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DETOXIFICAÇÃO DE FARELOS DE CRAMBE E TUNGUE E AVALIAÇÃO NA RESPOSTA NUTRICIONAL DO JUNDIÁ (*Rhamdia quelen*)

TESE DE DOUTORADO

Alexandra Pretto

Santa Maria, RS, Brasil.

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DETOXIFICAÇÃO DE FARELOS DE CRAMBE E TUNGUE E AVALIAÇÃO NA RESPOSTA NUTRICIONAL DO JUNDIÁ (*Rhamdia quelen*)

Alexandra Pretto

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Zootecnia, Área de Concentração em Produção Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Zootecnia.**

Orientador: Profª. Leila Picolli da Silva

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elaborada por
Alexandra Pretto

como requisito parcial para obtenção do grau de
Doutora em Zootecnia

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Santa Maria, 19 de Fevereiro de 2013.

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"Para realizar grandes conquistas, devemos
não apenas agir mas também sonhar;
não apenas planejar, mas também acreditar."
(Anatole France)

"Sucesso é a soma de pequenos esforços, repetidos o tempo todo".
(Robert Collier)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Zootecnia
Universidade Federal de Santa Maria

DETOXIFICAÇÃO DE FARELOS DE CRAMBE E TUNGUE E AVALIAÇÃO NA RESPOSTA NUTRICIONAL DO JUNDIÁ (*Rhamdia quelen*)

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Data e Local da Defesa: Santa Maria, 19 de Fevereiro de 2013.

Os farelos vegetais são apontados como as principais alternativas de fontes de proteína com ampla disponibilidade, baixo custo e potencial nutricional para substituir a farinha de peixe em dietas para piscicultura. No entanto, possuem menor conteúdo de proteína, maior teor de fibras e antinutrientes, necessitando de um estudo minucioso para aplicabilidade ampla e segura. Técnicas de processamento ou pré-tratamento podem extrair e/ou inativar antinutrientes melhorando o valor nutricional das fontes. Neste sentido, foram mensurados nutrientes e antinutrientes e digestibilidade proteica *in vitro* dos farelos de tungue e crambe *in natura* e após o tratamento químico em solução ácido-alcoólica. Este procedimento reduziu os teores de gordura, cálcio, fósforo, taninos condensados e ácido fítico no farelo de tungue, aumentou fibra em detergente neutro e não alterou proteína bruta, matéria mineral, compostos fenólicos, taninos totais e hidrolisáveis. Para o farelo de crambe o tratamento elevou fibra e matéria mineral e reduziu proteína bruta, gordura, cálcio, fósforo, fenóis totais, taninos (totais, hidrolisáveis e condensados) e ácido fítico. Não foi observado efeito do tratamento químico sobre a digestibilidade protéica *in vitro* dos farelos. Os farelos de crambe ou tungue também foram pré-tratados com enzimas microbianas exógenas, fitase não comercial (ação de fitase e tanase – 1400 U e 1100 U/Kg) e fitase comercial (Natuphos/BASF, 1400 U/Kg). A fitase comercial mostrou maior eficiência sobre o farelo de tungue, reduzindo a concentração de ácido fítico e a fitase não comercial atuou mais efetivamente sobre taninos no farelo de crambe. A substituição de farinha de peixe e farinha de carne e ossos pelos farelos de crambe ou de tungue *in natura* (forma integral) ou detoxificados quimicamente ou enzimaticamente na alimentação de jundiás (*Rhamdia quelen*) foi avaliada durante nove semanas, com o estudo do crescimento, parâmetros digestivos, metabólicos e deposição corporal de nutrientes. Crescimento similar ao controle foi observado desde o início do estudo nos animais alimentados com farelo de crambe nas formas integral ou detoxificada quimicamente. Estes animais mostraram mínimas alterações enzimáticas e metabólicas. A inclusão do farelo de tungue integral ou detoxificado quimicamente causou menor crescimento aos animais. Na dieta contendo o farelo integral, estes resultados foram observados até o final do experimento e foram somados às alterações enzimáticas digestivas, metabólicas e menor sobrevivência dos animais. A forma tratada quimicamente do farelo de tungue propiciou maior crescimento em relação ao farelo integral, possivelmente devido à remoção de antinutrientes e substâncias tóxicas. A inclusão dos farelos vegetais (ambas as formas de tratamento enzimático) reduziu o crescimento dos peixes desde a primeira avaliação, com resposta mais pronunciada nos animais que receberam o farelo de tungue. Aumento no índice digestivosomático e quociente intestinal pode demonstrar uma adaptação fisiológica destes animais ao consumo das dietas contendo o farelo de tungue, as quais apresentaram o maior teor de fibra. Quanto à composição corporal, cinzas, proteína bruta e fósforo não foram alterados entre os tratamentos contendo os farelos vegetais em estudo, mas dietas com inclusão de farelo de tungue resultaram em menor matéria seca e gordura corporal.

Palavras-chave: Antinutrientes. Crescimento. Deposição de nutrientes. Fontes de proteína. Melhorias nutricionais. Metabolismo. Peixe.

ABSTRACT

Animal Science Doctoral Thesis
Post-Graduate Program in Animal Science
Federal University of Santa Maria

DETOXIFICATION OF CRAMBE AND TUNG MEAL AND ASSESSMENT IN THE NUTRITIONAL RESPONSE OF SILVER CATFISH (*Rhamdia quelen*)

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The vegetable meals are pointed out as the main alternative sources of the protein with widespread availability, low cost and nutritional potential to replace the fish meal in aquaculture diets. However, they have lower protein content, higher fiber and antinutrients, requiring a thorough study for broad and secure applicability. Processing techniques or pretreatment may extract and/or inactivate antinutrients improving the nutritional value of the sources. In this sense, we measured nutrients and antinutrients and dynamics of in vitro protein digestibility of tung and crambe meal in nature and after chemical treatment in acid-alcohol solution. This procedure reduced the levels of fat, calcium, phosphorus, condensed tannins and phytic acid in tung meal, increased neutral detergent fiber and did not change crude protein, ash, phenolic compounds, total and hydrolysable tannins. For crambe meal the treatment increased the fiber and ash contents and reduced crude protein, fat, calcium, phosphorus, total phenols, tannins (total, hydrolysable and condensed) and phytic acid. However, there was no effect of chemical treatment on the in vitro protein digestibility of the meals. The crambe or tung meals were also pretreated with exogenous microbial enzymes, non-commercial phytase (phytase and tannase action – 1400 U and 1100 U/Kg) and commercial phytase (Natuphos/BASF, 1400 U/Kg). The commercial phytase showed higher efficiency on the tung meal, reducing phytic acid concentration and non-commercial phytase acted more effectively on the tannins in crambe meal. The replacement of fish meal and meat and bone meal by in natura crambe or tung meal (integral form) or chemically or enzymatically detoxified in feeding of silver catfish (*Rhamdia quelen*) was evaluated for nine weeks, with the study of growth, digestive parameters, metabolic and body nutrient deposition. Growth response similar to the control was observed since the beginning of the study in animals fed with crambe meal in the integral or chemically detoxified forms. These animals showed minimal enzymatic and metabolical changes. The inclusion of integral tung meal or chemically detoxified caused slower growth of animals. In the diet containing the integral form of meal, these results were observed until the end of the experimental period and were summed the lower digestive enzymatic and metabolic changes and lower survival of the animals. Tung meal chemically treated caused greater growth compared to the integral form, possibly due to removal of antinutrients and toxic substances. The inclusion of vegetable meals (both forms of enzymatically treatment) reduced fish growth since the first evaluation, but the response was more pronounced in animals that received tung meal. The increase in the digestivosomático index and intestinal quotient can demonstrate a physiological adaptation of these animals to the consumption of diets with tung meal, which showed the highest fiber content. With regard to body composition, ash, crude protein and phosphorus were not changed between treatments containing vegetable meals under study, but treatments with inclusion of tung meal provided lower dry matter and fat content in animals.

Keywords: Antinutrients. Growth. Nutrients deposition. Protein sources. Nutritional improvements. Metabolism. Fish.

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1 INTRODUÇÃO

No período de 2006 a 2011, a aquicultura mundial apresentou crescimento superior a 10% e já representa 63,6 milhões de toneladas de pescado produzidos ao ano (FAO, 2012). No Brasil a aquicultura teve aumento de 31,2% na produção de 2008 a 2010, com destaque para a piscicultura que cresceu 40% neste período (BRASIL, 2010). Este aumento na produção de pescado criado deve ser acompanhado por uma expressiva ampliação na produção de rações, a fim de melhorar as técnicas de criação e aprimorar o desempenho dos animais.

A principal fonte protéica utilizada na nutrição aquícola é a farinha de peixe devido sua elevada qualidade quanto ao perfil aminoacídico e de ácidos graxos essenciais (DREW et al., 2007). Em 2006 somente a atividade Aquícola mundial (produção de peixes e crustáceos) utilizou 68,2% da produção global de farinha de peixe (TACON; METIAN, 2006). Porém o aumento no custo deste ingrediente pode levar a reflexões quanto ao uso quase exclusivo desta fonte protéica na nutrição aquícola. Neste sentido, muitas pesquisas direcionam estudos para a identificação de novas fontes de proteína, que possam substituir a farinha de peixe e reduzir a dependência de ingredientes de origem animal na alimentação aquícola (DREW et al., 2007; GATLIN III et al., 2007; HARDY, 2010).

No Brasil, este direcionamento é consonante com o crescimento da cadeia de biocombustíveis, que tem gerado grande quantidade de coprodutos (farelos e cascas) a partir da exploração de diversas culturas como soja, algodão, girassol, canola (BIODIESELBR, 2010) e outras culturas como o crambe (*Crambe abyssinica*) e o tungue (*Aleurites fordii*), que em razão de seu elevado teor de óleo e características peculiares ao cultivo, não competem com as espécies já consolidadas e diversificam a disponibilidade de matérias-primas neste setor.

O crambe, oleaginosa pertencente à família Brassicaceae, tem se mostrado uma excelente alternativa para plantio de segunda safra no Brasil, atingindo produtividades em torno de 1000 a 1500 Kg/ha. No País, o principal estado produtor é o Mato Grosso do Sul, onde são cultivados cerca de 1800 ha desta oleaginosa. Esta cultura destaca-se principalmente pela adaptabilidade, rusticidade e precocidade e representa uma excelente alternativa para diversificar a matriz de óleos vegetais, já que o óleo extraído tem sido aplicado na indústria química e recentemente na produção de biodiesel (WANG et al., 2000; PITOL et al., 2010). O

grão inteiro apresenta entre 26 e 40% de óleo. A extração mecânica pode retirar em média 70% do óleo total, resultando na torta de crambe, um co-produto com teor de gordura superior a 12%. Já o farelo de crambe permanece com um teor de óleo em torno de 2-4% e o nível protéico varia de 35-40% (PITOL et al., 2010).

O tungue, planta perene da família Euphorbiaceae, tem vida produtiva superior a 30 anos, apresenta fácil cultivo e pode ser implantada para recuperação de áreas degradadas e onde a agricultura mecanizada torna-se difícil. Essa cultura é de clima temperado, e por esse motivo apresenta alto potencial de cultivo no Rio Grande do Sul, sendo o único Estado brasileiro a cultivar essa frutífera, embora ainda com pequena área plantada (163 ha) (IBGE, 2008). Apesar de sua produtividade média girar em torno de 2.472 kg/ha, alguns estudos têm demonstrado que com manejo cultural adequado (correção de acidez/adubação) essa pode alcançar 10.000kg/ha de fruto, sendo que 25% desse valor será convertido em óleo, muito utilizado na indústria química para produção de tintas e resinas e atualmente empregado para a produção de biodiesel (CARTER et al., 1998). Em relação ao farelo de tungue, resultante da extração do óleo, não há relatos de sua aplicação na alimentação animal devido à escassez de estudos a respeito de suas potencialidades e restrições nutricionais.

Após serem conhecidas suas características nutricionais e antinutricionais, farelos derivados de oleaginosas ou de cereais vêm sendo incluídos em dietas para peixes como fontes alternativas de proteína ou energia (HERNÁNDEZ et al., 2007; OVERLAND et al., 2009; CHENG et al., 2010). Contudo, têm sido apontadas limitações ao emprego destas fontes vegetais na formulação de dietas, devido ao seu menor conteúdo de proteína, maior teor de fibras e a presença de uma variedade de substâncias antinutricionais que podem reduzir a palatabilidade do alimento, a eficiência de utilização dos nutrientes, e em casos mais severos, provocar disfunções no aparelho digestório (FRANCIS et al., 2001; KROGDAHL et al., 2010). Uma alternativa para reduzir estas restrições é a utilização destes coprodutos após a extração ou inativação dos principais antinutrientes.

Alguns compostos antinutricionais como inibidores de protease, antivitaminas e lectinas são sensíveis às altas temperaturas, por isso podem ser extraídos ou inativados através de técnicas comuns de processamento que utilizam calor como secagem e tostagem (GATLIN III et al., 2007; KROGDAHL et al., 2010). No entanto, o ácido fítico, os compostos fenólicos (ácidos fenólicos, taninos hidrolisáveis e condensados, ligninas, lignanas), as saponinas e os polissacarídeos não amiláceos são estáveis ao calor e necessitam outra forma de detoxificação. Solventes como metanol, etanol, acetona e água, empregados de forma isolada ou combinada, são capazes de extrair compostos fenólicos e saponinas (NACZK; SHAHIDI,

2004; HASSAS-ROUDSARI et al., 2009; KROGDAHL et al., 2010). Outra alternativa é a utilização exógena de enzimas microbianas, capazes de atuar especificamente sobre antinutrientes de estrutura complexa liberando nutrientes absorvíveis (STECH et al., 2009; KUMAR et al., 2012). A aplicação da fitase microbiana no pré-tratamento de ingredientes ou diretamente sobre as rações tem se mostrado eficiente para disponibilizar fósforo inorgânico preso a molécula de ácido fítico e aperfeiçoar o desempenho dos peixes (PORTZ; LIEBERT 2004; BISWAS et al., 2007).

Neste sentido, as fontes protéicas de origem vegetal são alternativas que vem sendo estudadas e podem ser incluídas em dietas para peixes, de modo a garantir o crescimento e sustentabilidade da piscicultura nacional. Aliado a este fato, somam-se as ações de fomento à criação de espécies nativas, que tem contribuído de forma significativa ao crescimento da aquicultura no Brasil. Na região Sul, o jundiá (*Rhamdia quelen*) é apontado como uma espécie potencial levando em consideração sua aceitação comercial, características desejáveis para produção e crescimento satisfatório inclusive no inverno (RODRIGUES et al., 2012). Estudos desenvolvidos para avaliar a aceitabilidade e o aproveitamento de fontes de proteína, convencionais ou alternativas pelo jundiá, demonstraram que a combinação de ingredientes de origem animal (farinha de peixe, farinha de carne e ossos) e vegetal (farelo de soja, de canola, de girassol) proporcionou melhor crescimento em juvenis desta espécie, em detrimento à utilização de ingredientes de origem única, até mesmo após a suplementação das fontes com aminoácidos na forma livre (LAZZARI et al., 2006; VEIVERBERG, 2011). Assim, o desenvolvimento de estudos voltados à resposta nutricional do jundiá, com a utilização de fontes de proteína alternativa como os farelos vegetais, revela-se de grande importância.

1.1 Objetivos

1.1.1 Objetivo geral

Estudar o potencial de aproveitamento de coprodutos agroindustriais como fontes protéicas na alimentação do jundiá com ênfase na aplicação de técnicas de extração ou inativação de fatores antinutricionais presentes nestes farelos a fim de melhorar o aproveitamento do alimento pelos animais.

1.1.2 Objetivos específicos

- Caracterizar quimicamente os farelos de crambe e tungue, visando determinar o seu potencial nutricional e fatores antinutricionais na alimentação de jundiás;
- Aplicar tratamentos (químicos ou enzimáticos) a fim de retirar ou inativar antinutrientes presentes nos farelos de crambe e tungue;
- Realizar estudo de digestibilidade "*in vitro*" da proteína a fim de avaliar o efeito dos tratamentos químicos sobre a disponibilidade deste nutriente em farelos de crambe e tungue;
- Avaliar o valor nutricional dos farelos de crambe e tungue, resultantes da extração dos compostos antinutricionais (tratamentos químicos ou enzimáticos), sobre o desempenho, enzimas digestivas e parâmetros bioquímicos de jundiás;
- Avaliar o valor nutricional da carne dos peixes alimentados com farelos de crambe e tungue *in natura* ou detoxificados via tratamentos químicos ou enzimáticos;

Este estudo foi desenvolvido a partir da condução de dois ensaios biológicos, posteriormente a análise nutricional e antinutricional dos farelos pré-tratados química e enzimaticamente. Os resultados estão apresentados na forma de artigos científicos, em que o artigo 1 corresponde a composição química dos farelos *in natura* e após a aplicação do tratamento químico. A inclusão destes farelos sobre a resposta nutricional de jundiás (crescimento, digestão, metabolismo e composição corporal) compõe o artigo 2. O artigo 3 contempla a análise química dos farelos pré-tratados com fitase comercial e enzima não comercial, a inclusão na dieta e os efeitos sobre o crescimento e composição corporal dos peixes. O artigo 4 complementa os resultados do artigo 3 a medida que revela dados referentes a atuação de enzimas digestivas e respostas metabólicas nos peixes.

2 ARTIGO 1

Farelos de tungue e crambe: caracterização nutricional, antinutricional e detoxificação para uso na nutrição animal

Tung and crambe meal: nutritional, antinutritional characterization and detoxification for use in animal nutrition

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RESUMO

Associada à produção de biocombustíveis de fontes vegetais, há geração de vários coprodutos passíveis de utilização na nutrição animal, os quais necessitam de um estudo minucioso para aplicabilidade ampla e segura. Neste contexto, o presente estudo foi conduzido com o objetivo de mensurar grupos químicos de interesse (nutrientes e antinutrientes) e dinâmica de degradabilidade *in* vitro de farelos de tungue e crambe *in natura* e tratados quimicamente em solução ácido-alcoólica para a minimização/retirada de antinutrientes. Os resultados demonstraram que o tratamento químico causou redução nos teores de gordura, cálcio, fósforo, taninos condensados e ácido fítico no farelo de tungue; ao passo que o teor de fibra em detergente neutro aumentou e proteína bruta, matéria mineral, compostos fenólicos, taninos totais e hidrolisáveis não foram alterados. Para o farelo de crambe o tratamento elevou o teor de fibra e matéria mineral e reduziu a concentração de proteína bruta, gordura, cálcio e fósforo assim como compostos fenólicos, taninos (totais, condensados e hidrolisáveis) e ácido fítico. Embora o tratamento químico tenha alterado o balanço da maioria dos constituintes de interesse nutricional, o seu efeito não foi observado na digestibilidade proteica *in vitro.*

Palavras-chave: Proteína vegetal, redução de antinutrientes, valor nutricional

ABSTRACT

Associated with the production of biofuls from plant sources, there is generation of several coproducts capable of use in animal nutrition, which requires careful study for broad and safe applicability. In this context, the present study was conducted with the aim of measuring the chemical groups of interest (nutrients and antinutrients) and dynamic of in vitro degradability of tung and crambe meals *in natura* and chemically treated in acid-alcohol solution to remove the antinutrients. The results showed that chemical treatment reduced levels of fat, calcium, phosphorus, condensed tannins and phytic acid in the tung meal; while the content of neutral detergent fiber increased and crude protein, ash, phenolic compounds, total and hydrolysable tannins have not changed. For the crambe meal the treatment increased the fiber and ash content and reduced the concentration of crude protein, fat, calcium and phosphorus as well as phenolic compounds, tannins (total, condensed and hydrolysable) and phitic acid. Although the chemical treatment has changed the balance of the constituents of most of nutritional interest, its effect was not observed in protein digestibility *in vitro*.

Keywords: Plant protein, antinutrientes reduction, nutritional value.

Introdução

Historicamente as principais fontes geradoras de energia para o consumo mundial são o petróleo, o carvão e o gás natural, todas previsivelmente esgotáveis e poluentes. Assim, diversos países têm desenvolvido estudos sobre fontes energéticas alternativas, onde os biocombustíveis têm se destacado como uma das formas mais eficientes de diversificar a matriz energética de forma sustentável (LOFRANO, 2008). Porém, o aumento na produção de biocombustíveis tem gerado grande volume de coprodutos e resíduos agroindustriais (tortas, farelos, etc) que requerem estudos para avaliar o seu potencial de aproveitamento, a fim de não se tornarem entraves para a sustentabilidade das cadeias produtivas (BRASIL, 2005).

As fontes proteicas de origem vegetal têm sido pesquisadas como substitutas naturais àquelas de origem animal, a fim de reduzir custos e garantir fornecimento contínuo de nutrientes a diversas categorias animais. Dessa forma, alguns coprodutos agroindustriais como o farelo de soja, de algodão, de canola e de linhaça tem sido amplamente empregados na nutrição animal (TREVIÑO et al., 2000; BARROS et al., 2002; LIM e LEE, 2009). Mas coprodutos como o farelo de tungue e de crambe, que surgiram pelo fato destas culturas serem empregadas como matérias-primas alternativas na produção de biocombustíveis, ainda são pouco conhecidos e necessitam estudos para melhor avaliar seu potencial nutricional (KAUTZ et al., 2008; PITOL et al., 2010). De modo geral, a viabilidade de utilização destes produtos (sementes, frutos, farelos) nas diferentes cadeias produtivas só poderá ser avaliada a partir do estudo de sua composição nutricional.

Logo, a utilização de um alimento, seja para nutrição humana ou animal, passa pela análise de sua composição bromatológica. Dentre os macronutrientes, o teor de proteína é revelado como um dos mais importantes para escolha de um alimento, por representar geralmente um dos itens mais onerosos da dieta. Em relação às fontes proteicas, a proteína de origem vegetal é uma fonte alternativa àquela de origem animal, sendo um aspecto importante a ampla variedade de ingredientes proteicos vegetais que podem ser utilizados na nutrição animal (MOURE et al., 2006).

No entanto, trabalhos têm apontado algumas restrições quanto ao uso de farelos vegetais, normalmente associadas a fatores antinutricionais que diminuem a digestibilidade nos animais (FRANCIS et al., 2001; ALZUETA et al., 2002; DREW et al., 2007). Desta forma, a simples constatação de disponibilidade de fontes proteicas abundantes e baratas, advindas de coprodutos agroindustriais não garante seu uso imediato e intensivo no arraçoamento animal, pois os antinutrientes intrínsecos a essas fontes (taninos, glicosinolatos, fitatos, polifenóis, mucilagens, etc) podem afetar negativamente eventos digestivos (ação antitripsínica e indisponibilização de minerais e vitaminas), aumentando a excreção de poluentes via urina e fezes, conseqüentemente, contribuindo para a poluição ambiental (FRANCIS et al., 2001; MOYANO LÓPEZ et al., 1999; SANTIGOSA et al., 2008).

Nesse contexto, a retirada/inativação dos fatores antinutricionais através de tratamentos químicos, térmicos ou enzimáticos pode melhorar o valor nutricional e a digestibilidade de diversos farelos vegetais, podendo refletir-se sobre o desempenho animal e também, na minimização de impactos ambientais (ALZUETA et al., 2002; XU e DIOSADY, 2002; GONÇALVES et al., 2005; FURUYA et al., 2008). Desta forma, o objetivo deste estudo foi avaliar o potencial de aproveitamento dos farelos de tungue e de crambe por meio da análise de nutrientes e antinutrientes e ensaio de digestibilidade proteica *in vitro* e enfatizar a aplicação de técnicas de extração de fatores antinutricionais buscando melhorar o valor nutricional destes alimentos.

Material e Métodos

O estudo foi desenvolvido no Laboratório de Piscicultura/Departamento de Zootecnia da Universidade Federal de Santa Maria e correspondeu à avaliação de características nutricionais e antinutricionais de farelos vegetais, aplicação de metodologias para extração de antinutrientes e estudo de digestibilidade proteica *in vitro*.

Preparo prévio das amostras

As amostras *in natura* de farelo de tungue e torta de crambe, oriundas respectivamente da Ind. Óleos Varela LTDA (Fagundes Varela, Rio Grande do Sul) e da Fundação MS (Maracaju, Mato Grosso do Sul), indústrias extratoras de óleo para biodiesel, foram primeiramente peneiradas para remoção de parte das cascas das sementes de crambe e endocarpo dos frutos de tungue. No farelo de

tungue, a separação física foi feita utilizando-se peneiras de 1 e 0,59 mm obtendo-se, respectivamente, os seguintes rendimentos: 47,32 e 52,68%. A fração que passou pela peneira de 0,59 mm foi aproveitada. A preparação das amostras de farelo de crambe incluiu a separação física em peneira de 1 mm (rendimento de 90%) e a retirada de gordura. Para isso, porções de torta de crambe foram lavadas três vezes com hexano (proporção peso:volume 1:3) e após, mantidas em estufa de circulação de ar forçado a 60ºC até evaporação completa do solvente. O teor de gordura foi reduzido em 67%.

Tratamentos químicos

Para a extração dos principais compostos antinutricionais presentes nos farelos aplicou-se sequencialmente solução ácida (mistura de ácido sulfúrico concentrado e água até pH 1,0) e alcoólica (etanol comercial 92,8º). Os farelos foram misturados à solução ácida (proporção peso:volume 1:10) e mantidos sob agitação (mesa agitadora a 240 mov/min) durante 1 hora. A mistura resultante foi filtrada através de duas peneiras (100 e 25 µm) de modo a separar a parte líquida da sólida. Logo após, o farelo hidratado foi misturado à solução alcoólica (proporção peso:volume 1:7,5) e novamente agitado durante 1 hora. Antes da segunda filtração, foi elevado o pH da mistura até 7,0 com hidróxido de sódio (NaOH 1N). O meio líquido obtido após as filtragens continha frações protéico dissolvidas. De modo a recuperar esta fração, optou-se por concentrá-la através de mudanças no pH da solução (metodologia do ponto isoelétrico), primeiramente elevando o pH até 9,0 com NaOH (1N) e após reduzindo-o a 4,5 com ácido clorídrico (HCl 1N) ocasionando a precipitação e separação proteica. As medidas de pH foram feitas com auxílio de pHmetro de bancada digital (Servilab, Brasil, modelo MPA 210-P). A fração sólida do farelo e a fração protéica concentrada foram secas em estufa de circulação de ar forçado a 60ºC durante 24 horas e após foram combinadas a fim de constituir o farelo detoxificado/tratado.

Análises de nutrientes

Para estudo da composição nutricional, amostras de farelos de tungue e crambe foram analisadas quanto à matéria seca (MS - 105±2°C/24 horas), cinzas (550°C/6 horas) e proteína bruta (PB - determinação de nitrogênio pelo método micro Kjeldahl - N x 6,25) conforme as metodologias descritas pela AOAC (1995). A gordura residual presente nos farelos foi extraída e quantificada pelo método de extração a frio (BLIGH e DYER, 1959). O teor de fibra em detergente neutro (FDN) também foi quantificado (VAN SOEST et al.,1991). A análise de cálcio e fósforo incluiu a etapa de digestão dos minerais em solução (ácido sulfúrico e mistura catalítica de sulfato de cobre e sulfato de potássio a 375ºC por 4,5 hs) e a quantificação através de espectrofotometria de absorção atômica para o cálcio e na região do visível para o fósforo (reação colorimétrica do molibdato de amônio com o fósforo em presença de agente redutor, utilizando-se K_2HPO_4 como padrão (BAGINSKI et al., 1982).

Hidratação

Para o teste de capacidade de ligação à água dos farelos, as amostras foram hidratadas (proporção peso:volume 1:20) e mantidas em repouso durante 24 horas. A seguir as amostras foram centrifugadas (1300xg, durante 20 min), retirada a água na fração sobrenadante e a capacidade de hidratação calculada pela diferença de peso entre a amostra úmida e seca (MCCONNELL et al., 1974).

Análise de antinutrientes

A extração e determinação dos compostos fenólicos totais, bem como taninos condensados e totais foram realizadas de acordo com MAKKAR (2000). Previamente a extração, as amostras foram lavadas duas vezes com solução de éter etílico (acidificado a 1% com ácido acético) e centrifugadas (3000xg durante 10 min). Na fase de extração adicionou-se 10 mL de acetona 70% às amostras (por duas vezes), seguindo posterior incubação em banho de ultra som durante 20 min e centrifugação (3000xg, durante 10 min) para coleta acumulativa da fração sobrenadante. As determinações foram realizadas por ensaio colorimétrico. Para a análise de compostos fenólicos totais foi adicionado diretamente a uma alíquota do extrato obtido (50 uL) o reagente Folin-Ciocalteau (1N) e solução aquosa de carbonato de sódio 20%. Na determinação de taninos totais, antes da quantificação colorimétrica, esta fração foi extraída após adição do reagente PVPP (polivinilpolipirrolidona – 100 mg para 1 mL de solução) e centrifugação (3000xg, durante 10 min) coletando-se o sobrenadante. A absorvância das amostras foi monitorada em 740 nm, utilizando-se ácido tânico na concentração de 100 mg/L como padrão. Para a quantificação de taninos condensados adicionou-se ao extrato obtido inicialmente (extração com acetona 70%) 3 mL da mistura de butanol-ácido clorídrico (9:1) e 0,1 mL de solução de sulfato de ferro e amônio em ácido clorídrico. As amostras foram aquecidas (banho-maria 90-95ºC) durante 40 minutos e após, monitorada a absorvância em 550 nm. A concentração de taninos hidrolisáveis foi obtida através da diferença entre o conteúdo de taninos totais menos os taninos condensados.

