results
15 *4.1 Water parameters*
16 There was not observed differences between the treatments for any water
17 parameters measured (Table 2).
18 Table 2. Water quality parameters from rearing tanks of mullet fed with increasing
19 levels of exogenous enzyme cocktail in the soybean-base diets during 75 days (E0:
20 Control – 0 g ton^{-1}; E50: 50 g ton^{-1}, E100: 100 g ton^{-1}, E150: 150 g ton^{-1}, E200: 200 g
21 ton^{-1})

Water Parameters	E0	E50	E100	E150	E200
Temperature **(ºC)**	24.00 ± 0.02	24.06 ± 0.07	24.05 ± 0.01	24.05 ± 0.10	24.09 ± 0.08
DO **(mg L^{-1})**	7.07 ± 0.01	7.04 ± 0.04	7.03 ± 0.02	7.05 ± 0.04	7.05 ± 0.06
pH	7.88 ± 0.01	7.85 ± 0.05	7.87 ± 0.03	7.89 ± 0.01	7.88 ± 0.01
TAN **(mg L^{-1})**	0.60 ± 0.01	0.59 ± 0.01	0.63 ± 0.05	0.65 ± 0.09	0.59 ± 0.03
Alkalinity **(mg $CaCO_3$ L^{-1})**	103.85 ± 0.26	105.29 ± 0.95	103.56 ± 1.27	104.08 ± 0.80	104.42 ± 0.36
Salinity	20.2 ± 0.00	20.2 ± 0.00	20.2 ± 0.00	20.2 ± 0.00	20.2 ± 0.00

22 *Values are mean ± SD of triplicates groups. DO: Dissolved oxygen; TAN: Total
23 ammonium nitrogen.
24
25 *4.2 Growth parameters*

1 The date of growth parameters and biometric indexes are listed in the Table 3.
2 Significant difference (P < 0.05) was observed only for calcium retention, and no
3 differences (P > 0.05) were observed in the others growth parameters analyzed.
4 Table 3. Growth parameters of mullet *Mugil liza* juvenile fed with increasing levels of
5 exogenous enzyme cocktail in the diets (E0: Control – 0 g ton^{-1}; E50: 50 g ton^{-1}, E100:
6 100 g ton^{-1}, E150: 150 g ton^{-1}, E200: 200 g ton^{-1})

Parameters	E0	E50	E100	E150	E200
AW$_{initial}$	0.18 ± 0.005	0.18 ± 0.005	0.18 ± 0.005	0.18 ± 0.005	0.18 ± 0.005
AW$_{final}$	1.17 ± 0.10	1.40 ± 0.27	1.25 ± 0,22	1.37 ± 0.27	1.22 ± 0.09
WG	0.99 ± 0.09	1.22 ± 0.27	1.08 ± 0.21	1.19 ± 0.28	0.91 ± 0.21
SGR	2.70 ± 0.11	2.93 ± 0.29	2.83 ± 0.17	2.94 ± 0.28	2.76 ± 0.11
FI	3.01 ± 0.25	3.44 ± 0.54	3.17 ± 0.45	3.26 ± 0.43	3.32 ± 0.49
FCR	3.11 ± 0.41	2.88 ± 0.26	3.06 ± 0.30	2.83 ± 0.32	3.04 ± 0.15
PI	1.02 ± 0.08	1.13 ± 0.21	1.08 ± 0.15	1.11 ± 0.15	1.13 ± 0.17
PER	0.96 ± 0.12	1.02 ± 0.09	0.93 ± 0.10	1.03 ± 0.14	0.89 ± 0.08
PhR	4.41 ± 0.82	4.56 ± 0.96	3.61 ± 0.78	4.69 ± 1.71	3.70 ± 0.31
CaR	7.07 ± 0.36b	7.37 ± 1.28b	10.23 ± 0.62ab	11.14 ± 0.53a	9.20 ± 2.15ab
Survival	96.7 ± 5.77	95.0 ± 5.0	93.3 ± 5.77	96.7 ± 2.89	96.7 ± 2.89
			Biometric indexes		
K	1.26 ± 0.04	1.24 ± 0.01	1.14 ± 0.21	1.28 ± 0.03	1.22 ± 0.02
HSI	1.48 ± 0.40	1.22 ± 0.11	1.26 ± 0.13	1.18 ± 0.06	1.25 ± 0.064
VSI	14.61 ± 4.37	12.02 ± 0.24	13.95 ± 2.75	15.87 ± 3.78	12.11 ± 0.62

