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Taida Juliana Adorian

FIBRAS FUNCIONAIS DA LINHAÇA E SEUS IMPACTOS NA NUTRIÇÃO DE JUNDIÁS

Santa Maria, RS
2018

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Tese apresentada ao Curso de Doutorado do Programa de Graduação em Zootecnia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Zootecnia.**

Orientadora: Profª Drª Leila Picolli da Silva

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“A tarefa não é tanto ver aquilo que ninguém viu,
mas **pensar o que ninguém ainda pensou,**
sobre aquilo que todo mundo vê.”

Arthur Schopenhauer

RESUMO

FIBRAS FUNCIONAIS DA LINHAÇA E SEUS IMPACTOS NA NUTRIÇÃO DE JUNDIÁS

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Este estudo objetivou avaliar a ação prebiótica de fibras funcionais de linhaça com distintas proporções de fibra alimentar solúvel e insolúvel e seus impactos na nutrição e saúde de juvenis de jundiás (6,43g). Para isso, foram concentradas as frações solúvel e insolúvel de fibra da linhaça, a partir da utilização de técnicas físicas e químicas de concentração. Estas frações foram combinadas em diferentes proporções (1:0,5, 1:1, 1:2 e 1:4 fibra solúvel: insolúvel) para obtenção de fibras funcionais, que foram adicionadas a dietas e avaliadas em um ensaio biológico com juvenis de jundiá. O ensaio biológico teve duração de 45 dias e foi realizado em sistema de recirculação de água, composto por 20 tanques (290L), biofiltros e reservatório de água. Neste período os peixes foram alimentados até a saciedade aparente, três vezes ao dia. Ao final do período os peixes foram submetidos a jejum de 18 horas e biometria para coleta de dados de peso, comprimento, coleta de sangue, tecidos (fígado e trato digestivo), muco e digesta para determinação de parâmetros de desempenho, composição e deposição corporal, metabólitos plasmáticos, hepáticos, enzimas digestivas, indicadores imunológicos, histologia intestinal e produção de ácidos graxos de cadeia curta. Após a biometria final os peixes foram mantidos nas unidades experimentais por mais cinco dias e ao final deste período, submetidos a estresse agudo, com posterior coleta de sangue e muco para determinação de metabólitos e indicadores imunológicos. O delineamento experimental utilizado foi o inteiramente casualizado, composto cinco tratamentos e quatro repetições (600 peixes). Os resultados obtidos foram submetidos à teste de normalidade, seguido por análise de variância, sendo as médias comparadas pelo teste de Tukey ao nível de 5% de significância. As dietas com as proporções 1:2 e 1:4 proporcionaram maior ganho de peso, taxa de crescimento específico e deposição de proteína bruta corporal aos peixes, proteínas totais circulantes e globulinas, assim como o teor de mucoproteína, imunoglobulinas totais e pH do muco cutâneo. Já os níveis de cortisol e o pH intestinal foram mais baixos nestes tratamentos. A dieta 1:0,5 alterou a atividade de tripsina no intestino dos jundiás e juntamente com a dieta 1:4 proporcionou maior altura das vilosidades intestinais. Enquanto que altura total da vilosidade foi superior para os peixes que receberam fibra de linhaça na dieta, independente da proporção, o inverso foi observado para a espessura da camada muscular. Independente da proporção na dieta, o consumo de fibra de linhaça aumentou as imunoglobulinas totais no plasma e a atividade da fosfatase alcalina no plasma e muco cutâneo. A produção de ácido acético intestinal foi superior nos peixes alimentados com a dieta 1: 2, enquanto que de ácido butírico com a dieta 1:4 e ácido propiônico com a dieta controle. A dieta controle levou a menor contagem de células caliciformes. Após o estresse agudo, os peixes alimentados com as dietas contendo as proporções de fibra solúvel: insolúvel 1: 2 e 1: 4 apresentaram maior teor de proteína total, globulina e atividade de fosfatase alcalina do plasma, além de maior teor de mucoproteína no muco cutâneo dos peixes. Em conclusão, os resultados indicam que a fibra de linhaça tem ação prebiótica imunoestimulante para juvenis de jundiá, sendo que as proporções de 1:2 e 1:4 de fibra solúvel: insolúvel otimizam o sistema imune e a produção de ácidos graxos de cadeia curta, com reflexos positivos sobre o desempenho dos peixes. Além disso, nessas proporções ela ainda age como mitigadora de estresse.

Palavras-chave: Fibra alimentar. Linhaça. Prebiótico. *Rhamdia quelen*.

ABSTRACT

FUNCTIONAL FIBERS OF LINSEED AND ITS IMPACTS ON NUTRITION OF SILVER CATFISH

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This study aimed to evaluate the prebiotic action of functional linseed fibers with different proportions of soluble and insoluble dietary fiber and its impact on the nutrition and health of juveniles silver catfish (6.43g). For this, the soluble and insoluble fractions of linseed fiber were concentrated, using the use of physical and chemical concentration techniques. These fractions were combined in different proportions (1:0.5, 1:1, 1:2 and 1:4 soluble: insoluble fibre) to obtain functional fibers, which were added to diets and evaluated in a biological test with juveniles silver catfish. The biological assay lasted 45 days and was performed in a water recirculation system, composed of 20 tanks (290L), biofilters and water reservoir. At this time the fish were fed to apparent satiety three times a day. At the end of the period the fish were submitted to a 18 hour fast and biometry for data collection of weight, length, blood collection, tissues (liver and digestive tract), mucus and digesta for determination of performance parameters, composition and body deposition, plasma metabolites, hepatic enzymes, digestive enzymes, immunological indicators, intestinal histology and production of short chain fatty acids. After the final biometry the fish were kept in the experimental units for another five days and at the end of this period, submitted to acute stress, with subsequent collection of blood and mucus for determination of metabolites and immunological indicators. The experimental design was a completely randomized design, consisting of five treatments and four replications (600 fish). The results were submitted to the normality test, followed by analysis of variance, and the means were compared by the Tukey test at the 5% level of significance. Diets 1:2 and 1:4 provided greater weight gain, specific growth rate and crude protein deposition in fish, total circulating proteins and globulins, as well as mucoprotein content, total immunoglobulins and cutaneous mucus pH. Cortisol levels and intestinal pH were lower in these treatments. The 1:0.5 diet altered the trypsin activity in the silver catfish intestine and together with the 1:4 diet provided higher intestinal villi height. While total villus height was higher for the fish that received linseed fiber in the diet, regardless of the proportion, the inverse was observed for the thickness of the muscle layer. Regardless of dietary ratio, linseed fiber intake increased total plasma immunoglobulins and plasma alkaline phosphatase activity and cutaneous mucus. The production of intestinal acetic acid was higher in the fish fed with the 1:2 diet, whereas of the butyric acid with the 1:4 diet and propionic acid with the control diet. The control diet led to lower counts of goblet cells. After acute stress, the fish fed the diets containing soluble: insoluble fiber ratios 1:2 and 1:4 presented higher total protein, globulin and plasma alkaline phosphatase activity, as well as a higher mucoprotein content in the mucus of fish. In conclusion, the results indicate that linseed fiber has an immunostimulating prebiotic action for silver catfish juveniles, and the 1:2 and 1:4 ratios of soluble: insoluble fiber optimize the immune system and the production of short-chain fatty acids, with positive reflexes on fish performance. Moreover, in these proportions it still acts as a stress reliever.

Keywords: Dietary fiber. Linseed. Prebiotic. *Rhamdia quelen.*

LISTA DE TABELAS

ARTIGO I

ARTIGO II

ARTIGO III

SUMÁRIO

1 INTRODUÇÃO

As estatísticas mostram que a aquicultura tem crescido em ritmo acelerado em todo o mundo, destacando a importância da atividade na produção de proteína de origem animal. De acordo com a FAO, em 2017 a produção de pescado foi 43% superior a carne suína, sendo que deste total, quase metade foi proveniente da aquicultura, demonstrando um crescimento no cultivo mundial de 60% entre 2007 e 2017 (ANUÁRIO PeixeBR, 2018; FAO, 2017). No Brasil, o setor aquícola também tem crescido substancialmente, com aumento de 8% na produção somente em 2017 (691.700 toneladas produzidas no ano de referência). Tal crescimento foi alavancado pelos estados da região Sul do País (Paraná, Santa Catarina e Rio Grande do Sul), que juntos contribuíram com mais de 178.000 toneladas no ano.

Com este crescimento da atividade e a intensificação do cultivo objetivando alcançar altos índices de produtividade, os peixes acabam sendo expostos com maior frequência a situações estressantes. Altas densidades de estocagem, variações na qualidade da água, manejos frequentes inerentes a atividade, reduzem a resposta imune dos animais, tornando-os mais susceptíveis a doenças e, consequentemente, aumentando a mortalidade e diminuindo a viabilidade econômica do cultivo de peixes (URBINATI; CARNEIRO, 2004).

Para reduzir estes impactos, uma prática comum por anos foi o uso de antibióticos. Porém, devido a restrição ao uso destas moléculas, seja por promoverem resistência em microorganismos patogênicos, pelo acúmulo residual sobre o produto animal ou pela contaminação ambiental, buscam-se alternativas racionais, eficientes e ambientalmente seguras para substituição destes produtos (CYRINO et al., 2010), motivando os estudos com aplicação de moléculas orgânicas, assim como aditivos alternativos para uso zootécnico.

Como opção, destaca-se a suplementação das dietas com prebióticos, os quais são carboidratos seletivamente fermentáveis que permitem modificações na composição e/ou atividade da microbiota intestinal, resultando em melhorias na saúde e desempenho dos animais (ROBERFROID, 2007). Grande parte dos prebióticos comercialmente disponíveis são frações isoladas e parcialmente hidrolisadas (oligossacarídios), provenientes da fibra alimentar, porém estudos tem demonstrado que o uso de concentrados de fibra alimentar tem efeitos similares aqueles proporcionados por prebióticos comerciais usuais (ADORIAN et al. 2015; ADORIAN et al. 2016; GOULART et al. 2017; MOMBACH, 2015). Embora em início de desenvolvimento conceitual e tecnológico, estes estudos demonstram que a manipulação do teor e das proporções das frações de fibra alimentar nas dietas, resulta em efeitos positivos para os peixes, com maior racionalidade produtiva e ambiental.

A fibra alimentar é classificada de acordo com a sua solubilidade em água, em solúvel ou insolúvel (WENZEL, 2012). Na prática, ambas as frações da fibra alimentar são partes da dieta, porém seus efeitos dependem da variação de seus teores individuais, da predominância de uma fração em relação a outra, sua composição química e organização estrutural (MACAGNAN et al., 2016; MORRE et al., 1998).

Ao chegar no intestino, tanto a fibra solúvel quanto a insolúvel servem como substrato para fermentação microbiana (WENZEL, 2012). Nesse ambiente a fibra se depara com grande atividade bioquímica de bactérias, sendo que as espécies sacarolíticas ali presentes, participam de forma intensa da sua quebra e fermentação (FERREIRA, 2012). Nesse processo são gerados alguns produtos, como os ácidos graxos de cadeia curta (AGCC) acetato, butirato e propionato, bem como, ocorrerá liberação gradual dos compostos fenólicos ligados a fibra, os quais são parcialmente absorvidos pelas células epiteliais do intestino (FERREIRA, 2012; QUIRÓS-SAUCEDA et al., 2014; WENZEL, 2012). Além da ação antioxidante que previne danos em lipídios, proteínas e ácidos nucleicos, conservando a fluidez, permeabilidade e integridade celular (BARRERA, 2012; REPETTO et al., 2012; ZHANG et al., 2008), os compostos fenólicos também apresentam atividade anti-inflamatória, que inibe a produção de citoquinas, evitando doenças imunológicas resultantes da inflamação (LIU; LIN, 2013; VERES, 2012).