Para determinação de ácido fítico, inicialmente o antinutriente foi extraído das amostras em solução de ácido clorídrico 2,4% (proporção peso:volume 1:20) após 1 h de agitação a temperatura ambiente e centrifugação (1200xg, durante 10 min). Uma alíquota do sobrenadante foi diluída em água ultrapurificada (25X) e eluída em uma coluna contendo resina de troca aniônica (Bio-RAd AG1-X4). O ácido fítico retido na resina foi eluído em solução de cloreto de sódio (NaCl 0,7M), coletado e quantificado a 500 nm, com curva de calibração produzida a partir de sal de ácido fítico (SIGMA, pureza de 90%). A intensidade de coloração obtida pelo reagente de cor ou reagente Wade (cloreto férrico 0,03% e ácido sulfosalicílico 0,3% em água destilada) decresce com o aumento de ácido fítico da amostra (LATTA e ESKIN, 1980).

A presença de saponinas nos farelos de tungue e crambe foi verificada por meio do teste de formação de espuma e da análise do índice de hemólise. Para tanto, foi produzido um extrato das amostras, em que, a 0,5g de farelo foram adicionados 20 mL de água destilada e levado a fervura

durante 2 min. O sobrenadante foi filtrado em balão volumétrico e coletado de forma acumulativa, pois este procedimento foi repetido mais duas vezes. Uma fração do extrato (5 mL) foi transferido para dois tubos de ensaio, ficando o primeiro tubo com extrato puro e o segundo diluído duas vezes com água destilada. Para cada tubo disposto na sequência a diluição foi sendo duplicada. A prova de formação de espuma consistiu na medida (cm) do anel de espuma persistente nos tubos em repouso, 15 min após terem sido agitados vigorosamente durante 15 segundos. O resultado é considerado positivo quando o anel de espuma é persistente e tenha espessura igual ou maior do que 1 cm e negativo abaixo deste valor. Para verificar o índice de hemólise, NaCl foi acrescido ao extrato saponínico para tornar esta solução isotônica. Logo após, colocou-se 2 mL desta solução em dois tubos de ensaio, novamente o primeiro tubo ficou com extrato puro e no segundo a diluição foi de 2 vezes. Nos tubos dispostos em sequência (total de 10) a diluição foi duplicada gradualmente. Em todos os tubos de ensaio foi acrescentado 2 mL de uma suspensão de hemáceas (coletada de cordeiros) a 2% em solução fisiológica. Após completa homogeneização, os tubos permanecerem em repouso durante 1 hora para posterior análise da maior diluição capaz de provocar hemólise total.

O teste para atividade de urease foi realizado conforme metodologia proposta por CASKEY e KNAPP (1944) analisando alterações de pH após a liberação de nitrogênio da molécula de uréia em presença da enzima. Em cada béquer adicionou-se a 0,2 g de amostra dos farelos, 10 mL de solução tampão fosfato de potássio (0,05 N pH 7,0), duas gotas de fenolftaleína 0,1% e 0,3 g de uréia. Colocou-se a mistura em banho-maria a 30ºC durante 30 min, agitando as amostras a cada 5 min. A seguir verificou-se o pH dos sobrenadantes (pHmetro digital). Se a enzima estiver presente em quantidade suficiente, haverá aumento do pH da solução e a coloração ficará vermelha, caso contrário, se o pH permanecer inalterado a atividade da enzima é muito baixa.

Digestibilidade protéica in vitro

O ensaio de digestibilidade *in vitro* da proteína dos farelos de tungue e crambe nas formas *in natura* e detoxificada foi conduzido através de metodologia desenvolvida por MAURON (1973) e modificada por DIAS et al. (2010). O método é baseado na digestão da amostra pelas enzimas pepsina (1:10.000, Nuclear) e pancreatina (Sigma) e a digestibilidade resulta da relação entre o nitrogênio total da amostra, nitrogênio digerido, nitrogênio produzido pela autodigestão das enzimas e o nitrogênio solúvel originalmente no farelo. Desta forma, adiciona-se a 100 mg de amostra desengordurada, 10 mL de HCl 0,1N e 4 mg de pepsina (diluição em HCl 0,1N proporção peso:volume 10:1). As amostras são mantidas em banho maria a 37º sob agitação durante 1 hora. Após, o pH da solução é corrigido a 7,0 com NaOH 0,4N e adiciona-se a cada amostra 20 mg de pancreatina (diluição em tampão fosfato de sódio 0,1M pH 8,5 na proporção peso:volume 10:1). Novamente as amostras são mantidas a 37º e agora agitadas por 3 horas. Decorrida esta etapa, a reação é paralizada com ácido tricloroacético (concentração final de 5% de ácido em cada tubo). Primeiramente retira-se uma alíquota (2 mL) para determinação do nitrogênio total da amostra. A seguir centrifuga-se a fração restante (10000 rpm/10

min) para análise do nitrogênio digerido. Para cada amostra teste segue um branco, que não recebe as soluções enzimáticas, cujo objetivo é verificar o nitrogênio solúvel da amostra. Para descontar o nitrogênio vindo da autodigestão enzimática, segue um tubo que só recebe as soluções de pepsina e pancreatina. Determina-se o teor de nitrogênio em cada fração pelo método micro Kjeldahl. Para comparação da digestibilidade das amostras foi adotado como padrão a caseína (Synth, pureza de 90%).

As análises de composição nutricional, antinutricional, hidratação e digestibilidade da proteína *in vitro* foram realizadas nos farelos *in natura* e nos farelos detoxificados, obtidos após a extração de compostos antinutricionais.

Análise estatística

Os dados de análise nutricional (n=4) e análise antinutricional (n=3) são expressos como média ± desvio padrão (DP), sendo submetidos à ANOVA e teste F ($p<0,05$) para comparação das médias entre os farelos nas formas *in natura* e detoxificada. O software estatístico utilizado para estes procedimentos foi o SPSS 8.0.

Resultados e Discussão

Em relação à composição nutricional (Tabela 1), a forma *in natura* do farelo de tungue apresentou, em média, 27% de proteína bruta, 3,3% de óleo e 52% de FDN. O alto teor de fibra resulta da presença do endocarpo fibroso (fração mais interna do fruto que protege a amêndoa) no farelo, o qual é prensado e processado junto com a amêndoa na retirada do óleo, e persiste mesmo após a tentativa de separação física com vista à concentração de nutrientes. Ainda quanto aos nutrientes analisados, foi observado que a forma tratada do farelo de tungue apresentou valores semelhantes à forma *in natura* para a concentração de cinzas e proteína bruta, ocorrendo perda de gordura (-66%), cálcio (-32%) e fósforo (-56%) e aumento de FDN (11%) após o tratamento.

A avaliação nutricional do farelo de crambe *in natura* (Tabela 1) mostrou que para alguns nutrientes (proteína bruta, fibra, matéria mineral e fósforo) os valores médios foram próximos aos encontrados por outros autores, porém, o teor de cálcio foi 40-50% menor (ANDERSON et al., 1993; LEDOUX et al., 1999). O farelo de crambe apresentou níveis semelhantes ao farelo de canola em relação à proteína bruta, FDN, cinzas e cálcio (35-38%; 21-34%; 5,8-6,8% e 0,5-0,6%, respectivamente) visto que estas oleaginosas pertencem a mesma família – Brassicaceae (BELL e CAN, 1993; ROSTAGNO et al., 2005; PENA et al., 2010). De modo geral, a composição nutricional do farelo de crambe também é semelhante àquela do farelo de girassol (31 a 41% de proteína; 28 a 45% de FDN; 7 a 9% de cinzas e extrato etéreo de 0,7 a 3,6%) (VILLAMIDE e SAN JUAN, 1998). Já em relação ao farelo de soja (45 a 48% de proteína e 13 a 15% de FDN) o farelo de crambe revela, em média, teor 15% menor de proteína e concentração de FDN duas vezes maior (ROSTAGNO et al.,

2005). Este elevado teor de fibra no farelo de crambe resulta da presença de grande quantidade de cascas, que são prensadas junto com a semente no processo industrial de extração do óleo, o que contribui para reduzir a concentração de alguns macronutrientes podendo prejudicar a digestão e o seu aproveitamento (PITOL et al., 2010).

A aplicação do tratamento ácido-alcoólico no farelo de crambe acarretou redução de algumas frações como proteína (-21%), gordura (-20%), cálcio (-47%) e fósforo (-37%) e aumento de cinzas (50%) e FDN (15%). A redução do conteúdo proteico no farelo de crambe provavelmente pode ser explicado pela interação de proteínas hidrofílicas presente nos alimentos com a água, resultante da propriedade de hidratação ou retenção de água (MOURE et al., 2006) (Figura 1). Provavelmente este fato contribuiu para a maior perda proteica no farelo de crambe, mesmo após o processo de recuperação da fração proteica solúvel feito através de mudanças no pH, considerando o ponto isoelétrico da proteína. Tanto para o farelo de crambe quanto para o farelo de tungue, parte da gordura possivelmente foi carreada após a lavagem dos farelos com etanol e o teor de fósforo e cálcio reduzidos após a extração do ácido fítico. O aumento da fração de cinzas no farelo de crambe tratado pode estar associado à formação de complexos minerais resultantes do tratamento do ingrediente com ácido sulfúrico.

Além de medidas de interesse nutricional, as fontes protéicas de origem vegetal também devem ser adequadamente avaliadas quantos aos antinutrientes, que na maioria das vezes são os principais fatores que limitam seu amplo uso na nutrição animal. Assim, o conhecimento dos teores de antinutrientes e a aplicação de algumas técnicas de processamento podem melhorar o valor nutricional dos farelos vegetais antes de sua inclusão nas dietas (MOURE et al., 2006; DREW et al., 2007).

Os compostos fenólicos podem estar na forma de fenóis simples, ácidos fenólicos, flavonóides, taninos condensados e hidrolisáveis, lignanas e ligninas. Em relação aos antinutrientes (Tabela 2) nas formas *in natura* do farelo de tungue e crambe o teor de composts fenólicos observado foi 1,23 e 0,97%, respectivamente. No farelo de canola as concentrações ficam em torno de 1,7 a 1,8% (XU e DIOSADY, 1997; HASSAS-ROUDSARI et al., 2009). Análises na semente integral do girassol revelam resultados em torno de 1,4 a 1,8% e na amêndoa até 2% (ZILIC et al., 2010). No entanto, concentrações em torno de 2,9% em coproduto de girassol foram encontradas após a extração de óleo (WEIZ et al., 2009). Em sementes de soja os valores totais de compostos fenólicos são mais baixos comparados com as oleaginosas citadas acima, podendo variar de 0,27 a 0,48%, dependendo do cultivar analisado (MALENCIC et al., 2007). Os compostos fenólicos, no conjunto das várias moléculas que compõe este grupo, podem atuar como antinutrientes (adstringência, sabor amargo) assim como pigmentantes e antioxidantes (NACZK e SHAHIDI, 2006).

Em relação ao conteúdo de taninos totais e taninos condensados, as concentrações obtidas, respectivamente, para os farelos de tungue (1,092 e 0,025%) e crambe (0,665 e 0,040%) são inferiores às encontradas para o farelo de canola (de 1,5 a 3% de taninos totais e 0,667% de taninos condensados) (XU e DIOSADY, 2002; PENA et al., 2010). No entanto, variações nas concentrações

dos antinutrientes podem ocorrer em razão do solvente utilizado no procedimento de extração, bem como na metodologia empregada para a quantificação dos compostos (SHAHIDI e NACZK, 1992).

Para retirada dos fatores antinutricionais é preciso que estes sejam solúveis em meio líquido. A solubilidade dos compostos fenólicos depende do tipo de solvente utilizado, grau de polimerização dos fenóis e sua interação com outros constituintes do alimento podendo formar complexos insolúveis. Desta forma, após o tratamento químico, o farelo de tungue apresentou elevação nos teores de fenóis totais (12%), taninos totais (9,75%) e taninos hidrolisáveis (10,5%) e redução na concentração de taninos condensados (-28%) em relação à sua forma *in natura*. Para o farelo de crambe obteve-se redução nos níveis de todos os antinutrientes como fenóis totais (-41%), taninos totais (-32%), taninos condensados (-75%) e taninos hidrolisáveis (-29%) quando foi aplicado o tratamento com as soluções ácida e alcoólica. Compostos fenólicos insolúveis como a lignina, que faz parte da parede celular das estruturas vegetais, e ligada a outros componentes celulares, confere resistência à parede e proteção contra patógenos podem ser de difícil extração. Já os fenóis solúveis, encontrados dentro dos vacúolos das células vegetais provavelmente conferem menor resistência à retirada (NACZK e SHAHIDI, 2006). Desta forma, não existe um procedimento capaz de extrair todas as classes de fenóis de um ingrediente. Solventes convencionais como metanol, acetona, acetato de etila e suas combinações são utilizados, porém o emprego de metodologias de extração menos poluentes e efetivas como o uso de etanol ou sua combinação com água podem ser desejáveis (HASSAS-ROUDSARI et al., 2009; NACZK e SHAHIDI, 2004).

Neste estudo, a redução no teor de fenóis totais, taninos totais, condensados e hidrolisáveis na forma tratada do farelo de crambe e nos taninos condensados no farelo de tungue mostrou que o tratamento com etanol comercial, na proporção utilizada, foi eficiente. O etanol é um solvente de baixo custo, fácil acesso, e pode ser facilmente processado/recuperado após o uso para não resultar em contaminação ambiental. Da mesma forma, em outro estudo, a extração de fenóis no farelo de canola com a aplicação de etanol 95º foi 45% mais efetiva em relação à extração com água a altas temperaturas (80, 100 e 160ºC) (HASSAS-ROUDSARI et al., 2009), indicando que este solvente potencialmente pode ser utilizado para remoção de antinutrientes em farelos vegetais para uso na nutrição animal. O aumento no teor de fenóis totais e consequentemente taninos totais e hidrolisáveis no farelo de tungue, após o tratamento químico, possivelmente está relacionado com o aumento da concentração de fibra do farelo, já que a maior concentração destes compostos geralmente está presente nas frações fibrosas do alimento como cascas e pericarpo (SILVA e SILVA, 1999).

O ácido fítico, encontrado em maior concentração nos grãos de cereais e sementes oleaginosas foi outro antinutriente analisado neste estudo. Na forma *in natura* dos farelos estudados, as concentrações encontradas foram inferiores às descritas para a canola (3-6%) (PENA et al., 2010), soja (2,2%) e girassol (4,4%) (HAN, 1988; KUMAR et al., 2011). Este antinutriente reduz a qualidade nutricional de sementes protéicas, pois se liga fortemente a alguns minerais (cálcio e fósforo) e proteínas e os torna pouco disponíveis para serem absorvidos. A extração de ácido fítico das sementes

ou farelos vegetais tem sido pesquisada, de modo que os principais tratamentos são a aplicação de ácidos ou enzimas (HAN, 1988; KUMAR et al., 2011; HARLAND e MORRIS, 1995).

Neste estudo, a aplicação do tratamento ácido aquoso (H_2SO_4 até pH 1,0) permitiu a redução do teor de ácido fítico nos farelos de tungue (-62%) e crambe (-38%). Para o farelo de soja a forma ácida mais eficiente foi o ácido clorídrico na concentração 1N (87% extração) em relação aos demais ácidos - sulfúrico, fosfórico ou fórmico, não existindo uma relação clara entre o tipo de ácido testado e a concentração aplicada (0,01; 0,1; 1,0 N) na retirada deste antinutriente (HAN, 1988). Já, testes realizados com arroz demonstram que o ácido sulfúrico na concentração de 5% removeu a maior quantidade de ácido fítico comparado com ácido tricloroacético e ácido clorídrico nas concentrações de 10 e 3%, respectivamente (SAAD et al., 2011). Portanto, também pode ser testada a eficiência de outros ácidos para remoção de fitato nos ingredientes tungue e crambe, já que não são encontrados relatos em relação a este aspecto.

As saponinas, moléculas compostas por uma porção de carboidratos contendo glicose e outra porção aglicona (não-açúcar) chamada sapogenina, apresentam ocorrência em muitas plantas utilizadas como alimento. Possuem a capacidade de diminuir a tensão superficial da água e formar espuma sob agitação, além de provocar a lise celular/hemólise em glóbulos vermelhos (SCHENKEL et al., 1999; KROGDAHL et al., 2010). Foram obtidos resultados positivos para a formação de espuma, uma das provas qualitativas para detectar a presença de saponinas (Tabela 3). No farelo de tungue foi detectado resultado positivo quanto a presença de saponinas para a prova de formação de espuma apenas no extrato puro na forma *in natura* do farelo. Já, para o farelo de crambe, na forma *in natura* foi observado resultado positivo até a diluição de 4 vezes do extrato e na forma tratada deste farelo somente no tubo sem diluição do extrato.

Para animais pecilotérmicos como os peixes, a presença de saponinas é altamente prejudicial devido a ação destrutiva sobre o epitélio respiratório branquial e componentes das membranas biológicas (FRANCIS et al., 2001). Nos farelos de tungue e crambe foi possível observar através do teste de formação de espuma, provavelmente redução no teor de saponinas após a aplicação do tratamento químico, já que a quantidade de espuma formada diminuiu. Sendo assim, a redução deste antinutriente, bem como dos demais antinutrientes observados nos farelos detoxificados possivelmente contribuirá para a melhoria no aproveitamento destas fontes quando aplicadas na nutrição animal. A prova de hemólise, também empregada para detectar a presença de saponinas apresentou resultado negativo para ambos os farelos, tanto na forma *in natura* quanto na forma tratada, já que não foi observado o rompimento de hemáceas em nenhum caso. Possivelmente a concentração de saponinas presente nestes farelos é baixa e insuficiente para provocar hemólise. Para o teste de atividade de urease também foram observados resultados negativos para os farelos de tungue e crambe em ambas as formas, podendo indicar que a enzima foi inativada nestes ingredientes no processo aplicado para extração do óleo, que também emprega calor.

A análise de digestibilidade protéica *in vitro* revelou, em média, teor de 42% para o farelo de tungue e 59% para o farelo de crambe, em suas formas *in natura* (Figura 2), e o tratamento ácido-alcoólico aumentou para 48% e 64% a digestibilidade nos respectivos farelos, possivelmente devido a retirada de alguns antinutrientes. A digestibilidade protéica da caseína (92,32%) obtida neste estudo é semelhante à digestibilidade observada por outros autores (ANTUNES et al., 1995; PIRES et al., 2006). Além disso, as proteínas de origem animal são caracterizadas por apresentar maior digestibilidade do que aquelas de origem vegetal. A digestibilidade dos farelos de tungue e crambe (forma *in natura* e reduzida em antinutrientes) são próximas àquelas encontradas para cultivares de feijão (47-56%) *in natura*. Porém, nas sementes de feijão em que foi aplicado tratamento enzimático com protease comercial (tripsina) ou protease de *Bacillus* sp a digestibilidade chegou a valores próximos de 80% (DIAS et al., 2010). Em outro estudo, amostras de soja *in natura* de diferentes cultivares apresentaram digestibilidade protéica variando de 32 a 57% (SANT'ANA et al., 2011). Em cultivares de arroz foi observada digestibilidade *in vitro* da proteína variando de 82 a 89% (ZHANG et al., 2010). A menor digestibilidade proteica observada nos farelos de crambe e tungue com relação aos vegetais supracitados pode estar associada aos antinutrientes que refletem sobre a digestão e disponibilidade da proteína alimentar. Além disso, diferenças também podem ser decorrentes da metodologia utilizada (SANT'ANA et al., 2011).

Desta maneira, pode-se inferir que a aplicação do tratamento químico reduziu frações de alguns nutrientes (principalmente proteína e minerais), ao mesmo tempo em que concentrou outras, em detrimento da retirada dos antinutrientes. Assim, a aplicação das fontes (formas *in natura* e detoxificadas) na alimentação animal e o acompanhamento do desempenho destes poderão corroborar com o indicativo de melhorias no valor nutricional dos ingredientes após o tratamento químico.

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Tabela 1. Composição nutricional dos farelos de tungue e crambe nas formas *in natura* e reduzida em antinutrientes.

Frações alimentares - % base seca	Ingredientes			
	Tungue		Crambe	
	Farelo *in natura*	Farelo tratado quimicamente	Farelo *in natura*	Farelo tratado quimicamente
Matéria mineral	7,02±0,12	7,05±0,25	8,09±0,11	16,33±0,18*
Proteína bruta	27,96±0,52	27,57±1,08	38,19±0,51	30,13±0,59*
Gordura	3,34±0,41	1,12±0,04*	5,98±0,58	4,79±0,08*
FDN	52,25±1,04	58,66±0,24*	31,04±0,43	36,34±0,34*
Cálcio	0,38±0,03	0,25±0,03*	0,57±0,09	0,29±0,02*
Fósforo	0,49±0,01	0,21±0,02*	0,75±0,04	0,47±0,01*

Dados expressos como média±desvio padrão (n=4). (*) Diferença entre a forma integral e tratada em cada farelo vegetal através do teste F ($p<0,05$).

Tabela 2. Antinutrientes analisados em farelos de tungue e crambe nas formas *in natura* e reduzida em antinutrientes.

Antinutrientes - % base seca	Ingredientes			
	Tungue		Crambe	
	Farelo *in natura*	Farelo tratado quimicamente	Farelo *in natura*	Farelo tratado quimicamente
Fenóis totais	1,23±0,09	1,41±0,08	0,98±0,08	0,58±0,03*
Taninos totais	1,09±0,08	1,21±0,10	0,66±0,09	0,45±0,04*
Taninos condensados	0,025±0,003	0,018±0,002*	0,040±0,008	0,010±0,001*
Taninos hidrolisáveis	1,07±0,08	1,19±0,10	0,62±0,09	0,44±0,04*
Ácido fítico	1,63±0,135	0,61±0,07*	1,94±0,16	1,19±0,11*

Dados expressos como média±desvio padrão (n=3). (*) Diferença entre a forma integral e tratada em cada farelo vegetal através do teste F ($p<0,05$).

Tabela 3. Teste qualitativo para determinação da presença de saponinas em farelos de tungue e crambe nas formas *in natura* e reduzida em antinutrientes.

Ingrediente	Espuma (cm/g amostra)		
	Extrato puro	Diluições	
		2 vezes	4 vezes
Tungue			
Farelo *in natura*	1,03±0,16 (+)	(-)	(-)
Farelo tratado quimicamente	(-)	(-)	(-)
Crambe			
Farelo *in natura*	4,34±0,25 (+)	3,33±0,26 (+)	1,22±0,09 (+)
Farelo tratado quimicamente	1,45±0,74 (+)	(-)	(-)

Dados expressos como média±desvio padrão (n=3). (+) = indica resultado positivo e (-) resultado negativo para a presença de saponinas através do teste de formação de espuma.

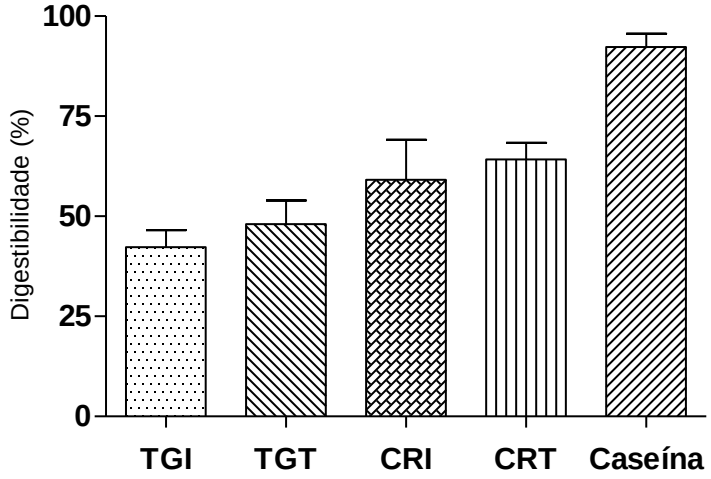

Figura 1. Capacidade de ligação à água dos farelos de tungue e crambe nas formas *in natura* e reduzida em antinutrientes. TGI= farelo de tungue *in natura*; TGT= farelo de tungue tratado quimicamente; CRI= farelo de crambe *in natura*; CRT= farelo de crambe tratado quimicamente. Dados expressos como média±desvio padrão (n=3). (*) Diferença entre a forma integral e tratada em cada farelo vegetal através do teste F ($p<0,05$).

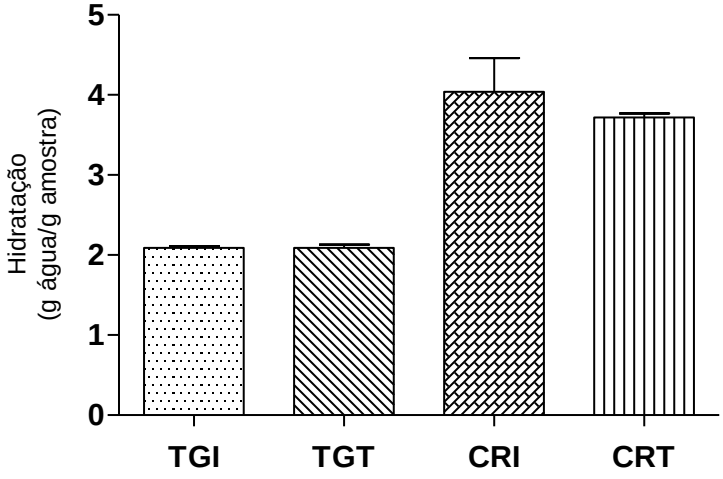

Figura 2. Digestibilidade protéica *in vitro* dos farelos de tungue e crambe nas formas *in natura* e reduzida em antinutrientes. TGI= farelo de tungue *in natura*; TGT= farelo de tungue tratado quimicamente; CRI= farelo de crambe *in natura*; CRT= farelo de crambe tratado quimicamente. Dados expressos como média±desvio padrão (n=3). (*) Diferença entre a forma integral e tratada em cada farelo vegetal através do teste F ($p<0,05$).

3 ARTIGO 2

Tung (Aleurites sp.) and Crambe (Crambe abyssinica) Meal In Nature and Detoxified Forms in the Omnivores Fish Diet

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Abstract

This study evaluated the effects of replacing fish meal and meat and bone meal by plant based meals from the biofuel production chain on growth, digestive enzymes, biochemical parameters and body nutrients deposition of silver catfish, Rhamdia quelen. The tung (Aleurites sp.) and crambe (Crambe abyssinica) meal were included in the silver catfish feed as an integral byproduct or in reduced antinutritional factors form, obtained after applying chemical treatment in integral meal. The inclusion of these ingredients was compared to the control treatment, with protein based in animal meal. The animals were fed for nine weeks and every three weeks the animals' growth was accompanied by biometrics besides analysis of digestive enzymes and parameters related to protein and energy metabolism in liver and plasma. Growth's results similar to control were observed in fish fed with both treatments containing crambe meal. These animals showed minimal changes in the digestive enzymes activity and plasmatic parameters. The diets containing tung meal caused lower fish's growth, alterations in acid protease, lipase and amylase activities and energy substrates as hepatic glycogen and glucose. However, fish fed reduced antinutrients form tung meal showed higher growth than inclusion of the integral form meal possibly due to removal of antinutrients and toxic substances.

Keywords: Agro-byproducts, animal protein, silver catfish, growth response, metabolic parameters

Introduction

The growing demand for fish meal and fish oil in the formulation of diets, especially for aquatic organisms in current years, has raised the cost of ingredients such as fish meal and

fish oil, considered sources of finite spectrum (Hardy 2010; Gatlin III et al. 2007). So research has been conducted for the purpose of finding ingredients for fish meal substitutes, such as soybean meal and soy concentrates or cereals such as wheat and corn (Santigosa et al. 2008; Olsen et al. 2007; Fournier et al. 2004). According to Gatlin III et al. (2007), the alternative ingredients use reduced 25-50% fish meal inclusion in diets for carnivorous fish species such as salmon, trout, sea bream and sea bass. For omnivorous species such as Rhamdia quelen, a South American native catfish, as the experimental model used in this study, the potential use of nutrients derived from plant sources may be even greater.

In Brazil, many studies are directed to search for information on oil seeds and fruits as oil production sources and cleaner fuels and biofuels use, featuring a worldwide demand. Some crops are exploited commercially as soybean, cotton and sunflower crops as many as peanut, rapeseed, crambe, radish, palm are being researched to determine the potential production and oil yield (MAPA 2012). After the production of biofuel, byproducts generated in this chain become available for application in several areas. Studies are needed to evaluate its effectiveness particularly in animal nutrition, with emphasis on the fish nutrition, because fish farming is an agricultural sector in extensive development in Brazil.

However, studies have pointed to some restrictions on the meals use, mainly in the integral form, due to lower vegetable protein concentration, antinutritional factors presence and non-starch polysaccharides, which reduce feed digestibility by the animals (Drew et al. 2007; Alzueta et al. 2002; Francis et al. 2001). Thus, one way to enhance the use and exploitation of these sources would be through the processing technologies application producing a product with higher protein concentration and lower antinutrients levels (fiber, tannins, phytates, saponins). However, the antinutritional factors removal/inactivation can be done by chemical treatments with use of the acids application, conventional solvents (methanol, acetone) or the use of less polluting methods of extraction and effective as the

ethanol use or their combination with water (Hassas-Roudsari et al. 2009; Naczk and Shahidi 2004). Enzymatic and heat treatment can also improve the nutritional value and digestibility of various vegetable meals, reflecting on the animal performance and also to minimize environmental impact (Kumar et al. 2011; Furuya et al. 2008; Gonçalves et al. 2005; Xu and Diosady 2002; Han 1988).