7 *Values are mean **±** SD of triplicates groups. AW, average weight; WG, weight gain;
8 SGR, specific growth rate; FI, feed intake; FCR, feed conversion rate; PI, protein
9 intake; PER, protein efficiency rate; K, condition factor; HSI, hepatic somatic index;
10 IVS, viscera somatic index.
11
12 *4.3. Proximal muscle composition*
13 Muscle initial and final compositions are listed in the Table 4. There was
14 significantly difference (P<0.05) only for bone Ca parameter. The E100 and E150
15 showed higher levels of bone Ca than Control and and E50 treatments.
16

1 Table 4. Proximal muscle composition of mullet *Mugil liza* juvenile fed with increasing
2 levels of exogenous enzyme cocktail in the soybean-base diets (E0: Control – 0 g ton^{-1};
3 E50: 50 g ton^{-1}, E100: 100 g ton^{-1}, E150: 150 g ton^{-1}, E200: 200 g ton^{-1})

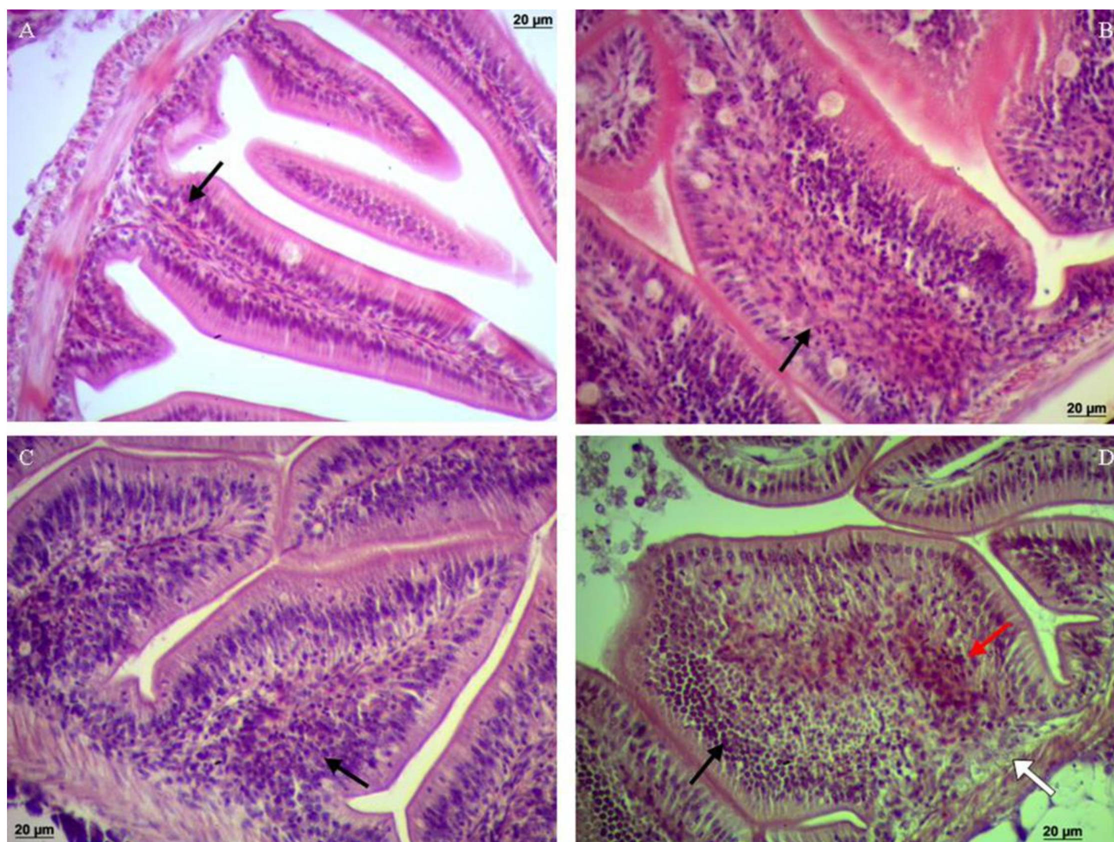
Proximate composition	Initial	Final E0	E50	E100	E150	E200
Dry Matter	21.45 ± 0.60	35.66 ± 5.67	33.60 ± 2.18	35.02 ± 3.58	39.97 ± 6.14	37.40 ± 4.76
Crude Protein	13.47 ± 0.91	19.61 ± 3.31	18.39 ± 1.12	18.54 ± 2.39	20.38 ± 2.89	20.62 ± 3.84
Ether Extract	5.55 ± 2.81	8.84 ± 2.37	9.61 ± 1.52	12.05 ± 2.92	11.02 ± 1.75	10.84 ± 1.53
Ashes	4.01 ± 0,07	4.83 ± 0.82	4.62 ± 0.54	4.66 ± 0.07	5.37 ± 0.63	5.15 ± 0.72
Bone P	2.56 ± 0.88	4.43 ± 0,50	4.98 ± 0.97	4.02 ± 0.76	4.32 ± 1.37	3.70 ± 0.39
Bone Ca	6.42 ± 1.21	7.08 ± 0.88b	7.24 ± 1.16b	10.38 ± 0.75a	11.22 ± 0.52a	8.95 ± 1.96ab