Como espécie com potencial de cultivo no Sul do Brasil, destaca-se o jundiá (*Rhamdia quelen*). Porém existem várias lacunas relacionadas a sua produção que precisam ser elucidadas para que a espécie se torne competitiva. Dentre as linhas de pesquisa que merecem atenção, estão as exigências nutricionais, ingredientes alternativos e sistemas de cultivos. Além disso, o desenvolvimento de aditivos alimentares para a espécie com foco principal na proteção e promoção da saúde é uma tendência que deve continuar crescendo nos próximos anos (VALLADÃO et al., 2018).

Trabalhos com jundiás demonstram que a adição de fibras alimentares concentradas nas dietas desta espécie, exercem ação efetivamente prebiótica, uma vez que otimizam o desempenho, metabolismo e sistema imunológico dos peixes (ADORIAN et al., 2015; ADORIAN et al., 2016; GOULART et al., 2017). Dentre as fontes de fibras testadas, os resultados de maior impacto foram obtidos com a adição de fibra de linhaça (*Linum uistatissimum L.*). A linhaça é reconhecida como uma fonte rica em fibra alimentar, que apresenta boa proporção de fibras solúveis e insolúveis (GALVÃO et al., 2008). A fibra solúvel, também conhecida como mucilagem, é composta por monossacarídeos como a

ramnose, galactose, frutose, xilose e arabinose. Já a fibra insolúvel, por celulose (monômeros de glicose) e lignina (álcoois aromáticos) (RAY et al., 2013; SHIM et al., 2014).

De acordo com Goulart et al. (2013), o farelo de linhaça in natura é uma fonte alternativa de proteína para fabricação de rações para jundiás. Segundo os autores, os bons resultados obtidos estão relacionados a presença da fibra solúvel, a qual pode ter exercido efeito prebiótico, refletindo de forma desejável no desempenho animal. Outras evidências da ação pebiótica da fibra de linhaça foram demonstradas por Goulart et al. (2017), ao suplementar mucilagem de linhaça em dietas para mesma espécie, a qual proporcionou maior ganho de peso e conversão alimentar. O que reforça essa ideia são os resultados de Adorian et al. (2015) e Adorian et al. (2016), onde peixes que receberam fibra de linhaça na dieta (solúvel + insolúvel) tiveram resultados iguais ou superiores ao que receberam dieta com prebiótico comercial (Actigen®).

Porém, as proporções de fibra solúvel e insolúvel ideais para otimizar tais resultados ainda não são conclusivas. Dessa forma, é perceptível a necessidade de aprofundar as pesquisas neste viés, focando na obtenção de fibras funcionais de linhaça, com inclusão de distintas proporções das frações solúvel e insolúvel.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Avaliar a ação prebiótica de fibras funcionais de linhaça com distintas proporções de fibra alimentar solúvel e insolúvel e seus impactos na nutrição e saúde de juvenis de jundiás.

1.1.2 Objetivos específicos

- Concentrar a fibra alimentar contida na linhaça para desenvolvimento de fibras funcionais com potencial prebiótico;

- Combinar e avaliar o potencial prebiótico das distintas proporções de fibra solúvel e insolúvel de linhaça (1:0,5; 1:1; 1:2; 1:4) em dietas para juvenis de jundiá, sobre os parâmetros de desempenho, metabólicos e imunológicos;

- Avaliar a resistência ao estresse de jundiás alimentados com distintas proporções de fibra solúvel e insolúvel de linhaça em dietas.

O presente estudo foi desenvolvido em duas fases. A primeira consistiu na obtenção das frações solúvel e insolúvel de fibra de linhaça e análise de sua composição química e propriedades físico-químicas. Na segunda fase, as frações foram combinadas em quatro distintas proporções de fibra solúvel: insolúvel (1:0,5, 1:1, 1:2 e 1:4), adicionadas a dietas para jundiás e avaliadas em ensaio biológico. Os resultados estão apresentados na forma de artigos científicos, onde o artigo I corresponde a avaliação das distintas proporções de fibra solúvel: insolúvel sobre o desempenho zootécnico, qualidade corporal, metabolismo e morfometria intestinal. No artigo II, avaliou-se o efeito das combinações sobre os parâmetros imunológicos e de crescimento. Enquanto que no artigo III, a ação imunoestimulante das fibras solúvel e insolúvel de linhaça foi avaliada em jundiás submetidos a estresse agudo.

É apresentado ainda um artigo no apêndice A, que corresponde a primeira fase do estudo, onde realizou-se a obtenção e caracterização química e de proriedades físico-químicas das frações solúvel e insolúvel de fibra de linhaça, assim como, de um concentrado proteico, avaliado em outra tese pertencente ao mesmo projeto do nosso grupo de pesquisa (“Alternativas de nutrientes e compostos bioativos: estudo do fracionamento da linhaça para nutrição de peixes”, registrado no CEUA pelo nº 8015120816).

2 ARTIGO I

O artigo científico intitulado “Functional linseed fibers and their impacts on silver catfish nutrition” foi submetido para a revista Animal Feed Science and Technology e está formatado segundo as normas descritas no Guia dos Autores (Anexo A).

Functional linseed fibers and their impacts on silver catfish nutrition

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Abstract

This study was conducted with the objective of evaluating the combination of different ratios of soluble and insoluble linseed fiber on the zootechnical performance, body quality and intestinal morphometry of young silver catfish. For this, the soluble and insoluble fractions of linseed fiber were concentrated separately and combined in four ratios (1:0.5, 1:1, 1:2, 1:4), which were added to silver catfish (6.43 ± 0.12 g) diets and evaluated in a bioassay, along with a control diet (without the addition of linseed fiber). After 45 days receiving the experimental diets, the animals were fasted and anesthetized in order to perform a biometry to collect data and tissues for further analysis. The experimental design was completely randomized, with five treatments and four replications. Data were submitted to analysis of variance and the means were compared by Tukey's test ($P < 0.05$). Diets 1:2 and 1:4 provided higher weight gain, specific growth rate and crude protein deposition to the fish, whereas only the 1:4 diet reflected higher crude body protein. The 1:0.5 diet altered the trypsin activity in the intestine of silver catfish and, together with the 1:4 diet, it provided higher intestinal villus height. While the total villus height was greater for the fish that received linseed fiber in their diet, regardless of the proportion, the opposite was observed for the muscle layer thickness. Body yield, somatic and digestive parameters, chymotrypsin activity and glucose, glycogen and liver protein were not altered, regardless of the experimental diets. In conclusion, the results indicate that linseed fiber acts effectively as a growth promoter in silver catfish diets, with the use of 1:2 and 1:4 ratios optimizing its prebiotic action on the animal organism.

Keywords: Rhamdia quelen, soluble fiber, insoluble fiber, *Linum usitatissimum*, prebiotic

Abbreviations: AFC, apparent feed conversion; CF, condition factor; CY, corporal yield; DSI, digestive somatic index; FBF, final body fat; FW, final weight; FBP, final body protein; HSI, hepatosomatic index; IBF, initial body fat; IBP, initial body protein; IQ, intestinal quotient; IW, initial weight; SCFA, short-chain fatty acids; SE, standard error; SGR, specific growth

rate; S:IF, soluble: insoluble fiber; TCA, trichloroacetic acid; UFSM, University of Santa Maria; VFI, visceral fat index.

1. Introduction

Food fiber consists of a complex and heterogeneous set of non-starch polysaccharides, oligosaccharides and minor compounds, which are resistant to the enzymatic digestion in the digestive tract of animals and which can, to varying degrees, be degraded and fermented into short chain fatty acids by intestinal microbiota (Buttriss and Stokes, 2008; Macagnan et al., 2016). Fibers are classified according to their solubility, as soluble or insoluble, and the relations between these fractions, their composition, organizational structure, physico-chemical characteristics and presence of bioactive compounds associated to the matrix, are determinant for their functional properties (Westenbrink et al., 2013, Macagnan et al., 2016). In practice, both fractions are found in diets, but the effects on the digestive and metabolic processes depend on both solubility variations and the chemical ratios and interactions between fractions (Van Soest et al., 1991; Morre et al., 1998; Silva and Walter, 2012).

In order to enhance the functional benefits, many authors suggest the application of dietary fiber hydrolysis techniques to obtain oligosaccharides, which are used as prebiotic agents in diets (Gullón et al., 2011; Chen et al., 2013; Gómez et al., 2014). However, in fish nutrition, studies have shown that the use of non-hydrolysed food fiber concentrates (linseed, brewer's yeast and citrus pulp) has equivalent or greater effects than consolidated commercial prebiotics, optimizing the immune system and acting as a growth promoter (Adorian et al., 2015; Mombach, 2015; Adorian et al., 2016; Goulart et al., 2017). This demonstrates that the functional agents for fish can be obtained with simpler and lower cost technology than oligosaccharides that make up the vast majority of commercial prebiotics.

For linseed (*Linum usitatissimum* L.), total dietary fiber concentration techniques were applied and the resulting fibrous concentrates were successfully tested on fish nutrition (Adorian et al., 2015; Adorian et al., 2016, Goulart et al., 2018). It is possible to believe, however, that there is still scope to optimize the results of these studies, through the direct application of different ratios of soluble and insoluble fibers, which can be extracted from isolated fractions and independently combined in fish diets. In this contex, this study was conducted to evaluate the combination of different soluble and insoluble linseed fiber ratios (1:0.5, 1:1, 1:2, 1:4) on the growth performance, body quality and intestinal morphometry of silver catfish (*Rhamdia quelen*).

2. Material and methods

The study was conducted at the Laboratory of Fish Farming of the Department of Animal Science of the Federal University of Santa Maria (UFSM), Rio Grande do Sul, Brazil (Latitude: 29º 41’ 03’’ S; Longitude: 53º 48’ 25’’ W), after being approved by the Ethics Committee on Animal Trials of this University, under the process number 8015120816.

2.1 Preparation of functional fibers

Linseed fiber was obtained in two distinct stages. In the first stage, soluble fiber of linseed (mucilage) was obtained by soaking the whole grain in water at a concentration of 10% w/v, maintaining the reaction between 60 ºC and 80 ºC under constant stirring for 150 min. Subsequently, the soluble fiber was separated from the grains by sieving, followed by addition of ethanol, for the precipitation of this fraction following the method described by Goulart et al. (2013). The resulting soluble fiber of this process was dried in an air circulating oven at 55ºC for 48 hours and ground in a micro-grinder (Marconi, model MA-630/1) to obtain particles smaller than 590 μm, representing the Linseed soluble fiber.

In the second stage, the insoluble fiber contained in the linseed was extracted. The demucilaged grain was defatted with hexane at a ratio of 1:2 (w/v), performing for 30 min washes. After defatted, the protein content of the residue was reduced by dispersion in distilled water at room temperature at the ratio of 1:30 (w/v), sifted and dried in an air circulating oven at 55 ºC for 24 h. The Linseed insoluble fiber obtained in this stage was ground in a micro-grinder (Marconi, model MA-630/1) to obtain particles smaller than 590 μm.

2.2 Experimental diets

Five experimental diets (Table 1) were formulated to achieve the nutritional requirements of juvenile silver catfish, according to Meyer and Fracalossi (2004). The experiment comprised the following treatments: Addition of functional fibers in the diet in proportions of 1:0.5, 1:1, 1:2, 1:4 of soluble: insoluble fiber (S:IF) and control diet (without addition of fiber). The diets were produced in the Laboratory of Fisheries of UFSM. The dry ingredients were weighed and manually homogenized, then water was added and pelleting with matrix of 3 mm in diameter. They were dried in an oven with forced air circulation for 24 h at a temperature of 55 ºC. After drying, the diets were milled and selected according to the fish ingestion capacity. Diets were stored under a temperature of −20 ºC throughout the experimental period. The diets composition and physicochemical properties were determined based on analyses of crude protein (method 960.52), total, insoluble and soluble dietary fibers (method 991.43) (AOAC, 1995), fat (Bligh and Dyer, 1959), hydration capacity and fat binding capacity (Wang and Kinsella, 1976), copper binding (McBurney, 1983) and phenolic compounds (Waterhouse, 2003).