To evaluate the response of *Rhamdia quelen* juveniles to the inclusion of crambe or tung meals in integral or reduced antinutritional factors forms, we studied some growth indicators such as weight gain, feed conversion, specific growth rate. Furthermore, relevant parameters to be evaluated in feeding studies are the fish digestive enzymes profile and metabolic response in tissues as plasma and liver, which are related to nutrients digestion and absorption by the animal organism.

Material and methods

Ingredient chemical treatment

The ingredient with a lower content of antinutrients (treated ingredient) was obtained after applying the following treatment: to integral meal applied sequentially the acid solution (a concentrated sulfuric acid and water mixture until pH 1.0) and alcohol (ethanol commercial 92.8º GL). The meal was mixed with acid solution (1:10 ratio weight:volume) and kept under stirring (shaker to 240 movements per minute) for one hour. The resulting mixture was filtered through two sieves (25 and 100 µm) to separate the liquid portion from the solid. After, the hydrated meal was mixed with alcohol (1:7.5 ratio weight:volume) and again stirred for one hour. Prior to the second filtration, the pH of the mixture was raised to 7.0 values with sodium hydroxide (NaOH 1N). The liquid medium obtained after filtering contained dissolved protein fractions. To recover this fraction, we chose to concentrate it by changing the solution pH, raising the pH to 9.0 with NaOH (1N) and after reducing it to 4.5 with

chloridric acid (HCl 1N) (isoelectric point) using digital pHmeter (Servilab, Brazil, MPA 210-P). The bran solid fraction and the concentrated protein fraction were dried in air forced circulation oven at 60ºC for 24 hours.

Fish, facilities and experimental design

All procedures involving animals were conducted in accordance with standards approved by the Ethics Committee for Animal Welfare, Universidade Federal de Santa Maria, by protocol number 026/2011. In this study 255 silver catfish juveniles obtained from the Experimental Fish Culture Station in Cruz Alta, Rio Grande do Sul, Brazil were utilized. At the experiment beginning, the weight and total length were 22±0.46 g and 13±0.10 cm (average ± standard deviation). The animals were randomly distributed in 15 tanks or experimental units (five diets and three replicates for each diet) with capacity of 125 L, being distributed 17 fish per tank, in a water recirculation system with two biological filters containing crushed rock and controlling the water temperature through two electrical resistance (1.000 W). The rearing system was mounted in a closed room containing air conditioner to control the air temperature to assist in maintaining the water temperature, since the experiment was conducted in the winter.

Fish were acclimated to the experimental conditions for one week and subsequently fed with the experimental diets during nine weeks. Diet was provided around 2-3% biomass from each tank, divided into three meals (0900, 1300 and 1700 h). Tanks were cleaned by siphoning out feces and food residues daily at 0800 and 1530 h, renewing about 10% water of each tank.

Water quality

The culture system water quality was monitored daily for temperature (morning – 21.42±0.88ºC and afternoon – 22.09±0.82ºC) and weekly for other parameters. Dissolved oxygen and pH were monitored with handheld dissolved oxygen instrument (YSI®, Yellowsprings, USA – model 550A) and pHmeter (Servilab, Brazil – model MPA 210-P) and results were, respectively, 6.02±0.71 mg/L and 6.82±0.14 units. Ammonia was quantified according to Verdouw et al. (1978), mean value of 0.11±0.09 mg/L. Nitrite, alkalinity and hardness were determined according to Boyd and Tucker (1992) and the values found were, respectively, 0.05±0.01 mg/L, 20.78±5.32 mg/L $CaCO_3$ and 43.56±10.28 mg/L $CaCO_3$. All parameters remained within the range suitable for silver catfish (Baldisserotto and Silva 2004).

Feed manufacturing and formulation

To evaluate the inclusion of vegetable protein sources in the diet of silver catfish, two simultaneous experiments were conducted with the following treatments: EXP. I: ICR – integral form crambe meal inclusion; TCR – reduced antinutrients form crambe meal inclusion; EXP. II: ITG – integral form tung meal inclusion; TTG – reduced antinutrients form tung meal inclusion. A control (CON) in which the basic protein was meat and bone meal and fish meal was conducted as comparative to treatments with inclusion of vegetable meals. In diets containing meal to be tested, the inclusion percentage these corresponded to the replacement of 20% of animal protein meal by the plant protein. Diets were formulated to contain approximately 34% of crude protein and 3100 Kcal/Kg of digestible energy (Table 1). Lysine and methionine requirements were based in Montes–Girao and Fracalossi (2006) and when necessary these amino acids were supplemented. Dry ingredients were ground, mixed and added of soybean oil. The mixtures were pelleted in a meat grinder with water (addition

149 at 50% of the ingredients dry weight), dried with forced air circulation at 50ºC for 24 h.
150 Pellets were packed in plastic bags and kept at -18ºC until use.
151
152 Growth performance and feed utilization and sampling of animals
153 To monitor the growth was performed biometry every three weeks. Before the
154 biometry, fish fasted for 18 h and were anesthetized with eugenol (20µL pure extract/L water)
155 (Cunha et al. 2010). After were found final average weight (LW, g); total length (TL, cm);
156 condition factor (CF) = (100*(LW*TL^3)); daily average gain (DAG, g/day) = ((final weigth –
157 initial weigth)/period); specific growth rate (SGR, %/day) = ([ln (final weigth) – ln (initial
158 weigth)/period)]); biomass (BM, g) = (total weight animals tank); survival (S, %) = (live
159 animals in the period/initial number animals in the tank). The feed conversion rate (FCR) also
160 was calculated as follow = (g dry feed given/g live weight gain).
161 Each three-week period two fish per tank (six for treatment) were captured. The
162 animals fasted for 18 h before each collection for emptying of digestive tract. Blood was
163 quickly collected from the caudal vein using heparinized syringes. Fish were killed by spinal
164 cord excision behind operculum and eviscerated to remove the digestive tract, liver and fat,
165 after weighed to obtain data about the digestivesomatic index (DSI), hepatosomatic index
166 (HSI) and visceral fat index (VFI) as a whole weight percentage. The length of the digestive
167 tract was measured to determine the intestinal quotient (IQ), parameter related to the total
168 length of the animal. Thereafter, quickly the digestive tract and liver were placed on ice and
169 frozen at -20ºC for digestive enzymes and biochemical parameters analysis, respectively.
170 Plasma aliquots were separated after blood centrifugation at room temperature for 10 min at
171 1200x*g* for plasmatic metabolic parameters posterior determination.
172
173 Tissue homogenates and digestive enzyme assay

The entire digestive tract was separated into stomach and total intestine. Each section was dissected in a Petri dish containing saline solution (0.9% NaCl) to remove any digestive content remnants and after homogenized (1:20 proportion tissue:homogenization buffer) in Turrax tissue disintegrator/homogenizer (Marconi, Brazil – model MA 102). The homogenization buffer solution was 0.02 M Tris/0.01 M phosphate, pH 7.5 in 50% (v/v) glycerol. The homogenates were centrifuged at 1200x*g* for 10 min and the supernatants were used as the enzyme source.

The acid protease activity was measured in the stomach using non-specific substrate (casein) according to Hidalgo et al. (1999). The assay was carried using 0.2 M KCl buffer, pH 1.8 and the samples were incubated at 30ºC for 40 min. The reaction was stopped by adding 15% trichloroacetic acid and the absorbance was recorded at 280 nm. All samples were assayed in duplicate and readings corrected for blank solutions. Tyrosine was used as standard, and one unit of protease was defined as the amount of enzyme needed to catalyze the formation of 1.0 µg of tyrosine/min/mg protein.

The trypsin and chymotrypsin alkaline proteases activity as well as α-amylase and lipase were determined in the intestine. Trypsin activity was assayed with α-*p*-toluenosulphonyl-L-arginine methyl ester hydrochloride (TAME). Crude extracts were incubated for 2 min (25ºC) in 2 mL of buffer (0.2 M Tris/0.01 M $CaCl_2$), pH 8.1. Chymotrypsin activity was assayed with benzoyl tyrosine ethyl ester (BTEE). Crude extracts were incubated for 2 min in 1 mL of buffer (0.1 M Tris/0.1 M $CaCl_2$), pH 7.8. Both trypsin and chymotrypsin were assayed in duplicate and enzyme activities were recorded at 247 and 256 nm, respectively, according to the protocol described by Hummel (1959). One unit of enzyme was defined as the amount of enzyme needed to hydrolyze 1 µmol of substrate (TAME or BTEE) min/mg protein.

198 The α-amylase activity was assayed in 0.2 M phosphate-citrate buffer, pH 7.0, with
199 0.5% NaCl and a starch concentration of 2.5%. The reaction was stopped by adding $Ba(OH)_2$
200 0.3N and $ZnSO_4$ 5%. The experimental protocol used was described by Bernfeld (1955)
201 modified by Hidalgo et al. (1999). The starch hydrolysis determination was done following
202 Park and Johnson (1949). The absorbance was recorded at 660 nm. One unit of enzyme was
203 defined as 1 µmol of glucose released from starch/min/mg protein.

204 Lipase activity was measured according to Gawlicka et al. (2000). Homogenates were
205 incubated with 0.4 mM *p*-nitrophenyl myristate in 24 mM ammonium bicarbonate solution,
206 pH 7.8, containing 0.5% Triton X-100 as an emulsifying agent. Substrate hydrolysis reaction
207 was stopped by addition of 24 mM NaOH. Absorbance change was measured at 405 nm after
208 incubation for 30 min at 30ºC. One unit was defined as micromole of substrate
209 hydrolyzed/min/ mg protein. Crude extracts protein content was determined by the Bradford
210 (1976) method using bovine serum albumin as a standard.

211

212 Tissue extracts and biochemical analysis

213 Liver glycogen levels were determined according to Bidinotto et al. (1998). The tissue
214 was weighed (50 mg) and was added KOH and ethanol (1 and 3 mL, respectively) for
215 hydrolysis and precipitation of glycogen. For protein analysis, the tissues were heated at
216 100ºC with KOH and centrifuged at 1000x*g* for 10 min. Supernatant was used to estimate the
217 total protein level by method described by Bradford (1976), using bovine serum albumin as
218 standard. For soluble sugar and ammonia determination, tissue samples were homogenized by
219 adding 10% TCA using a Turrax tissue disintegrator/homogenizer and centrifuged at 1000*g*
220 for 10 min for proteins flocculation. The completely deproteinated supernatant was used for
221 soluble sugar determination according to Dubois et al. (1956) with phenol-sulfuric acid.
222 Hepatic ammonia was determined by the Verdouw et al. (1978) protocol after ammonia

reaction with phenol and hypochlorite forming a blue-coloured indophenol compound. To measure hepatic amino acids and transaminases, liver samples were mechanically disrupted by adding 1 mL phosphate buffer 20 mM, pH 7.5 and the homogenate was centrifuged at 1000x*g* for 10 min. The neutral supernatant extract was used for amino acid colorimetric determination according to Spies (1957), using ninhydrin 1.5% in isopropyl alcohol as the color reagent.

The neutral extract for amino acids quantification was used to measure the hepatic transaminases concentration, but was necessary to dilute the crude extract in homogenization buffer for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) quantification, respectively, two and ten times. The enzymes were determined by using colorimetric procedures following the protocols described in the kits (Doles Reagents and Laboratory Equipment Ltda. Goiania, Goiás, Brazil). ALT or AST concentration was expressed as UI enzyme/mg hepatic tissue. All hepatic extracts were prepared using the 1:20 proportion tissue:homogenization buffer.

For the plasmatic metabolic parameters analysis as glucose, total protein, albumin, triglycerides and cholesterol were used colorimetric kits (Doles) and the procedures followed the specific protocols for each parameter. The amino acids in plasma were also quantified by Spies (1957) method.

Body composition analysis

In each collection period, two fish per tank (six per treatment) were sampled for whole body composition analysis after the fish being ground using a food processor. Moisture concentration (60ºC overnight and after to 105±2ºC for 24 h), ash (550ºC for 6 h) and crude protein (nitrogen determination by micro Kjeldahl method - N x 6.25) were determined as

AOAC (1995). Fat was extracted and quantified following the method proposed by Bligh and Dyer (1959).

Statistical analysis

The data were tested for normality (Shapiro-Wilk test), submitted to one-way ANOVA and comparison of treatments including vegetable meals with the control, in each experiment, was performed using Dunnett's test. The F test was used to compare means between the treatments containing the meal integral form in relation to the treated form. Data were expressed as mean ± mean standard error and the differences were considered to be significant at a probability level of $P<0.05$. Statistical analysis was performed by the Statistical Analysis System SAS® version 8.2 (2001).

Results

After three, six and nine weeks of feeding diets containing tung meal, the results related to silver catfish growth performance showed that integral form tung (ITG) fed animals presented lower live weight (LW), total length (TL), biomass (BM), daily average gain (DAG) and specific growth rate (SGR), including negative results for the last two parameters, compared to animals fed diets containing reduced antinutrients form tung meal (TTG) and with the control treatment (CON) (Table 2). TTG treatment provide underperformed to control after three and six weeks where was observed less LW, TL, DAG and SGR. The feed conversion rate (FCR) was higher in TTG treatment compared to control only after three weeks feeding period. TTG treatment did not cause difference in fish growth performance in relation to the CON treatment animals at the end of the experimental period. In the treatment containing integral tung meal it was not possible to calculate the feed conversion rate due to the mortality (68.62% survival) and extremely low growth in animals that received the diet.

In the CON treatment the survival (S) was 98% and 100% in the TTG treatment. Condition factor in fish that received ITG treatment differed in all evaluation periods of control diet and after six weeks differed also from the animals of TTG treatment. Crambe meal fed animals, integral or treated, showed no differences between them in none evaluation period, for the growth's variables studied. Inclusion of integral crambe meal (ICR) and treated crambe meal (TCR) only it differed from control relative to TL after three weeks of feeding (Table 2). The survival in these treatments was 100%.

In both treatments with tung meal inclusion was observed lower hepatosomatic index (HIS) after three weeks compared to control. In this period, fish showed lower visceral fat index (VFI) in ITG treatment than in TTG and control (Table 3) and after six weeks lower intestinal quotient (IQ) was observed in fish fed with diet ITG. In the diet with treated crambe meal was found lower HSI in relation to control after three weeks (Table 3). Silver catfish fed with diets containing crambe, presented differences for digestivesomatic index (DSI), HIS and IQ after nine weeks, wherein TCR treatment caused reduction in these parameters compared to animals that received ICR diet.

Regarding the digestive enzymes activity, the ITG diet increased amylase activity until six weeks of feeding in relation to TTG and CON treatments (Table 4). Lipase activity remained lower than CON until the end of the experiment in animals that received TGI treatment. After nine weeks, ITG diet caused a reduction in acid protease compared to CON and TTG treatments. After three and nine weeks it was also observed lower lipase activity for the TTG treatment animals in relation to the CON. Furthermore, fish fed diet TTG exhibited lower amylase, trypsin and chymotrypsin activities in relation to ITG, as it was observed increased acid protease activity after nine weeks of feeding. In the treatments with crambe meal inclusion was only observed an increase in lipase activity in TCR compared to ICR and

296 CON after six weeks and reduction in acid protease in the last evaluation period in ICR
297 compared to TCR and CON treatments (Table 4).

298 In plasma of fish fed diets with ITG was observed reduced triglycerides (three weeks),
299 protein (six weeks) levels and lower glucose, amino acids, triglycerides and cholesterol (nine
300 weeks) concentration compared to the CON (Table 5). Fish fed TTG diet exhibited, after three
301 weeks, increase in amino acids levels compared to CON and higher protein and triglycerides
302 concentration in relation to ITG treatment. However, after nine weeks there was a reduction in
303 amino acids levels in relation to CON diet. In diets containing crambe meal (integral and
304 treated) after three-weeks feeding fish showed an increase in plasmatic amino acids
305 concentration in relation to the CON treatment (Table 5). ICR diet caused a reduction in
306 cholesterol levels after six weeks compared to TCR and after nine weeks in relation to CON
307 treatment. Albumin levels were not changed significantly between the diets with vegetable
308 meals in relation to control, following the experimental periods (first period: 0.55 to 0.74;
309 second period: 0.81 to 0.94; third period: 0.68 to 0.74 - mean values in g/dL).

310 In hepatic tissue, after three weeks, the animals fed diet ITG exhibited reduced
311 glycogen and glucose levels and ALT increased compared to CON (Table 6). In addition, ITG
312 treatment increased ammonia after six weeks and increase protein after nine weeks, while
313 glycogen and glucose remained reduced until the end of the feeding period. In relation to
314 CON, animals fed diet TTG showed reduction in glycogen and increase in protein and
315 ammonia after three weeks, glycogen and glucose reduced after six weeks and ALT decreased
316 after nine weeks. In the last assessment period the animals that received TTG diet had levels
317 of glycogen, glucose, protein and AST similar to control and thus differing from the results
318 presented by the animals fed ITG diet (Table 6). Fish consumed diets with treated crambe
319 meal exhibited some changes after the six-weeks period as glycogen and glucose levels
320 decrease. These changes remained after nine weeks as there was a protein and AST increase,

compared to control in this period (Table 6). Similarly to the results found in plasma, the hepatic amino acids levels were not changed significantly in the diets with protein plant in relation to control, following the experimental periods (first period: 88.8 to 98.1; second period: 131.4 to 154.7; third period: 91.4 to 113.1 - mean values in μmol/g tissue).

The proximate body composition of silver catfish fed the ITG diet revealed higher ash concentration and lower fat percentage in relation to TTG and CON. The crude protein composition was not altered in experimental diets. In crambe meal diets any body composition parameter has changed (Table 7).

Discussion

Several studies show that vegetable meals addition to a certain level reduces the fish growth performance. For example, reduction of 20% in the inclusion of fish meal in the Salmo salar diet and increase in the inclusion of corn, wheat gluten and soy concentrate, significantly reduced the body weight, weight gain and feed intake (Pratoomyot et al. 2010). Similarly, replacement of 75% of protein from fish meal by soybean meal in tilapia specimens caused no loss in growth response, but above this percentage the final body weight, feed intake, SGR and FCR were changed (Lin and Luo 2011). Canola meal inclusion in seabass, Lateolabrax japonicus diets reduced growth, feed efficiency, digestive enzymes and survival demonstrating that fish meal protein replacement by this meal in the diet should not exceed 20%.

After three and six weeks of feeding, the tung meal inclusion reduced growth compared to diet based in animal source protein. However, the late evaluation stages the animals fed with TTG showed similar growth response to animals that received the control diet. The growth performance results observed in the first evaluation period can be related to influences in the flavor and low palatability in diets containing the tung meals in both forms.

Although there are no studies with silver catfish, the animal organism may require adaptation period, physiologic or metabolic, before accepting diets containing vegetable ingredients, in the same way as with the salmon (Torstensen et al. 2008). Animals fed both crambe meal forms exhibited growth similar to CON treatment after three, six and nine weeks of feeding. However, the growth results obtained in this study (weight, SGR and FCR) were lower than those found by Lazzari et al. (2006) at 30 and 60 days when silver catfish juveniles (initial weight of 15g) were fed with diets based on the fish meal or meat and bone meal with soybean meal combination (average data showed by animals after 30 and 60 days - weight 43 and 85g; SGR 3.3 and 2.6; FCR 1.1 and 1.5).

Adding about 30% of soybean meal in combination with animal protein sources appears to have resulted in the amino acids and other nutrients best balance contributing to the high species growth. In this study, the lower growth response was also observed in control diet. This fact may be related to the protein sources proportions used in fish diet, in which the greater inclusion was meat and bone meal compared to fish meal, due to their greater availability and lower cost. However, the major problem of this protein source is uneven composition, which in some cases can result in poor digestibility (Bureau et al. 2000).

In our study, the vegetable meal inclusion in each specific treatment corresponded to 20% animal protein substitution. Furthermore, unlike other studies, the vegetable ingredient was the meal to be tested, in integral or reduced antinutritional factors forms not correspond to a plant sources mixture such as meals or concentrated ingredients, because we wanted to test the inclusion effect of each ingredient separately in the diet. However, some studies have pointed that the plant protein sources mixture is more appropriated to obtain adequate amino acid profile compared to single plant protein source incorporation (Hansen et al. 2007; Olsen et al. 2007). Thus, this fact coupled with lower fish meal inclusion in the diets may have

resulted in a lower amino acids balance, primarily essential amino acids, slowing the fish growth.

The worst performance and mortality observed in fish fed with diet containing integral tung meal is probably associated with the antinutritional factors presence and toxic compounds as phorbol esters found in Euphorbiaceae specimens. These substances can be released in the fish intestine during digestion process damaging the growth performance, as causing organs or mucous cells pathological lesions (Kumar et al. 2010). The chemical treatment applied to tung meal removed 68% of phytic acid and concentrated compounds such as phenols (increase of 12%) and totals tannins (increase of 9.75%) due to the fiber concentration increase in the meal after treatment, because these molecules are bound fractions fibrous food as shells and pericarp (data not shown). Furthermore, the phorbol esters it should have been reduced or totally removed in the diet containing treated tung meal, because was not observed mortality and the animals showed similar growth to that control diet after nine weeks of fed. Likewise, the detoxification of Jatropha curcas kernel meal (heat treatment and solvent extraction for phorbol esters extraction) in diets for common carp fingerlings allowed to replace 50% of the protein derived from fish meal resulting in performance similar to the control group (Kumar et al. 2010). Relative the diets containing crambe meal, the chemical treatment applied decreased total phenols levels (-41%), total tannins (-32%) and phytic acid (-38%) (data not shown), but few changes related to growth response (initially for TL) were observed between the animals of these treatments along the experimental period.

The major changes regarding the digestive enzymes activity were observed in the treatment ITG, reducing the acid protease and lipase action and increasing the intestinal amylase activity. Furthermore, intestinal lipase activity reduction or increase and acid protease reduction was also observed, respectively, in TTG, TCR and ICR diets at different

evaluation periods experimental. The digestive enzymes concentration and profile may vary based on the type, source and amount of nutrients in the diet (Lundstedt et al. 2004). The Jatropha curcas kernel meal use partly detoxified (shorter time for removal phorbol esters) in diets for common carp fingerlings strongly reduced the intestinal amylase, lipase and protease enzymes activity, unlike the results obtained with the full detoxified meal inclusion (higher enzyme activity) (Kumar et al. 2011). An in vitro study showed digestive enzymes inhibition by adding tannin to graded levels in intestinal homogenates of Labeo hohita. Tannin levels (6.25-200 µg) can inhibit and/or lower the protease, amylase and lipase activities. However, protease and lipase activities were reduced more compared with amylase activity (Maitra and Ray 2003). Other studies have shown low digestive enzymes activity, especially proteases with the plant protein inclusion in the diet (Lin and Luo 2011; Lazzari et al. 2010; Santigosa et al. 2008). So, the results showed in our study also can be related to action of phorbol esters and other antinutrientes as tannins and phytate found in highest concentration in the integral form of meals and partially reduced after applying chemical treatment of meals. However, the amylase activity increase in silver catfish fed to integral tung meal may have been the strategy adopted by the animal organism as an attempt to gain energy through the diet that was consumed in small amounts, possibly due to reduced palatability and high antinutrients concentration. In this case, the lipase activity decrease also contributed to the low energy precursor's absorption. So, the animals have the capacity to adapt their digestive physiology in response to changes in their nutrition requirements or dietary profile to some extent. Additionally, the enzyme activity will reflect the nutrients digested and available for absorption.

Blood is a dynamic tissue in the body, able to quickly reflect changes in physiological, nutritional and health and may be tools to indicate stress response (Kader et al. 2012). Therefore, measurement of plasma parameters related to protein and energy metabolism are

indicators of animal response to a particular food. Reduction in plasma cholesterol, found in studies including dietary plant protein sources to replace animal protein has been attributed to the hypocholesterolemic effect of vegetable ingredients, and may be caused by an increase in bile salts excretion or intestinal cholesterol absorption inhibition. In this study, results related to cholesterol may be linked to this fact, however this situation was observed only in fish fed ICR and ITG diets. Reduction in this and other plasmatic parameters as glucose, triglycerides and even protein is reflected by low feed intake combined with low nutrients digestibility and absorption, leading to a condition of energy deficit in the organism, resulting in poor growth and even animals death fed ITG diet. In plasma also was verified amino acids concentration increase in TTG, ICR and TCR treatments. This result may be related to the fact that the plant origin protein digestion have occurred more quickly in relation to animal protein. In addition, some studies show that the timing between amino acids that reach the bloodstream is impaired in diets with vegetables meals inclusion in relation to diets based on fish meal and this difference in plasma amino acids profile results in increase amino acid deamination and reduce protein synthesis (Larsen et al. 2012; Wacyk et al. 2012). This fact may have contributed to the lower growth observed in animals fed diets containing tung meal compared to the control treatment.

Few studies relate the animals growth response given diet to metabolic pattern appears in organs such as liver, through the study of parameters related to protein and energy metabolism. These parameters can indicate the best nutrients use since that the liver acts on the receipt and distribution of nutrients coming from the digestive process. As well as in plasma, in this study the most changes in liver tissue also occurred in relation to the animals that received the diets containing tung meal. Increased activity of the enzymes ALT or AST was also observed in Fournier et al. (2004) that replaced fish meal by lupin, wheat gluten and corn gluten meal. The authors reported these results to the increase in the ammonia excretion

445 in these treatments. In our case, the increase in transaminases concentrations observed in fish
446 fed ITG or TCR diets are involved with the reduction in the concentrations of the major
447 energy reserves, glycogen and glucose. In this situation, the organism adopts a metabolic
448 strategy, the gluconeogenic rout, starting to get energy to keep blood glucose levels and
449 replenish glycogen storage, mainly through amino acids, releasing ammonia and increasing
450 transaminase activity, enzymes involved in this process. Increased liver protein levels may
451 possibly be assigned the highest ALT or AST enzymes concentration. The reduction in
452 glycogen concentration may explain the lowest liver weight or HSI observed in some
453 treatments of this study. Robaina et al. (1995) through histological study also observed
454 reduction in glycogen storage in the liver as increased lipid deposition with increased soybean
455 meal levels in diets for sea bream, Sparus aurata.

456 In relation to nutrient body deposition, highest ash concentration observed in animals
457 fed a diet ITG may have resulted of the concentration of this fraction due to low animal's
458 body growth in this treatment. These animals also exhibited decrease in whole body fat
459 content and along with the results of the decrease in plasma triglyceride concentrations may
460 suggest significant lipid mobilization in this group that exhibited poor growth rate. Similar
461 results were found for yellowtail in diets including more than 20% corn gluten meal (Regost
462 et al. 1999). Moisture and fat usually vary inversely in fish flesh. This fact was observed in
463 fish receiving the diet TTG that had higher body fat (although not differing from the control
464 treatment) and 9% more dry matter in carcass. This result also was found by Elangovan and
465 Shim (2000) and Kaushik et al. (2004) with the replacement of animal meal by vegetable
466 meal. Already, body protein concentration did not differ among treatments compared to
467 control because the protein is a body component more constant. This is a significant result in
468 relation to nutrition and fish farming.

469

Conclusion

The treated tung meal inclusion allowed to reach similar growth results to the control and higher than those for the diet containing the integral form of meal, emphasizing the importance of removing antinutrients from this ingredient. For crambe meal, the results observed for animal performance were similars for both, integral or treated form. Thus, the integral crambe meal inclusion in the silver catfish diet represents a lower cost protein source, because didn't received the chemical treatment, but growth performance and body composition satisfactory compared to the control diet. In addition, the results of the growth response, digestion and metabolism, presented by animals consuming ICR and TCR diets, revealed little change showing that in the conditions of this study, the level of animal protein replacement used did not impair the performance of silver catfish, omnivorous specie tending to carnivory.

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640 Table 1. Formulation and proximate composition of the experimental diets (%).

Ingredients	Treatments[a]				
		EXP. I		EXP. II	
	CON	ICR	TCR	ITG	TTG
Fish meal	12.50	9.84	10.20	9.84	9.73
Meat and bone meal	43.50	34.80	35.50	35.00	34.80
Integral crambe meal	-	20.84	-	-	-
Treated crambe meal	-	-	25.00	-	-
Integral tung meal	-	-	-	32.45	-
Treated tung meal	-	-	-	-	32.45
Wheat flour	12.95	6.20	7.65	3.80	3.20
Corn grain	16.24	10.60	5.93	5.90	2.95
Corn starch	2.04	5.00	7.80	3.80	7.21
Soybean oil	4.00	4.15	4.89	6.18	6.62
Salt	0.50	0.50	0.50	0.50	0.50
Dicalcium phosphate	-	0.65	-	-	-
Vitamin/mineral mixture[b]	2.00	2.00	2.00	2.00	2.00
Methionine	0.54	0.503	0.488	0.50	0.53
Calcium carbonate	-	0.02	0.04	0.03	0.01
Inert	5.73	4.89	-	-	-
	Diet composition (%)				
Crude protein[c]	33.91	33.92	33.92	33.84	33.97
Fat[c]	12.45	11.86	12.50	13.38	13.00
Ash[c]	15.14	13.48	16.00	13.79	13.82
Neutral detergent fiber[c]	6.62	9.84	12.03	17.28	19.94
Carbohydrates[d]	14.16	12.39	6.83	8.26	5.39
Digestible energy[e] (Kcal/Kg)	3070	3077	3078	3048	3063
Calcium[c]	2.26	2.87	3.51	3.07	2.30
Total phosphorus[c]	1.37	1.72	2.14	1.87	1.40
Ca:P ratio	1.66	1.67	1.64	1.64	1.64
Lysine[c]	1.78	1.75	1.82	1.69	1.70
Methionine[c]	1.25	1.26	1.25	1.25	1.26

641 [a]CON=control diet, protein with the base consisting of animal meal; ICR= integral crambe meal inclusion;
642 TCR=treated crambe meal inclusion; ITG= integral tung meal inclusion; TTG=treated tung meal inclusion.
643 [b]Vitamin/mineral (kg product): Folic acid: 300mg; Choline: 100g; Inositol: 10g; Niacin: 9000mg;
644 Pantothenic acid: 3000mg; Biotin: 0.1mg; Vit.A: 1000000UI; Vit. B1: 1500mg; Vit. B2: 1500mg; Vit. B6:

150mg; Vit. B12: 2000mg; Vit. C: 15g; Vit. D3: 240000UI; Vit. E: 10000mg; Vit. K3: 400mg; Copper: 1000mg; Iron: 6000mg; Iodine: 45mg; Manganese: 8000mg; Selenium: 60mg; Zinc: 14g.