4 *Values are mean **±** SD of triplicates groups.
5
6 *4.4 Intestinal histological analysis*
7 The semi-quantitative score of intestinal lesions are listed below in the Table 5.
8 In the control group fishes showed lesions in the scores I, II, III and IV, while in all
9 treatment with enzyme inclusion the fishes exhibited only lesions in the score I (Table
10 5) (Figure 1).
11 Table 5. Observed intestinal lesions in mullet *Mugil liza* juvenile fed with increasing
12 levels of exogenous enzyme cocktail in the soybean-base diets (E0: Control – 0 g ton^{-1};
13 E50: 50 g ton^{-1}, E100: 100 g ton^{-1}, E150: 150 g ton^{-1}, E200: 200 g ton^{-1})

Treatment	**Observations**	**Score levels I**	**II**	**III**	**IV**
Control	*n = 9*	1	3	2	3
E50	*n = 6*	4	2		
E100	*n = 4*	4			
E150	*n = 4*	4			
E200	*n = 4*	4			

14
15 The values of lipid accumulation in the gastrointestinal tissue are shown in the
16 Figure 2. Fishes from control group showed significantly ($P < 0.05$) less lipid
17 accumulation into the peritoneal areal than fishes from all enzyme groups, which not
18 differs between them.
19
20 *4.5 Bacterial count*
21 Four bacterial morphotypes were recognized along the counts: cocci (Cc), vibrio
22 (Vb), bacilli (Bc) and filamentous (Fl).

1 There were not observed interactions ($P > 0.05$) between the levels of enzyme
2 cocktail and the bacterial count in the different tract sections. However differences ($P >$
3 0.05) were observed when comparisons were made isolating the tract sections inner the
4 treatments. In E0 total bacteria counts, the lower value was observed in the mid
5 intestine; cocci morphotype in the proximal intestine; vibrio and bacilli in the mid
6 intestine; filamentous were less abundant in the mid and distal segments. In E50
7 treatment, were observed less total bacteria and bacilli in the proximal intestine;
8 filamentous bacterial was higher in stomach in comparison to others intestinal
9 segments. E100 treatment exhibited lower counts in the proximal intestine for total
10 bacteria, cocci and vibrio morphotypes, while the bacilli was observed less abundant in
11 the proximal and mid intestine. The E150 and E200 treatment did not show significant
12 ($P > 0.05$) differences (Figure 3).
13



1 Figure 1. Histology screening from intestinal folds of *Mugil liza* juveniles fed with
2 increasing levels of exogenous enzyme cocktail in the soybean-base diets, showing
3 lymphocyte infiltrate (black arrow), necrosis (white arrow), hemorrhagic lesions (red
4 arrow) and deformation of intestinal folds (B, C and D); in (A), a normal intestinal fold.
5 In clockwise: A – score I; B – score II; C – score III; D – score IV.