2.3 Animals and feed

Six-hundred juveniles of silver catfish with an average initial weight of 6.43 ± 0.12 g were distributed randomly in 20 polypropylene tanks with 290 liters capacity (30 animals per experimental unit). Each tank had individual water inlet and outlet, arranged in a water recirculation system comprised of a decanter, two mechanical and biological filtering and a water reservoir with a capacity for 2000 liters, equipped with a heating system. During the experimental period, the fish were fed with the experimental diet until apparent satiation three times a day (9:00, 13:00 and 17:00 o'clock) for 45 days.

2.4 Water quality

Prior to the first and last meals (8:00 and 15:00 o'clock), fecal residues were removed from the tanks by siphoning twice a day. During the experimental period, the water quality parameters were monitored using colorimetric kits and maintained as follows: morning temperature of 23.33 ± 1.71ºC; afternoon temperature of 24.90 ± 1.37ºC; pH: 7.45 ± 0.20; alkalinity: 37.25 ± 4.95 mg $CaCO_3$/L; hardness: 36.75 ± 11.25 mg $CaCO_3$/L; total ammonia: 0.28 ± 0.10 mg L^{-1}; nitrite: 0.02 ± 0.14 mg L^{-1} and oxygen: 7.75 ± 0.88 mg L^{-1}.

2.5 Data collection and performance evaluation

In the early and late experimental period, a biometric assessment was performed to collect data from the animals, which had fasted for 18 h and were anesthetized with Benzocaine (100 mg/L), to estimate the following: individual weight gain (g); total length (cm); specific growth rate (SGR): [(ln (final weight) − ln (initial weight))/days] × 100,where: ln= Neperian logarithm; condition factor (CF): weight/(total length) 3 × 100; apparent feed conversion (AFC): feed intake/weight gain and consumption (g). The daily feed intake (g)

was recorded to calculate the total feed intake estimated per experimental unit at the end of the experiment.

2.6 Corporal composition and nutrient deposition

For the analysis of proximate corporal composition, eight animals per treatment were used. Crude protein was determined by the micro-Kjeldahl method (method 960.52) using the N x 6.25 factor, and the moisture content and ash content were determined according to AOAC (1995). Fat was extracted and quantified according to the method described by Bligh and Dyer (1959).

The nutrients deposition was calculated according to the following equations:

- Body deposition protein (g): [FW × (% FBP/100)] – [IW × (% IBP/100)];
- Body deposition fat (g): [FW × (% FBF/100)] – [IW × (% IBF/100)];

Where: FW = final weight; IW = initial weight; IBP = initial body protein; FBP = final body protein; IBF = initial body fat; FBF = final body fat.

2.7 Corporal yield and digestive index

For the analysis of the somatic parameters, eight animals per treatment were euthanized by benzocaine overdose (10%, 250 mg/L) (AVMA, 2013). This fish were used for determining the digestive somatic index (DSI): (weight of the digestive tract/weight of the whole fish) × 100; hepatosomatic index (HSI): (weight of the liver/weight of the whole fish) × 100; visceral fat index (VFI): (weight of visceral fat/whole weight) × 100; intestinal quotient (IQ): length of the digestive tract/total fish length; and corporal yield (CY): ((eviscerated weight with head and gills)/(whole weight)) × 100. Subsequently, the intestine and liver of these fish were used for determination of digestive enzymes and hepatic metabolites.

166 *2.8 Analysis of digestive enzymes*

167 Eight fish per treatment were used to determine the activity of trypsin and
168 chymotrypsin enzymes. The intestines collected were homogenized in a buffer solution
169 (10mM phosphate/20mM Tris). The samples were then centrifuged, and the supernatants were
170 used in the assays as enzyme source for determining intestine trypsin and chymotrypsin
171 enzymes. To determine the trypsin enzyme activity, TAME (α-ρ-toluenesulphonyl- L-arginin
172 e methyl ester hydrochloride) was used as substrate. The intestine extracts were incubated for
173 two minutes in a 2-ml buffer solution of Tris/$CaCl_2$, pH 8.1. For determining chymotrypsin,
174 the substrate used was BTEE (benzoyl-L-tyrosine ethyl ester). The extracts were incubated for
175 two minutes in a 2-ml buffer solution of Tris/$CaCl_2$ (2 ml), pH of 7.8. The trypsin activity was
176 expressed in µmol of hydrolyzed TAME/minute/mg of protein, and the chymotrypsin activity
177 in µmol of BTEE/minute/mg protein. Readings were taken in a spectrophotometer,
178 absorbance of 247 and 256 nm respectively, following the methodology described by
179 Hummel (1959).

180

181 *2.9 Histological parameters*

182 Anterior intestine was collected (four fish/ treatment) and prepared for light
183 microscopy. Histological samples were fixed in 10% formalin and preserved in 70% ethanol
184 and subjected to the histological routine, following the method described by Gressler et al.
185 (2016). The material was sent to go through the histological routine for dehydration in
186 increasing ethanol series (70%–99% alcohol) and embedded in methacrylate glycol resin
187 (Technovit 7100). From this material, slits of 2 µm were obtained from rotary microtome
188 (LEICA RM2245) to subsequent coloration with hematoxylin-eosin. For morphological
189 examination, the slides were observed and documented in light microscopy (ZEISS PrimoStar
190 with AxioCam ERc5s) and analyzed through the software ZEN LITE (Carl Zeiss). At each

repetition villus height, total villus height, epithelium thickness and muscle layer thickness were estimated using Image J® software. The slides were thoroughly examined in order to determine the presence of histopathological alterations.

2.10 Hepatic metabolites

Hepatic metabolites were determined in the liver samples (50 mg), which were heated to 100 ºC with KOH to estimate the protein content according to the technique described by Bradford (1976). In an aliquot of this extract, ethanol was added to hydrolyze and precipitate glycogen, and after centrifugation at 1000g for 10 min, the glucose content was determined (Park and Johnson, 1949). The liver samples (50 mg) were homogenized in 10% trichloroacetic acid (TCA) and centrifuged (1000g, 10 min), and the supernatant was used for glucose quantification (Park and Johnson, 1949).

2.11 Statistical analysis

Initially, the data were analyzed for outlier identification. The experimental design was completely randomized with five treatments and four replications. The data were subjected to analysis of variance and means were compared by Tukey's test. Differences were considered significant at the level of $P<0.05$

3. Results

3.1 Performance parameters

Fish performance was significantly influenced by the tested soluble and insoluble fiber ratios (Table 2). Diets with 1:2 and 1:4 S:IF diets given greater weight gain (P= 0.041) and specific growth rate (P= 0.048) in animals when compared to other treatments tested. The total length was also higher (P= 0.015) for fish fed a ratio of 1:2 in the diet, but not different

from animals fed the diet containing ratio of 1:0.5, 1:1 and 1:4. Condition factor, food consumption and apparent feed conversion were not influenced by the diets tested (P>0.05).

3.2 Corporal composition and nutrient deposition

Corporal composition and nutrient deposition were influenced by the diets tested (Table 3). Diets with ratio of 1:4 S:IF provided higher corporal crude protein (P= 0.041) for fish, when compared to the control diet. The same diet provided greater corporal dry matter (P= 0.023) than fish fed with the diet containing ratio of 1:0,5. Diets with ratio of 1:2 and 1:4 caused greater deposition of crude protein in the body (P= 0.003), compared to the other treatments. There was no significant difference in corporal fat, ash and fat deposition (P>0.05).

3.3 Corporal yield and digestive index

Diets containing different proportions of soluble and insoluble fiber no influenced significantly in corporal yield, somatic and digestive parameters (P>0.05) (Table 4).

3.4 Digestive enzymes

Diets containing different proportions of soluble and insoluble fiber no influenced significantly chymotrypsin activity (P>0.05) (Table 5). However, trypsin activity was higher for fish fed with ratio of 1:0,5 S:IF in diet (P= 0.007). Fish fed with diet containing ratio of 1:2 and 1:4 showed lower trypsin activity.

3.5 Histological parameters

Linseed fiber ratios significantly influenced the development of the silver catfish intestine. Villus height was higher for fish that received fiber in their diet (P<0.001),

regardless of the ratio. The opposite was observed for the muscular layer thickness (P<0.001), which was superior for the fish fed on the control diet. The total villus height was higher for the fish fed on the 1:0.5 and 1:4 S:IF diets (P= 0.003), not differing significantly from the 1:1 and 1:2 diets. On the other hand, the epithelium thickness was lower in fish fed on the 1:2 diet, differing only from those fed on the 1:0.5 diet (P= 0.020).

3.6 Hepatic metabolites

Diets containing different proportions of soluble and insoluble fiber no influenced significantly (P>0.05) in the levels of glucose, glycogen and protein in fish liver (Table 7).

4. Discussion

The results obtained in this study present a new perspective for the use of dietary fiber in fish nutrition. The simple inclusion of 10% of dietary fiber from linseed, without protein-energy changes or constitutional ingredients in the diet, promoted a mean increase of 28.5% in the weight gain of the animals compared to the control diet (Table 2). Among the tested soluble: insoluble fiber ratios, 1:2 and 1:4 promoted higher specific weight gain and growth rate, without affecting the consumption and feed conversion of fish (Table 2), truly acting as growth promoters.

In recent years, studies have shown that sensible dietary fiber inclusions optimize the immune system and animal production, with an emphasis on the prebiotic action (Cerezuela et al., 2013; Yarahmadi et al., 2014; Adorian et. al., 2015; Adorian et al., 2016; Goulart et al., 2017,). While the incorporation of more refined substances such as scFOS, XOS and GOS do not present growth effects for several species of fish (Grisdale-Helland, et al., 2008; Buentello, et al., 2010; Burr, et al. 2010; Hoseinifar, et al., 2014; Guerreiro, et al., 2015; Guerreiro, et al., 2015; Hoseinifar et al., 2016; Guerreiro et al., 2018). These results

266 demonstrate the clear need for a change in perspectives on this food fraction in fish nutrition,
267 which can no longer be seen as a diluent of energy and antinutrient, but rather as a fraction
268 that deserves to be studied in detail, in order to express its functional effects the animal health
269 and production.

270 The positive effects of linseed fiber consumption on fish are possibly reflective of the
271 stimulus it exerts on the intestinal microbiota, similar to that reported for humans (Wenzel,
272 2012; Merrifield and Ringø, 2014). Since dietary fiber is resistant to the enzymatic digestion
273 and reaches the intestine while still being intact, it acts as a substrate for microbial
274 fermentation. In this fermentation process, short-chain fatty acids (SCFA) are produced; they
275 enter several metabolic pathways, generating energy and releasing bioactive compounds
276 bound to fiber (Ferreira, 2012; Wenzel, 2012; Quirós-Sauceda et al., 2014; Ríos-Covián et al.,
277 2016; Celi et al., 2017).

278 This release of bioactive compounds may have contributed to the higher performance
279 of the fish that received the 1:2 and 1:4 S:IF in diets, because they have higher phenolic
280 compound (Table 1) contents, which follow the physiological processes that are common to
281 fiber, producing a synergic effect in the gastrointestinal tract (Goñi et al., 2009), promoting an
282 antioxidant environment and the maintenance of the intestinal integrity (Saura-Calixto, 2011;
283 Quirós-Sauceda et al., 2014). This fact shows that the functional effects of fiber are not only
284 related to their ratios, but also to characteristics that are intrinsic to their source of origin.

285 However, it is important to highlight that the use of diets with a higher degree of fiber
286 solubility (1:0.5 and 1:1) do not lead to significant differences in animal performance,
287 compared to the control diet (Table 2); this indicates that silver catfish tolerate high levels of
288 soluble fiber in their diet (51.9-68.3 g/kg). However, under these conditions, the prebiotic
289 action of linseed fiber appears to be inhibited.

290 Considering the above demonstrated aspects, it is clear that linseed fiber is a functional

ingredient, with the ability to improve performance when properly administered. Evidence of its functional role had already been reported for juvenile silver catfish, where the administration of soluble linseed fiber (mucilage) provided greater weight gain and feed conversion (Goulart et al., 2017), similarly to what occurred with juvenile Nile tilapias (*Oreochromis niloticus*) (Mombach, 2015). These results demonstrate that the formulation of diets can be manipulated in order to balance the amount of dietary fiber, in order to obtain positive results from its presence. However, it is important to emphasize that these authors only evaluate food fiber concentrates from isolated fractions (soluble), without considering that the combination of different ratios of soluble and insoluble fiber could boost their action.