[c]Calculated based on the ingredients analysis;

[d]Neutral detergent soluble Carbohydrate calculated: 100-(crude protein+ash+fat+neutral detergent fiber +moisture).

[e]Calculated from the formula: (Protein*5,64*0,83)+(fat*9,44*0,88)+(Carbohydrate*4,11*0,65))*10 (Meyer et al. 2004).

678 Table 2. Performance of silver catfish juvenile fed a diet based in animal protein (control) and
679 diet containing crambe or tung meal in the integral or reduced antinutritional factors (treated)
680 forms.

Variables[§]	Treatments[‡]				
	EXP. I			EXP. II	
	CON	ICR	TCR	ITG	TTG
	Three weeks				
LW (g)	34.53±0.49	32.14±0.57	32.11±0.90	21.27±0.85[a]	30.31±0.68 [a]*
TL (cm)	15.27±0.06	14.72±0.13[A]	14.71±0.03[A]	13.39±0.10[a]	14.72±0.16 [a]*
CF	0.97±0.01	1.01±0.01	1.01±0.02	0.88±0.02[a]	0.95±0.03
DAG (g/day)	0.59±0.01	0.47±0.03	0.49±0.04	-0.03±0.03[a]	0.41±0.02 [a]*
SGR (%/day)	2.11±0.02	1.76±0.11	1.82±0.13	-0.16±0.16[a]	1.59±0.04 [a]*
FCR	1.16±0.03	1.50±0.12	1.35±0.10	ND	1.68±0.13[a]
BM (g)	587.0±8.3	546.4±9.6	545.8±15.3	307.6±46.0[a]	515.3±11.6*
	Six weeks				
LW (g)	41.00±1.84	37.24±1.41	36.98±2.83	21.35±0.54[a]	35.28±1.48[a]*
TL (cm)	16.34±0.22	15.66±0.19	15.79±0.24	13.53±0.14[a]	15.54±0.21[a]*
CF	0.94±0.01	0.97±0.003	0.94±0.03	0.86±0.01[a]	0.94±0.01*
DAG (g/day)	0.45±0.04	0.36±0.04	0.36±0.07	-0.014±0.01[a]	0.32±0.03[a]*
SGR (%/day)	1.46±0.10	1.23±0.10	1.24±0.19	-0.069±0.04[a]	1.15±0.06[a]*
FCR	2.36±0.22	3.05±0.39	3.01±0.51	ND	3.28±0.34
BM (g)	614.9±27.6	558.6±21.1	554.8±42.4	237.5±58.4[a]	529.2±22.2*
	Nine weeks				
LW (g)	52.16±3.49	46.91±3.00	44.23±4.07	21.95±0.86[a]	43.69±2.46*
TL (cm)	17.59±0.29	16.89±0.33	16.68±0.41	13.88±0.18[a]	16.59±0.32*
CF	0.96±0.002	0.97±0.01	0.95±0.02	0.82±0.001[a]	0.96±0.01*
DAG (g/day)	0.48±0.05	0.39±0.05	0.35±0.07	-0.0003±0.01[a]	0.35±0.03*
SGR (%/day)	1.35±0.10	1.18±0.11	1.10±0.15	-0.0035±0.06[a]	1.10±0.06*
FCR	3.03±0.63	3.52±0.61	4.00±0.72	ND	3.79±0.47
BM (g)	662.8±59.6	609.8±39.0	574.9±52.9	184.4±44.4[a]	567.9±32.0*
S (%)	98.0±1.9	100±0.0	100±0.0	68.0±16.9	100±0.0

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682 [‡]CON=control diet, protein with the base consisting of animal meal; ICR= integral crambe meal inclusion;
683 TCR=treated crambe meal inclusion; ITG= integral tung meal inclusion; TTG=treated tung meal inclusion.
684 [§]LW=live weight; TL=total length; CF= condition factor; DAG= daily average gain; SGR=specific growth rate;
685 FCR=feed conversion rate; BM=biomass; S=survival. ND= not determined. Values expressed as mean ±

686 standard error of mean (n=3). Capital letter indicate difference in I experiment (ICR or TCR treatments) and
687 lower case indicate difference in II experiment (ITG or TTG treatments) in relation to the control by Dunnett's
688 test ($p<0.05$). *Indicate difference between treated form and integral form of the meal by F test ($p<0.05$).
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Table 3. Somatic parameters of silver catfish juveniles fed a diet based in animal protein (control) and diet containing crambe or tung meal in the integral or reduced antinutritional factors (treated) forms.

Variables[§]	Treatments[‡]				
	EXP. I			EXP. II	
	CON	ICR	TCR	ITG	TTG
	Three weeks				
DSI (%)	3.14±0.21	3.27±0.21	3.19±0.16	2.83±0.21	3.10±0.30*
HSI (%)	1.48±0.04	1.32±0.05	1.26±0.07[A]	0.94±0.06[a]	1.09±0.07[a]
VFI (%)	3.25±0.45	2.51±0.29	2.91±0.50	1.54±0.42[a]	3.81±0.71*
IQ	1.10±0.06	1.26±0.07	1.22±0.07	1.11±0.06	1.11±0.11
	Six weeks				
DSI (%)	2.42 ±0.08	2.15 ±0.13	2.42 ±0.08	1.93 ±0.22	2.63 ±0.17*
HSI (%)	1.10±0.08	0.89±0.04	1.07±0.05*	1.04±0.13	1.02±0.09
VFI (%)	3.35±0.52	2.00±0.54	2.44±0.48	2.54±0.96	2.93±0.59
IQ	0.98 ±0.05	0.93 ±0.09	0.95 ±0.05	0.79 ±0.05[a]	0.96±0.05*
	Nine weeks				
DSI (%)	2.55 ±0.30	2.87 ±0.13	2.22 ±0.10*	2.53 ±0.19	2.91 ±0.23
HSI (%)	1.12±0.07	1.13±0.14	0.87±0.07*	0.98±0.06	1.18±0.13
VFI (%)	2.52±0.31	2.97±0.53	2.58±0.51	0.75±0.28	2.29±0.27
IQ	1.20 ±0.08	1.26 ±0.10	0.94 ±0.09*	0.96 ±0.03	1.10 ±0.09

[‡]CON=control diet, protein with the base consisting of animal meal; ICR= integral crambe meal inclusion; TCR=treated crambe meal inclusion; ITG= integral tung meal inclusion; TTG=treated tung meal inclusion. [§]DSI= digestivesomatic index; HSI=hepatosomatic index; VFI=visceral fat index; IQ= intestinal quotient. Values expressed as mean ± standard error of mean (n=6). Capital letter indicate difference in I experiment (ICR or TCR treatments) and lower case indicate difference in II experiment (ITG or TTG treatments) in relation to the control by Dunnett's test ($p<0.05$). *Indicate difference between treated form and integral form of the meal by F test ($p<0.05$).

Table 4. Digestive enzymes activity of silver catfish juvenile fed a diet based in animal protein (control) and diet containing crambe or tung meal in the integral or reduced antinutritional factors (treated) forms.

Variables[§]	Treatments[‡]				
		EXP. I		EXP. II	
	CON	ICR	TCR	ITG	TTG
	Three weeks				
Amylase	0.13±0.02	0.20±0.03	0.18±0.02	0.34±0.06[a]	0.15±0.02*
Lipase	10.33±0.54	8.42±1.45	10.90±0.93	6.45±1.02[a]	7.24±0.54[a]
Acid protease	122.0±9.0	130.3±11.6	114.3±6.8	116.5±8.7	120.6±10.7
Trypsin	10.17±0.71	10.38±0.79	10.62±0.65	10.32±0.74	10.83±0.84
Chymotrypsin	7.88±0.42	7.63±0.35	8.21±0.55	8.26±0.65	8.51±0.70
	Six weeks				
Amylase	0.30±0.02	0.32±0.02	0.38±0.03	0.48±0.06[a]	0.30±0.04*
Lipase	12.14±0.84	12.40±1.17	16.49±1.15[A]*	6.96±0.33[a]	12.09±1.24*
Acid protease	100.4±3.9	101.4±5.2	106.3±3.7	102.9±7.8	113.0±7.3
Trypsin	9.43±0.28	10.43±0.45	10.45±0.74	11.95±1.63	9.73±0.46
Chymotrypsin	7.39±0.24	8.54±0.30	8.12±0.60	7.78±0.21	7.67±0.41
	Nine weeks				
Amylase	0.33±0.06	0.40±0.05	0.44±0.07	0.43±0.03	0.21±0.04*
Lipase	12.57±0.69	12.68±1.22	10.33±0.88	9.52±0.56[a]	9.38±0.33[a]
Acid protease	119.9±7.6	99.6±4.2[a]	121.8±4.6*	97.1±6.4[a]	138.9±5.1*
Trypsin	8.63±0.64	7.89±0.59	7.28±0.39	9.05±0.43	7.17±0.41*
Chymotrypsin	6.54±0.41	6.54±0.35	6.74±0.56	7.25±0.49	5.48±0.19*

[‡]CON=control diet, protein with the base consisting of animal meal; ICR= integral crambe meal inclusion; TCR=treated crambe meal inclusion; ITG= integral tung meal inclusion; TTG=treated tung meal inclusion. [§]Amylase=µmol glucose hydrolysed/min/mg protein; lipase=µg substrate hydrolysed/min/mg protein; acid protease=µg tyrosine hydrolysed/min/mg protein; trypsin=µmol TAME hydrolysed/min/mg protein; chymotrypsin=mmol BTEE hydrolysed/min/mg protein. Values expressed as mean ± standard error of mean (n=6). Capital letter indicate difference in I experiment (ICR or TCR treatments) and lower case indicate difference in II experiment (ITG or TTG treatments) in relation to the control by Dunnett's test (p<0.05). *Indicate difference between treated form and integral form of the meal by F test (p<0.05).

Table 5. Plasma biochemistry of silver catfish juvenile fed a diet based in animal protein (control) and diet containing crambe or tung meal in the integral or reduced antinutritional factors (treated) forms.

Variables[§]	Treatments[‡]				
	EXP. I			EXP. II	
	CON	ICR	TCR	ITG	TTG
	Three weeks				
Glucose	42.3±3.2	48.4±3.5	49.4±0.7	36.8±3.8	42.6±2.8
Total protein	3.61±0.10	3.78±0.14	3.84±0.17	3.32±0.04	3.89±0.14*
Amino acids	3.53±0.18	4.29±0.13^{A}	4.44±0.28^{A}	3.87±0.12	4.37±0.20^{a}
Triglycerides	545.3±40.2	403.1±54.3	494.3±61.1	207.5±28.1^{a}	551.4±107.5*
Cholesterol	169.3±11.7	162.8±11.1	184.8±12.4	164.5±11.7	156.1±14.8
	Six weeks				
Glucose	40.9±2.5	43.6±2.3	44.0±3.3	33.4±3.2	36.0±1.7
Total protein	3.41±0.15	3.22±0.08	3.21±0.04	2.70±0.36^{a}	3.39±0.08*
Amino acids	2.80±0.24	2.40±0.21	2.87±0.20	2.38±0.21	2.73±0.22
Cholesterol	162.7±14.2	140.1±11.1	188.8±9.7*	144.1±7.1	165.1±6.2
	Nine weeks				
Glucose	55.9±3.7	47.8±1.8	49.7±2.3	34.1±2.6^{a}	47.5±2.1*
Total protein	3.10±0.07	3.26±0.14	3.09±0.04	2.90±0.12	3.13±0.09
Amino acids	4.38±0.04	4.24±0.16	4.26±0.20	3.89±0.12^{a}	3.93±0.18^{a}
Triglycerides	429.6±56.9	271.5±85.5	272.6±39.4	115.3±20.6^{a}	237.2±89.3
Cholesterol	172.1±8.1	137.8±8.4^{A}	154.5±8.6	125.6±8.1^{a}	158.4±17.6

[‡]CON=control diet, protein with the base consisting of animal meal; ICR= integral crambe meal inclusion; TCR=treated crambe meal inclusion; ITG= integral tung meal inclusion; TTG=treated tung meal inclusion. [§]Glucose, triglycerides, cholesterol =mg/dL; total protein=g/dL; amino acids=mmol/dL. Values expressed as mean ± standard error of mean (n=6). Capital letter indicate difference in I experiment (ICR or TCR treatments) and lower case indicate difference in II experiment (ITG or TTG treatments) in relation to the control by Dunnett test ($p<0.05$). *Indicate difference between treated form and integral form of the meal by F test ($p<0.05$).

Table 6. Hepatic metabolism of silver catfish juvenile fed a diet based in animal protein (control) and diet containing crambe or tung meal in the integral or reduced antinutritional factors (treated) forms.

Variables[§]	Treatments[‡]				
	EXP. I			EXP. II	
	CON	ICR	TCR	ITG	TTG
	Three weeks				
Glycogen	366.8±36.0	384.2±17.3	340.5±38.8	115.8±20.1[b]	275.5±18.8[a]*
Glucose	426.6±39.0	460.2±32.7	527.0±40.9	320.0±24.2[a]	424.3±20.4*
Protein	44.0±2.9	55.7±5.1	48.7±3.7	67.9±2.3[a]	59.9±2.4[a]*
Ammonia	5.17±0.20	5.39±0.26	6.13±0.45	5.96±0.40	6.10 ± 0.14[a]
ALT	20.8±2.3	18.7±1.8	17.9±1.2	29.6±2.5[a]	21.8±2.0*
AST	1122.7±73.0	1225.7±66.0	1109.4±78.5	1078.3±49.3	1272.8±117.9
	Six weeks				
Glycogen	442.5±43.5	343.6±35.9	216.3±48.6[A]	34.5±2.3[a]	205.1±41.3*[a]
Glucose	387.7±21.5	367.1±15.9	291.7±33.7[A]	100.2±7.9[a]	171.5±30.2[a]
Protein	45.9±4.4	56.5±3.2	58.8±4.1	52.8±6.	62.7±5.0
Ammonia	5.45±0.41	5.36±0.30	5.25±0.31	6.89±0.41[a]	5.27±0.25*
ALT	24.5±2.2	33.1±2.1	28.2±3.8	23.7±3.8	28.6±2.4
AST	782.2±106.4	839.2±57.2	869.2±73.6	1104.2±162.0	833.9±112.0
	Nine weeks				
Glycogen	446.0±34.2	332.8±30.8	153.9±16.2[A]*	93.1±7.9[a]	479.8±73.2*
Glucose	379.4±33.2	423.4±16.5	255.9±20.3[A]*	156.4±13.2[a]	430.9±32.8*
Protein	49.9±4.2	56.7±4.5	64.3±2.5[A]	73.7±3.7[a]	42.8±1.9*
Ammonia	5.11±0.14	5.77±0.32	5.83±0.34	4.93±0.50	5.47±0.21
ALT	35.7±4.4	29.1±5.0	32.7±3.1	25.9±2.2	24.7±1.7[a]
AST	687.9±107.2	861.7±60.7	1028.6±87.7[A]	838.8±50.8	691.8±25.5*

[‡]CON=control diet, protein with the base consisting of animal meal; ICR= integral crambe meal inclusion; TCR=treated crambe meal inclusion; ITG= integral tung meal inclusion; TTG=treated tung meal inclusion. [§]Glycogen, glucose=µmol glucose/g tissue; protein=mg/g tissue; ammonia= µmol/g tissue; ALT (alanine aminotransferase), AST (aspartate aminotransferase)=UI/mg tissue. Values expressed as mean ± standard error of mean (n=6). Capital letter indicate difference in I experiment (ICR or TCR treatments) and lower case indicate difference in II experiment (ITG or TTG treatments) in relation to the control by Dunnett test ($p<0.05$). *Indicate difference between treated form and integral form of the meal by F test ($p<0.05$).

768 Table 7. Body composition of silver catfish juvenile at the beginning and after nine weeks of
769 feeding with diet based in animal protein (control) and diet containing crambe or tung meal in
770 the integral or reduced antinutritional factors (treated) forms.

Treatments‡	Variables (%)			
	Dry matter	Ash	Crude protein	Fat
Initial	25.69 ± 0.27	3.34 ± 0.16	14.30 ± 0.26	8.20 ± 0.40
Nine weeks				
CON	26.17±0.76	3.05±0.10	15.47±0.10	9.05±0.57
ICR	27.33±0.37	3.04±0.18	15.64±0.48	8.88±0.58
TCR	26.85±0.39	3.29±0.11	15.70±0.23	8.43±0.48
ITG	26.85±0.60	3.65±0.18[a]	15.91±0.55	6.76±0.31[a]
TTG	28.57±0.87	3.14±0.13*	15.59±0.34	11.14±0.77*

771 ‡CON=control diet, protein with the base consisting of animal meal; ICR= integral crambe meal inclusion;
772 TCR=treated crambe meal inclusion; ITG= integral tung meal inclusion; TTG=treated tung meal inclusion.
773 Values expressed as mean ± standard error of mean (n=6). Capital letter indicate difference in I experiment (ICR
774 or TCR treatments) and lower case indicate difference in II experiment (ITG or TTG treatments) in relation to
775 the control by Dunnett test ($p<0.05$). *Indicate difference between treated form and integral form of the meal by
776 F test ($p<0.05$).
777

4 ARTIGO 3

Enzymatic treatment in crambe and tung meal and application in diets for *Rhamdia quelen*

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Abstract

The use of enzymes, directly on rations or in the pretreatment of ingredients, in fish diets which include vegetable meals, can improve the availability and utilization of nutrients by the animal. In this sense, the study aimed to improve the nutritional value of crambe (CR) and tung (TG) meal by the pretreatment with non-commercial enzymatic preparation phytase (phytase and tannase action, 1400 U and 1100 U/Kg respectively – PHY+TAN) or commercial phytase (Natuphos/BASF, 1400U/Kg – PHY). The effectiveness of treatments was observed from the analysis of antinutrients like phytic acid and tannins (total, hydrolysable and condensed). The commercial phytase showed higher efficiency on tung meal, but the non-commercial phytase acted more effectively on crambe meal. Later the meals were used in formulating diets for *Rhamdia quelen* (silver catfish) as partial substitute to animal meal. The assay lasted nine weeks with assessments to the growth response in every three weeks and at the final analysis of body composition and nutrient deposition. The inclusion of tung or crambe meal (both forms of pretreatment) in the diets reduced the growth of fish from the first evaluation period, but the response was more pronounced in animals receiving treatments containing tung meal. This meal has a high concentration of fiber and this may be the reason for the reduced growth. A rise in the digestive somatic index and in the intestinal quotient in animals of these treatments (TG PHY and TG PHY+TAN) can demonstrate a physiological adaptation to feed consumed. Treatments with crambe meal (both forms of pretreatment) inclusion although causing less growth compared to control diet did not alter these somatic indices. The body composition did not change any treatment containing crambe or tung meal compared to control for the ash and crude protein content in the carcass and ash and phosphorus in the bones. Animals fed with

tung meals had lower dry matter content and fat body. Additionally, treatments containing crambe or tung meal provided lower retention and deposition of protein and fat in the carcass of silver catfish.

Keywords: Plant protein, antinutrients, phytase, tannase, growth response, body composition.

1. Introduction

Growth in fish production in Brazil (2% per year) is due to largely increase in the continental aquaculture that shows annual growth rate around 17% (MPA, 2010). This growth must be supported by the increase in feed production, which is prepared with high percentages of fish meal (Drew et al., 2007). For example, in 2006 year the consumption of fish meal only for world aquaculture activity represented 3.7 million tons, consumption equivalent to 68% of the global supply of this ingredient (Hardy, 2010).

In this sense, the growth of fish farming motivates the identification of alternatives that can meet the feed industry ensuring efficiency in aquaculture production, since the lack of fish meal is already announced (Gómez-Requeni et al., 2004; Hardy, 2010). Many plant ingredients researched for use in fish nutrition are meals and pies, coproducts generated in large quantities from the agroindustrial processing and biofuels chain. These sources have higher availability and studies of their applicability are needed in order to contribute to sustainable development of several production chains. However, in relation to the fish meal, the vegetable meals have some disadvantages as: low protein concentration, carbohydrate complex structure

that reduces the digestibility of feed and energy reserve in the form of starch, which has lower utilization by the fish. Still, the presence of antinutritional factors such as protease inhibitors, lectins, saponins, non-starch polysaccharides can contribute to reduce the palatability of the feed and nutrient utilization efficiency (Drew et al., 2007; Gatlin III et al., 2007; Krogdahl et al., 2010). Moreover, other molecules deserve special attention because of its harmful action on the several nutritional fractions from feed. Phytic acid is an important antinutrient found in seeds and cereal grains, formed during maturation of these as a way of storing energy, phosphorus and cations (Ravindran et al., 1994). Along this molecule, 50 to 80% of the total phosphorus becomes unavailable to monogastric animals such as fish, as well as proteins, amino acids, starch, lipids and minerals may be complex in this molecule (Francis et al., 2001; Kumar et al., 2012). In the same way, tannins have great ability to form complexes with proteins, starch, cellulose and minerals. This feature reflects the inhibitory action of tannins on proteases, amylases and lipases digestive and helps to reduce the digestibility of nutrients in legumes and cereals, which are rich in tannins (Silva and Silva, 1999; Francis et al., 2001; Battestin et al., 2004; Belmares et al., 2004).

To minimize the undesirable effects of these antinutrients the application of exogenous enzymes proved to be efficient. Furthermore, this form of treatment can prevent losses of minerals and other nutrients which occur for example, after addition of extractant solutions (Ai et al., 2007; Kumar et al., 2012). The enzymes can be used in the treatment of vegetable ingredient (prior to pelletizing) or sprayed onto the pellets (Cao et al., 2007). In this sense, phytases produced by fungi, bacteria and yeasts are the main sources of microbial phytase responsible for the removal of orthophosphate groups of inositol and providing free inorganic phosphorus available for absorption of animals

(Cao et al., 2007; Kumar et al., 2012). Additionally, the tannin acyl hydrolase or tannase hydrolyze esters and side ports of hydrolysable tannins generating glucose and gallic acid, but does not hydrolyze condensed tannins (Rodríguez-Durán et al., 2011). The action of tannase in vegetable meals can reduce the formation of tannin-protein complex once the tannins are degraded into simple compounds, unable to produce this effect (Battestin et al., 2004).

In many studies on fish nutrition, the use of these and other enzymes in diets with inclusion of vegetable meals enable improvement in growth, feed efficiency and body composition of nutrients (Storebakken et al., 1998; Sajjadi and Carter, 2004; Liebert and Portz, 2005). Silver catfish (*Rhamdia quelen*) is a native catfish found in southern Brazil, that has their cultivation stimulated mainly by their ability to take advantage of vegetable protein in combination with animal protein sources (Lazzari et al., 2007). The use of enzymes for this specie so as to improve the utilization of the nutrients has been little exploited. The aim of the study was to improve the nutritional value of crambe and tung meals by exogenous treatment with phytases (commercial and non-commercial) enzymes and then incorporate them into the diet in order to partially replace animal meal. In this sense, growth parameters, use and disposal of body nutrients were evaluated.

2. Material and methods

2.1 Meals enzymatic treatment

The enzymatic treatment of crambe and tung meals was performed according to the modified method proposed by Storebakken et al. (1998). The meals were pretreated with commercial phytase produced by *Aspergillus niger* (product BASF Natuphos®

10000 U/g) or non-commercial enzyme with phytase and tannase activity produced by *Paecilomyces variotti* (460 U phytase and 360 U tannase/mg) developed in the Laboratory of Food Biochemistry of the Faculty of Food Engineering /UNICAMP (Madeira et al., 2011). The concentrations used were 1400 U phytase (concentration very close to the level used by Rocha et al. (2007) in study with silver catfish fingerlings) and 1100 U tannase/Kg meal. The meals were added to warm water (40ºC) (1:3 weight:volume ratio), and enzyme (previously dissolved in water at the same temperature). The resulting pH of the mixture was 6.06 and 5.77 for crambe and tung meal, respectively. The incubation was continued for 2 hours in water bath (40°C) with constant stirring of the mixture through manual electric mixer for 5 min every 10 min interval. After completion of the incubation, the mixture was taken to an oven at 55ºC until achieve less than 12% moisture. After cooled, the meals were ground, chemically analyzed and later used to prepare rations.

2.2 Chemical analysis

The meals subjected to enzymatic treatment and other ingredients used in the production of the diets were analyzed for dry matter (105±2°C for 24 h), ash (550°C for 6 h) and crude protein (nitrogen determination by micro Kjeldahl method - N x 6.25) according to the AOAC (1995). Fat was extracted and quantified following the method proposed by Bligh and Dyer (1959) and content of neutral detergent fiber was determined as Van Soest et al. (1991). Analysis of the content of calcium and phosphorus included the digestion of minerals (sulfuric acid and catalytic mixture of copper and potassium sulfate at 375ºC for 4.5 h) and quantification by atomic absorption spectrophotometry for calcium and in the visible region for phosphorus

(colorimetric reaction of ammonium molybdate with phosphorus in the presence of reducing agent) using as standard K_2PO_4 (Baginski et al., 1982).

The extraction and determination of total phenolic compounds and tannins (total and condensed) were performed according to Makkar (2000). For extraction was added acetone 70% in the samples (10 mL for twice) followed incubation in ultrasonic bath for 20 min and centrifugation (3000x*g* for 10 min) for accumulative collection of the supernatant fraction. The determinations were performed by colorimetric assay. For the analysis of phenolic compounds was added directly to an aliquot of the extract obtained (50 µL) the Folin-Ciocalteau reagent (1N) and aqueous sodium carbonate solution (20%). In the determining total tannin, before of the colorimetric quantification, this fraction was extracted with PVPP (polyvinylpolypyrrolidone – 100 mg/mL) reagent and centrifugation (3000x*g* for 10 min) collecting the supernatant. The absorbance was monitored at 740 nm, using tannic acid (100 mg/L) as standard. For quantification of condensed tannins it was initially added to the extract obtained 3 mL of butanol-hydrochloric acid (9:1) and ammonium iron sulfate in hydrochloric acid (0.1 mL). The samples were heated (bath at 90-95ºC) for 40 min and thereafter, the absorbance was monitored at 550 nm. The concentration of hydrolysable tannins was obtained by difference between the total tannin content less condensed tannins.

For determination of phytic acid initially antinutrient was extracted from the samples in a hydrochloric acid solution 2.4% (1:20 weight:volume ratio) after 1 hour of stirring at room temperature and centrifugation (1200x*g* for 10 min). An aliquot of the supernatant was diluted in ultrapurified water (25 times) and eluted in a column containing anion exchange resin (Bio-Rad AG1-X4). Phytic acid retained on the resin was eluted in sodium chloride solution (0.7 M), collected and measured colorimetrically

by spectrophotometry at 500 nm with a calibration curve produced from phytic acid salt (Sigma, purity 90%). The staining intensity obtained by the color reagent (Wade – 0.03% ferric chloride and 0.3% sulfosalicylic acid in distilled water) decreases with increasing phytic acid in the sample (Latta and Eskin, 1980).

2.3 Feed manufacturing and formulation

The vegetable meals pretreated enzymatically were incorporated into diets for silver catfish to replace 20% of animal protein meal by the plant protein. Thus, two simultaneous experiments were conducted with the following treatments: EXP. I: CR PHY – crambe meal inclusion treated with phytase; CR PHY+TAN – crambe meal inclusion treated with phytase and tannase; EXP. II: TG PHY – tung meal inclusion treated with phytase; TG PHY+TAN – tung meal inclusion treated with phytase and tannase. A control (CON) in which the basic protein was meat and bone meal and fish meal was conducted as comparative to treatments with inclusion of vegetable meals. Diets were formulated to contain approximately 36% of crude protein and 3200 Kcal/Kg of digestible energy (Table 2). Lysine and methionine requirements were based in Montes–Girao and Fracalossi (2006) and when necessary these amino acids were supplemented. Dry ingredients were ground, mixed and added of soybean oil. The mixtures were pelleted in a meat grinder with water (addition at 50% of the ingredients dry weight), dried with forced air circulation at 50ºC for 24 h. Pellets were packed in plastic bags and kept at -18ºC until use.

2.4 Fish, facilities and experimental design

All procedures involving animals were conducted in accordance with standards approved by the Ethics Committee for Animal Welfare, Federal University of Santa Maria, by protocol number 026/2011. In this study were utilized 500 silver catfish fingerlings obtained from the Experimental Fish Culture Station of the University of Passo Fundo, Rio Grande do Sul, Brazil. At the experiment beginning, the average weight and the total length were, respectively, 7.6±0.1 g and 9.5±1.0 cm. The animals were randomly distributed in 20 experimental units (five diets test and four replicates for each diet) with capacity of 125 L, being distributed 25 fish per experimental unit, in a water recirculation system with two biological filters containing crushed rock and controlling the water temperature through the two electrical resistance (1000 W). The rearing system was mounted in a closed room containing air conditioner to control the air temperature to assist in maintaining the water temperature, since the experiment was conducted in the winter.