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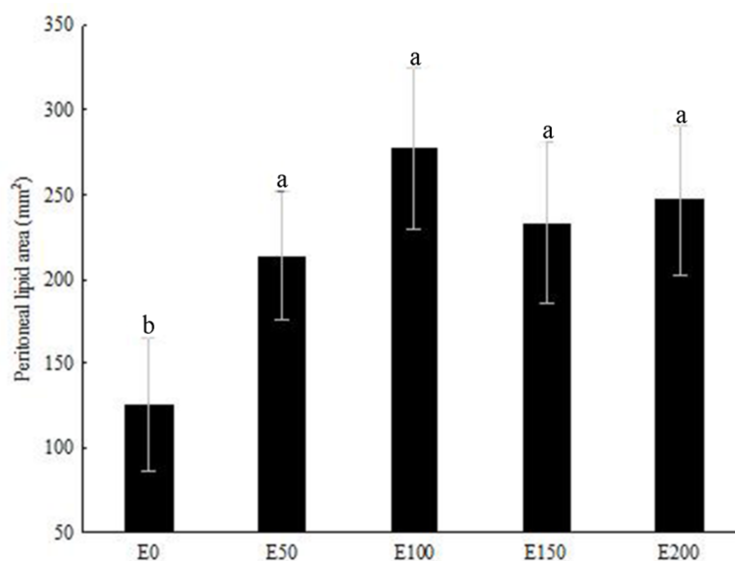
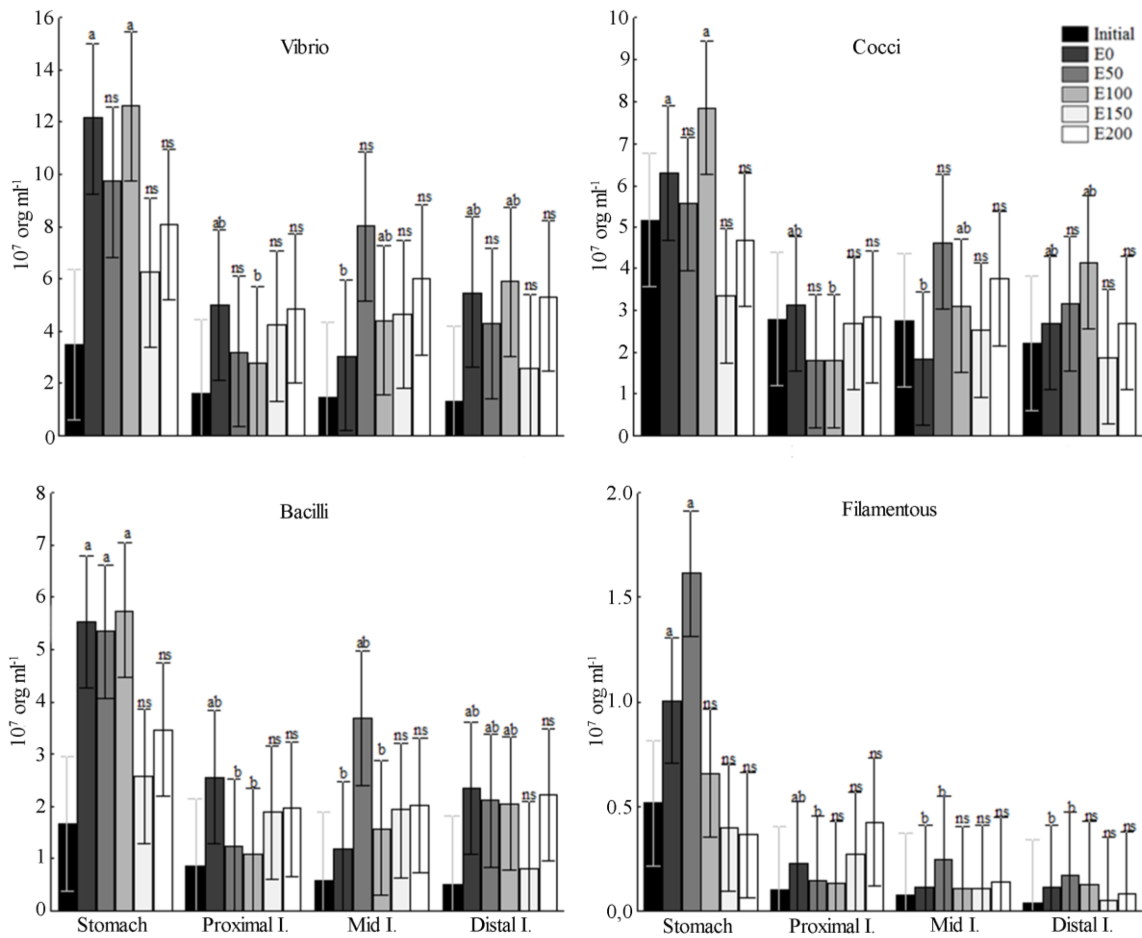