Our results show that the effects of linseed fiber are not only limited to improvements in the performance of the animals, since their supplementation in diets leads to positive changes in the body composition of fish and in the pattern of nutrient deposition in the body. This is clear from the higher crude protein content (1:4) and protein deposition (1:2 and 1:4) provided by diets (Table 3). These fiber ratios may have stimulated the production of SCFA by the intestinal microbiota, providing an additional amount of energy for animal metabolism. This may reflect in improvements in the mucosal morphology, increasing intestinal villus and absorptive area, and avoiding possible infections by opportunistic microorganisms (Topping, 1996; Park and Floch, 2007). Thus, the energy saved by the reduction of cell turnover can be destined to protein deposition (Merrifield et al., 2010; Ferreira, 2012). These results demonstrate that, in spite of being less efficient compared to glucose metabolism, potentially fermentable fibers can contribute to nutrient deposition.

It is worth highlighting that the supplementation of linseed fiber at the tested ratios did not cause physiological and metabolic changes in silver catfish (Table 4 and 7). However, the higher hydration capacity of the 1:0.5 S:IF in diet (Table 1), may have caused an increase in the viscosity of the digesta, to the point of hindering the enzyme-substrate interaction

316 (Easwood, 1992; Sinha et al., 2011). In an attempt to compensate for this situation, digestive
317 metabolism may have increased the secretion and activity of trypsin (Table 5) which, during a
318 culture cycle, could reflect on adaptations of the gastrointestinal tract.

319 The functional effect of linseed fiber is also evidenced by the positive changes in the
320 intestinal histological parameters of the silver catfish (Table 6). These results show that the
321 consumption of this fiber stimulates the development of intestinal villi, providing a greater
322 absorptive area, which may have contributed to the better performance and nutritional
323 deposition observed in the fish that received the 1:2 and 1:4 diets. In addition, larger villi
324 reduce the susceptibility of fish to diseases caused by intestinal pathogens (Brumano and
325 Gattás, 2009; Ferreira, 2012). Goulart et al. (2017) report similar results when supplementing
326 soluble fiber of linseed and β-Glican + Mananas in diets. The authors also point out that the
327 higher the villi height, the better the digestion and absorption of nutrients, reflecting greater
328 zootechnical performance, as occurred in this study.

329 The greater thickness of the intestinal epithelium of silver catfish fed on the 1:0.5 diet
330 corroborates the idea that its greater hydration capacity hinders the absorption of nutrients by
331 fish, which occurs not only because it has effects on the viscosity of the digesta, but also
332 according to intestinal histological changes, since the greater thickness of the epithelium
333 demands greater metabolic efforts for the absorption of the nutrients. However, the lower
334 thickness of the muscle layer resulting from the consumption of linseed fiber diets is directly
335 related to the higher villus height, which as well as increasing the absorptive area, has a
336 protective function (Ferreira, 2012). As the control diet provided less development of the villi,
337 there was a need to thicken the muscular layer, in order to maintain its physiological role in
338 protecting against the invasion of pathogens, since this layer consists of a dense network of
339 macrophages (Bauer, 2008).

340 Finally, it is important to consider that each fiber source has its peculiarities and it is

essential to study them more thoroughlly and to establish the correct levels and ratios of inclusion, since its beneficial effects can be easily compromised by their excess in the diet, whereas, when balanced, they may improve animal performance and the functionality of the gastrointestinal tract (Celi et al., 2017).

5. Conclusion

These results allow concluding that linseed fiber acts effectively as a growth promoter in silver catfish diets, and the use of the 1:2 and 1:4 ratios of soluble: insoluble linseed fiber optimizes its prebiotic action in the animal organism. However, it is necessary to conduct further studies in the area, which allow understanding the action of each fiber fraction, as well as its effects on immunological parameters.

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Table 1. Dietary formulations and proximate composition of the experimental diets (g/kg)

	Treatments[1]				
Ingredients	1:0.5	1:1	1:2	1:4	Control
Fish meal[2]	582.00	577.00	571.00	567.00	621.00
Maize starch	100.00	100.00	100.00	100.00	100.00
Linseed soluble fiber	93.70	64.80	35.80	13.80	
Linseed insoluble fiber	43.20	73.00	102.80	125.50	
Microcrystalline cellulose					105.70
NaCl	5.00	5.00	5.00	5.00	5.00
Soybean oil	50.00	46.00	42.00	39.00	54.00
Vitamin and mineral mixture[3]	30.00	30.00	30.00	30.00	30.00
BHT[4]	0.10	0.10	0.10	0.10	0.10
Inert[5]	96.00	104.10	113.30	119.60	84.20
Total	1000	1000	1000	1000	1000
Analyzed nutrient					
Crude protein	381.40	382.80	382.40	383.40	377.80
Calculated energy (MJ/kg)[6]	13.41	13.42	13.42	13.43	13.41
Lipids	116.30	115.20	116.50	116.50	119.00
Total dietary fiber	102.90	103.90	103.10	103.30	103.50
Soluble fiber	68.30	51.90	35.00	21.30	02.70
Insoluble fiber	34.60	52.00	68.10	82.00	100.80
Physicochemical properties[7]					
Hydration capacity	2.40	1.79	1.30	1.43	1.51
Fat binding capacity	0.94	0.91	0.97	0.96	1.05
Copper binding capacity	10.80	10.96	10.52	11.02	10.70
Phenolic compounds (mg EAG/g)[8]	55.77	68.80	77.80	86.21	

[1]Ratio soluble: insoluble fiber.
[2]Waste flour tilapia/Copisces-Paraná/ Brazil.
[3]Composition (kg): folic acid 997.50 mg; pantothenic acid 9975.00 mg; biotin 159.60 mg; cobalt 39.90 mg; copper 2800.00 mg; etoxiquin 24.78 g; iron 19.62 g; iodine 120.00 mg; manganese 5200.00 mg; niacin 19.95 g; selenium 119.70 mg; zinc 28.00 g; vit.A 1995000 UI; vit. B1 4987.50 mg; vit. B12 5985,00 mg; vit. B2 4987.50g; vit. B6 4987.50 mg; vit. C 70.00 g; vit. D3 198000.05 UI; vit. E 19950.00 UI; vit. K 997.50 mg.
[4]Butyl hydroxy toluene (BHT).
[5]Sand.
[6]Digestible energy calculated according to ingredient analysis = [(crude protein × 5640 kcal/kg × 0.9) + (fat × 9510 kcal/kg × 0.85) + (Carbohydrates soluble in neutral detergent × 4110 kcal/kg ×0.50)] (Jobling, 1983).
[7]Hydration capacity: g water/g sample; Fat binding capacity: g fat/g sample; Copper binding: mg Cu/ g sample.
[8]Calculated

561 **Table 2.** Performance parameters of *Rhamdia quelen* fed with different ratio soluble:
562 insoluble linseed fiber in the diet

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Weight gain (g)	31.23^{ab}	27.77^{b}	33.12^{a}	33.88^{a}	24.44^{b}	1.22	0.041
Total length (cm)	15.78^{ab}	15.23^{ab}	15.70^{a}	15.55^{ab}	15.06^{b}	0.06	0.015
Condition factor	0.94	0.93	0.97	0.96	0.94	0.01	0.407
Specific growth rate (%/day)	4.45^{ab}	4.36^{ab}	4.65^{a}	4.56^{a}	4.13^{b}	0.06	0.048
Consumption (g)	982.56	936.56	1088.31	1173.49	889.51	28.33	0.066
Apparent feed conversion	1.06	1.12	1.08	1.11	1.21	0.02	0.229

563 [1]Ratio soluble: insoluble fiber. Values are expressed as mean. SE: standard error. Different letters on the rows indicate
564 significant difference by the Tukey's test ($P<0.05$).
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567 **Table 3.** Corporal composition (g/kg) and body deposition of protein and fat (g) of juvenile
568 *Rhamdia quelen*

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Crude protein	156.50^{ab}	157.10^{ab}	157.80^{ab}	160.30^{a}	152.20^{b}	0.29	0.041
Fat	87.20	88.80	89.30	94.80	93.60	0.17	0.235
Dry matter	243.00^{b}	253.30^{ab}	253.20^{ab}	264.06^{a}	252.60^{ab}	0.19	0.023
Ash	28.80	26.50	29.60	27.70	25.70	0.07	0.476
	Body deposition (g)						
Protein	4.30^{b}	4.59^{ab}	5.21^{a}	4.95^{a}	4.03^{b}	0.12	0.003
Fat	2.42	2.33	2.62	2.70	2.21	0.07	0.274

569 [1]Ratio soluble: insoluble fiber. Values are expressed as mean. SE: standard error. Different letters on the rows
570 indicate significant difference by the Tukey's test ($P<0.05$).
571

572 **Table 4.** Corporal yield and digestive index (g/kg) of juvenile silver catfish (*Rhamdia quelen*)

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Corporal yield	845.20	867.20	859.80	863.60	867.10	0.25	0.113
Hepatosomatic index	15.10	14.70	15.10	16.60	14.90	0.03	0.226
DSI	44.30	38.10	39.40	38.10	35.90	0.09	0.135
Intestinal quotient	12.30	11.20	10.60	10.80	10.70	0.02	0.713
Visceral fat index	16.40	22.10	19.50	24.90	25.00	0.14	0.976

573 [1]Ratio soluble: insoluble fiber. DSI: Digestive somatic index. Values are expressed as mean. SE: standard error.
574 Different letters on the rows indicate significant difference by the Tukey's test ($P<0.05$).
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580 **Table 5.** Activity of digestive enzymes of juvenile *Rhamdia quelen* receiving the
581 experimental diets

Treatments[1]	Chymotrypsin (µmol/btee/min/mg protein)	Trypsin (µmol/tame/min/mg protein)
1:0.5	8318.93	13.05[a]
1:1	7521.85	9.11[ab]
1:2	5886.04	7.20[b]
1:4	6222.31	6.98[b]
Control	7367.09	8.55[ab]
Standard error	478.75	0.61
P-value	0.522	0.007

582 [1]Ratio soluble: insoluble fiber. Values are expressed as mean. Means with different letters in the column indicate
583 significant differences by Tukey test (P<0.05).
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586 **Table 6.** Intestinal histology of juvenile *Rhamdia quelen* fed with different ratio soluble:
587 insoluble linseed fiber in the diet

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Total villus height	802.04[a]	691.19[ab]	736.54[ab]	802.07[a]	631.84[b]	16.57	0.003
Villus height	694.91[a]	677.92[a]	647.02[a]	708.32[a]	539.87[b]	13.12	<0.001
Epithelium thickness	101.92[a]	86.72[ab]	83.45[b]	96.80[ab]	95.58[ab]	2.01	0.020
Muscle layer thickness	49.02[b]	47.49[b]	41.42[b]	46.85[b]	58.82[a]	1.05	<0.001

588 [1]Ratio soluble: insoluble fiber. Total villus height, villus height, epithelium thickness and muscle layer thickness:
589 µm. Values are expressed as mean. SE: standard error. Different letters on the rows indicate significant
590 difference by the Tukey's test (P<0.05).
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593 **Table 7**. Hepatic metabolites of juvenile *Rhamdia quelen* receiving the experimental diets

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Glucose	220.45	211.74	242.48	212.06	247.75	25.20	0.323
Glycogen	14.23	10.90	14.03	13.11	12.45	0.75	0.646
Protein	65.72	69.17	63.24	60.40	58.94	1.50	0.246

594 [1]Ratio soluble: insoluble fiber. Glucose: (µmol glucose/g tissue); Glycogen: (µmol glucose/g tissue); Protein:
595 (mg protein/g tissue). Values are expressed as mean. SE: standard error. Different letters on the rows indicate
596 significant difference by the Tukey's test ($P<0.05$).
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3 ARTIGO II

O artigo científico intitulado “Functional linseed fibers in improves the immune functions and performances of juveniles of silver catfish” foi submetido para a revista Animal Feed Science and Technology e está formatado segundo as normas descritas no Guia dos Autores (Anexo A).