Fish were acclimated to the experimental conditions for one week and subsequently fed with the experimental diets during nine weeks. Diet was provided around 4-5% biomass from each experimental unit, divided into three meals (08:00, 13:00 and 17:00 h). Experimental units were cleaned by siphoning out feces and food residues daily at 09:00 and 15:30 h, renewing about 10% water of each tank.

2.5 Water quality

The culture system water quality was monitored daily for temperature (morning – 25.5±0.7ºC and afternoon – 26.0±1.0ºC) and weekly for other parameters. Dissolved oxygen and pH were monitored with handheld dissolved oxygen instrument (YSI®, Yellowsprings, USA – model 550A) and pHmeter (Servilab, Brazil – model MPA 210-

P) and results were, respectively, 6.52±0.47 mg/L and 7.08±0.25 units. Ammonia was quantified according to Verdouw et al. (1978), mean value of 0.23±0.06 mg/L. Nitrite, alkalinity and hardness were determined according to Boyd and Tucker (1992) and the values found were, respectively, 0.10±0.05 mg/L, 27.44±4.70 mg/L $CaCO_3$ and 39.00±7.01 mg/L $CaCO_3$. All parameters remained within the range suitable for silver catfish (Baldisserotto and Silva, 2004).

2.6 Growth performance and feed utilization and somatic parameters

To monitor the growth and food intake estimation by animals, biometry was performed each three weeks. Before the biometry, fish fasted for 18 h and anesthetized with eugenol (20 µL pure extract/L water) (Cunha et al., 2010). After, it was determined final average weight (LW, g); total length (TL, cm); condition factor (CF) = [K= (weight x 100)/(length total^3)]; daily average gain (DAG, g/day) = ((final weight – initial weight)/period); specific growth rate (SGR, %/day) = ([ln (final weight) – ln (initial weight)/period]); biomass (BM, g) = (total weight of animals in the tank); survival (S, %) = (live animals in the period/initial number of animals in the tank). The feed conversion rate (FCR) also was calculated as follow = (g dry feed given/g live weight gain).

For each three-week period, two fish per tank (eight for treatment) were captured. The animals fasted for 18 h before each collection for emptying of digestive tract. Fish were killed by spinal cord excision behind operculum, weighed and measured and then eviscerated for calculation of the carcass yield (CY, %) = (carcass weight/fish weight x 100). The digestive tract, liver and fat were weighed to obtain data about the digestivesomatic index (DSI), hepatosomatic index (HSI) and visceral fat index (VFI)

as a whole weight percentage. The length of the digestive tract was measured to determine the intestinal quotient (IQ), which is related to the total length of the animal. The protein efficiency ratio (PER) was calculated based on body weight gain relative to the amount of protein consumed.

2.7 Body composition analysis

In each collection period, eight fish per treatment were sampled for whole body composition analysis after the fish being ground using a food processor. Moisture concentration, ash and crude protein (nitrogen determination by micro Kjeldahl method - N x 6.25) were determined as AOAC (1995). Fat was extracted and quantified following the method proposed by Bligh and Dyer (1959). From the results of protein and fat were calculated the following parameters:

- protein retention coefficient (PRC, %) = [100 x ((FBW x FBP)-(IBW x IBP))]/FI x CPD;
- body protein deposition (BPD, g) = [FBW x (FBP/100)] – [IBW x (IBP/100)];
- body fat deposition (BFD, g) = [FBW x (FBF/100)] – [IBW x (IBF/100)];

where: FBW=final body weight; FBP=final body protein; IBW=initial body weight; IBP=initial body protein; FI= feed intake; CPD= crude protein diet; FBF=final body fat; IBF=initial body fat.

For ash and phosphorus analysis in the bones, eviscerated carcasses were wrapped in aluminum foil and placed in an electric oven for 15 min at 180ºC. The flesh was removed and the bones washed in distilled water, dried with forced air circulation at 60ºC for 24 h and stored at -18ºC. For measuring the ash content part of bone structure was maintained at 550ºC for 6 h. Analysis of the content of phosphorus in

bones was performed following the method proposed by Baginski et al. (1982) using as standard K_2PO_4.

2.8 Statistical analysis

The data were tested for normality (Shapiro-Wilk test), submitted to one-way ANOVA and comparison of treatments including vegetable meals with the control, in each experiment, was performed by Dunnett's test. The F test was used to compare means between the treatments containing the meal treated with phytase in relation to the meal treated with phytase and tannase. Data were expressed as mean ± mean standard error and the differences were considered to be significant at a probability level of $P<0.05$. Nutritional and antinutritional compounds data of the meal treated with phytase in relation to the meal treated with phytase and tannase (crambe or tung) are expressed as mean ± standard deviation (n=4) and were compared by F test ($p<0.05$). Statistical analysis was performed by the Statistical Analysis System SAS® version 8.2 (2001).

3. Results

The analysis of antinutritional compounds (Table 1) revealed reduction of 23 and 25% in the concentration of total and hydrolysable tannins in crambe meal treated with non-commercial phytase (phytase and tannase action – PHY+TAN) in relation to commercial phytase (PHY), respectively. However, the phenolic compounds and condensed tannins concentrations were similar regardless of the type of phytase used. The concentration of phytic acid in crambe meal was reduced in 29% after treatment with PHY over the meal treated with PHY+TAN and the integral form of meal (2.26% phytic acid). For tung meal no changes were observed in relation to the content of

phenols and tannins (total, hydrolysable and condensed) among treatments (Table 1). It was found reduction (-58%) in the concentration of phytic acid after treatment with PHY regarding meal treated with PHY+TAN and the integral form of meal (1.93% phytic acid). For tung and crambe meals the enzymatic treatments reduced, on average, 20 and 35% the total and hydrolysable tannins concentration regarding integral forms (tung – 1.09% and crambe – 0.66%).

Replacement of animal meal by crambe meal (both enzymatic treatments) caused less weight gain in fish after three (-15%), six (-20%) and nine weeks (-23%) (Table 3). Other performance parameters such as total length (TL), daily average gain (DAG), specific growth rate (SGR) and biomass (BM) also showed similar results. The feed conversion rate (FCR) differ from control only after three weeks of feeding for the animals fed with diet containing crambe meal treated with PHY. The condition factor (CF) did not differ between treatments containing crambe and the control. Among the treatments CR PHY and CR PHY+TAN, no differences were observed with respect to growth performance parameters throughout the study. Compared to control, the treatments containing tung meal enzymatically treated also provide lower growth after three (-34%), six (-50%) and nine weeks (-56%) (Table 3). Similarly, it was observed lower TL, DAG, SGR, CF, BM and higher FCR in these treatments until the end of the experimental period. In addition it was observed that after six weeks fish receiving the treatment TG PHY+TAN showed higher SGR and BM and after nine weeks exhibited greater live weight (LW), TL, DAG and SGR compared to treatment TG PHY (Table 3).

In relation to somatic parameters, the treatments containing crambe meal did not caused changes in CY, DSI, HSI, VFI and IQ when compared to control in all three

periods (Table 4). Among the treatments CR PHY and CR PHY+TAN only was observed higher HIS after six weeks in fish undergoing to CR PHY+TAN treatment. Up to six weeks of feeding, the fish receiving the treatments TG PHY and TG PHY+TAN did not alter the parameters carcass yield (CY), digestivesomatic index (DSI), hepatosomatic index (HSI) and visceral fat index (VFI) compared to control treatment (Table 4). From this period intestinal quotient (IQ) was higher with TG PHY+TAN treatment and after nine weeks the DSI and IQ proved to be high for both treatments in relation to control. The VFI was lower for fish that received the TG PHY treatment at the end of the experiment when compared to control. This change was also observed when compared to TG PHY+TAN treatment. Between the treated forms with PHY or PHY+TAN for the tung meal it was also verified difference after six week of experiment where PER was higher for fish that received TG PHY+TAN treatment compared to TG PHY.

Considering the results for body composition it was observed lower percentage of fat and dry matter in fish carcass of both treatments containing tung meal compared to control (Table 5). These parameters did not change in the animals who received both treatments with inclusion of crambe meal. The concentration of ash and crude protein did not differ in relation to the control for the treatments containing vegetable meals under study. Deposition of nutrients studied through the PRC, BPD and BFD showed lower concentrations in treatments containing vegetable meals (crambe and tung) compared to control. The enzymatic treatments applied in crambe meal – PHY or PHY+TAN did not alter the rates of deposition in fish. However, animals that received the treatments containing tung meal had higher PRC and BPD in TG PHY+TAN compared to TG PHY treatment. The BFD did not differ between treatments containing

tung meal. Ash and phosphorus in the bones did not differ among treatments with inclusion of crambe or tung compared to control. Similarly, the percentage of these nutrients did not differ between the two forms of enzymatic treatment for each meal included in the diet.

4. Discussion

The effect of non-commercial phytase (PHY+TAN) in the crambe meal was demonstrated only on the concentrations of total and hydrolysable tannins. This enzyme did not reduce the level of phytic acid as the commercial phytase (PHY). For the tung meal the PHY+TAN enzyme did not reduce concentrations of total and hydrolysable tannins nor phytic acid. However, the PHY enzyme provided greater reduction in phytic acid content in this meal compared to crambe meal. For the enzymes PHY and PHY+TAN incubation on the crambe or tung meal it was used the 1:3 weight:volume ratio, temperature of 40°C and 2 hours incubation for 2 hours. According to Storebakken et al. (1998) this protocol increased the concentration of soluble phosphorus and reduced phytic acid content in samples of soy concentrate. Most phytases have an optimal pH in the range of 4.5-6.0 and a temperature range of 45-60ºC. Phytase produced by *Aspergillus niger* has pH and temperature ranges varying from 5.0-5.5 and 55-58ºC (Cao et al., 2007). According to Battestin and Macedo (2007) the temperature of 40 to 65ºC and pH 4.5 to 6.5 were optimum for tannase activity and stability produced by *Paecilomyces variotii*. However, Schons et al. (2011) applied tannase (non-commercial) and phytase (commercial) on sorghum at a 1:6 meal:water ratio at a temperature of 34ºC for 24 hrs of incubation and obtained reduction of the content of total phenol and hydrolysable tannins by 87% and 43%, respectively. The

concentration used for each enzyme was 100 U/Kg meal. The inefficient action of the enzyme PHY+TAN on the tung meal and lower action on crambe meal can be due to the type of substrate, to lower dilution used and reduced incubation time of the enzyme to act on the substrate. This enzyme also did not alter the concentration of condensed tannins in the meals tested. According Rodríguez-Durán et al. (2011) tannase does not affect the bonds between carbon molecules and thus hardly hydrolyzes condensed tannins.

Most studies show that the inclusion of enzymes such as phytase in diets containing vegetable ingredients enhances the growth response, the digestibility and deposition of nutrients by the animal (Biswas et al., 2007; Rocha et al., 2007; Portz and Liebert, 2004). However, the results reveal that even with the reduction of some antinutrients, crambe or tung meals enzymatically treated did not provide similar performance to control treatment when replaced 20% of protein of animal source (fish meal and meat and bone meal). Similar results were found when Fortes-Silva et al. (2011) partially replaced fish meal by soybean meal (31%) in the diet of sea bass. Even with the addition of phytase (1500 FTU/Kg) animals continued to show less weight gain and specific growth rate in this treatment compared to diet containing fish meal as protein base. Lim and Lee (2009) also noted that the inclusion of phytase (1000 FTU/Kg) in diets with replacement of 20 or 30% of animal protein by mixing soybean meal and cottonseed meal no had effect on the growth response of parrot fish (*Oplegnathus fasciatus*). Possibly the incorporation of meal may have provide lower digestibility and nutrient absorption due to persistence of antinutrients not metabolized by the enzymes applied. Furthermore, fibers characterized another antinutritional fraction which were found in high percentage in the sources under study and contribute

to reduce the nutritional value of food. The changes in the content of antinutrients present in crambe meal after the action of enzymes caused no change in growth response when this meal was included in the ration (CR PHY or CR PHY+TAN treatments). Possibly the reduction in the concentration of antinutrients was bland and did not allow the animals to express on growth parameters and nutrient deposition. Already, the animals fed with tung meal demonstrated the worst growth performance among the treatments containing the vegetable meals. This ingredient has mainly lower protein concentration and increased amount of fiber in relation to crambe meal and in this case needed to be included in the diet at a higher percentage than the other meal, resulting in a higher percentage of fiber in these treatments. Moreover, the higher concentration of phenolic compounds and total and hydrolysable tannins certainly gives the tung meal low palatability caused by astringency and bitter taste of these molecules.

The best performance demonstrated by fish fed with TG PHY+TAN diet from the sixth week, considering SGR and FCR, may be related to a metabolic and physiological adaptation fastest to animals in this treatment compared to those who received the TG PHY treatment. In addition, it was observed that after six weeks of experiment, silver catfish fed with tung meal treated with PHY+TAN showed an increase in IQ in relation to control treatment. At the end of the experimental test animals from TG PHY+TAN and TG PHY treatments had higher IQ and DSI compared to the control. This fact can be related to a higher concentration of fiber in the diets containing tung meal and the results may indicate an adaptation of the gastrointestinal tract to the type of feed ingested. The inclusion of soluble non-starch polysaccharides (0 - 80g/Kg) in diet of African catfish caused increase in the digest viscosities, reduced nutrient digestibilities and increased the digestive organs weight. According to the

composition of the ingredients, the digestive tract of the fish can increase in size and/or volume in attempt to increase the area of contact with the food, and improve digestibility (Leenhouwers et al., 2006). However, the treatments containing crambe meal caused no change in somatic indexes as CY, DSI, HIS, VFI and IQ in silver catfish compared to control. But, the treatments with inclusion of tung meal also reduced PER in relation to the animal control treatment. This response was also verified in other studies with incorporation of vegetable protein in place of animal protein sources (Pratoomyot et al., 2010; Lim and Lee, 2009; Hansen et al., 2007; Gaber, 2006). Increased levels of digestible and indigestible carbohydrates (starch/fiber), reduced palatability, presence of antinutritional factors and imbalance in the concentration of amino acids and phosphorus available in the diet are the main causes of lower growth and nutrient use efficiency by protein.

The absorption of nutrients by fish depends on the rate at which these can be taken up from intestinal lumen and the time in which are in contact with the absorptive epithelium (Shiau et al., 1988). So, the final answer to the use of a diet for fish is reflected by the body deposition of nutrients. As in other studies, no differences were found in ash and crude protein levels in carcass of silver catfish in treatments with inclusion of vegetable meals compared to control (Hansen et al., 2007; Gaber, 2006; Kaushik et al., 2004). The concentration of dry matter and fat was not altered in fish fed crambe meal in the diet. However, the treatments containing tung meal led to lower percentage of body dry matter and fat. Deposition of fat in the carcass may vary with the level and/or type of vegetable meal included in the diet. The answer may correspond to an increase of body fat (muscular and perivisceral), usually associated with inefficient use of protein sources and amino acids in the diet (Pratoomyot et al., 2010).

Additionally, the lowest concentration of carcass fat, is related in some studies, to the fiber content present in vegetable meals which limit feed intake or prevented the efficient utilization of nutrients contributing to the lower energy release in the diet. Or even fiber fractions of certain ingredients may have hypocholesterolemic effect and affect metabolic pathways such as lipogenesis (Rodrigues et al., 2012; Dias et al., 2005; Shiau et al., 1988). The results obtained may possibly be due to these facts. Rates of nutrient deposition analyzed in this study as PCR, BPD and BFD followed the same trend observed in the growth parameters for the animals fed with inclusion of crambe or tung meal in diets and showed lower values compared to control. Fish fed with the TG PHY+TAN diet, which showed higher growth than in those undergoing TG PHY treatment, presented higher PRC and BPD and possibly this growth did not result in greater BFD, since in this treatment it was observed higher VFI. As observed by Gaber (2006) in this study the inclusion of crambe and tung meal in diets probably reduced the available energy for protein synthesis resulting in lower growth and nutrient utilization. Unlike the results found in other studies (Fortes-Silva et al., 2011; Liebert and Portz, 2005; Portz and Liebert, 2004), these data show that the application of enzymes on the crambe and tung meals did not alter the total deposition of minerals and phosphorus in the bones of animals.

In the present study, the lowest response observed in parameters such as fish performance may indicate that the use of supplemental phytases (commercial and non-commercial) at crambe or tung meal in the levels used is not feasible and the other antinutritional fractions as fiber may contribute more effectively on the results found than the concentrations of phytic acid and tannins present in the meals studied. Suggest that future studies addressing higher level of inclusion of enzymes in the treatment of

456 these proteins sources and their incorporation to a lesser percentage in combination with
457 other plant sources such as soybean, an ingredient of higher nutritional value.
458
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467
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622 Table 1: Chemical composition analyzed of crambe and tung meals after enzymatic treatment
623 with phytase or phytase and tannase.

	Crambe		Tung	
Nutrientes (%)	PHY	PHY+TAN	PHY	PHY+TAN
Ash	8.08±0.07	8.21±0.01	6.72±0.03	6.73±0.04
Crude protein	37.10±0.18	37.03±0.16	28.60±0.34	28.74±0.57
Fat	7.23±0.10	7.00±0.07	2.77±0.18	2.63±0.15
NDF	31.72±0.87	30.62±0.72	53.81±1.23	55.21±0.91
Calcium	0.95±0.02	0.93±0.02	0.63±0.02	0.60±0.01
Total phosphorus	0.89±0.05	0.99±0.03	0.50±0.03	0.57±0.04
Antinutrients (%)				
Phenolic compounds	0.98±0.03	0.90±0.03	1.24±0.02	1.22±0.01
Total tannins	0.43±0.01	0.33±0.03*	0.87±0.01	0.85±0.01
Hydrolysable tannins	0.394±0.002	0.292±0.027*	0.861±0.002	0.841±0.012
Condensed tannins	0.035±0.002	0.035±0.001	0.012±0.001	0.012±0.001
Phytic acid	1.59±0.01	2.24±0.03*	0.79±0.04	1.88±0.03*

624 Values expressed as mean ± standard deviation (n=4). *Indicates difference between the meal treated
625 with phytase in relation to the meal treated with phytase and tannase by F test ($p<0.05$).

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636 Table 2: Formulation and proximate composition of the experimental diets (%).

Ingredients	Treatments[a]				
	EXP. I			EXP. II	
	CON	CR PHY	CR PHY+TAN	TG PHY	TG PHY+TAN
Fish meal	26	22.23	22.24	22.24	22.24
Meat and bone meal	30.50	24.85	24.85	24.85	24.85
Crambe meal+phytase	-	18.90	-	-	-
Crambe meal+phytase+tannase	-	-	19.00	-	-
Tung meal+phytase	-	-	-	24.36	-
Tung meal+phytase+tannase	-	-	-	-	24.78
Wheat flour	13.57	7.58	8.25	7.65	7.56
Corn grain	11.80	8.00	6.45	4.55	4.25
Corn starch	4.43	7.50	8.10	5.85	5.20
Soybean oil	5.80	4.90	5.88	7.45	7.95
Salt	0.50	0.50	0.50	0.50	0.50
Dicalcium phosphate	0.55	0.45	-	-	0.25
Vitamin/mineral mixture[b]	2.00	2.00	2.00	2.00	2.00
Methionine	0.40	0.40	0.40	0.42	0.42
Calcium carbonate	-	-	-	-	-
Inert	4.45	2.69	2.33	0.13	-
	Diet composition (%)				
Crude protein[c] (%)	36.00	36.00	36.00	35.73	35.70
Fat[c] (%)	13.16	12.13	13.04	13.91	14.36
Ash[c] (%)	14.42	13.26	13.31	13.34	13,33
Neutral detergent fiber[c] (%)	7.68	10.23	10.05	16.46	16.64
Carbohydrates[d] (%)	10.07	9.29	5.70	6.34	5.80
Digestible energy[e] (Kcal Kg^{-1})	3.200	3.200	3.200	3.199	3.199
Calcium[c] (%)	2.44	2.20	2.09	2.06	2.12
Total phosphorus[c] (%)	1.49	1.36	1.30	1.23	1.29
Ca:P ratio	1.63	1.62	1.60	1.68	1.64
Lysine[c] (%)	2.16	2.10	2.10	2.02	2.03
Methionine[c] (%)	1.50	1.46	1.46	1.43	1.44

637 [a]CON=control diet, protein with the base consisting of animal meal; CR PHY=crambe meal inclusion treated
638 with phytase; CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal
639 inclusion treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase and tannase.
640 [b]Vitamin/mineral (kg product): Folic acid: 300mg; Choline: 100g; Inositol: 10g; Niacin: 9000mg;
641 Pantothenic acid: 3000mg; Biotin: 0.1mg; Vit.A: 1000000UI; Vit. B1: 1500mg; Vit. B2: 1500mg; Vit. B6:
642 150mg; Vit. B12: 2000mg; Vit. C: 15g; Vit. D3: 240000UI; Vit. E: 10000mg; Vit. K3: 400mg; Copper:
643 1000mg; Iron: 6000mg; Iodine: 45mg; Manganese: 8000mg; Selenium: 60mg; Zinc: 14g. [c]Calculated based
644 on the ingredients analysis; [d]Neutral detergent soluble Carbohydrate calculated: 100-(crude

645 protein+ash+fat+neutral detergent fiber +moisture). [e]Calculated from the formula:
646 (Protein*5,64*0,83)+(fat*9,44*0,88)+(Carbohydrate*4,11*0,65))*10 (Meyer et al., 2004).
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674 Table 3: Performance of silver catfish juvenile fed with a diet based in animal protein
675 (control) and diet containing crambe or tung meal treated with phytase or with
676 phytase and tannase.

Variables[§]	Treatments[‡]				
	EXP. I			EXP. II	
	CON	CR PHY	CR PHY+TAN	TG PHY	TG PHY+TAN
	Three weeks				
LW (g)	16.97±0.22	14.16±0.46^{A}	14.87±0.29^{A}	11.05±0.17^{a}	11.23±0.16^{a}
TL (cm)	12.31±0.09	11.62±0.11^{A}	11.72±0.06^{A}	11.01±0.11^{a}	11.01±0.07^{a}
CF	0.91±0.01	0.90±0.01	0.92±0.05	0.83±0.01^{a}	0.84±0.01^{a}
DAG (g/day)	0.45±0.01	0.31±0.02^{A}	0.35±0.02^{A}	0.16±0.06^{a}	0.17±0.07^{a}
SGR (%/day)	3.85±0.07	2.96±0.17^{A}	3.19±0.13^{A}	1.77±0.07^{a}	1.87±0.06^{a}
FCR	0.94±0.02	1.36±0.11^{A}	1.24±0.08	2.56±0.12^{a}	2.49±0.03^{a}
BM (g)	419.9±3.9	354.3±11.4^{A}	371.7±7.2^{A}	276.3±4.4^{a}	278.0±1.4^{a}
S (%)	99.0±2.0	100±0.0	100±0.0	100±0.0	99.0±2.0
	Six weeks				
LW (g)	33.20±0.44	26.09±1.40^{A}	27.24±1.23^{A}	16.13±0.48^{a}	17.56±0.36^{a}
TL (cm)	15.09±0.03	13.99±0.23^{A}	14.15±0.22^{A}	12.35±0.21^{a}	12.63±0.07^{a}
CF	0.97±0.05	0.95±0.01	0.96±0.01	0.86±0.03^{a}	0.87±0.02^{a}
DAG (g/day)	0.61±0.01	0.44±0.03^{A}	0.47±0.03^{A}	0.20±0.01^{a}	0.24±0.01^{a}
SGR (%/day)	3.52±0.05	2.92±0.14^{A}	3.03±0.13^{A}	1.79±0.07^{a}	2.00±0.05^{a}*
FCR	1.08±0.05	1.44±0.09	1.39±0.12	2.98±0.15^{a}	2.44±0.12^{a}*
BM (g)	755.4±14.6	600.1±32.3^{A}	620.5±33.9^{A}	362.6±9.4^{a}	394.9±9.7^{a}
S (%)	99.0±2.0	100±0.0	98.91±2.17	98.91±2.17	97.83±2.2
	Nine weeks				
LW (g)	56.25±2.41	42.33±3.11^{A}	43.27±2.61^{A}	22.76±0.54^{a}	25.70±0.55^{a}*
TL (cm)	17.59±0.25	16.02±0.38^{A}	16.06±0.26^{A}	13.53±0.12^{a}	13.97±0.08^{a}*
CF	1.03±0.01	1.02±0.01	1.04±0.02	0.91±0.07^{a}	0.94±0.01^{a}
DAG (g/day)	0.77±0.04	0.55±0.05^{A}	0.57±0.04^{A}	0.24±0.08^{a}	0.29±0.01^{a}*
SGR (%/day)	3.18±0.08	2.71±0.12^{A}	2.75±0.11^{A}	1.74±0.03^{a}	1.94±0.04^{a}*
FCR	1.31±0.08	1.61±0.10	1.63±0.11	3.04±0.11^{a}	2.66±0.16^{a}
BM (g)	1166.8±50.1	888.9±65.3^{A}	899.4±62.1^{A}	461.17±15.81^{a}	508.27±22.02^{a}
S (%)	99.0±2.0	100±0.0	98.91±2.17	96.43±2.74	94.05±4.55

677 [‡]CON=control diet, protein with the base consisting of animal meal; CR PHY=crambe meal inclusion treated
678 with phytase; CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal
679 inclusion treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase and tannase.
680 [§]LW=live weight; TL=total length; CF=condition factor; ADG=average daily gain; SGR=specific growth
681 rate; FCR=feed conversion rate; BM=biomass; S=cumulative survival. Values expressed as mean ± standard
682 error of mean (n=4). Capital letter indicate difference in I experiment (CR PHY or CR PHY+TAN) and

683 lower case indicate difference in II experiment (TG PHY or TG PHY+TAN) in relation to the control by
684 Dunnett's test ($p<0.05$).*Indicate difference of the meal treated with phytase in relation to the meal treated
685 with phytase and tannase by F test ($p<0.05$).

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Table 4: Somatic parameters of silver catfish juvenile fed with a diet based in animal protein (control) and diet containing crambe or tung meal treated with phytase or with phytase and tannase.

Variables[§]	Treatments[‡]				
	EXP. I			EXP. II	
	CON	CR PHY	CR PHY+TAN	TG PHY	TG PHY+TAN
	Three weeks				
CY (%)	85.05±0.58	85.15±0.48	8.36±0.34	85.95±0.40	85.09±0.68
DSI (%)	3.71±0.34	3.44±0.19	3.52±0.23	3.47±0.21	3.71±0.29
HSI (%)	1.40±0.07	1.38±0.08	1.42±0.09	1.34±0.07	1.27±0.08
VFI (%)	3.10±0.47	3.57±0.41	3.47±0.55	2.54±0.39	2.31±0.20
IQ	1.20±0.06	1.28±0.07	1.33±0.09	1.23±0.05	1.39±0.11
PER	3.14±0.07	2.23±0.16[a]	2.46±0.14[A]	1.17±0.06[a]	1.20±0.01[a]
	Six weeks				
CY (%)	87.79±0.29	87.77±0.71	86.99±0.43	86.24±0.65	87.42±0.48
DSI (%)	3.32±0.14	3.34±0.29	3.10±0.10	3.99±0.20	3.62±0.26
HSI (%)	1.33±0.11	1.17±0.05	1.46±0.05*	1.27±0.04	1.28±0.03
VFI (%)	3.86±0.30	3.27±0.34	3.91±0.41	3.15±0.37	3.56±0.52
IQ	1.16±0.05	1.22±0.07	1.34±0.08	1.35±0.06	1.43±0.08[a]
PER	2.74±0.15	2.10±0.12[A]	2.22±0.17[A]	1.01±0.05[a]	1.24±0.06[a]*
	Nine weeks				
CY (%)	86.89±0.48	86.94±0.71	86.63±0.57	87.26±0.70	86.92±0.23
DSI (%)	2.87±0.13	3.02±0.18	2.95±0.21	3.95±0.25[a]	3.63±0.14[a]
HSI (%)	1.40±0.08	1.29±0.13	1.19±0.04	1.32±0.09	1.29±0.06
VFI (%)	4.55±0.49	4.57±0.49	4.59±0.38	2.30±0.34[a]	3.66±0.34*
IQ	1.21±0.08	1.21±0.06	1.37±0.10	1.49±0.05[a]	1.48±0.04[a]
PER	2.27±0.13	1.87±0.11[A]	1.88±0.12[A]	0.99±0.04[a]	1.14±0.07[a]

[‡]CON=control diet, protein with the base consisting of animal meal; CR PHY=crambe meal inclusion treated with phytase; CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal inclusion treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase and tannase. [1]CY=carcass yield; DSI=digestivesomatic index; HIS=hepatosomatic index; VFI=visceral fat index; IQ= intestinal quotient; PER= protein efficiency ratio. Values expressed as mean ± standard error of mean (n=8), for PER (n=4). Capital letter indicate difference in I experiment (CR PHY or CR PHY+TAN) and lower case indicate difference in II experiment (TG PHY or TG PHY+TAN) in relation to the control by Dunnett's test ($p<0.05$).*Indicate difference of the meal treated with phytase in relation to the meal treated with phytase and tannase by F test ($p<0.05$).

728 Table 5: Body composition of silver catfish juvenile after nine weeks of feeding with diet
729 based in animal protein (control) and diet containing crambe or tung meal
730 treated with phytase or with phytase and tannase.