17 Figure 2. Peritoneal lipid area of *Mugil liza* juvenile fed with increasing levels of
18 exogenous enzyme cocktail in the soybean-base diets (E0: Control – 0 g ton^{-1}; E50: 50 g
19 ton^{-1}, E100: 100 g ton^{-1}, E150: 150 g ton^{-1}, E200: 200 g ton^{-1}). Different letters indicates
20 significant differences ($P < 0.05$) between the treatments.

1



2 Figure 3: Bacterial morphotypes counts from digestive tract of *Mugil liza* juvenile fed
3 with increasing levels of exogenous enzyme cocktail in the soybean-base diets (E0:
4 Control – 0 g ton^{-1}; E50: 50 g ton^{-1}, E100: 100 g ton^{-1}, E150: 150 g ton^{-1}, E200: 200 g
5 ton^{-1}). Different letters indicates significant differences ($P < 0.05$) between sections
6 from same treatments.

7

8

5. Discussion

The results of enzyme utilization in aquaculture are still contradictory and may be dependent of the studied species, feeding habit, type and the mix of the enzyme applied and plant source utilized. In this work, were not observed significant results with inclusion of enzyme cocktail in the animal performance, and the same results can be found in the literature (Dalsgaard *et al.*, 2012; Yigit and Olmez, 2011; Fahangi and Carter, 2007; Ogunkoya *et al.*, 2006; Stone *et al.*, 2003).

Phytase requires low pH (2.5 – 5.5) to access optimum activities and in fish diets the differences observed in the efficiency relies mainly in the diversity of digestive fish systems, mainly because of stomach pH (Cao *et al.* 2007). Despite that phosphorus retention and bone phosphorus was not different between the treatments, it is well known that phytate can chelate with others minerals, such calcium, reducing both availability and bone mineralization (Singh and Satyanarayana, 2014). Calcium retention was different between the treatments, which was higher in the E150 when compared with the Control and E50 treatments, which could indicate that the phytase was not fully underutilized by the mullets, and more, there is a maximum level that the fishes could utilizes it for calcium absorption, being it 150 g ton^{-1} in this study.

The fiber fraction present in the soybean meal is about 18%, represented by cellulose, hemicellulose and pectin substances, being the last most cited and as well named as non-starch polysaccharide (Burssens *et al.*, 2011; Banaszkiewicz, 2011). Non starch-polysaccharides are antinutritional factors that impair fish digestion by increasing the tract viscosity (Leenhouwers *et al.*, 2007), gut physiology and morphology (Hossain *et al.*, 2001; Leenhouwers *et al.*, 2006) among others effects. It is well known that soybean meal diets induces the infiltration of inflammatory leukocyte cells in the intestinal mucosa tissue and shortening of mucosal folds in salmonid fish species (Krogdahl *et al.*, 2010; Bakke-McKellep *et al.*, 2007; Ostaszewska *et al.*, 2005; Krogdahl *et al.*, 2003) and described by Baeverfjord and Krogdahl (1996) as a non-infectious subacute enteritis.

Previously, Ramos *et al.* (2015) observed in mullets fed with citrus pectin intestinal lesions-like as the expressed in salmonids fed with soybean meal. In the present study, some fishes from control treatment manifest moderate to serious infiltration of inflammatory cells, alterations of villus morphology and in some cases, necrosis of intestinal tissue whilst fishes from all enzyme fed group shown only slight inflammatory cell, which is normal in healthy fishes. However, the animal performance

1 was not impaired during the 75 days of experiment, with the final weight higher 6.5
2 times than the initial weight, and perhaps for more prolonged periods it is could be more
3 dangerous, as seen by Francesco *et al*. (2004) for rainbow trout, in a feed regime with
4 plant proteins during 157 days. The enzymes present in the cocktail, most of
5 carbohydrases, includes enzymes that hydrolyzes non-starch polysaccharides
6 (xylanases, pectinases, β-glicosidades) might have helped to hamper the effects
7 observed in intestinal tissue of fishes fed with control soybean-base diet.