Functiona linseed l fibers in improves the immune functions and performances of juveniles of silver catfish

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Abstract

This study was conducted to evaluate the prebiotic action of distinct linseed functional fibers in the diets of juvenile silver catfish, under immunological and growth parameters. For this, soluble and insoluble fractions of linseed fiber were concentrated separately and combined in four ratios (1:0.5, 1:1, 1:2, 1:4), which were added to silver catfish diets (6.43 ± 0.12 g) and evaluated in a bioassay, along with a control diet (without the addition of linseed fiber). After 45 days receiving the experimental diets, the animals were submitted to biometry for data collection and samples for further analysis. The experimental design was completely randomized, with five treatments and four replications; data were submitted to analysis of variance and the means were compared by Tukey's test ($P < 0.05$). Total circulating proteins and globulins were higher in the plasma of fish fed on diets 1:2 and 1:4, as well as mucoprotein content, total immunoglobulins and cutaneous mucus pH. Cortisol levels and intestinal pH were lower in these treatments. Regardless of the dietary ratio, the linseed fiber intake increased total plasma immunoglobulins and plasma alkaline phosphatase activity and cutaneous mucus. The production of intestinal acetic acid was higher in fish fed on the 1:2 diet, whereas the production of butyric acid was higher with the 1:4 diet and the propionic acid with the control diet. The control diet led to lower counts of goblet cells. Fish performance was higher for the group that received the 1:2 and 1:4 diets. In conclusion, the results indicate that linseed fiber has an immunostimulating action for juvenile silver catfish; ratios of 1:2 and 1:4 soluble: insoluble fiber optimize the immune system and the production of SCFA, with positive effects on fish performance.

Keywords: SCFA, *Rhamdia quelen*, dietary fiber, *Linum usitatissimum*, prebiotic.

Abbreviations: DWG, daily weight gain; SCFA, short-chain fatty acids; SGR, specific growth rate; SE, standard error; S:IF, soluble:insoluble fiber; IgT, total immunoglobulin; UFSM, University of Santa Maria;

1. Introduction

Fiber is a food constituent with distinct functional properties in the animal organism, which are intrinsically associated with the proportions of its water-soluble or -insoluble fractions (Macagnan et al., 2016). The effects of its consumption are directly related to the fermentability of the fiber by the intestinal microbiota, as well as by the bioactive compounds associated with it, which promote improvements in the gastrointestinal environment, impacting on the health and performance of animals (Giuntini and Menezes, 2011; Saura-Calixto, 2011).

The short chain fatty acids (SCFA) resulting from fiber fermentation are absorbed and metabolically used as an energy source, positively influencing metabolic and physiological processes (Guillon and Champ, 2000; Ferreira, 2012). They further promote luminal pH reduction, avoiding infections by opportunistic microorganisms, potentiate the immune system and provide maintenance of the mucosal integrity (Radecki and Yokoyama, 1991; Park and Floch, 2007; Ferreira, 2012).

The intensive production of fish for commercial interests constantly exposes them to stressful conditions and unfavorable environments, which increase the susceptibility to a diversity of pathogens (Yokoyama et al., 2005). Therefor, the use of substances that modulate the immune system and improve immunocompetence may promote the activation of defense mechanisms and increase resistance and tolerance to unfavorable conditions, avoiding deleterious effects for fish and reflecting improvements in their performance, as occurs with prebiotic supplementation for different animal species (Silva and Nörnberg, 2003; Yokoyama et al., 2005; Saurabh and Sahoo, 2008; Merrifield et al., 2010; Ringø et al., 2010; Freitas, et al., 2014).

In this context, this study aims at evaluating the prebiotic action of different soluble and insoluble linseed fiber ratios (1:0.5, 1:1, 1:2, 1:4) in juvenile silver catfish (*Rhamdia quelen*) diets under immunological parameters and growth.

2. Material and methods

The study was conducted at the Laboratory of Fish Farming of the Department of Animal Science of the Federal University of Santa Maria (UFSM), Rio Grande do Sul, Brazil (Latitude: 29º 41’ 03’’ S; Longitude: 53º 48’ 25’’ W), after being approved by the Ethics Committee on Animal Trials of this University, under the process number 8015120816.

2.1 Preparation of functional fibers

Linseed fiber is obtained in two distinct stages. In the first stage, soluble fiber of linseed (mucilage) was obtained by soaking the whole grain in water at a concentration of 10% w/v, maintaining the reaction between 60◦C and 80◦C under constant stirring for 150 min. Subsequently, the soluble fiber was separated from the grains by sieving, followed by addition of ethanol, for the precipitation of this fraction following the method described by Goulart et al. (2013). The resulting soluble fiber of this process was dried in an air circulating oven at 55ºC for 48 hours and ground in a micro-grinder (Marconi, model MA-630/1) to obtain particles smaller than 590μm, representing the Linseed soluble fiber.

In the second stage, the insoluble fiber contained in the linseed was extracted. The demucilaged grain was defatted with hexane at a ratio of 1:2 (w/v), performing for 30 min washes. After defatted, the protein content of the residue was reduced by dispersion in distilled water at room temperature at the ratio of 1:30 (w/v),sifted and dried in an air circulating oven at 55 ºC for 24 h. The Linseed insoluble fiber obtained in this stage was

ground in a micro-grinder (Marconi, model MA-630/1) to obtain particles smaller than 590μm.

2.2 Experimental diets

Five experimental diets (Table 1) were formulated to achieve the nutritional requirements of juvenile silver catfish, according to Meyer and Fracalossi (2004). The experiment comprised the following treatments: Addition of functional fibers in the diet in proportions of 1:0.5, 1:1, 1:2, 1:4 of soluble: insoluble fiber (S:IF) and control diet (without addition of fiber). The diets were produced in the Laboratory of Fisheries of UFSM. The dry ingredients were weighed and manually homogenized, then water was added and pelleting with matrix of 3 mm in diameter. They were dried in an oven with forced air circulation for 24 h at a temperature of 55 ºC. After drying, the diets were milled and selected according to the fish ingestion capacity. Diets were stored under a temperature of −20 ºC throughout the experimental period. The diets composition and physicochemical properties were determined based on analyses of crude protein (method 960.52), total, insoluble and soluble dietary fibers (method 991.43) were determined according to the methodologies described by AOAC (1995), fat (Bligh and Dyer, 1959), hydration capacity and fat binding capacity (Wang and Kinsella, 1976), copper binding (McBurney, 1983) and phenolic compounds (Waterhouse, 2003).

2.3 Animals and feed

Six-hundred juveniles of silver catfish with an average initial weight of 6.43 ± 0.12 g were distributed randomly in 20 polypropylene tanks with 290 liters capacity (30 animals per experimental unit). Each tank had individual water inlet and outlet, arranged in a water recirculation system comprised of a decanter, two mechanical and biological filtering and a

water reservoir with a capacity for 2000 liters, equipped with a heating system. During the experimental period, the fish were fed with the experimental diet until apparent satiation three times a day (9:00, 13:00 and 17:00 o'clock) for 45 days.

2.4 Water quality

Prior to the first and last meals (8:00 and 15:00 o'clock), fecal residues were removed from the tanks by siphoning twice a day. During the experimental period, the water quality parameters were monitored using colorimetric kits and maintained as follows: morning temperature of 23.33 ± 1.71ºC; afternoon temperature of 24.90 ± 1.37ºC; pH: 7.45 ± 0.20; alkalinity: 37.25 ± 4.95 mg $CaCO_3$/L; hardness: 36.75 ± 11.25 mg $CaCO_3$/L; total ammonia: 0.28 ± 0.10 mg L^{-1}; nitrite: 0.02 ± 0.14 mg L^{-1} and oxygen: 7.75 ± 0.88 mg L^{-1}.

2.5 Plasma analyzes

Blood samples were collected randomly (eight fish/treatment) by tail vein puncture using heparinized syringes. The samples were placed in microcentrifuge tubes for centrifuging (1000g, 10 min at room temperature). The plasma was stored under refrigerated (- 8 ºC) to determine the concentrations of total proteins (g/dL), albumin (g/dL), globulin (g/dL)= total protein−albumin), glucose (mg/gL), triglycerides (mg/dL) and cholesterol (mg/gL). These tests were carried out in automation system (Labmax 100) using commercial kits Labtest®. The activity of alkaline phosphatase was carried out using commercial kit Doles®.

Total immunoglobulin (IgT) levels were measured using the method described by Hoseinifar et al. (2015). Briefly, total protein content was measured using commercial kits for total circulating proteins (g/dL) Labtest®. Thereafter, the immunoglobulin molecules precipitated down using a 12% solution of polyethylene glycol (Sigma®). The difference in

protein contents prior and after immunoglobulin molecules precipitation is considered as the IgT content.

The concentration of cortisol in fish plasma was determined by enzyme immunoassay for ELISA, using commercial kit DBC®. The test principle follows a typical scenario of competitive binding between an unlabeled antigen and an enzyme labeled with antigen. The assay was performed on a 96-well microplates and the absorbance read on Plate Reader (Eppendorf, AF2200) at 450 nm.

2.6 Skin mucus analyzes

Fish skin mucus samples were collected randomly (eight fish/treatment) using the methods of Ross et al. (2000) and Palaksha et al. (2008), with modifications. The fish were transferred to polyethylene bags containing 10 mL of 50 mM NaCl and these were gently shaken (manually) for 60 seconds to release the mucus. The bags were placed on ice to euthanize the fish by hypothermia. After euthanasia was observed, skin mucus was collected by soft scraping of the dorsolateral surface, avoiding contamination with urinary-genital and intestinal excretions. The mucus samples were transferred to amber glass tubes, homogenized and stored (-20 ° C) for further analysis.

The levels of mucoprotein (glycoprotein) were determined using a Bioclin® commercial kit. The principle of this methodology is the protein precipitation in a solution of perchloricacid, resulting in a glycoprotein fraction denominated seromucoid and/or mucoproteins. These are, then, precipitated in the filtrate with phosphotungstic acid and subsequently dissolved and dosed by means of the tyrosine content.

Skin mucus total immunoglobulin levels were measured using the method described by Hoseinifar et al. (2015). Briefly, mucus total protein content was measured according to the technique described by Bradford (1976). Thereafter, the immunoglobulin molecules

precipitated down using a 12% solution of polyethylene glycol (Sigma). The difference in protein contents prior and after immunoglobulin molecules precipitation is considered as the IgT content. The pH of the fish skin mucus was determined with the aid of a digital pHmeter. The activity of alkaline phosphatase was carried out using commercial kit Doles®.

2.7 Parameters of gut contents

The gut contents of sixteen fish treatment were collected for determination of pH (eight fish/treatment) and short-chain fatty acids (eight fish/treatment). For this, the fish were previously fed the experimental diets and euthanized by benzocaine overdose (10%, 250 mg/L) (AVMA, 2013). The gut contents were collected after section of the intestine and the pH samples were added with 5 mL of distilled water and immediately measured with a digital pHmeter. The gut contents samples for the determination of SCFA were stored in sterile plastic tubes and kept at -20 ° C until analysis.

The SCFA determination was performed based on modified Bianchi et al. (2011) method. The fish gut contents (0.5 mg) was added of 2.5 mL distilled water and 0.25g of sodium chloride, then it was homogenized for 1 min in a vortex homogenizer and centrifuged at 3586 ×g for 10 min. Subsequently, 1.5 mL of the supernatant was transferred to an reaction tube and was added 20 μL of a 0.9 M H_2SO_4 solution (pH 2). The samples were centrifuged at 3586 ×g for 5 min, and then 1 mL of supernatant was transferred to a 4 mL vial, and sealed with a PTFE/rubber septum. The SCFA were extracted by the headspace solid-phase microextraction (HS-SPME) technique using a Car/PDMS fiber (Carboxen-polydimethylsiloxane) (10 mm × 75 μm of film thickness, Supelco, Bellefonte, PA, USA).