Variables[§]	Treatments[‡]					
		EXP. I			EXP. II	
	Initial	CON	CR PHY	CR PHY+TAN	TG PHY	TG PHY+TAN
		Carcass				
Dry matter	22.76±1.17	29.58±0.50	29.08±0.52	29.51±0.38	26.11±0.38[a]	26.57±0.76[a]
Ash	2.41±0.27	2.74±0.10	3.05±0.11	2.96±0.08	3.04±0.10	2.99±0.13
Crude protein	13.15±1.26	15.02±0.24	15.19±0.16	15.44±0.12	14.78±0.19	14.85±0.15
Fat	6.81±0.82	12.74±0.76	12.40±0.62	13.35±0.52	8.52±0.68[a]	8.85±0.93[a]
PRC	ND	1.74±0.08	1.46±0.05[A]	1.51±0.04[A]	0.87±0.02[a]	1.02±0.02[a]*
BPD	ND	7.49±0.33	5.45±0.30[A]	5.70±0.24[A]	2.39±0.08[a]	2.84±0.05[a]*
BFD	ND	6.67±0.51	4.78±0.42[A]	5.23±0.23[A]	1.42±0.16[a]	1.77±0.25[a]
		Bones				
Ash	ND	59.39±0,69	60.26±0.66	60.27±0.62	56.98±1.00	57.48±0.88
Phosphorus	ND	14.70±0.31	15.20±0.13	15.00±0.15	14.82±0.13	14.49±0.29

731 [‡]CON=control diet, protein with the base consisting of animal meal; CR PHY=crambe meal inclusion
732 treated with phytase; CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG
733 PHY=tung meal inclusion treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase
734 and tannase. [1]Dry matter, ash, crude protein, fat and protein retention coefficient (PRC) = (%); body protein
735 deposition (BPD) and body fat deposition (BFD) = (g). ND = no determined. Values expressed as mean ±
736 standard error of mean (n=8) in natural matter in carcass and in basis of dry matter in the bones. Capital
737 letter indicate difference in I experiment (CR PHY or CR PHY+TAN) and lower case indicate difference in
738 II experiment (TG PHY or TG PHY+TAN) in relation to the control by Dunnett's test (p<0.05).*Indicate
739 difference of the meal treated with phytase in relation to the meal treated with phytase and tannase by F test
740 (p<0.05).

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5 ARTIGO 4

Enzymatically pretreated crambe or tung meals in diets for *Rhamdia quelen* juveniles: study of digestive and biochemical parameters

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Abstract

The nutritional value of plant protein sources to replace animal protein has been tested in several fish species, seeking to maintain similar performance to the animals with the use of lower-cost ingredients. However, changes at digestive and metabolic level can occur in this situation. The objective of this study was to evaluate possible changes in the digestive enzymes activity and biochemical parameters in plasma and liver of silver catfish juveniles fed with crambe or tung meals, pretreated enzymatically (commercial phytase Natuphos® or non-commercial enzyme with phytase and tannase activity), in partial replacement of animal protein (meat and bone meal and fish meal). The results showed reduction in acid protease and intestinal lipase in animals who received both treatments containing tung meal. Chymotrypsin activity was reduced and after increased in these treatments. These enzymatic changes possibly interfered to reduce plasmatic cholesterol and total protein and hepatic glycogen, glucose and aminotransferases. The inclusion of crambe meal treated with non-commercial enzyme caused a reduction in lipase activity and altered glycogen, glucose and aminotransferases in liver throughout the study. Treatment containing crambe meal treated with commercial phytase only reduced hepatic glucose compared to control. The inclusion of crambe or tung meal in the diet of silver catfish reduced the weight gain of the animals. However, fish fed with tung meal showed more significant reduction, which coincided with a greater change in the digestive enzymes activity and an increase in intestinal quotient possibly as a strategy to increase the digestive capacity and obtain nutrients.

Keywords: protein plant, silver catfish, phytase, tannase, metabolic response.

Introduction

Among protein sources available for animal feed, plant protein appear as the alternative ingredients most used to replace fish meal in aquaculture diets (Cheng et al. 2010; Gaber 2006). For omnivorous some studies show that the replacement of fish meal by single source of vegetable protein can reach up to 50% without affecting animal performance (Lin and Luo 2011; Kumar et al. 2010; Gaber 2006). For carnivores, the combination of several plant proteins can replace similar fish meal percentages to those found in diets for omnivores (Bonaldo et al. 2011; Gomes et al. 1995).

However, the main obstacles to the use of vegetable meals in diet formulation refers to the lower protein content and low palatability in relation to sources of animal origin, higher fiber content and the presence of a variety of secondary compounds (Drew et al. 2007). These molecules can affect protein digestion and availability of amino acids, minerals and vitamins impairing growth and can functionally affect organs of the animals (Krogdahl et al. 2010). Some antinutrients as protease inhibitors, lectins and antivitamins can be destroyed after the application of heat, with greater efficiency in the process in the presence of moisture (Aregheore et al. 1998). Other molecules such as phenolic compounds, tannins, saponins and phytic acid can be reduced by applying acid, basic or alcoholic extracting solution (Ochanda et al. 2010; Han 1998). However, the use of these solutions can solubilize minerals and other nutrients as well as change the structural conformation of amino acids reducing their availability (Friedman 1996; Ai et al. 2007; Kumar et al. 2012). Thus, an alternative is the use of exogenous enzymes such as phytase, produced by fungi, bacteria and yeasts which are commercially available to catalyze the hydrolysis of phytic acid. They release phosphorus for absorption. Tannase can reduce the formation of tannin-protein complex in vegetable

ingredients, once the tannins are degraded into simple compounds as glucose and gallic acid, and can be other alternative (Battestin et al. 2004).

Regarding the performance of growth, digestibility and nutrients utilization upon application of enzymes in the diet, some results show efficiency (Biswas et al. 2007; Portz and Liebert 2004) and others did not provide improvements (Lim and Lee 2009; Vielma et al. 2000). The first step in the utilization of ingested nutrients is the presence of enzymes at appropriate locations along the wall and the lumen of the digestive tract (Tengjaroenkul et al. 2000). The profile or the concentration of digestive enzymes can be altered by the type, source and amount of nutrients ingested (Lundstedt et al. 2004). The inclusion of vegetable meals in the diets coupled with the presence of antinutrients inhibit protease, lipase and amylase digestive (Maitra and Ray 2003) that can help to understand the occurrence of reduced growth in animals. Moreover, also may be useful indicators of animal performance parameters related to protein and energy metabolism in plasma and liver, characterized as the central processing and distribution of nutrients in the organism. The concentration of protein or amino acids plasmatic and hepatic aminotransferases can be changed according to the use of dietary proteins (Hansen et al. 2007; Lin and Luo 2011). Then, the aim of this study was to evaluate the inclusion of crambe or tung meals, enzymatically treated (phytase or phytase+tannase), in the diet of silver catfish juveniles (*Rhamdia quelen*), an omnivorous species with great potential for cultivation in Southern Brazil. The response to treatments was monitored through the digestive enzymes activity and biochemical parameters in plasma and liver and related to weight gain of the animals.

Material and methods

The enzymatic treatment of crambe and tung meals was performed according to the modified method proposed by Storebakken et al. (1998). The meals were pretreated with commercial phytase produced by *Aspergillus niger* (product BASF Natuphos® 10000 U/g) or non-commercial enzymatic preparation with phytase and tannase activity produced by *Paecilomyces variotti* (460 U phytase and 360 U tannase/mg) developed in the Laboratory of Food Biochemistry of the Faculdade de Engenharia de Alimentos/UNICAMP (Madeira et al. 2011). The concentrations used were 1400 U phytase and 1100 U tannase/Kg meal. The meals were added to warm water (40ºC) (1:3 weight:volume ratio), and enzyme (previously dissolved in water at the same temperature). The resulting pH of the mixture was 6.06 and 5.77 for crambe and tung meal, respectively. The incubation was continued for 2 hours in water bath (40°C) with constant stirring of the mixture through manual electric mixer for 5 min every 10 min interval. After completion of the incubation, the mixture was taken to an oven at 55ºC until achieve less than 12% moisture. After cooled, the meals were ground, chemically analyzed and later used to prepare rations.

The chemical analyses carried out in meals subjected to enzymatic treatment were: total and condensed tannins, performed according Makkar (2000). For extraction was added acetone 70% in the samples (10 mL for twice) followed incubation in ultrasonic bath for 20 min and centrifugation (3000x*g* for 10 min) for accumulative collection of the supernatant fraction. In determining total tannin, before of the colorimetric quantification, this fraction was extracted with PVPP (polyvinylpolypyrrolidone – 100 mg/mL) reagent and centrifugation (3000x*g* for 10 min) collecting the supernatant. The absorbance was monitored at 740 nm, using tannic acid (100 mg/L) as standard. For quantification of condensed tannins, it was initially added to the extract obtained 3 mL of butanol-hydrochloric acid (9:1) and ammonium

iron sulfate in hydrochloric acid (0.1 mL). The samples were heated (bath at 90-95ºC) for 40 min and thereafter, the absorbance was monitored at 550 nm. The concentration of hydrolysable tannins was obtained by difference between the total tannin content less condensed tannins.

Phytic acid was extracted from the samples in a hydrochloric acid solution 2.4% (1:20 weight:volume ratio) after 1 hour of stirring at room temperature and centrifugation (1200x*g* for 10 min) (Latta and Eskin 1980). An aliquot of the supernatant was diluted in ultrapurified water (25 times) and eluted in a column containing anion exchange resin (Bio-Rad AG1-X4). Phytic acid retained on the resin was eluted in sodium chloride solution (0.7 M), collected and measured at 500 nm with a calibration curve produced from phytic acid salt (Sigma, purity 90%). The staining intensity obtained by the color reagent (Wade – 0.03% ferric chloride and 0.3% sulfosalicylic acid in distilled water) decreases with increasing phytic acid in the sample.

The vegetable meals pretreated enzymatically were incorporated into diets for silver catfish to replace 20% of animal protein meal by the plant protein. Thus, two simultaneous experiments were conducted with the following treatments: EXP. I: CR PHY – crambe meal inclusion treated with phytase; CR PHY+TAN –crambe meal inclusion treated with phytase and tannase; EXP. II: TG PHY – tung meal inclusion treated with phytase; TG PHY+TAN – tung meal inclusion treated with phytase and tannase. A control (CON) in which the basic protein was meat and bone meal and fish meal was conducted as comparative to treatments with inclusion of vegetable meals. Diets were formulated to contain approximately 36% of crude protein and 3200 Kcal/Kg of digestible energy (Table 2). Lysine and methionine requirements were based in Montes–Girao and Fracalossi (2006) and when necessary these amino acids were

supplemented. Dry ingredients were ground, mixed and added of soybean oil. The mixtures were pelleted in a meat grinder with water (addition at 50% of the ingredients dry weight), dried with forced air circulation at 50ºC for 24 h. Pellets were packed in plastic bags and kept at -18ºC until use.

All procedures involving animals were conducted in accordance with standards approved by the Ethics Committee for Animal Welfare, Federal University of Santa Maria, by protocol number 026/2011. In this study were utilized 500 silver catfish with weight average of 7.6±0.1 g randomly distributed in 20 experimental units with capacity of 125 L (25 fishes per experimental unit, to test five diets and four replicates for each diet) in a water recirculation system with two biological filters containing crushed rock and controlling the water temperature through the two electrical resistance (1000 W). The rearing system was mounted in a closed room containing air conditioner to control the air temperature to assist in maintaining the water temperature, since the experiment was conducted in the winter. Fish were acclimated to the experimental conditions for one week and subsequently fed with the experimental diets during nine weeks. Diet was provided around 4-5% biomass from each experimental unit, divided into three meals (08:00, 13:00 and 17:00 h). Experimental units were cleaned by siphoning out feces and food residues daily at 09:00 and 15:30 h, renewing about 10% water of each tank.

The culture system water quality was monitored daily for temperature (morning – 25.5±0.7ºC and afternoon – 26.0±1.0ºC) and weekly for other parameters. Dissolved oxygen and pH were monitored with handheld dissolved oxygen instrument (YSI®, Yellowsprings, USA – model 550A) and pHmeter (Servilab, Brazil – model MPA 210-P) and results were, respectively, 6.52±0.47 mg/L and 7.08±0.25 units. Ammonia concentration was quantified according to Verdouw et al. (1978), mean value of 0.23±0.06 mg/L. Nitrite, alkalinity and hardness were determined according to Boyd

and Tucker (1992) and the values found were, respectively, 0.10±0.05 mg/L, 27.44±4.70 mg/L $CaCO_3$ and 39.00±7.01 mg/L $CaCO_3$. All parameters remained within the range suitable for silver catfish (Baldisserotto and Silva 2004).

For each three-week period, eight fish per treatment were captured. The animals fasted for 18 h before each collection to empty the digestive tract. Blood was quickly collected from the caudal vein using heparinized syringes after six and nine weeks. Fish were anesthetized with eugenol (20µL pure extract/L water) (Cunha et al. 2010) killed by spinal cord excision behind operculum and eviscerated to remove the digestive tract and liver. Digestive tract was measured to determine the intestinal quotient (IQ), which measure is related to the total body length. Thereafter, the digestive tract and liver were quickly placed on ice and frozen at -20ºC for digestive enzymes and biochemical parameters analysis, respectively. Plasma aliquots were separated after blood centrifugation at room temperature for 10 min at 1200x*g* for posterior determination of plasmatic metabolic parameters. For each collect, the remaining fish were weighed and measured to assess growth.

The digestive tract was separated in stomach and total intestine. Each section was dissected in a Petri dish containing saline solution (0.9% NaCl) to remove any digestive content remnants and after homogenized (1:20 tissue:homogenization buffer proportion) in Turrax tissue disintegrator/homogenizer (Marconi, Brazil – model MA 102). The homogenization buffer solution was 0.02 M Tris/0.01 M phosphate, pH 7.5 in 50% (v/v) glycerol. The homogenates were centrifuged at 1200x*g* for 10 min and the supernatants were used as the enzyme source. In the stomach the acid protease activity was measured using non-specific substrate (casein) according to Hidalgo et al. (1999). The assay was carried using 0.2 M KCl buffer, pH 1.8 and the samples were incubated at 30ºC for 40 min. The reaction was stopped by adding trichloroacetic acid 15%. The

enzyme extract absorbance was recorded at 280 nm. All samples were assayed in duplicate and readings corrected for blank solutions. Tyrosine was used as standard, and one unit of protease was defined as the amount of enzyme needed to catalyze the formation of 1.0 µg of tyrosine/min/mg protein. In the intestine, the trypsin and chymotrypsin alkaline proteases activity such as α-amylase and lipase were determined. Trypsin activity was assayed with α-*p*-toluenosulphonyl-L-arginine methyl ester hydrochloride (TAME). Crude extracts were incubated for 2 min (25ºC) in 2 mL of buffer (0.2 M Tris/0.01 M $CaCl_2$), pH 8.1. Chymotrypsin activity was assayed with benzoyl tyrosine ethyl ester (BTEE). Crude extracts were incubated for 2 min in 1 mL of buffer (0.1 M Tris/0.1 M $CaCl_2$), pH 7.8. Both trypsin and chymotrypsin were assayed in duplicate and enzymes activities were recorded at 247 and 256 nm, respectively, according to described protocol by Hummel (1959). One unit of enzyme was defined as the amount of enzyme needed to hydrolyze 1 µmol of substrate (TAME or BTEE)/min/mg protein. The α-amylase activity was assayed in 0.2 M phosphate-citrate buffer, pH 7.0, with 0.5% NaCl and a starch concentration of 2.5%. The reaction was stopped by adding $Ba(OH)_2$ 0.3N and $ZnSO_4$ 5%. The experimental protocol used was described by Bernfeld (1955) modified by Hidalgo et al. (1999). The starch hydrolysis determination was done following Park and Johnson (1949). The absorbance was recorded at 660 nm. One unit of enzyme was defined as 1 µmol of glucose released form starch/min/protein mg. Lipase activity was measured according to Gawlicka et al. (2000). Homogenates were incubated with 0.4 mM *p*-nitrophenyl myristate in 24 mM ammonium bicarbonate solution, pH 7.8, containing 0.5% Triton X-100 as an emulsifying agent. Substrate hydrolysis reaction was stopped by addition of 24 mM NaOH. Absorbance change was measured at 405 nm after incubation for 30 min at 30ºC. One unit was defined as micromole of substrate hydrolyzed/min/protein mg.

Crude extracts protein content was determined by the Bradford (1976) method using bovine serum albumin as a standard.

Liver glycogen levels were determined according to Bidinotto et al. (1998). The tissue was weighed (50 mg) and was added KOH and ethanol (1 and 3 mL, respectively) for hydrolysis and precipitation of glycogen. For protein analysis, the tissues were heated at 100°C with KOH and centrifuged at 1000x*g* for 10 min. Supernatant was used to estimate the total protein level by the method described by Bradford (1976), using bovine serum albumin as standard. The soluble sugar tissue samples were homogenized by adding 10% TCA using a Turrax tissue disintegrator/homogenizer and centrifuged at 1000x*g* for 10 min for proteins flocculation. The completely deproteinated supernatant was used for soluble sugar determination according to Dubois et al. (1956) with phenol-sulfuric acid. To measure hepatic transaminases, liver samples were mechanically disrupted by adding 1 mL phosphate buffer 20 mM, pH 7.5 and the homogenate was centrifuged at 1000x*g* for 10 min. This neutral extract was used to measure the hepatic transaminases concentration, but was necessary to dilute the crude extract in homogenization buffer for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) quantification, respectively, two and ten times. The enzymes were determined by using colorimetric procedures following the protocols described in the kits (Doles Reagents and Laboratory Equipment Ltda. Goiania, Goiás, Brazil). ALT or AST concentration was expressed as UI enzyme/mg hepatic tissue. All hepatic extracts were prepared using the 1:20 tissue:homogenization buffer proportion. For the plasmatic metabolic parameters analysis as glucose, total protein and cholesterol were used colorimetric kits (Doles) and the procedures followed the specific protocols for each parameter.

The data were tested for normality (Shapiro-Wilk test), submitted to one-way ANOVA and comparison of treatments including vegetable meals with the control, in each experiment, was performed using Dunnett's test. The F test was used to compare means between the treatments containing the meal treated with phytase in relation to the meal treated with phytase and tannase within each experiment. Data were expressed as mean ± mean standard error and the differences were considered to be significant at a probability level of $p<0.05$. Antinutritional compounds data of the meal treated with phytase in relation to the meal treated with phytase and tannase (crambe or tung) are expressed as mean ± standard deviation (n=4) and were compared by F test ($p<0.05$). Statistical analysis was performed by the Statistical Analysis System SAS® version 8.02 (2001).

Results

In relation to antinutritional compounds (Table 1) occurred reduction of 23 and 25% in the concentration of total and hydrolysable tannins in crambe meal treated with non-commercial phytase (phytase and tannase action – PHY+TAN) in relation to commercial phytase (PHY), respectively. However, condensed tannins concentrations were unchanged. The concentration of phytic acid in crambe meal was reduced in 29% after treatment with PHY over the meal treated with PHY+TAN and the integral form of meal (2.26% phytic acid). For tung meal no changes were observed in relation to the content tannins (total, hydrolysable and condensed) among treatments (Table 1). It was found reduction (-58%) in concentration of phytic acid after treatment with PHY regarding meal treated with PHY+TAN and the integral form of meal (1.93% phytic acid).

The activity of digestive enzymes revealed significant differences for lipase and chymotrypsin between animals fed with diets containing tung meal after three weeks when compared to the control treatment. Protease and trypsin activity was not different in relation to control (Table 3). After six weeks, in both treatments with tung meal inclusion there was a reduction in protease activity and a significant increase in the activity of chymotrypsin in the treatment TG PHY+TAN in relation to control. Although it was not statistically significant, the results for trypsin in fish receiving treatment TG PHY+TAN and trypsin and chymotrypsin for TG PHY exhibit increase of 34, 17.8 and 21%, respectively, after six weeks (Table 3). In this period, lipase did not change but after nine weeks, the animals exhibited lower lipase intestinal activity in both treatments including tung meal compared to control and fish fed with TG PHY+TAN diet exhibited higher trypsin and chymotrypsin activity compared to TG PHY treatment. Amylase did not differ between treatments containing tung meal and compared to control along to study. Diets containing crambe meal did not cause alteration in the digestive enzyme studied between treatments and when compared to control values along the experiment, it was only observed reduction in lipase activity after nine weeks in CR PHY+TAN in relation to control (Table 3).

After six weeks there were no changes for plasmatic glucose, total proteins and cholesterol among fish fed with diets containing vegetable meals and control treatment. Only higher total protein level was verified for fish that received the treatment CR PHY+TAN regarding CR PHY (Table 4). Lower concentration of total protein and cholesterol was observed in animals that received TG PHY treatment when comparing with the control and also lower plasmatic cholesterol levels in relation to TG PHY+TAN treatment. Diets with crambe meal inclusion did not alter these plasmatic parameters compared to control (Table 4).

In the liver, after three weeks, fish fed with diets containing tung meal exhibited lower levels of glycogen and glucose in relation to control (Table 5). In the second evaluation period, the glycogen storage remained reduced in animals receiving TG PHY diet and there was reduced concentration of ALT in the TG PHY+TAN treatment, both changes compared to the control. After nine weeks of feeding, despite having higher concentrations than in the previous periods, the fish subjected to treatments containing tung meal continued exhibiting lower levels of glucose and glycogen in the liver compared to control. Likewise, the ALT enzyme presents lower concentration in TG PHY+TAN and AST, which were reduced in both treatments where tung meal was included in diets in relation to the control. Between treatments containing crambe meal, after three weeks, CR PHY reduced glucose level and CR PHY+TAN reduced the concentration of ALT in fish compared to control (Table 5). Still, fish fed with diet CR PHY+TAN had a lower concentration of aminotransferases compared to those receiving CR PHY diet. After six weeks, CR PHY+TAN treatment resulted in increased levels of hepatic glycogen and glucose and reduced ALT concentration compared to control. In relation to the treatment CR PHY, besides the increase in energy reserves, changed the concentration of aminotransferases, increasing AST and maintaining reduced ALT. By the sixth week the level of protein in the liver was not altered by the treatments containing crambe or tung meal. At the end of the experiment, the CR PHY treatment resulted in less glucose level compared to control and lower concentrations of glycogen and glucose in relation to animals fed with CR PHY+TAN diets. Animals subjected to CR PHY+TAN treatment had lower protein content compared to the control.

Replacement of animal meal by crambe meal (both enzymatic treatments) caused less weight gain in fish after three (-15%), six (-20%) and nine weeks (-23%). Likewise, the treatments containing tung meal enzymatically treated also provide lower

322 growth after three (-34%), six (-50%) and nine weeks (-56%) (Fig. 1). Furthermore, the
323 intestinal quotient was changed in animals receiving tung meal in the diet, after six
324 weeks in the TG PHY+TAN treatment and with the end of the trial period in both
325 treatments with tung meal inclusion compared to animals of control treatment (Fig. 2).
326

327 **Discussion**

328 The lower growth in fish fed with vegetable meals as substitutes for animal meal
329 also have been demonstrated in other studies. This response has been associated with
330 lower digestive enzymes activity and nutrient digestibility plus imbalance in the
331 concentration of amino acids absorbed and reduced palatability (Lin and Luo 2011; Ai
332 et al. 2010; Pratoomyot et al. 2010; Hansen et al. 2007). These effects are associated
333 with the presence of antinutrients from plant ingredients introduced into the diets
334 (Francis et al. 2001; Krogdahl et al. 2010). In addition, tannins are known for causing
335 astringency, influencing the feed consumption (Becker and Makkar 1999). Tung meal
336 has higher total and hydrolyzable tannin content against the crambe meal. Furthermore,
337 the application of non-commercial phytase+tanase, in the concentration used was not
338 efficient in reducing the concentration of tannins and phytic acid in tung meal. This
339 enzyme was more efficient for the crambe meal, but acted only on the concentration of
340 total and hydrolysable tannins. Already, the commercial phytase reduced more
341 effectively the phytic acid for tung meal over the crambe meal. Treatments containing
342 vegetable meals, especially those with inclusion of tung meal had a higher fiber content.
343 In general, other antinutrients as fiber also appear to have affected the growth of silver
344 catfish in this study beyond tannins and phytic acid, since the answer in weight gain was
345 minimally changed over study between treatments with inclusion of crambe or tung
346 meal subjected to pre-treatment with different microbial enzymes.

The ratio of the response obtained in the activity of the digestive enzymes and metabolism of proteic and energetic nutrients can be important tools for understanding the efficiency of nutrient's diet utilization by animal as well as verify metabolic adaptation of fish to dietary changes (Rodiles et al. 2012). Treatments with tung meal inclusion led first to reduction in lipase and chymotrypsin activity and after reduction in protease activity. In an *in vitro* study, it was observed inhibition in protease, lipase and amylase activity after incubation with tannin extracted from *Acacia auriculiformes*. However, even at low concentrations, the activity of the enzymes protease and lipase was more affected by this antinutrient (Maitra and Ray 2003). Cheng et al. (2010) found a reduction in the activity of these enzymes after incorporating increasing levels of canola meal (50%) in the diet of seabass (*Lateolabrax japonicas*). The presence of tannins was related to the decrease in the activity of these enzymes. The inclusion of detoxified *Jatropha curcas* kernel meal and soybean meal in the diet of common carp also reduced amylase, protease and lipase activity. The authors relate these results with the presence of phytic acid (Kumar et al. 2011). In this study, the reduction in the activity of digestive enzymes may be more related to the presence of tannins, which are already found in higher concentrations in the tung meal. In addition, both treatments showed similar inhibitory response in the enzymes probably because have very similar content in this antinutrient. After six weeks of feeding, reduction in protease activity in silver catfish receiving treatments with tung meal inclusion was associated with an increase in the chymotrypsin activity and also with a tendency to trypsin increase. This response was also found in *Solea senegalensis* juveniles when fed by soybean meal, soybean protein concentrate, soybean protein isolate, wheat gluten meal or pea protein concentrate in partial substitution of fish meal. These changes in the distribution and activity of digestive enzymes have been identified as a compensatory mechanism of

digestion process (Rodiles et al. 2012). Similarly, silver catfish juveniles fed with tung meal appear to develop mechanisms in the digestive process in order to obtain nutrients in these diets that seem to be less digestible than others in this study. In addition to alterations in the activity of digestive enzymes, the increase of intestinal quotient observed from the sixth week also may be acting as a physiological compensatory mechanism. Kumar et al. (2011) also found that common carp fingerlings increased the intestine relative length with the inclusion of vegetable protein in the diet to increase digestibility from the highest retention time and contact of digestive enzymes with feed. This response also was observed in trout (*Oncorhynchus mykiss*) and seabream (*Sparus aurata*) after replacement of fish meal for vegetable protein (Santigosa et al. 2008). Now, animals fed treatments containing crambe meal showed very similar results between these when compared to control. This fact may be associated with a small reduction in the phytic acid and tannins concentration after treatment with commercial or non-commercial phytase on crambe meal. The reduction of antinutrients in treated crambe meals was not sufficient to provide changes in digestive response of animals between treatments. Similarly, the lowest concentration of tannin and fiber in crambe meal contributed the improved performance in weight gain compared to animals fed with treatments containing tung meal. Lower lipase and proteases activity may have contributed to lower uptake of amino acids and plasma protein formation and reduced energy molecules absorption as cholesterol in animals that received TG PHY treatment.

At the lowest concentration of glycogen and glucose in liver found in most of the experimental period in the animals fed with diets containing tung meal compared to control is related to the lower digestive enzyme activity, possibly caused by the presence of antinutrients in the diet. The lower uptake of energetic compounds in the diet leads to use of other nutrients for their production. For example, Lundstedt et al.

(2004) found that a diet with a lower protein content for *Pseudoplatystoma corruscans* decreased glycogen, glucose, pyruvate and lactate while increased the free amino acids content in the liver, suggesting that this class of nutrients supplied the metabolic demand for energy. Are involved in this process aminotransferases as ALT and AST which catabolize amino acids for transfer amino groups to alpha-keto acids reversibly (Lin and Luo 2011). Thus, the increase in the activity of these enzymes with higher ammonia excretion was correlated to the decrease in the protein efficiency when juvenile turbot (*Psetta máxima*) were fed with vegetable meals as partial substitutes for fish meal (including 20% in diet) (Fournier et al. 2004). In this study it was observed fluctuations in aminotransferases concentration in all treatments during the evaluation period. Compared to control, in some periods there was a reduction in the concentration of these enzymes in the animals that received treatments containing tung meal (both forms of enzyme treatment) and crambe meal treated with non-commercial phytase. The aminotransferases are specific for one or at most a few donors of amino groups. Commonly ALT acts on the amino group of the alanine and AST of the aspartate (Médale and Guillaume 2001). However, as noted in other studies, reduction in the activity of these enzymes can occur due to a decrease in available substrates for transamination, as a result of the reduced supply of these nutrients in the diet (Hansen et al. 2007; Lin and Luo 2011). The results of the present study seem to agree with these facts. As described by Cowey et al. (1981) replacement of fish meal by corn gluten or casein in diets for rainbow trout, increased the level of amino acids available for gluconeogenesis, lipogenesis or direct oxidation when used proteins of low biological value because protein synthesis was hampered by lack of one or more essential amino acids. Furthermore, according to Larsen et al. (2012) in *Oncorhynchus mykiss* juveniles fed with diet based on animal protein (fish meal and porcine blood meal), the absorption

was more synchronous while diets with partial replacement by a combination of vegetable meals (pea protein, wheat gluten, field beans, sunflower and soybean meal), absorption is less synchronized.