8 The gastrointestinal microbial community of fishes plays an important role in
9 nutrition and despite this, it is neglected in most studies with feed enzymes. Even in the
10 monogastric nutrition, e.g. poultry and swine that the use of feed enzymes is already
11 common, few articles have focused in the effects of enzyme inclusions and microbiota
12 in their nutrition (Bedford and Cowieson, 2012). Despite the some studies with enzyme
13 addition have been made recently, only Zhou *et al.* (2013) evaluated the diets over the
14 intestinal microbiota and the growth of grass carp, and the results suggest that cellulase
15 has changed the bacterial species and density.

16 In the present work, the enzyme supplementation in mullets feed did not
17 exhibited influence between the distributions of bacteria across the tract sections in the
18 control and enzyme inclusions levels. The effects observed were independent of enzyme
19 inclusion and singly in total count were observed a stomach richer in bacteria than
20 proximal and mid intestine in E50, E100 and E0, respectively. And when morphotypes
21 were analyzed isolated the same previously trend could be observed for Cocci (E0 and
22 E100), vibrio (E0 and E100), bacilli (E0, E50 and E100) and filamentous E0 and E50.

23 Recently, Ramos *et al.* (2015) also observe that mullets feed with citrus pectin
24 and control diets (no pectin) exhibited the similar microbial behavior. The authors have
25 suggested that this fish species could exhibit a ruminant-like feed habit. Thus, another
26 hypothesis would help to explain the lack of results in fish growth in this study relies on
27 the breakdown of complexes carbohydrates from food by enzymes into monomers and
28 oligomers, which can be fermented by the microorganisms (Castillo and Gatlin, 2014;
29 Sinha *et al.*, 2011), being this hypothesis more acceptable since that the accumulation of
30 lipids in the peritoneal area in the fed enzyme treatments was higher than the control,
31 and could be a reflex of volatile fatty acids produced for bacterial fermentation into the
32 tract by bacteria and subsequently absorbed by the fish.
33
34 ## 6. Conclusion

1 The enzyme inclusion did not exhibit any improvements in the animal
2 performance, body composition and microbial tract community, but was observed a
3 higher calcium deposition and retention in the treatments with enzymes supplemented-
4 diet. This present study shows as well that the enzyme supplementation, even the lower
5 addition level, reduces the intestinal damage caused by soybean meal diet.
6
7 ## 7. Acknowledgments

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14
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DISCUSSÃO GERAL

A utilização de polissacarídeos não-amiláceos da dieta, tanto purificados, como aqueles contidos em fontes vegetais usadas na aquicultura, ainda é um tanto contraditória. Os efeitos de PNAs são dependentes da espécie, tipo de polissacarídeo, idade do animal, hábito alimentar e do nível de inclusão (Sinha *et al.* 2011). O uso cada vez mais frequente de produtos vegetais em dietas para peixes devido à pressão para reduzir o consumo da farinha de peixe reforçam a necessidade de avaliar os efeitos dos fatores antinutricionais nos organismos e fomas de se mitigar esses danos (Francis *et al.* 2001).

Grande parte dos aglutinantes utilizados em dietas para peixes são de origem vegetal. Esses polímeros apresentam grande capacidade de coesão das partículas de nutrientes, o que as mantêm unidas por tempo mais prolongado e reduzindo a perda dos nutrientes e consequentemente a poluição da água (Paolucci *et al.* 2012). Contudo, apesar de muitas vezes tratados como aditivos inertes, seus efeitos biológicos mutas vezes adversos já foram descritos na literatura (Sinha *et al.* 2011; Krogdahl *et al.* 2010; Gatlin *et al.* 2007; Francis *et al.* 2001).