The extraction was carried out at 40 °C, with agitation by stir bar for 30 min of fiber exposition. Previously to the extraction, the samples were kept up for 10 min without fiber exposition at the same extraction temperature. The analyses were carried out by a gas

chromatography equipped with a flame ionization detector (GC-FID), Varian Star 3400CX (CA, USA). The SPME fiber was desorbed in a split/split less operated in a split less mode with a period of 1.30 min at 230 °C. The carrier gas used was hydrogen, under a constant pressure of 10 psi. The separation was made in a ZB-WAX Plus column (Chrompack, USA) of 30 m × 0.25 mm i.d. × 0.25 μm film thickness). The oven temperature was programmed at an initial temperature of 50 °C maintained for 1 min, and then increased to 110 °C at a rate of 5 °C min^{-1}, after that, the temperature increased to 250 °C at 15 °C min-1 and was maintained for 10 min. The detector temperature was held at 230 °C. The SCFA identification was achieved by the comparison of the SCFA, acetic acid, butyric acid and propionic acid with their authentic standards retention times (Sigma Aldrich). The quantification was performed by a five-point external calibration curve.

2.8 Goblet cell counts

Anterior intestine was collected (four fish/ treatment) and prepared for light microscopy. Histological samples were fixed in 10% formalin and preserved in 70% ethanol and subjected to the histological routine, following the method described by Gressler et al. (2016). The material was sent to go through the histological routine for dehydration in increasing ethanol series (70%–99% alcohol) and embedded in methacrylate glycol resin (Technovit 7100). From this material, slits of 2 μm were obtained from rotary microtome (LEICA RM2245) to subsequent coloration with hematoxylin-eosin. For morphological examination, the slides were observed and documented in light microscopy (ZEISS PrimoStar with AxioCam ERc5s) and analyzed through the software ZEN LITE (Carl Zeiss). Goblet cells were counted in 500 μm of villus and the results expressed in μm. The slides were thoroughly examined in order to determine the presence of histopathological alterations.

2.9 Performance

In the early and late experimental period, a biometric assessment was performed to collect data from the animals, which had fasted for 18 h and were anesthetized with Benzocaine (100 mg/L), to estimate the following: biomass (g): final biomass - initial biomass; daily weight gain(g): average weight gain/ 45 days; and fish survival (%). The daily feed intake (g) was recorded to calculate the total feed intake estimated per experimental unit at the end of the experiment.

2.10 Statistical analysis

Initially, the data were analyzed for outlier identification. The experimental design was completely randomized with five treatments and four replications. The data were subjected to analysis of variance and means were compared by Tukey's test. Differences were considered significant at the level of $P<0.05$.

3. Results

3.1 Plasma parameters

Plasma parameters were significantly influenced by the functional fibers tested (Table 2). The total circulating proteins ($P= 0.020$) and globulins ($P<0.001$) were higher in the plasma of fish fed diets containing ratio 1:2 and 1:4 of S:IF, while cortisol ($P= 0.005$) had reductions in treatments. Diets with functional fibers showed higher content of total immunoglobulins ($P<0.001$) and alkaline phosphatase activity ($P= 0.005$) in plasma.

3.2 Skin mucus parameters

Skin mucus parameters were significantly influenced by the functional fibers tested (Table 3). The mucoprotein ($P= 0.046$), total immunoglobulins ($P= 0.043$) e pH ($P= 0.004$)

248 were higher in skin mucus of fish fed diets containing ratio 1:2 and 1:4 of S:IF. The skin
249 mucus protein was superior (P= 0.012) in fish fed diet containing ratio 1:4, not differing
250 significantly only from fish fed 1:2 diet. Diets containing functional fibers resulted in
251 increased (P= 0.005) alkaline phosphatase activity in skin mucus, compared to control diet.
252
253 *3.3 Parameters of gut contents*
254 The gut content of silver catfish was significantly influenced by the fiber ratios
255 consumed in the diet (Table 4). The pH of the gut content of fish fed on diets 1:2 and 1:4 of
256 S:IF was significantly lower (P= 0.003). Acetic acid production was higher in the gut content
257 of fish fed on the 1:2 diet (P= 0.043), and it was not significantly different from those fed on
258 the 1:1 and 1:4 diets of S:IF. On the other hand, the production of butyric acid was higher for
259 fish fed on the 1:4 diet (P= 0.002). The production of propionic acid was higher in the gut
260 content of fish fed on the control diet (P= 0.048).
261
262 *3.4 Goblet cell counts*
263 Distinct counts of intestinal goblet cells were found in fish fed with the experimental
264 diets (Table 5). Diets with ratio of 1:0,5 S:IF provided higher intestinal goblet cell counts, not
265 differing from those given the 1: 2 diet. Fish fed with the control diet present lower goblet cell
266 counts (P>0.001).
267
268 *3.5 Performance parameters*
269 Diets with ratio of 1:2 and 1:4 S:IF provided higher biomass (P= 0.014) and daily weight
270 gain (P= 0.027) in fish when compared to the other treatments tested (Table 6). Feed intake
271 and fish survival were not influenced by the diets tested.
272

3. Discussion

Up to now, there are no studies evaluating the effects of different ratios of soluble and insoluble dietary fiber on fish diets. These results show that linseed fiber supplementation in 1:2 and 1:4 ratios truly acts as a prebiotic, stimulating the immune system, SCFA production, and juvenile silver catfish performance.

This is clear from the fact that fish fed on diets with 1:2 and 1:4 S:IF had higher total protein contents and globulin levels in their plasma. These fiber ratios may have stimulated the production of protective proteins in plasma, such as globulins, lysozyme, complementary proteins and other peptides, with proven immune action and bactericidal activity (Alexander and Ingram, 1992; Maqsood et al., 2009; Misra et al., 2009). Similar results were reported by Adorian et al. (2016) when evaluating different dietary fiber concentrates in diets for the same species.

As well as promoting the activation of plasma immune functions, diets containing 1:1, 1:2 and 1:4 of S:IF promote the reduction of plasma cortisol levels, which is the main indicator of stress for fish (Urbinati et al., 2014). This result indicates a higher tolerance to adverse culture conditions, as well as higher immunocompetence, since high levels of cortisol lead to the depression of the immune system, with reflexes on growth (Mommsen et al., 1999; Wendelaar Bonga, 2011; Urbinati et al., 2014).

Regardless of the dietary ratio, linseed fiber promotes higher levels of total immunoglobulins and of alkaline phosphatase in plasma, indicating a real immunostimulatory action. This is because immunoglobulins are involved in the systemic immunity of fish, and IgM (more common in the plasma of teleosts) promotes activation of the complement system that smooths and opsonizes pathogens, acting as mediator in the agglutination for phagocytosis and the removal of pathogens (Hatten et al., 2001; Zhao et al., 2008; Ye et al., 2013; Mashoof and Criscitiello, 2016).

In the same way, the greater activity of alkaline phosphatase indicates improvements in the immune system, since it is a hydrolase that has a protective role, with the capacity to dephosphorylate certain molecules, removing phosphate groups (Calhau et al., 1999; Mota et al. 2008; Ghahderijani et al., 2015). These results are in agreement with those of Goulart et al. (2017), which highlight the prebiotic ability of soluble linseed fibers to provide increased IgM in silver catfish plasma, indicating an increase in the immune function. Studies by Yarahmadi et al. (2014) also demonstrate the immunostimulatory action of dietary fibers, reporting higher lysozyme activity and expression of the immunological genes of rainbow trouts (*Oncorhynchus mykiss*).

Skin mucus presents a large number of immunological substances, serving as an indicator of the prebiotic action of ingredients used in fish nutrition (Esteban, 2012; Nigam et al., 2012; Guardiola et al., 2014ab; Guardiola et al., 2015). Our results demonstrate that the use of linseed fiber in the diet promotes positive changes in the skin mucus components of silver catfish, strengthening the first defense line against pathogens. The production of mucoprotein was stimulated by ratios 1:2 and 1:4 of S:IF, giving greater adhesive and viscoelastic action. The main action mode of mucoproteins is the uptake of foreign particles, which are removed by the continuous secretion of mucus by goblet cells (Roussel and Delmotte, 2004; Lang et al., 2007; Esteban, 2012).

The higher production of immunoglobulins in the skin mucus resulting from the consumption of 1:2 and 1:4 diets indicates a higher mucus capacity to eliminate pathogens, avoiding colonization. However, the lower pH observed in these treatments hinders the invasion by opportunistic pathogens, which usually require a neutral to alkaline environment (Balebona et al., 1995; Zhang et al., 2010; Gonçalves et al., 2016). In addition, a higher pH promotes the deterioration of biologically active mucus molecules, such as lysozyme,

reducing the antimicrobial activity, leading to impaired immune responses (Al-Arifa, et al., 2011).

Silver catfish fed with 1:4 also have a higher protein content in their mucus, indicating a positive influence on the production of the different substances that compose this fraction. Among them, lysozyme, antimicrobial peptides, protease enzymes and lectins stand out; they show a lytic activity against many bacteria, preventing the colonization by pathogens, cleaving proteins and interacting with the superficial structures of pathogens, resulting in the increase of phagocytosis (Subramanian et al., 2007; Saurabh and Sahoo, 2008; Esteban, 2012; Najafian and Babji, 2012; Gomez et al., 2013; Beck and Peatman, 2015). Regardless of the ratios, linseed fiber promotes an increase in alkaline phosphatase, which is an immunologically active enzyme, acting as an antimicrobial agent and in the regeneration of the skin at the early stages of healing, under stress and parasitic infection (Bates et al., 2007; Beck and Peatman, 2015).

The results observed in this study demonstrate that linseed fiber acts effectively as an immunostimulant of the plasma and skin mucus functions of silver catfish, and can be used as a prebiotic in diets for the species. It is suggested that these results are a consequence of the higher fermentative production of acetic and butyric acid, which was reflected on the reduction of intestinal pH (Table 4). Our results show that the fermentability profile is altered by the fiber ratios contained in the diet; higher ratios of insoluble fiber (1:2 and 1:4) reflect a higher production of acetic and butyric acid. However, it is important to observe that, regardless of the ratios of linseed fiber, acetic acid was produced in greater abundance, followed by propionic and butyric acid, similarly to what was reported by Ding et al. (2015) when studying the in vitro fermentability profile of linseed fiber for pigs.

The higher production of acetic and butyric acid may also have contributed to the better performance of fish fed on diets 1:2 and 1:4 (Table 6), which presented a daily weight

gain 28% higher than those fed on the control diet (same nutritional density as the test diets). It is known that the SCFA generated in the intestinal fermentation are used as a source of maintenance energy, which optimizes the use of the nutrients ingested in the diets for growth functions, making the body energetically more efficient for muscle production (Fukuda et al., 2011; Koh et al., 2016). This is highlighted by different authors, who show that dietary fiber has a growth promoting and nutritional deposition effect, similar to those provided by consolidated commercial prebiotics (Adorian et al., 2015; Mombach, 2015; Adorian et al., 2016; Goulart et al., 2017).

The abundance of propionic acid in the gut content of fish fed on the control diet is probably related to the absence of fermentable fibrous compounds, which reflects in the use of other components as a source of energy by the microbiota. Studies with species of the same food habit demonstrate that corn starch is not fully digested by the fish and its residues reach the posterior intestine, stimulating the development of specific bacteria that generate propionic acid (Heinitzet et al., 1996; Van Soest et al., 1991). Propionic acid inhibits lipogenesis, which is not desired in fish at this stage, as it may compromise animal development (Morrison and Preston, 2016).

Intrinsic characteristics of linseed fiber may have contributed to the fermentability profile of the diets and their reflexes on fish performance. This is because, among the monosaccharides found in linseed fiber, xylose, galactose and arabinose oligomers are found in larger amounts (Goulart et al., 2017). These monosaccharides are responsible for promoting the growth of beneficial bifidobacteria that contribute to the growth of the animal and act directly on some populations of pathogenic bacteria through competitive exclusion (Ringo et al., 2010; Freitas et al., 2014).