Thus, the lowest weight gain observed for juvenile silver catfish fed with crambe meal and more pronounced with tung meal may be related to impaired digestion and absorption of proteins and energetic compounds in these treatments compared to control diet. Additionally, animals seem to develop metabolic and physiological mechanisms of compensation to achieve a balance digestive and growth rates on diets with inclusion of vegetable meals.

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Table 1 Antinutrients analyzed in crambe and tung meals after enzymatic treatment with phytase or phytase and tannase.

	Crambe meal		Tung meal	
Antinutrientes	PHY	PHY+TAN	PHY	PHY+TAN
Total tannins	0.43±0.01	0.33±0.03*	0.87±0.01	0.85±0.01
Hydrolysable tannins	0.394±0.002	0.292±0.027*	0.861±0.002	0.841±0.012
Condensed tannins	0.035±0.002	0.035±0.001	0.012±0.001	0.012±0.001
Phytic acid	1.59±0.01	2.24±0.03*	0.79±0.04	1.88±0.03*

Values expressed as mean ± standard deviation (n=4). *Indicate difference of the meal treated with phytase in relation to the meal treated with phytase and tannase by F test (p<0.05).

Table 2 Formulation and proximate composition of the experimental diets (%).

Ingredients	Treatments[a]				
	EXP. I			EXP. II	
	CON	CR PHY	CR PHY+TAN	TG PHY	TG PHY+TAN
Fish meal	26	22.23	22.24	22.24	22.24
Meat and bone meal	30.50	24.85	24.85	24.85	24.85
Crambe meal+phytase	-	18.90	-	-	-
Crambe meal+phytase+tannase	-	-	19.00	-	-
Tung meal+phytase	-	-	-	24.36	-
Tung meal+phytase+tannase	-	-	-	-	24.78
Wheat flour	13.57	7.58	8.25	7.65	7.56
Corn grain	11.80	8.00	6.45	4.55	4.25
Corn starch	4.43	7.50	8.10	5.85	5.20
Soybean oil	5.80	4.90	5.88	7.45	7.95
Salt	0.50	0.50	0.50	0.50	0.50
Dicalcium phosphate	0.55	0.45	-	-	0.25
Vitamin/mineral mixture[b]	2.00	2.00	2.00	2.00	2.00
Methionine	0.40	0.40	0.40	0.42	0.42
Calcium carbonate	-	-	-	-	-
Inert	4.45	2.69	2.33	0.13	-
Diet composition (%)					
Crude protein[c]	36.00	36.00	36.00	35.73	35.70
Fat[c]	13.16	12.13	13.04	13.91	14.36
Ash[c]	14.42	13.26	13.31	13.34	13,33
Neutral detergent fiber[c]	7.68	10.23	10.05	16.46	16.64
Carbohydrates[d]	10.07	9.29	5.70	6.34	5.80
Digestible energy[e] (Kcal/Kg)	3.200	3.200	3.200	3.199	3.199
Calcium[c]	2.44	2.20	2.09	2.06	2.12
Total phosphorus[c]	1.49	1.36	1.30	1.23	1.29
Ca:P ratio	1.63	1.62	1.60	1.68	1.64
Lysine[c]	2.16	2.10	2.10	2.02	2.03
Methionine[c]	1.50	1.46	1.46	1.43	1.44

[a]CON=control diet, protein with the base consisting of animal meal; CR PHY=crambe meal inclusion treated with phytase; CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal inclusion treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase and tannase. [b]Vitamin/mineral (kg product): Folic acid: 300mg; Choline: 100g; Inositol: 10g; Niacin: 9000mg; Pantothenic acid: 3000mg; Biotin: 0.1mg; Vit.A: 1000000UI; Vit. B1: 1500mg; Vit. B2: 1500mg; Vit. B6: 150mg; Vit. B12: 2000mg; Vit. C: 15g; Vit. D3: 240000UI; Vit. E: 10000mg; Vit. K3: 400mg; Copper: 1000mg; Iron: 6000mg; Iodine: 45mg; Manganese: 8000mg; Selenium: 60mg; Zinc: 14g. [c]Calculated based on the ingredients analysis; [d]Neutral detergent soluble Carbohydrate calculated: 100-(crude protein+ash+fat+neutral detergent fiber +moisture). [e]Calculated from the formula: (Protein*5,64*0,83)+(fat*9,44*0,88)+(Carbohydrate*4,11*0,65))*10 (Meyer et al. 2004).

627 **Table 3** Digestive enzymes activity of silver catfish juvenile fed with a diet based in
628 animal protein (control) and diet containing crambe or tung meal treated with phytase or
629 with phytase and tannase.

Variables[§]	Treatments[‡]				
	EXP. I			EXP. II	
	CON	CR PHY	CR PHY+TAN	TG PHY	TG PHY+TAN
	Three weeks				
Amylase	0.15±0.02	0.15±0.03	0.19±0.03	0.25±0.04	0.16±0.03
Lipase	13.51±1.57	11.19±1.02	13.33±1.73	9.43±0.83[a]	9.15±0.88[a]
Acid protease	117.5±8.4	127.2±7.7	128.4±13.2	114.3±4.4	105.8±6.1
Trypsin	12.52±0.69	12.41±1.07	14.07±0.87	14.31±1.13	13.23±0.82
Chymotrypsin	11.64±0.67	10.20±0.77	11.37±0.61	9.26±0.55[a]	9.34±0.61[a]
	Six weeks				
Amylase	0.15±0.03	0.14±0.01	0.13±0.02	0.20±0.03	0.18±0.02
Lipase	14.38±1.13	14.67±1.13	16.83±1.09	13.11±0.72	14.67±1.38
Acid protease	116.3±6.7	112.3±4.7	109.5±6.2	96.1±5.4[a]	92.4±4.7[a]
Trypsin	11.63±0.87	11.27±0.69	11.12±0.56	13.61±1.73	15.61±1.20
Chymotrypsin	9.85±0.70	10.26±0.65	10.66±0.72	12.78±1.16	13.93±1.02[a]
	Nine weeks				
Amylase	0.20±0.01	0.15±0.02	0.20±0.02	0.16±0.01	0.19±0.02
Lipase	13.46±0.57	11.70±0.66	10.85±0.85[A]	9.08±0.52[a]	9.88±0.48[a]
Acid protease	78.4±4.5	87.5±8.3	74.9±7.5	95.0±5.4	92.0±5.2
Trypsin	7.07±0.39	7.06±0.58	7.15±0.49	6.15±0.38	7.68±0.30*
Chymotrypsin	8.58±0.24	8.50±0.52	8.14±0.55	7.84±0.30	9.16±0.47*

630 [‡]CON=control diet, protein with the base consisting of animal meal; CR PHY=crambe meal inclusion treated
631 with phytase; CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal
632 inclusion treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase and tannase.
633 [1]Amylase=µmol glucose hydrolysed/min/mg protein; lipase=µg substrate hydrolysed/min/mg protein; acid
634 protease=µg tyrosine hydrolyse/min/mg protein; trypsin=µmol TAME hydrolysed/min/mg protein;
635 chymotrypsin=mmol BTEE hydrolysed/min/mg protein. Values expressed as mean ± standard error of mean
636 (n=8). Capital letter indicate difference in I experiment (CR PHY or CR PHY+TAN) and lower case indicate
637 difference in II experiment (TG PHY or TG PHY+TAN) in relation to the control by Dunnett's test
638 (p<0.05).*Indicate difference of the meal treated with phytase in relation to the meal treated with phytase and
639 tannase by F test (p<0.05).

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Table 4 Plasmatic biochemistry of silver catfish juvenile fed with a diet based in animal protein (control) and diet containing crambe or tung meal treated with phytase or with phytase and tannase.

Variables[§]	Treatments[‡]				
		EXP. I		EXP. II	
	CON	CR PHY	CR PHY+TAN	TG PHY	TG PHY+TAN
			Six weeks		
Glucose	42.7±2.1	41.4±2.4	45.5±1.5	39.2±2.6	41.1±3.5
Total protein	3.80±0.16	3.45±0.08	4.21±0.20*	3.63±0.16	3.75±0.16
Cholesterol	190.1±12.9	152.1±10.8	185.5±20.0	154.7±13.6	172.7±12.8
			Nine weeks		
Glucose	40.4±2.9	42.4±1.0	41.2±2.1	34.1±2.9	38.0±1.7
Total protein	4.09±0.16	3.75±0.17	3.62±0.07	3.15±0.17[a]	3.60±0.18
Cholesterol	191.6±8.9	159.0±15.4	167.2±9.8	125.4±10.9[a]	161.4±12.3*

[‡]CON=control diet, protein with the base consisting of animal meal; CR PHY=crambe meal inclusion treated with phytase; CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal inclusion treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase and tannase.[1]Glucose, cholesterol=mg/dL; total protein=g/dL. Values expressed as mean ± standard error of mean (n=8). Capital letter indicate difference in I experiment (CR PHY or CR PHY+TAN) and lower case indicate difference in II experiment (TG PHY or TG PHY+TAN) in relation to the control by Dunnett's test ($p<0.05$).*Indicate difference of the meal treated with phytase in relation to the meal treated with phytase and tannase by F test ($p<0.05$).

668 **Table 5** Hepatic metabolism of silver catfish juvenile fed with a diet based in animal
669 protein (control) and diet containing crambe or tung meal treated with phytase or with
670 phytase and tannase.

Variables[§]	Treatments[‡]				
	EXP. I			EXP. II	
	CON	CR PHY	CR PHY+TAN	TG PHY	TG PHY+TAN
	Three weeks				
Glycogen	280.1±35.7	331.4±51.7	236.8±30.3	157.2±18.2[a]	121.1±12.3[a]
Glucose	381.4±20.5	273.7±24.5[A]	299.0±25.2	174.6±21.5[a]	140.5±25.1[a]
Protein	69.7±5.7	77.8±6.9	75.9±4.4	69.8±4.8	65.3±3.0
ALT	56.6±4.8	62.9±4.9	25.9±3.1[A]*	49.6±13.2	45.1±3.2
AST	1105.6±24.0	1264.3±64.9	1018.2±21.7*	1349.6±109.9	1078.3±20.7
	Six weeks				
Glycogen	202.6±28.0	174.9±18.6	442.4±28.9[A]*	85.3±11.0[a]	155.2±19.0*
Glucose	311.5±29.0	325.3±24.5	499.3±17.6[A]*	288.3±27.1	232.3±15.2
Protein	41.1±2.8	44.6±3.3	38.5±1.7	43.2±1.5	47.6±1.5
ALT	31.9±2.5	35.0±3.2	21.1±2.6[a]*	41.2±3.1	14.9±2.6[a]*
AST	880.4±59.6	729.8±61.7	996.7±42.8*	753.1±40.8	853.2±44.5
	Nine weeks				
Glycogen	573.3±45.7	487.3±23.8	587.9±20.7*	322.9±36.6[a]	349.1±22.2[a]
Glucose	490.1±15.8	377.6±20.0[A]	489.3±25.4*	301.0±15.2[a]	309.8±9.4[a]
Protein	57.5±1.5	50.2±2.4	48.6±2.5[A]	53.0±2.7	55.3±1.5
ALT	79.6±4.7	72.4±4.7	75.7±4.3	77.7±9.8	48.8±4.9[a]*
AST	1397.7±55.2	1285.9±60.8	1255.9±83.9	997.4±70.5[a]	1004.7±109.3[a]

671 [‡]CON=control diet, protein with the base consisting of animal meal; CR PHY=crambe meal inclusion treated
672 with phytase; CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal
673 inclusion treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase and
674 tannase.[1]Glycogen, glucose=µmol glucose/g tissue; protein=mg/g tissue; ALT (alanine aminotransferase),
675 AST (aspartate aminotransferase)=UI/mg tissue. Values expressed as mean ± standard error of mean (n=8).
676 Capital letter indicate difference in I experiment (CR PHY or CR PHY+TAN) and lower case indicate
677 difference in II experiment (TG PHY or TG PHY+TAN) in relation to the control by Dunnett's test
678 (p<0.05).*Indicate difference of the meal treated with phytase in relation to the meal treated with phytase and
679 tannase by F test (p<0.05).

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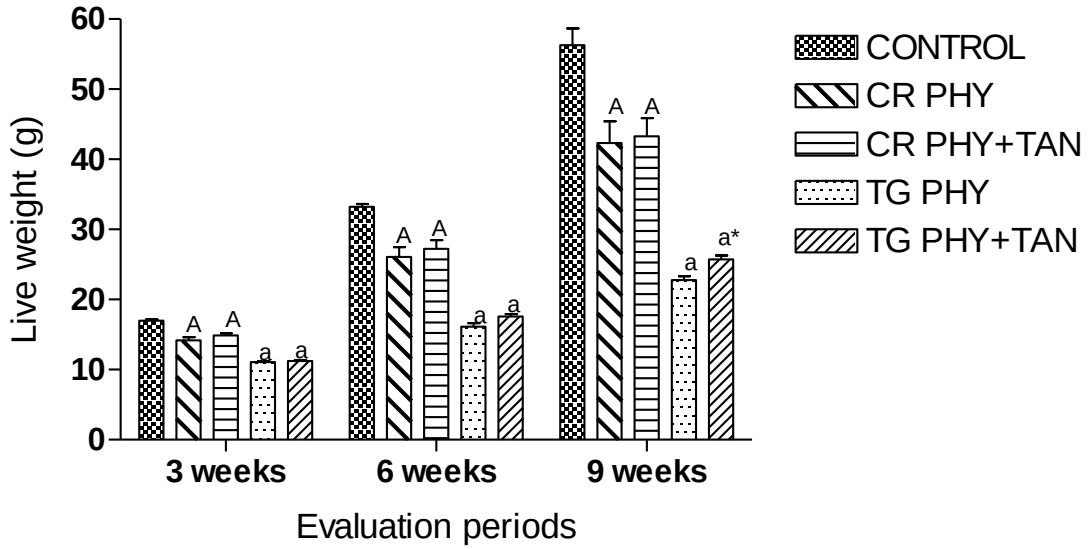

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688 **Figure 1** Live weight of silver catfish juvenile fed with a diet based in animal protein (control) and diet
689 containing crambe or tung meal treated with phytase or with phytase and tannase. CON=control diet, protein
690 with the base consisting of animal meal; CR PHY=crambe meal inclusion treated with phytase; CR
691 PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal inclusion treated
692 with phytase. Values expressed as mean ± standard error of mean (n=4). Capital letter indicate difference in I
693 experiment (CR PHY or CR PHY+TAN) and lower case indicate difference in II experiment (TG PHY or
694 TG PHY+TAN) in relation to the control by Dunnett's test (p<0.05).*Indicate difference of the meal treated
695 with phytase in relation to the meal treated with phytase and tannase by F test (p<0.05).

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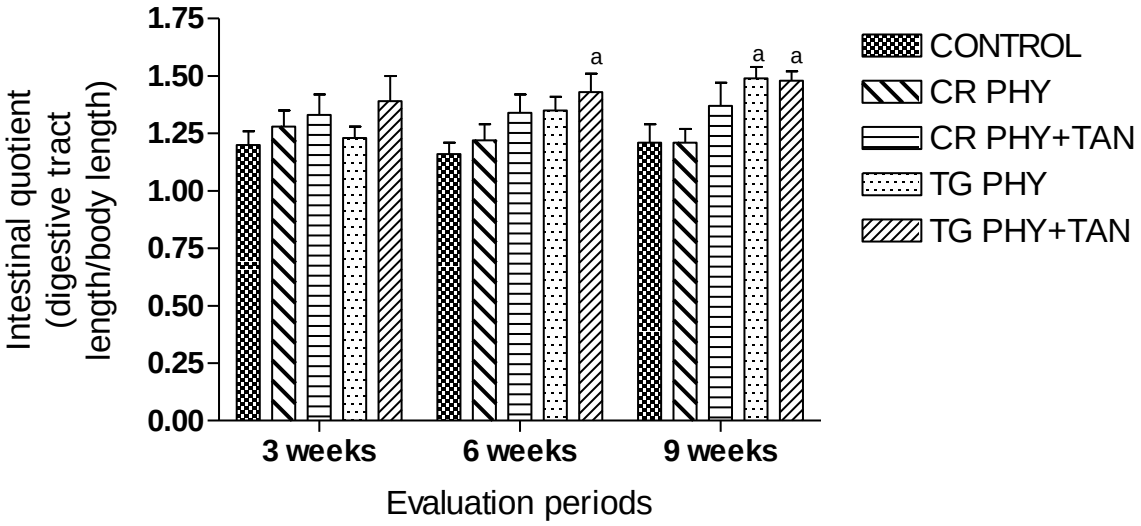

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709 **Figure 2** Intestinal quotient of silver catfish juvenile fed with a diet based in animal protein (control) and
710 diet containing crambe or tung meal treated with phytase or with phytase and tannase. CON=control diet,
711 protein with the base consisting of animal meal; CR PHY=crambe meal inclusion treated with phytase;
712 CR PHY+TAN=crambe meal inclusion treated with phytase and tannase; TG PHY=tung meal inclusion
713 treated with phytase; TG PHY+TAN=tung meal inclusion treated with phytase and tannase. Values
714 expressed as mean ± standard error of mean (n=4). Capital letter indicate difference in I experiment (CR
715 PHY or CR PHY+TAN) and lower case indicate difference in II experiment (TG PHY or TG PHY+TAN)
716 in relation to the control by Dunnett's test (p<0.05).*Indicate difference of the meal treated with phytase
717 in relation to the meal treated with phytase and tannase by F test (p<0.05).

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6 DISCUSSÃO GERAL

Os farelos vegetais são apontados como as principais alternativas de fontes de proteína de ampla disponibilidade, baixo custo e com potencial nutricional de substituir a farinha de peixe, a principal fonte proteica empregada na dieta de espécies aquícolas, que apresenta um ritmo de produção mais lento em relação à quantidade demandada pela indústria (ESPE et al., 2012). As restrições ao uso dos farelos vegetais podem ser minimizadas a partir da extração e/ou inativação dos principais antinutrientes, através de técnicas de processamento ou pré-tratamento dos ingredientes, alcançando melhorias no valor nutricional destas fontes proteicas.

A substituição de fontes proteicas de origem animal pelas formas *in natura*/integral ou detoxificada com tratamento químico nos farelos de tungue ou crambe, na alimentação do jundiá (artigo 2), revelou crescimento inferior nos animais alimentados com as dietas contendo farelo de tungue (ambas as formas) até o final do experimento em relação ao tratamento controle. Resultados semelhantes também foram encontrados na criação de salmão (*Salmo salar*), seabass (*Lateolabrax japonicus)* e turbot (*Psetta máxima*) após a inclusão de farelos vegetais na alimentação destas espécies (FOURNIER et al., 2004; CHENG et al., 2010; PRATOOMYOT et al., 2010). A avaliação de grupos de interesse (nutrientes e antinutrientes) nos farelos de tungue e crambe (artigo 1) revelou que a forma *in natura* do farelo de tungue apresentou teores menores que o crambe em relação a proteína (-26%), matéria mineral (-13%), gordura (-44%), cálcio (-33%) e fósforo (-35%) e valor 40% superior em termos de FDN. Entre os antinutrientes, o farelo de tungue apresenta maior concentração de compostos fenólicos, taninos totais e taninos hidrolisáveis (20, 39 e 41,5%, respectivamente) e menor teor de taninos condensados e ácido fítico (-37 e -16%, respectivamente) em relação ao farelo de crambe. Assim, para substituir 20% da proteína de origem animal, a inclusão média de farelo de tungue foi de 35%, o que conferiu à estes tratamentos os teores mais elevados de FDN e possivelmente de antinutrientes. No entanto, a forma detoxificada do farelo de tungue propiciou maior crescimento e 100% de sobrevivência em relação à forma integral, onde foi observada sobrevivência de 68%. Os peixes alimentados com farelo de tungue tratado na dieta, apesar de demonstrarem em alguns momentos alterações digestivas como menor atividade de lipase e metabólicas, como redução de glicogênio e glicose hepáticos e aumento de proteína e amônia neste tecido, ao final do

ensaio, apresentaram crescimento semelhante ao tratamento controle. Conforme mencionado no artigo 1, o tratamento ácido-alcoólico aplicado sobre o farelo de tungue resultou em composição semelhante quanto a proteína bruta e cinzas, mas reduziu gordura (-66%), cálcio (-32%), fósforo (-56%), taninos condensados (-28%) e ácido fítico (-62%) e aumentou FDN (11%), fenóis totais (12%), taninos totais (9,75%) e taninos hidrolisáveis (10,5%). Apesar do aumento observado em antinutrientes da classe dos compostos fenólicos, possivelmente associado ao aumento de FDN, o tratamento ácido alcoólico permitiu a redução de outras moléculas antinutricionais analisadas e pode ter removido também compostos tóxicos como ésteres de forbol encontrados em espécies da família Euphorbiaceae, a qual pertence o tungue. Outros pesquisadores também revelam que após completa retirada de ésteres de forbol do farelo de *Jatropha curcas*, exemplares de *Cyprinus carpio* apresentaram crescimento similar ao controle e melhor que os demais grupos em que o farelo não foi completamente detoxificado, (tempo de tratamento com solventes orgânicos de 60 minutos em comparação ao tempo de 30 minutos) (KUMAR et al., 2010; KUMAR et al., 2011).

O menor crescimento dos peixes no tratamento com inclusão de farelo de tungue integral também pode ser atribuído a um desbalanceado perfil de aminoácidos vindo da dieta (ESPE et al., 2012). Assim, acompanhando este resultado foram observadas as maiores alterações em relação a atividade de enzimas digestivas com redução de lipase, protease ácida e aumento de amilase intestinal. Neste tratamento os animais apresentaram reduzido consumo de alimento, possivelmente devido a baixa palatabilidade da ração, que associado a redução na ação de enzimas digestivas, acarretou uma carência na absorção de nutrientes e precursores energéticos sinalizada pela redução nas concentrações plasmáticas de aminoácidos, colesterol e triglicerídeos. Esta condição influenciou o estoque de glicogênio hepático e a concentração de glicose, que nestes animais sempre estiveram em concentrações reduzidas em relação aos peixes do tratamento controle. Para contornar esta carência energética ou até mesmo para manter a glicemia em alguns momentos, a utilização de aminoácidos para a produção de glicose através do processo de gliconeogênese possivelmente ocorreu nestes animais no decorrer do estudo. Corroboram com esta afirmativa o aumento na concentração das transaminases (ALT ou AST), amônia e proteína no fígado. Fournier et al. (2004) também observaram que juvenis de turbot (*P. máxima*) aumentaram a excreção de amônia acompanhando a inclusão de farelos vegetais na dieta, sugerindo aumento no catabolismo protéico. De acordo com Hansen et al. (2007) o aumento nas concentrações de amônia podem ser um indicador de reduzida síntese de proteínas, expressa por menor crescimento e retenção de proteínas. No tratamento contendo farelo de tungue integral também ocorreram algumas

alterações somáticas como o menor índice hepatossomático, que pode ser relacionado à redução na estocagem de glicogênio no fígado e menor quociente intestinal e índice de gordura visceral, possivelmente associado à menor ingestão de alimento e captação de nutrientes. As alterações digestivas e metabólicas no tratamento com inclusão de tungue integral influenciaram na deposição de gordura e cinzas na carcaça, resultando respectivamente, em menor e maior percentual em relação ao controle e ao tratamento com tungue na forma tratada.

Nos tratamentos com inclusão de farelo de crambe os animais apresentaram crescimento semelhante ao controle ao longo do estudo. Possivelmente este desempenho se deve às características nutricionais deste farelo, que conforme verificado no artigo 1 apresentou maior concentração de nutrientes (principalmente proteína) e menor teor de compostos fenólicos e taninos em relação ao farelo de tungue, sendo incluído em menores percentuais na dieta de jundiás. Alterações não persistentes ocorreram em relação a atividade das enzimas digestivas como o aumento na ação de lipase após seis semanas de alimentação com o farelo de crambe tratado e redução de protease no tratamento com inclusão da forma integral do farelo ao final do estudo. Os animais recebendo o tratamento contendo farelo de crambe tratado apresentaram no fígado menor concentração de glicogênio e glicose a partir da sexta semana de alimentação, sendo esta alteração persistente até o final do estudo e no último período de avaliação também foi verificado aumento de proteína e AST possivelmente indicando que poderá ocorrer uma condição de gliconeogênese, a partir da utilização de aminoácidos. Conforme verificado, após o tratamento químico aplicado no farelo de crambe, ocorreu redução de proteína (-21%), gordura (-20%), cálcio (-47%), fósforo (-37%) fenóis totais (-41%), taninos totais (-32%), taninos condensados (-75%), taninos hidrolisáveis (-29%) e ácido fítico (-38%) e aumento de cinzas (50%) e FDN (15%). Por isso, a inclusão do farelo tratado (25%) foi superior à inclusão do farelo integral (20,84%) contribuindo para aumentar o teor de FDN neste tratamento, porém ainda mantendo menores as concentrações de antinutrientes. Apesar de não ocorrer alterações nos parâmetros de crescimento entre os tratamentos contendo farelo de crambe, algumas alterações somáticas foram verificadas no tratamento com farelo de crambe tratado como menor índice hepatossomático, resultado que concorda com o menor valor de glicogênio hepático. Neste tratamento também foi verificado menor índice digestivossomático e quociente intestinal. Seu maior teor de FDN pode ter aumentado a velocidade do trânsito gastrointestinal, reduzindo o aproveitamento de nutrientes e a manutenção das reservas energéticas. Contrariamente, Santigosa et al. (2008) observaram que exemplares de truta (*Oncorhynchus mykiss*) e seabream (*Sparus aurata*) desenvolveram

um mecanismo compensatório à inibição da atividade das enzimas digestivas após a substituição da farinha de peixe por fontes protéicas vegetais que foi o aumento no comprimento intestinal. Nos tratamentos contendo farelo de crambe (ambas as formas), as alterações digestivas e metabólicas não causaram mudanças em relação aos nutrientes depositados na carcaça em comparação ao tratamento controle.

No segundo ensaio biológico, o desempenho de jundiás alimentados com os farelos de tungue ou crambe tratados enzimaticamente com fitase comercial (Natuphos) ou enzima não comercial com ação de fitase e tanase (artigo 3) seguiu a mesma tendência observada no primeiro ensaio, em que os peixes tratados com farelo de tungue também demonstraram menor crescimento em comparação ao controle e os animais recebendo as dietas com farelo de crambe mostram maior crescimento percentual em relação aos anteriores, porém agora não atingiram crescimento semelhante ao controle no decorrer do estudo. Vielma et al. (2000) observaram que o tratamento de proteínas derivadas de soja (farelo e concentrado protéico) com fitase, em substituição à farinha de peixe não influenciou o crescimento de exemplares de truta (*O. mykiss*). No segundo experimento foram desenvolvidas dietas contendo maior inclusão de farinha de peixe a fim de atingir maior percentual protéico, pois trabalhou-se com animais mais jovens (peso inicial de 7,6g) e em condições de temperatura de água mais elevada (26ºC). Estas condições podem ter possibilitado crescimento mais acelerado, principalmente dos animais do tratamento controle desde o primeiro período de avaliação (3 semanas) e mantendo esta tendência até o final do estudo. No primeiro experimento o baixo crescimento até mesmo dos peixes recebendo o tratamento controle possivelmente está associado à formulação das dietas (com menor teor protéico e inclusão de farinha de peixe), temperatura mais baixa da água (22ºC) e animais em estágio de desenvolvimento mais avançado, em que são necessários experimentos de maior duração para que os animais expressem as diferenças no crescimento de acordo com a dieta. Piedras et al. (2004) observaram que juvenis de jundiá (média de 24g) quando expostos a temperaturas da água entre 20 a 26ºC, apresentam o melhor crescimento na temperatura de 23,7ºC e sinalizam que o ótimo de temperatura de exemplares jovens é mais elevado que o de adultos.

O tratamento enzimático realizado com fitase comercial reduziu 29% do ácido fítico em relação ao farelo de crambe tratado com a enzima não comercial, em que as concentrações deste antinutriente permaneceram semelhantes à amostra integral do farelo de crambe. A enzima não comercial reduziu 23 e 25% a concentração de taninos totais e hidrolisáveis, mas não alterou o teor de fenóis totais, taninos condensados e ácido fítico. Os peixes alimentados com as dietas contendo farelo de crambe (ambos os tratamentos enzimáticos) não

apresentaram diferenças entre si em relação aos parâmetros de crescimento avaliados ao longo do estudo. Quanto aos índices zootécnicos somente foi observado maior índice hepatossomático para os animais do tratamento contendo farelo de crambe tratado com enzima não comercial após 6 semanas, resultado que pode estar associado a maior estocagem de glicogênio encontrada no fígado neste período. Nos tratamentos contendo farelo de crambe ao final do estudo, os animais já apresentavam taxa de eficiência protéica semelhante aos animais do controle. Em relação a atividade das enzimas digestivas, nas dietas contendo farelo de crambe somente foi observado menor ação de lipase na dieta contendo o farelo tratado com enzima não comercial após 9 semanas, comparado ao controle. Quanto ao metabolismo energético e protéico no fígado, na dieta contendo o farelo tratado com fitase comercial ocorreu redução de glicose hepática, mas o tratamento com enzima não comercial resultou em alterações mais persistentes como o acúmulo de glicogênio e altos níveis de glicose e baixas concentrações de ALT. O acúmulo de reservas energéticas não parece estar relacionado com uma situação gliconeogênica, pois a concentração das transaminases mostrou-se reduzida ou não diferiu do controle. A menor concentração das transaminases neste caso pode indicar uma redução nos substratos disponíveis para transaminação como uma consequência do reduzido nível destes aminoácidos na dieta, situação também observada em estudo desenvolvido por Hansen et al (2007) e Lin e Luo (2011). As alterações metabólicas não acarretaram diferenças sobre a composição corporal em nutrientes, porém os índices de deposição de proteína, gordura e coeficiente de retenção protéica foram inferiores em relação ao controle, fatos que estão relacionados provavelmente a um desbalanço de aminoácidos provenientes da dieta, resultando em menor síntese proteica. Os resultados de desempenho muito similares entre os animais alimentados com ambas as formas do farelo de crambe tratado enzimaticamente podem ser decorrentes do nível próximo de antinutrientes, incapaz de proporcionar alterações significativas entre os dois tratamentos.