Em peixes, ações negativas foram reportadas para diversas espécies como o salmão do Atlântico, truta arco-íris, tilápia do Nilo, bagre africano, carpa e no presente estudo, na tainha (Krogdahl *et al.* 2010; Leenwhouvers *et al.* 2006, 2007a, 2007b; Hossain *et al.* 2001, 2003; Amirkolaie *et al.* 2001; Siddhuraju *et al.* 2001; Storebakken 1985). No Capítulo 1 da presente tese, foram observados efeitos negativos no crescimento na tainha *Mugil liza* quando alimentada com goma guar. Seus efeitos negativos são derivados da natureza solúvel desse composto que quando no trato, impede a digestão e a absorção dos nutrientes por fomar uma "proteção" contra as enzimas digestivas (Paolucci *et al.* 2012; Sinha *et al.* 2011; Leenwhouver *et al.* 2006).

A pectina cítrica, apesar de também ser um polissacarídeo solúvel, não causou efeitos negativos sobre o crescimento da tainha como observado no Capítulo 2. Comparações com a literatura são difíceis pela ausência de estudos avaliando a utilização desse aglutinante na dieta de pexies, contudo, em dietas para lagostas, seu uso foi promissor pela melhora da qualidade dos pellets e pelo melhor desempenho dos animais em comparação a outros aglutinantes (Volpe *et al.* 2008, 2012).

No Capítulo 3, dietas baseadas em farelo de soja como a principal fonte protéica e dietas suplementadas com um coquetel de enzimas exógenas, compostas principalmente por enzimas degradadoras de PNAs (carboidrases) foram utilizadas,

1 contudo, não foram observados efeitos positivos provenientes da inclusão enzimática,
2 tão pouco efeitos negativos provenientes do farelo de soja. É comum na literatura não
3 obter resultados satisfatórios quando há a inclusão de enzimas exógenas nas dietas
4 (Dalsgaard *et al.*, 2012; Yigit and Olmez, 2011; Fahangi and Carter, 2007; Ogunkoya *et*
5 *al.*, 2006; Stone *et al.*, 2003). Existem algumas hipóteses que justificam essa ausência
6 de resposta. De acordo com Castillo e Gatlin (2014), a primeira hipótese surge da
7 ineficiência de algumas espécies de peixes não conseguirem metabolizar a glicose
8 disponibiliza em grandes quantidades quando dietas são suplementadas com
9 carboidrases; a segunda aponta que os nutrientes liberados da digestão podem passar a
10 ser utilizados pelos microoganismos intestinais; e a terceira, justifica que em alguns
11 estudos, existe a necessidade de limitar os nutrientes presentes na dieta para que a
12 aplicação das enzimas tenha algum reflexo no crescimento.
13 Até o momento, apenas um estudo foi realizado avaliando o efeito de enzimas
14 exógenas em dietas com restrição nutricional em peixes. Kumar *et al.* (2006).forneceu
15 duas dietas com níveis sub-otimos de proteína, com e sem a gelatinização do amido,
16 para juvenis de carpa com suplementação de uma única enzima exógena, a α-amilase.
17 Nesse caso, os efeitos positivos ocorreram apenas na dieta em que o amido não foi
18 gelatinizado, e segundo os autores, isso ocorreu porque a liberação lenta da glicose do
19 amido não cria o estresse metabólico causado pela rápida absorção da glicose, que por
20 sua vez é favorecido pelo fornecimento do amido gelatinizado. Sabe-se que a tainha é
21 tolerante a altos níveis de glicose proveniente da dieta como pode ser observado no
22 estudo de Zamora-Sillero *et al.* (2013). Tainhas alimentadas com elevados níveis de
23 dextrina não demosntraram redução no crescimento e alterações na bioquímica do
24 plasma e do glicogênio hepático. A teoria da utilização dos nutrientes pelos
25 microoganismos pode justificar os resultados do Capítulo 3.
26 A comunidade microbiana tem uma importante participação na nutrição de
27 peixes através da síntese de vitaminas e no provimento de enzimas exógenas e pela
28 manutenção da saúde intestinal através da competição com bactérias patogênicas por
29 exclusão competitiva (Ganguly *et al.* 2012; Gómes e Balcázar 2008). PNAs não são
30 passíveis de digestão por enzimas animais, contudo, podem ser fermentados por
31 microoganismos intestinas e seus produtos, os ácidos graxos voláteis, absorvidos pelo
32 hospedeiro e utilizados como fonte de energia suplementar (Sinha *et al.* 2011; Lunn &
33 Buttriss, 2007; Montagne *et al.* 2003; Wenk, 2001; Alles *et al.*, 1999; Asp, 1996;
34 McDougall *et al.*, 1996). Interessantemente, nos três estudos realizados, foram

observadas quantidades de bactérias relativamente grandes no estômago, ultrapassando significativamente como com a inclusão de goma guar, o total de bactérias nos segmentos intestinais. Isso pode indicar uma estratégia alimentar, como a observada em ruminantes terrestres (Allison, 1993).