In addition to the aforementioned effects, the consumption of linseed fiber also contributed to intestinal homeostasis. The higher number of goblet cells in the intestinal

epithelium of the fish that received the 1:0.5, 1:2 and 1:4 diets, respectively, support the idea that linseed fiber has an immunostimulatory action, since goblet cells are responsible for the production of intestinal mucus, composed mainly of mucins that bind to membranes and provide an additional layer of defense to protect epithelial cells (Lang et al., 2007). Moreover, they create a viscous gel that hinders microbial penetration by protecting and lubricating the lining of the intestine (Junqueira and Carneiro, 2013).

In addition to mucins, other important substances are found in the intestinal mucus, including innate and adaptive immune factors, such as immunoglobulins. Among the immunoglobilins produced by fish, IgT is strategically designed to help teleosts to maintain homeostasis with the microbiota, since it excludes unwanted luminal bacteria, avoiding their colonization (Zhang et al., 2010; Gonçalves et al., 2016; Salinas et al., 2011).

4. Conclusion

Our results allow concluding that linseed fiber has an immunostimulating action for juvenile silver catfish, with the ratios of 1:2 and 1:4 soluble: insoluble fiber optimizing the immune system and the production of SCFA, with positive effects on the performance of fish. More studies need to be conducted with this source of fiber in order to accurately determine its action mode.

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610 **Table 1**. Dietary formulations and proximate composition of the experimental diets (g/kg)

	Treatments[1]				
Ingredients	1:0.5	1:1	1:2	1:4	Control
Fish meal[2]	582.00	577.00	571.00	567.00	621.00
Maize starch	100.00	100.00	100.00	100.00	100.00
Linseed soluble fiber	93.70	64.80	35.80	13.80	
Linseed insoluble fiber	43.20	73.00	102.80	125.50	
Microcrystalline cellulose					105.70
NaCl	5.00	5.00	5.00	5.00	5.00
Soybean oil	50.00	46.00	42.00	39.00	54.00
Vitamin and mineral mixture[3]	30.00	30.00	30.00	30.00	30.00
BHT[4]	0.10	0.10	0.10	0.10	0.10
Inert[5]	96.00	104.10	113.30	119.60	84.20
Total	1000	1000	1000	1000	1000
Analyzed nutrient					
Crude protein	381.40	382.80	382.40	383.40	377.80
Calculated energy (MJ/kg)[6]	13.41	13.42	13.42	13.43	13.41
Lipids	116.30	115.20	116.50	116.50	119.00
Total dietary fiber	102.90	103.90	103.10	103.30	103.50
Soluble fiber	68.30	51.90	35.00	21.30	02.70
Insoluble fiber	34.60	52.00	68.10	82.00	100.80
Physicochemical properties[7]					
Hydration capacity	2.40	1.79	1.30	1.43	1.51
Fat binding capacity	0.94	0.91	0.97	0.96	1.05
Copper binding capacity	10.80	10.96	10.52	11.02	10.70
Phenolic compounds (mg EAG/g)[8]	55.77	68.80	77.80	86.21	

611 [1]Ratio soluble: insoluble fiber.
612 [2]Waste flour tilapia/Copisces-Paraná/ Brazil.
613 [3]Composition (kg): folicacid 997.50 mg; pantothenic acid 9975.00 mg; biotin 159.60 mg; cobalt 39.90 mg;
614 copper 2800.00 mg; etoxiquin 24.78 g; iron19.62 g; iodine 120.00 mg; manganese 5200.00 mg; niacin 19.95 g;
615 selenium 119.70 mg; zinc 28.00 g; vit.A 1995000 UI; vit. B1 4987.50 mg; vit. B12 5985,00 mg; vit. B2
616 4987.50g; vit. B6 4987.50 mg; vit. C 70.00 g; vit. D3 198000.05 UI; vit. E 19950.00 UI; vit. K 997.50 mg.
617 [4]Butylhydroxytoluene (BHT).
618 [5]Sand.
619 [6]Digestible energy calculated according to ingredient analysis = [(crude protein × 5640 kcal/kg × 0.9) + (fat ×
620 9510 kcal/kg × 0.85) + (Carbohydrates soluble in neutral detergent × 4110 kcal/kg ×0.50)] (Jobling, 1983).
621 [7]Hydrationcapacity: g water/g sample; Fat binding capacity: g fat/g sample; Copper binding: mg Cu/ g sample.
622 [8]Calculated

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628 **Table 2.** Plasma parameters of juvenile *Rhamdia quelen* receiving the experimental diets

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Total proteins	3.23^{ab}	3.18^{b}	3.70^{a}	3.71^{a}	3.09^{b}	0.07	0.020
Albumin	0.54	0.72	0.68	0.67	0.71	0.02	0.210
Globulin	2.55^{b}	2.91^{ab}	3.05^{a}	3.21^{a}	2.53^{b}	0.06	<0.001
Glucose	51.83	52.87	57.28	54.42	51.71	2.20	0.938
Triglycerides	553.57	661.67	618.83	658.33	653.49	20.83	0.216
Cholesterol	131.14	184.50	157.12	188.14	178.71	7.08	0.510
Alkaline phosphatase	21.85^{a}	21.77^{a}	22.78^{a}	22.23^{a}	18.85^{b}	0.35	0.008
IgT	1.98^{a}	2.18^{a}	2.14^{a}	2.10^{a}	1.45^{b}	0.05	<0.001
Cortisol	17.98^{ab}	12.43^{b}	13.85^{b}	12.68^{b}	19.10^{a}	0.78	0.005

629 [1]Ratio soluble: insoluble fiber. Total proteins (g/dL); Albumin (g/dL); Globulin (g/dL); Glucose (mg/gL);
630 Triglycerides (mg/dL); Cholesterol (mg/gL); Alkaline phosphatase (U.I/L); IgT: Total immunoglobulin (mg/dL)
631 and Cortisol (µg/dL). Values are expressed as mean.SE: standard error. Different letters on the rows indicate
632 significant difference by the Tukey's test (P<0.05).
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637 **Table 3.** Skin mucus parameters of juvenile *Rhamdia quelen* fed with different ratio soluble:
638 insoluble linseed fiber in the diet

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Mucoprotein	3.82^{ab}	4.12^{ab}	4.38^{a}	4.56^{a}	3.56^{b}	0.12	0.046
Protein	66.12^{b}	66.14^{b}	69.76^{ab}	77.17^{a}	63.16^{b}	1.39	0.012
IgT	32.82^{b}	35.34^{ab}	37.67^{a}	37.16^{a}	31.98^{b}	1.18	0.043
pH	6.56^{ab}	6.51^{ab}	6.45^{a}	6.41^{a}	6.71^{b}	0.31	0.004
Alkaline phosphatase	34.14^{a}	30.62^{a}	31.40^{a}	31.80^{a}	24.07^{b}	0.91	0.005

639 [1]Ratio soluble: insoluble fiber. Mucoprotein (mg/dL); Protein (mg protein/g mucus); IgT: Total immunoglobulin
640 (mg protein/g mucus); Alkaline phosphatase (U.I/L).Values are expressed as mean· SE: standard error. Different
641 letters on the rows indicate significant difference by the Tukey's test ($P<0.05$).
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644 **Table 4**. pH and concentration of short-chain fatty acids (μmol/g) in gut contents of *Rhamdia*
645 *quelen*

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
pH	7.37^{ab}	7.36^{ab}	7.29^{b}	7.21^{b}	7.45^{a}	0.10	0.003
	Short-chain fatty acids (μmol/g)						
Acetic acid	4.75^{c}	6.85^{ab}	7.32^{a}	6.37^{ab}	5.73^{b}	1.07	0.043
Butyric acid	0.02^{c}	0.02^{c}	0.04^{ab}	0.06^{a}	0.03^{bc}	0.01	0.002
Propionic acid	0.11^{b}	0.09^{b}	0.11^{b}	0.10^{b}	0.20^{a}	0.02	0.048
Total SCFA	4.88	6.96	7.47	6.60	5.96		

646 [1]Ratio soluble: insoluble fiber. Values are expressed as mean.SE: standard error. Different letters on the rows
647 indicate significant difference by the Tukey's test (P<0.05).
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650 **Table 5**. Effect of different proportions of soluble and insoluble fiber on intestinal goblet cell
651 counts (cells/g) in silver catfish

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	EP	P
Goblet cell counts	26.50^{a}	18.25^{bc}	23.50^{ab}	19.50^{b}	12.66^{c}	0.58	<0.001

652 [1]Ratio soluble: insoluble fiber. Goblet cell counts in 500 µm. Values are expressed as mean.SE: standard error.
653 Different letters on the rows indicate significant difference by the Tukey's test (P<0.05).
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Table 6. Parameters of performance and survival of *Rhamdia quelen* receiving the experimental diets

Treatments[1]	Biomass (g)	DWG (g)	Feed intake (g)	Survival (%)
1:0.5	936.90^{ab}	0.58^{ab}	982.56	97
1:1	833.57^{b}	0.55^{b}	936.56	96
1:2	993.94^{a}	0.65^{a}	1088.31	98
1:4	1016.42^{a}	0.61^{a}	1173.49	97
Control	733.75^{b}	0.49^{b}	889.51	96
Standard error	22.27	0.02	28.33	0.29
P-value	0.014	0.027	0.066	0.073

[1]Ratio soluble: insoluble fiber. DWG: daily weight gain. Values are expressed as mean. SE: standard error. Means with different letters in the column indicate significant differences by Tukey test (P<0.05).

4 ARTIGO III

O artigo científico intitulado “Functional linseed fibers enhance the immune functions of silver catfish in response to acute stress” foi submetido para a revista Aquaculture Research e está formatado segundo as normas descritas no Guia dos Autores (Anexo B).

Functional linseed fibers enhance the immune functions of silver catfish in response to acute stress

Linseed fiber improves immunity of fish under stress

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Abstract

This study was conducted to evaluate the immunostimulating activity of diets supplemented with different rations of soluble and insoluble linseed fiber to *Rhamdia quelen* under hypoxia-induced acute stress. For this reason, soluble and insoluble fractions of linseed fiber were concentrated separately and combined into four ratios (1:0.5; 1:1; 1:2; 1:4), which were added to the diets of silver catfish (6.43 ± 0.12 g) and evaluated in a biological assay, along with a control diet (without addition of linseed fiber). After being fed the experimental diets for 45 days, specimens of silver catfish were submitted to hypoxia-induced acute stress. They were kept out of water for 60 seconds. Immediately afterwards, blood and cutaneous mucus were collected for subsequent determination of immunological indicators and stress. The experimental design was completely randomized with five treatments and four replications. The data underwent analysis of variance and the means were compared by Tukey's test ($P < 0.05$). The fish fed diets containing the 1:2 and 1:4 soluble: insoluble fiber ratios, showed higher total protein content, globulin and plasma alkaline phosphatase activity, in addition to higher mucoprotein content in the cutaneous mucus of the fish. Regardless of their ratio in the diet, linseed fiber provided higher plasma levels of total immunoglobulins and reduction of cortisol levels. The 1:1, 1:2 and 1:4 diets led to higher levels of total immunoglobulins and alkaline phosphatase activity in cutaneous mucus. The results indicate that linseed fiber has a stress-reduction and immunostimulant effect on silver catfish, and the 1:2 or 1:4 soluble: insoluble fiber ratios provided greater stimulation of the target immunological indicators.

Keywords: *Rhamdia quelen*, dietary fiber, immunostimulant, stress.

1. Introduction

Fish farming is an ever-expanding business. It produces animal protein on a large scale and at a fast pace. However, intensive fish farming causes great stress to animals, i.e., osmoregulatory, metabolic and immunologic disorders, induced by the combined action of cortisol and catecholamines (Wedemeyer, Barton & McLeay, 1990; Mommsen, Vijayan & Moon, 1999; Urbinati, Zanuzzo & Biller-Takahashi, 2014). These disorders reduce immune responses, which results in infectious diseases that inhibit the effective development of fish farming (Plant & Lapatra, 2011).