Sobre o farelo de tungue a fitase comercial reduziu 58% da concentração de ácido fítico em relação ao tratamento com a enzima não comercial, que não alterou o teor deste e dos demais antinutrientes analisados. Até a terceira semana de alimentação observou-se resultados semelhantes em todos os parâmetros de crescimento e índices somáticos analisados para ambos os tratamentos contendo farelo de tungue tratado com enzimas. Porém, a partir da sexta semana observa-se melhor conversão alimentar, maior taxa de crescimento específico e quociente intestinal nos animais do tratamento em que foi incluído o farelo tratado com a enzima não comercial. Esta melhoria em alguns indicadores de crescimento permaneceu até o final do estudo, porém no último período de avaliação o aumento no quociente intestinal

estendeu-se também aos animais alimentados com a dieta contendo o farelo tratado com a enzima comercial. Após três semanas de alimentação, ambos os tratamentos contendo farelo de tungue acarretaram redução na atividade de quimotripsina; após seis semanas, redução de protease e aumento de quimotripsina; e ao final do estudo houve redução na ação de lipase intestinal. No plasma a menor atuação das enzimas reduziu a captação de substratos energéticos e no fígado reduziu o estoque de glicogênio e a liberação de glicose em relação ao tratamento controle. A redução na atividade de enzimas é observada em vários estudos à medida que se eleva a inclusão de farelos vegetais na dieta sendo associada e presença de antinutrientes (CHENG et al., 2010; KUMAR et al., 2011; RODILES et al., 2012). A menor ação das enzimas digestivas também é relacionada ao menor crescimento e digestibilidade e absorção de nutrientes. Nossos resultados estão de acordo com estas afirmativas, pois foram verificadas as maiores alterações enzimáticas e metabólicas nos tratamentos contendo o farelo de tungue, podendo-se sugerir que este ingrediente apresenta menor valor nutricional em relação ao farelo de crambe. Desta forma, os juvenis de jundiá desenvolveram alguns mecanismos de compensação quando alimentados com as dietas contendo farelo de tungue como aumentar a atividade de uma enzima digestiva em resposta a menor ação das demais, aumentar a área de contato dos substratos com as enzimas através do aumento no comprimento relativo do intestino, melhorando a captação de nutrientes da dieta para permitir uma taxa de crescimento, mesmo que mais reduzida em relação à dieta controle. Semelhantes mecanismos de compensação foram observados em juvenis de tilápia (*Oreochromis niloticus x O. aureus*), truta (*O. mykiss*) e *Solea senegalensis* (SANTIGOSA et al., 2008; LIN; LUO, 2011; RODILES et al., 2012).

7 CONCLUSÕES GERAIS

Com base nos resultados obtidos neste trabalho, pode-se concluir que:

- A análise nutricional e antinutricional realizada sobre os farelos revelou que o farelo de crambe apresenta maior concentração nutricional e menor teor em vários dos antinutrientes analisados, sendo apontado como um ingrediente de melhor valor nutricional na alimentação de peixes como o jundiá;
- O tratamento químico aplicado sobre os farelos de tungue e crambe retirou parte dos antinutrientes nestes farelos, mas também removeu algumas frações nutricionais;
- O tratamento químico aplicado sobre os farelos de tungue e crambe não alterou a digestibilidade protéica destes ingredientes, verificada *in vitro*;
- A retirada de antinutrientes através da via química proporcionou melhorias no desempenho e sobrevivência dos animais alimentados com o farelo de tungue nesta forma em relação à inclusão do farelo de tungue integral na dieta de jundiás, ocasionando alterações digestivas e metabólicas mais brandas. Para o farelo de crambe o tratamento químico não propiciou melhorias no desempenho em relação à forma integral e mínimas alterações digestivas e metabólicas foram observadas, sugerindo que este ingrediente pode ser incorporado na dieta sem a aplicação prévia do tratamento químico para extração de antinutrientes;
- As enzimas fitase comercial e não comercial (ação de fitase e tanase) atuaram de forma diferenciada de acordo com o farelo; sobre o crambe não ocorreram diferenças no desempenho e leves alterações digestivas e metabólicas indicando que a atuação das enzimas não modificou suficientemente a concentração dos antinutrientes a fim de serem verificadas melhorias; sobre o tungue, a enzima não comercial não demonstrou atuação em relação aos antinutrientes, mas sugere-se que o desenvolvimento de mecanismos digestivos compensatórios pelos animais deste tratamento possibilitou melhor crescimento e menores alterações metabólicas em relação ao tratamento com fitase comercial;
- A via química ou enzimática de tratamento aplicada sobre os farelos de tungue e crambe não alterou o valor nutricional da carne dos peixes criados em relação a concentração de proteína bruta.

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ANEXOS

ANEXO 1 – Normas de publicação da Revista Semina:Ciências Agrárias

Categorias dos Trabalhos

a) Artigos científicos: no máximo 20 páginas incluindo figuras, tabelas e referências bibliográficas;

b) Comunicações científicas: no máximo 12 páginas, com referências bibliográficas limitadas a 16 citações e no máximo duas tabelas ou duas figuras ou uma tabela e uma figura;

b) Relatos de casos: No máximo 10 páginas, com referências bibliográficas limitadas a 12 citações e no máximo duas tabelas ou duas figuras ou uma tabela e uma figura;

c) Artigos de revisão: no máximo 25 páginas incluindo figuras, tabelas e referências bibliográficas.

Apresentação dos Trabalhos

Os originais completos dos artigos, comunicações, relatos de casos e revisões podem ser escritos em português, inglês ou espanhol, no editor de texto Word for Windows, com espaçamento 1,5, em papel A4, fonte Times New Roman, tamanho 11 normal, com margens esquerda e direita de 2 cm e superior e inferior de 2 cm, respeitando-se o número de páginas, devidamente numeradas, de acordo com a categoria do trabalho. Figuras (desenhos, gráficos e fotografias) e Tabelas serão numeradas em algarismos arábicos e devem estar separadas no final do trabalho.

As figuras e tabelas deverão ser apresentadas nas larguras de 8 ou 16 cm com altura máxima de 22 cm, lembrando que se houver a necessidade de dimensões maiores, no processo de editoração haverá redução para as referidas dimensões. As legendas das figuras deverão ser colocadas em folha separada obedecendo à ordem numérica de citação no texto. Fotografias devem ser identificadas no verso e desenhos e gráfico na parte frontal inferior pelos seus respectivos números do texto e nome do primeiro autor. Quando necessário deve ser indicado qual é a parte superior da figura para o seu correto posicionamento no texto.

Preparação dos manuscritos

Artigo científico: Deve relatar resultados de pesquisa original das áreas afins, com a seguinte organização dos tópicos: Título; Título em inglês; Resumo com Palavras-chave (no máximo seis palavras); Abstract com Key words (no máximo seis palavras); Introdução; Material e Métodos; Resultados e Discussão com as conclusões no final ou Resultados, Discussão e Conclusões separadamente; Agradecimentos; Fornecedores, quando houver e Referências Bibliográficas. Os tópicos devem ser escritos em letras maiúsculas e minúsculas e destacados em negrito, sem numeração. Quando houver a necessidade de subitens dentro dos tópicos, os mesmos devem receber números arábicos. O trabalho submetido não pode ter sido publicado em outra revista com o mesmo conteúdo, exceto na forma de resumo de congresso, nota prévia ou formato reduzido.

A apresentação do trabalho deve obedecer à seguinte ordem:

1. *Título do trabalho,* acompanhado de sua tradução para o inglês.
2. *Resumo e Palavras-chave*: Deve ser incluído um resumo informativo com um mínimo de 150 e um máximo de 300 palavras, na mesma língua que o artigo foi escrito, acompanhado de sua tradução para o inglês (*Abstract e Key words*).
3. *Introdução*: Deverá ser concisa e conter revisão estritamente necessária à introdução do tema e suporte para a metodologia e discussão.

4. *Material e Métodos*: Poderá ser apresentado de forma descritiva contínua ou com subitens, de forma a permitir ao leitor a compreensão e reprodução da metodologia citada com auxílio ou não de citações bibliográficas.
5. *Resultados e discussão com conclusões ou Resultados, Discussão e Conclusões:* De acordo com o formato escolhido, estas partes devem ser apresentadas de forma clara, com auxílio de tabelas, gráficos e figuras, de modo a não deixar dúvidas ao leitor, quanto à autenticidade dos resultados, pontos de vistas discutidos e conclusões sugeridas.
6. *Agradecimentos*: As pessoas, instituições e empresas que contribuíram na realização do trabalho deverão ser mencionadas no final do texto, antes do item Referências Bibliográficas.

Observações:
Quando for o caso, antes das referências, deve ser informado que o artigo foi aprovado pela comissão de bioética e foi realizado de acordo com as normas técnicas de biossegurança e ética.
Notas: Notas referentes ao corpo do artigo devem ser indicadas com um símbolo sobrescrito, imediatamente depois da frase a que diz respeito, como notas de rodapé no final da página.
Figuras: Quando indispensáveis figuras poderão ser aceitas e deverão ser assinaladas no texto pelo seu número de ordem em algarismos arábicos. Se as ilustrações enviadas já foram publicadas, mencionar a fonte e a permissão para reprodução.
Tabelas: As tabelas deverão ser acompanhadas de cabeçalho que permita compreender o significado dos dados reunidos, sem necessidade de referência ao texto.
Grandezas, unidades e símbolos: Deverá obedecer às normas nacionais correspondentes (ABNT).
7. Citações dos autores no texto: Deverá seguir o sistema de chamada alfabética seguidas do ano de publicação de acordo com os seguintes exemplos:

a) Os resultados de Dubey (2001) confirmam que .....
b) De acordo com Santos et al. (1999), o efeito do nitrogênio.....
c) Beloti et al. (1999b) avaliaram a qualidade microbiológica.....
d) [...] e inibir o teste de formação de sincício (BRUCK et. al., 1992).
e) [...] comprometendo a qualidade de seus derivados (AFONSO; VIANNI, 1995).

Citações com três autores
Dentro do parêntese, separar por ponto e vírgula.
Ex: (RUSSO; FELIX; SOUZA, 2000).
Incluídos na sentença, utilizar vírgula para os dois primeiros autores e (e) para separar o segundo do terceiro.
Ex: Russo, Felix e Souza (2000), apresentam estudo sobre o tema....

Citações com mais de três autores
Indicar o primeiro autor seguido da expressão et al.
Observação: Todos os autores devem ser citados nas Referências Bibliográficas.
8. Referências Bibliográficas: As referências bibliográficas, redigidas segundo a norma NBR 6023, ago. 2000, da ABNT, deverão ser listadas na ordem alfabética no final do artigo. Todos os autores participantes dos trabalhos deverão ser relacionados, independentemente do número de participantes (única exceção à norma – item 8.1.1.2). A exatidão e adequação das referências a trabalhos que tenham sido consultados e mencionados no texto do artigo, bem como opiniões, conceitos e afirmações são da inteira responsabilidade dos autores.

As outras categorias de trabalhos (Comunicação científica, Relato de caso e Revisão) deverão seguir as mesmas normas acima citadas, porem, com as seguintes orientações adicionais para cada caso:

Comunicação científica: Uma forma concisa, mas com descrição completa de uma pesquisa pontual ou em andamento (nota prévia), com documentação bibliográfica e metodologia completas, como um artigo científico regular. Deverá conter os seguintes tópicos: Título (português e inglês); Resumo com Palavras-chave; Abstract com Key words; Corpo do trabalho sem divisão de tópicos, porém seguindo a seqüência – introdução, metodologia, resultados (podem ser incluídas tabelas e figuras), discussão, conclusão e referências bibliográficas.

Relato de caso: Descrição sucinta de casos clínicos e patológicos, achados inéditos, descrição de novas espécies e estudos de ocorrência ou incidência de pragas, microrganismos ou parasitas de interesse agronômico, zootécnico ou veterinário. Deverá conter os seguintes tópicos: Título (português e inglês); Resumo com Palavras-chave; Abstract com Key-words; Introdução com revisão da literatura; Relato do (s) caso (s), incluindo resultados, discussão e conclusão; Referências Bibliográficas.

Artigo de revisão bibliográfica: Deve envolver temas relevantes dentro do escopo da revista. O número de artigos de revisão por fascículo é limitado e os colaboradores poderão ser convidados a apresentar artigos de interesse da revista. No caso de envio espontâneo do autor (es), é necessária a inclusão de resultados relevantes próprios ou do grupo envolvido no artigo, com referências bibliográficas, demonstrando experiência e conhecimento sobre o tema. O artigo de revisão deverá conter os seguintes tópicos: Título (português e inglês); Resumo com Palavras-chave; Abstract com Key-words; Desenvolvimento do tema proposto (com subdivisões em tópicos ou não); Conclusões ou Considerações Finais; Agradecimentos (se for o caso) e Referências Bibliográficas.

Outras informações importantes

1 A publicação dos trabalhos depende de pareceres favoráveis da assessoria científica "*Ad hoc*" e da aprovação do Comitê Editorial da Semina: Ciências Agrárias, UEL.

2. Não serão fornecidas separatas aos autores, uma vez que os fascículos estarão disponíveis no endereço eletrônico da revista (http://www.uel.br/revistas/uel).

3. Os trabalhos não aprovados para publicação serão devolvidos ao autor.

4. Transferência de direitos autorais: Os autores concordam com a transferência dos direitos de publicação do referido artigo para a revista. A reprodução de artigos somente é permitida com a citação da fonte e é proibido o uso comercial das informações.

5. As questões e problemas não previstos na presente norma serão dirimidos pelo Comitê Editorial da área para a qual foi submetido o artigo para publicação.

6. Informações devem ser dirigidas a:

Universidade Estadual de Londrina Centro de Ciências Agrárias Departamento de Medicina Veterinária Preventiva Comitê Editorial da Semina Ciências Agrárias	**ou Universidade Estadual de Londrina** Coordenadoria de Pesquisa e Pós-graduação Conselho Editorial das revistas Semina Campus Universitário - Caixa Postal 600186051-990

Campus Universitário - Caixa Postal 600186051-990 Londrina, Paraná, Brasil. Informações: Fone: 0xx43 33714709 Fax: 0xx43 33714714 Emails: vidotto@uel.br; csvjneve@uel.br	Londrina, Paraná, Brasil. Informações: Fone: 0xx43 33714105 Fax: Fone 0xx43 3328 4320 Emails: eglema@uel.br;

Condições para submissão:
Como parte do processo de submissão, os autores são obrigados a verificar a conformidade da submissão em relação a todos os itens listados a seguir. As submissões que não estiverem de acordo com as normas serão devolvidas aos autores.

1. A contribuição é original e inédita, e não está sendo avaliada para publicação por outra revista; caso contrário, deve-se justificar em "Comentários ao Editor".
2. **Devem ser preenchidos dados de autoria de todos os autores no processo de submissão**.
   Utilize o botão "**incluir autor**"
3. **No passo seguinte preencher os metadados em inglês.**
   Para incluí-los, após salvar os dados de submissão em português, clicar em "**editar metadados**" no topo da página - alterar o idioma para o inglês e inserir: titulo em inglês, abstract e key words. Salvar e ir para o passo seguinte.
4. A **identificação de autoria** do trabalho foi removida do arquivo e da opção Propriedades no Word, garantindo desta forma o critério de sigilo da revista, caso submetido para avaliação por pares (ex.: artigos), conforme instruções disponíveis em Assegurando a Avaliação Cega por Pares.
5. Os arquivos para submissão estão em formato Microsoft Word, Open Office ou RTF (desde que não ultrapassem 2MB)
6. O texto está em espaço 1,5; fonte Times New Roman de tamanho 11; emprega itálico em vez de sublinhado (exceto em endereços URL);
   O texto segue os padrões de estilo e requisitos bibliográficos descritos em Diretrizes para Autores, na seção Sobre a Revista.
7. URLs para as referências foram informadas quando necessário.

ANEXO 2 – Normas de publicação do Journal of the World Aquaculture Society

The *Journal of the World Aquaculture Society* is an international scientific journal that publishes original, peer-reviewed, English-language papers concerned with the culture of aquatic plants and animals. Subjects appropriate for the *Journal* would include, but not necessarily be limited to, nutrition, diseases, genetics and breeding, physiology, environmental quality, culture systems engineering, husbandry practices, and economics and marketing. Papers in which relevance to aquaculture is not clearly demonstrated will not be considered for publication. There are no page charges for papers published in the *Journal*.
Four categories of papers are published in the *Journal*. **Review Articles** provide objective synthesis of a subject of importance in aquaculture. Reviews should not simply summarize knowledge but should strive to interpret that knowledge and provide sound conclusions based on the available literature. Authors wishing to prepare a review article should contact the Managing Editor to discuss the suitability of the subject for the Journal. **Research Articles** are full scientific reports of original research. Research articles are typically of broad scope and interest to other workers in the relevant discipline. **Research Notes** differ from research articles on the basis of scope, not quality. Notes are printed in essentially the same format as full articles but are shorter and of narrower scientific focus. Notes should have no more than four tables or figures. **Communications** are brief reports of new observations, concepts, or methodologies. Communications should contain no more than two tables or figures.

Checklist for Manuscript Preparation

I. General Instructions

Format manuscripts for 22 x 28 cm (8½ x 11 inch) or A4 (21 x 30 cm) paper. Number *all* pages sequentially. Number *all* lines in the text beginning with the title page. Use any standard 12 pt font. Do not use italic, bold, or other non-standard type. Underline words to be italicized. Do not justify right margins. Indent the first sentence of all paragraphs. Double-space throughout, including title page, abstract, literature cited, tables, and figure legends. Leave at least a 2.5-cm (1-inch) margin on all sides. Use metric units of measurement. When needed, English equivalents may be given in parentheses. Abbreviations accepted without definition are listed on the inside back cover of the *Journal*. Designate temperature as 20 C. Define all other abbreviations the first time they are used. Express ratios by using a slant line (e.g., mg/L). Scientific names should accompany common names in the title and when they are first mentioned in the abstract and in the text. Authority for scientific names need not accompany the genus and species unless needed for clarity. Spell out one to ten unless followed by a unit of measurement (e.g., four fish, 4 kg, 14 fish). Do not begin a sentence with a numeral. Use 1,000 instead of 1000; 0.13 instead of .13; and % instead of percent. Use the 24-hour clock for dial time: 0830, not 8:30 a.m. Calender date should be day month year (7 August 1990).

Each reference cited in the text must be listed in the Literature Cited section, and vice versa. Literature citations in the text follow the name-and-year system.
1. One author: Jones (1994) or (Jones 1994)
2. Two authors: Smith and Jones (1994) or (Smith and Jones 1994)
3. Three or more authors: Smith et al. (1994) or (Smith et al. 1994)
4. Manuscripts accepted for publication: Jones (in press) or (Jones, in press)
5. Reference to unpublished data or personal communications is strongly discouraged. If necessary, cite as R. Ishihara (Humboldt State University, unpublished data) or R. Ishihara (Humboldt State University, personal communication).

6. Within parentheses, use a semicolon to separate multiple citations of literature and figures and tables (Smith1991; Jones 1994) (Table 1; Fig. 2). Cite multiple references within parentheses by year, with the oldest first.
Use "Figure" only to start a sentence; otherwise use "Fig." or "Figs." (e.g., Fig. 5; Figs. 5, 6). Spell out "Table" in all usages.

Assemble the manuscript in this order: title page, abstract page, text, literature cited, tables, figure legends, figures.

II. Title Page (Page 1)
Near the middle of the page, type the title of the paper, centered, in capital and lower case letters (e.g., Acute Toxicity of Copper Sulfate to Channel Catfish Ictalurus punctatus). Below the title, type the author(s) names, affiliation(s), and unabbreviated complete address (es). If the author is currently at another location, include a superscript number after the name and provide the full present address as a footnote. In papers written by authors at different addresses, type the name and address of the first author, the name and address of the second author, and so on. In multiauthored papers, type "Corresponding author:" and follow with the full mailing address of the author responsible for correspondence. Type this near the bottom of the page, but above any footnotes.

III. Abstract page (Page 2)
Type the heading "Abstract," centered, at the top of the page. Abstract must be one paragraph. Do not cite references or use abbreviations other than those listed on the back cover of the *Journal*. Be concise (normally not more than 3% of the text length) but include why you did the study, how you did it, the results of the study, and what the results mean. "Communications" do not have an abstract.

IV. Text (Beginning on page 3 for full papers; on page 2 for Communications)
Follow general instructions in Section I. Begin with an introduction that concisely establishes the purpose and importance of the work. Do not use a heading for this section. Subsequent sections in the text should include centered headings in capital and lower case letters. Typical main headings are Materials and Methods, Results, Discussion, and Acknowledgments. Do not start these sections with a new page. Second level headings (if required) are centered, in capital and lower case letters, and underlined. Do not use third level headings. Acknowledgments should contain grant and contribution numbers. Acknowledge only those people and institutions that contributed directly to the research or manuscript quality.

V. Literature Cited
Start this section at the top of a new page. Spell out journal names in full. Verify all entries against citations in the text. Verify the accuracy of all entries against the original sources, especially journal titles, authors, pages, and spelling. Start the first line of each entry at the left margin and indent other lines. Alphabetize entries first by the surnames of the senior authors and first word or acronym of corporate authors; second by the initials of senior authors with the same surname (e.g., Smith, B. F. precedes Smith, J. W.); and third, by the surnames of the junior authors. Single authored citations precede multiauthored works by the same senior author regardless of date. List multiple works by the same authors by date. Distinguish papers by the same author in the same year by putting lower case letters after the date (e.g. 1994a, 1994b). Be sure that such date citations within the text correspond to the dates in the Literature Cited. The following illustrates some common citation formats.

Journal Article:
Xu, D. and W. A. Rogers. 1993. Oxytetracycline residues in hybrid striped bass muscle. Journal of the World Aquaculture Society 24:466-472.
Book:
Boyd, C. E. 1982. Water quality management for pond fish culture. Elsevier Scientific Publishing Company, Amsterdam, The Netherlands.
Stickney, R. R., editor. 1986. Culture of non salmonid freshwater fishes. CRC Press, Inc., Boca Raton, Florida, USA.
Article or chapter in a book:
Ward, P. D. 1982. The development of bacterial vaccines for fish. Pages 47-58 in R. J. Roberts, editor. Microbial diseases of fish. Academic Press, New York, New York, USA.
Dissertation or Thesis:
Hymel, T. M. 1985. Water quality dynamics in commercial crawfish ponds and toxicity of selected water quality variables to Procambarus clarkii. Master's thesis. Louisiana State University, Baton Rouge, Louisiana, USA.

VI. Tables (Continue page numbering)
Start each table on a new sheet. Double space everything, including title, column headings, and all entries. Do not reduce type size in an effort to fit the table on one page. Use the same size type as the text. Print tables broadside, if necessary, to allow adequate margins. In extreme instances, continue the table on a second page. Type the table caption at the top of the page. Start at the left margin with the table number, which should be in arabic followed by a period (e.g., Table 4.). Follow with the table title using sentence-style capitalization (not title-style). Place a single horizontal line beneath the table title. Use single horizontal lines to separate column heads. Use a single horizontal line to indicate the end of the table. Do not use vertical lines in the table. Indicate footnotes by lowercase superscript letters (a, b, c, etc.).

VII. Figure Legend (Continue page numbering)
Put captions on the same page as the figure. Type the first line at the margin for each entry. Indent other lines. Spell out "Figure" followed by an Arabic number. Use sentence-style capitalization of the caption. Figure 1. Growth of Peneaus setiferus over time at various combinations of water exchange and stocking density. Do not include symbols (dots, circles, triangles, etc.) in the figure captions. Label them in the figure or refer to them by name in the caption. Do not refer to magnification of photomicrographs in the caption: figures will be reduced when printed so they will be wrong if given in the caption. Place a bar scale directly on each photo and give its equivalent length in the caption (e.g., bar = 25 μm).

VIII. Illustrations Line Drawings
Submit electronic copies of line drawings with the initial manuscript submission. Save files in TIFF or EPS format at 300dpi.resolution or higher. Lettering should be clear and large enough to withstand at least 50% reduction without becoming illegible. A clean sans serif typeface (such as Helvetica or Univers) is preferred. Lettering on a figure 20 cm wide should be at least 4.5 mm high (18-point type) to withstand reduction.

IX. What and Where to Submit
Completed manuscripts should be submitted online via the website (http://mc.manuscriptcentral.com/jwas). New users should go to the tab in the upper right hand corner to "Create an Account". A User ID and password will be sent via email within a few minutes. Follow the online directions for submission of your manuscript.

ANEXO 3 – Normas de publicação da Revista Aquaculture

Aquaculture is an international journal for **freshwater and marine researchers** interested in the exploration, improvement and management of all **aquatic food resources.** It publishes original research on **farming of aquatic animals** and plant organisms including finfish, mollusks, crustaceans and aquatic plants for human consumption.

Types of paper

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere. Articles are expected to contribute new information (e.g. novel methods of analysis with added new insights and impacts) to the knowledge base in the field, not just to confirm previously published work. *Review Articles* can cover either narrow disciplinary subjects or broad issues requiring interdisciplinary discussion. They should provide objective critical evaluation of a defined subject. Reviews should not consist solely of a summary of published data. Evaluation of the quality of existing data, the status of knowledge, and the research required to advance knowledge of the subject are essential. *Short Communications* are used to communicate results which represent a major breakthrough or startling new discovery and which should therefore be published quickly. They should not be used for
preliminary results. Papers must contain sufficient data to establish that the research has achieved reliable and significant results. *Technical Papers* should present new methods and procedures for either research methodology or culture-related techniques.
The *Letters to the Editor* section is intended to provide a forum for discussion of aquacultural science emanating from material published in the journal.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.
Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.
Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.
Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.
Results
Results should be clear and concise.
Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.
Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself. The abstract should be not longer than 400 words.

Keywords

Immediately after the abstract, provide a maximum of 4-6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature and units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI. You are urged to consult IUPAC: Nomenclature of Organic Chemistry: http://www.iupac.org/ for further information.

1. Authors and editors are, by general agreement, obliged to accept the rules governing biological nomenclature, as laid down in the International Code of Botanical Nomenclature, the International Code of Nomenclature of Bacteria, and the International Code of Zoological Nomenclature.
2. All biota (crops, plants, insects, birds, mammals, etc.) should be identified by their scientific names when the English term is first used, with the exception of common domestic animals.
3. All biocides and other organic compounds must be identified by their Geneva names when first used in the text. Active ingredients of all formulations should be likewise identified.
4. For chemical nomenclature, the conventions of the International Union of Pure and Applied Chemistry and the official recommendations of the IUPAC IUB Combined Commission on Biochemical Nomenclature should be followed.

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text). Give the meaning of all symbols immediately after the equation in which they are first used. In chemical formulae, valence of ions should be given as, e.g. Ca2+ and not Ca++. Isotope numbers
should precede the symbols, e.g., 18O. The repeated writing of chemical formulae in the text is to be avoided where reasonably possible; instead, the name of the compound should be given in full. Exceptions may be made in the case of a very long name occurring very frequently or in the case of a compound being described as the end product of a gravimetric determination (e.g., phosphate as P2O5).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes: Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Save text in illustrations as 'graphics' or enclose the font.
• Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.

• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Produce images near to the desired size of the printed version.
Submit each figure as a separate file. A detailed guide on electronic artwork is available on our website: http://www.elsevier.com/artworkinstructions.**You are urged to visit this site; some excerpts from the detailed information are given here.**
Formats
Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as 'graphics'.
TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required. If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.
Please do not:
• Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.
Color artwork
Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.
Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.
Text graphics
Text graphics may be embedded in the text at the appropriate position. Further, high-resolution graphics files must be provided separately whether or not the graphics are embedded. See further under Electronic artwork.

Tables
Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address
• Full postal address
• Telephone and fax numbers

All necessary files have been uploaded, and contain:

• Keywords
• All figure captions
• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print
• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes

For any further information please visit our customer support site at http://support.elsevier.com.

ANEXO 4 – Normas de publicação da Revista Fish Physiology and Biochemistry

Fish Physiology and Biochemistry is an international journal publishing original research papers in all aspects of the physiology and biochemistry of fishes. Coverage includes experimental work in such topics as biochemistry of organisms, organs, tissues and cells; structure of organs, tissues, cells and organelles related to their function; nutritional, osmotic, ionic, respiratory and excretory homeostasis; nerve and muscle physiology; endocrinology; reproductive physiology; energetics; biochemical and physiological effects of toxicants; molecular biology and biotechnology and more. The journal presents full papers, brief communications, rapid communications, unsolicited and invited reviews and editorial comments and announcements.

Title Page
The title page should include: The name(s) of the author(s); A concise and informative title; The affiliation(s) and address(es) of the author(s); The e-mail address, telephone and fax numbers of the corresponding author

Abstract
Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords
Please provide 4 to 6 keywords which can be used for indexing purposes.

Text Formatting
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