Em ruminantes, a câmara fermentativa, rúmen, é o nicho bacteriano de fermentação do material fibroso ingerido por esses animais. Além dos ácidos graxos voláteis absorvidos pela parede ruminal, a biomassa microbiana fornece a parte mais importante da proteína dietética desses animais (Allison, 1993). O mesmo pode ocorrer no trato da tainha, e isso é reforçado pelos níveis de glicogênio hepático maiores com a inclusão de goma guar e pectina (12%), e pela maior deposição lipídica entérica de peixes alimentados de rações com inclusão de enzimas. No ambiente, essa espécie alimenta-se principalmente de detritos, que é a matéria orgânica em decomposição, pobre em macronutrientes e rica em materiais fibrosos e microorganismos que os decompõem, logo, mais do que detritivoria, a estratégia alimentar pode ser de fato bacteriófaga, onde o peixe se alimenta na realidade de bactérias aderidas aos detritos (Seeliger *et al.,* 1997). Bactérias não possuem organelas citoplasmáticas e outras estruturas internas comum aos eucariontes, e por sua vez, possuem grande parte de seu conteúdo formado por proteínas e ácidos nucléicos, e relativamente poucos carboidratos estruturais, dessa forma, possuem menor relação C:N e C:P, sendo nutricionalmente mais importantes (Strom 2000).

A ocorrência de enterite em salmonídeos alimentados com farelo de soja é muito bem reportada na literatura (Krogdahl *et al.*, 2010; Bakke-McKellep *et al.*, 2007; Ostaszewska *et al.*, 2005; Krogdahl *et al.*, 2003; Baeverfjord e Krogdahl 1996). Apesar de ainda se desconhecer o agente causador, a substitução da dieta por formas mais processadas do farelo, como o isolado e o concentrado protéico de soja, é suficiente para mitigar a ocorrência dessa patologia (Krogdahl *et al.* 2010). O processamento da soja consiste na eliminação de carboidratos, que em sua maioria é composto de polissacarídeos péctinicos (Choct 1997). O fornecimento de dietas com pectina cítrica e com farelo de soja como fonte protéica para tainha provocaram a ocorrência de infiltratos inflamatórios e deformações das vilosidades intestinais, além de necrose. Contudo, os animais não apresentaram redução de desempenho em nenhum dos casos. O tempo de duração dos experimentos (60 e 75 dias) pode não ter sido significativo para que não se tenha observado um efeito sobre o desempenho dos animais (de Francesco *et al.* 2004), ou, pelo tamanho relativamente grande do intestino desses animais, os danos

nesse orgão podem não ter sido significativos a ponto de representar uma perda na capacidade absortiva dos nutrientes. Os peixes que foram alimentados com rações suplementadas por enzimas exógenas, representadas principalmente por carboidrases, não apresentaram patologias intestinais e possivelmente essas enzimas reduziram o potencial antinutritivo do farelo de soja, mesmo no menor nível de suplementação.

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CONCLUSÃO GERAL

Baseado nos resultados desta Tese conclui-se:

1. A inclusão de goma guar em dietas para juvenis de *tainha* causou efeito antinutricional aos animais. É recomendado que sua inclusão não ultrapasse os 4%.
2. A inclusão de pectina cítrica em dietas para juvenis de tainha não afetou o desempenho dos animais. Contudo, a ocorrência de enterite nos animais faz com que o uso desse polissacarídeo nas dietas de peixes como aglutinante deve ser feita com cautela.
3. A inclusão de enzimas exógenas em dietas baseadas no farelo de soja para juvenis de tainha não melhorou nenhum dos parâmetos de desempenho, mas a retenção de cálcio ósseo foi maior. Contudo, foi observada a ocorrência de enterite nos animais alimentados com farelo de soja, e a inclusão de enzimas, mesmo no menor nível de suplementação, foi o suficiente para mitigar a ocorrência dessa patologia.