In this scenario, maintaining the health of cultivated species is essential for sustainable growth of the industry. Management of functional food supplements has been a

sustainable approach to minimize the use of chemicals in aquaculture (Guardiola, Cuesta & Esteban, 2016).

The use of immunostimulants has been considered as an environment-friendly method to prevent diseases in farming systems (Carbone & Faggio, 2016). Among stress reduction techniques, the use of prebiotics stands out as a promising alternative to minimize stress in intensive farming. Normally, commercial prebiotics are concentrated medium-chain oligosaccharides, obtained by partial hydrolysis of non-starch polysaccharides (NSPs) present in plant dietary fiber.

Concentrated plant fiber also showed beneficial effects on the health of different animal species (Yarahmadi, Miandare, Farahmand, Mirvaghefi & Hoseinifar, 2014; Adorian et al., 2015; Mombach, 2015; Adorian et al., 2016; Goulart et al., 2017). However, the use of plat fiber as a food supplement is still controversial because it may possibly increase the viscosity of the digesta, which undermines the digestibility of the diets.

Previous studies conducted by our research group have shown that the sources and solubility of fibers cause different effects on animal metabolism and growth (Adorian et al., 2015; Adorian et al., 2016; Goulart et al., 2017). Among the researched fiber sources, linseed fiber has demonstrated excellent prebiotic functionality (Adorian et al., 2015; Adorian et al., 2016). However, its ideal degree of solubility for dietary inclusion is not yet known, and its immunomodulating effects in stressful situations are little explored.

Therefore, the present study was conducted to evaluate the stress-reducing effect of diets supplemented with different ratios of soluble and insoluble linseed fiber on silver catfish (*Rhamdia quelen*) under acute stress.

2. Material and methods

The study was conducted at the Laboratory of Fish Farming Department of Animal Science, Federal University of Santa Maria (UFSM), Rio Grande do Sul, Brazil (Latitude: 29º 41’ 03’’ S; Longitude: 53º 48’ 25’’ W), after being approved by the Ethics Committee on Animal Experiments of this University, under protocol number 8015120816.

2.1 Preparation of functional fibers

Linseed fiber was obtained in two distinct stages. In the first stage, soluble fiber of linseed (mucilage) was obtained by soaking the whole grain in water at a concentration of 10% w/v, maintaining the reaction between 60 ºC and 80 ºC under constant stirring for 150 min. Subsequently, the soluble fiber was separated from the grains by sieving, followed by

addition of ethanol for precipitation of this fraction, following the method described by Goulart et al. (2013). The resulting soluble fiber of this process was dried in an air circulating oven at 55ºC for 48 hours and ground in a micro-grinder (Marconi, model MA-630/1) to obtain particles smaller than 590 μm, representing the Linseed soluble fiber.

In the second stage, the insoluble fiber contained in the linseed was extracted. The demucilaged grain was defatted with hexane at a 1:2 (w/v) ratio in a 30 min wash. After defatting, the protein content of the residue was reduced by dispersion in distilled water at room temperature at a 1:30 (w/v) ratio, sifted and dried in an air circulating oven at 55 ºC for 24 h. The linseed insoluble fiber obtained in this stage was ground in a micro-grinder (Marconi, model MA-630/1) to obtain particles smaller than 590 μm.

2.2 Experimental diets

Five experimental diets (Table 1) were formulated to achieve the nutritional requirements of juvenile silver catfish, according to Meyer and Fracalossi (2004). The experiment consisted of the following treatments: Addition of functional fibers in the diet in the following soluble: insoluble fiber ratios (S:IF): 1:0.5; 1:1; 1:2; 1:4 and control diet (without addition of fiber). The diets were produced in the Laboratory of Fisheries, UFSM. The dry ingredients were weighed and manually homogenized, then water was added and pelleted with a matrix of 3 mm in diameter. They were dried in a forced air circulation oven for 24 h at a temperature of 55 ºC. After drying, the diets were milled and selected according to fish ingestion capacity. Diets were stored under a temperature of −20 ºC throughout the experimental period. The composition and physicochemical properties of the diets were determined, based on analyses of crude protein (method 960.52), while total, insoluble and soluble dietary fibers (method 991.43) were determined according to the methodologies described by AOAC (1995); fat (Bligh & Dyer, 1959), hydration capacity and fat binding capacity (Wang & Kinsella, 1976), copper binding (McBurney, 1983) and phenolic compounds (Waterhouse, 2003) were also determined.

2.3 Animals and feed

Six hundred silver catfish juveniles with average initial weight of 6.43 ± 0.12 g were distributed randomly into 20 polypropylene tanks with 290 liter capacity (30 animals per experimental unit). Each tank had individual water inlet and outlet, arranged in a water recirculation system comprised of a decanter, two mechanical and biological filters and a water reservoir with a 2000 liter capacity, equipped with a heating system. During the

experimental period, the fish were fed with the experimental diet until apparent satiation three times a day (9:00, 13:00 and 17:00 o'clock) for 45 days.

2.4 Water quality

Prior to the first and last meals (8:00 and 15:00 o'clock), fecal residues were removed from the tanks by siphoning twice a day. During the experimental period, water quality parameters were monitored by using colorimetric kits and maintained as follows: morning temperature of 23.33 ± 1.71ºC; afternoon temperature of 24.90 ± 1.37ºC; pH: 7.45 ± 0.20; alkalinity: 37.25 ± 4.95 mg $CaCO_3$/L; hardness: 36.75 ± 11.25 mg $CaCO_3$/L; total ammonia: 0.28 ± 0.10 mg L^{-1}; nitrite: 0.02 ± 0.14 mg L^{-1} and oxygen: 7.75 ± 0.88 mg L^{-1}.

2.5 Stress

After being fed the experimental diets for 45 days, specimens of silver catfish were submitted to hypoxia-induced acute stress, according to the methodology described by Barcellos, Kreutza & Quevedo (2006). Such methodology consisted of catching fish from each tank with the aid of a dip net and removing them from the water, keeping them under hypoxia for 60 seconds. Immediately after stress, blood and mucus were collected from the fish for subsequent analysis.

2.6 Plasma collection and analysis

Blood samples were collected randomly (eight fish/treatment) by tail vein puncture using heparinized syringes. The samples were placed in micro-centrifuge tubes and centrifuged (1000g, 10 min). Plasma was stored under refrigeration (- 8 ºC) to determine the concentrations of total circulating proteins (g/dL), albumin (g/dL), globulin (g/dL)= total protein−albumin), glucose (mg/gL), triglycerides (mg/dL) and cholesterol (mg/gL). These tests were carried out in an automation system (Labmax 100), using Labtest® commercial kits. Alkaline phosphatase activity was determined by using a Doles® commercial kit.

Total immunoglobulin (IgT) levels were measured by using the method described by Hoseinifar et al. (2015). Briefly, total protein content was measured by using Labtest® commercial kits for total circulating proteins (g/dL). Thereafter, the immunoglobulin molecules precipitated down by using a 12% solution of polyethylene glycol (Sigma®). The difference in protein contents prior and after immunoglobulin molecule precipitation is considered as IgT content.

Cortisol concentration in fish plasma was determined by enzyme immunoassay for ELISA, using a DBC® commercial kit. The test principle follows a typical scenario of competitive binding between an unlabeled antigen and an enzyme-labeled antigen. The assay was performed on a 96-well microplate while absorbance was read on a PlateReader (Eppendorf, AF2200) at 450 nm.

2.7 Skin mucus collection and analysis

Fish skin mucus samples were collected randomly (eight fish/treatment) by using the methods of Ross, Firth, Wang, Burka & Johnson (2000) and Palaksha, Shin, Kim & Jung (2008), with modifications. The fish were transferred to polyethylene bags containing 10 mL of 50 mMNaCl and they were gently shaken (manually) for 60 seconds to release the mucus. The bags were placed on ice to euthanize the fish by hypothermia. After occurrence of euthanasia, skin mucus was collected by soft scraping of the dorsolateral surface, avoiding contamination with urinary-genital and intestinal excretions. The mucus samples were transferred to amber glass tubes, homogenized and stored (-20 ° C) for further analysis.

The levels of mucoprotein (glycoprotein) were determined with a Bioclin® commercial kit. The principle of this methodology is protein precipitation in a perchloric acid solution, which results in a glycoprotein fraction referred to as seromucoid and/or mucoproteins. Then, they are precipitated in the filtrate with phosphotungstic acid and subsequently dissolved and dosed by means of tyrosine content.

Total immunoglobulin levels in skin mucus were measured with the method described by Hoseinifar et al. (2015). Briefly, mucus total protein content was measured according to the technique described by Bradford (1976). Thereafter, the immunoglobulin molecules precipitated down by using a 12% solution of polyethylene glycol (Sigma®). The difference in protein contents prior and after immunoglobulin molecules precipitation was considered as IgT content. The pH of the fish skin mucus was determined with the aid of a digital pHmeter. Alkaline phosphatase activity was determined with a Doles® commercial kit.

2.8 Statistical analysis

Initially, the data were analyzed for outlier identification. The experimental design was completely randomized with five treatments and four replications. The data were subjected to analysis of variance and means were compared by Tukey's test. Differences were considered significant at the level of $P<0.05$.

3. Results

3.1 Plasma parameters

Diets with 1:2 and 1:4 S:IF ratios provided higher total protein content (P= 0.012) and plasma globulin (P= 0.045) in fish when compared to the other treatments (Table 2). Plasma alkaline phosphatase activity was significantly influenced by the functional fibers tested (P= 0.030). Fish fed diets containing 1:2 and 1:4 S:IF showed higher alkaline phosphatase activity than the other treatments. Albumin, glucose, triglycerides and cholesterol content of the plasma was not influenced by the functional fibers tested (Table 2). Total immunoglobulin content was higher in the plasma of fish fed diets containing functional linseed fiber (P= 0.002) when compared to fish fed the control diet (Table 2). Plasma cortisol level of fish was significantly higher in fish fed the control diet (P= 0.039) (Figure 1).

3.2 Skin mucus parameters

Mucoprotein, total immunoglobulins and alkaline phosphatase activity of fish skin mucus were influenced by the consumption of functional fibers in the diets (Table 3). Mucoprotein was higher (P= 0.007) in the skin mucus of fish fed diets containing 1:2 and 1:4 S:IF. Total immunoglobulins and alkaline phosphatase activity of fish skin mucus were higher (P= 0.039) in fish fed diets containing 1:1, 1:2 and 1:4 S:IF (Table 3). Protein content and pH of the fish skin mucus was not influenced by the functional fibers (P>0.05) (Table 3).

4. Discussion

Aquaculture production based on intensive farming systems allows an increase in fish yield per area, and greater control of food and animal health (Lima et al. 2006). However, routine activities of these systems, even when properly executed, submit the fish to stressful situations, thus affecting their immune response and causing damage to production. Therefore, knowledge about immunological responses triggered by acute stress favors the development of dietary strategies that mitigate the adverse effects of stress on the health of farmed fish. Our results showed that when adding fibers from an adequate source (linseed) to isonutritive diets, a simple change in the ratios of soluble and insoluble fractions promotes significant immunological gains for silver catfish juveniles. This is indicative that this group of indigestible components should receive greater attention in aquaculture.

When adding linseed fiber to the diets of silver catfish, we found that the 1:2 and 1:4 S:IF ratios produced a positive response from the immune system, increasing plasma total protein levels, which is positively related to several immunological components (globulins,

lysozyme, complement and other peptides (Alexander & Ingram, 1992; Misra, Das & Mukherjee, 2009; Maqsood, Samoon & Singh, 2009; Choudhury et al., 2005; Jha et al., 2007). Additionally, alkaline phosphatase, whose activity is protective for fish as a result of its antimicrobial effect (Ghahderijani, Hajimoradloo, Ghorbani & Roohi, 2015) was also increased. These responses show that fish fed those diets responded effectively to the stress stimuli by adapting their immune system to prevent damage to their body.

Results showed that dietary supplementation with linseed fiber had an immunomodulatory effect on silver catfish in situations of acute stress (diets 1:2 e 1:4 S:IF) thus increasing plasma total immunoglobulin content, one of the main components of innate immunity of fish (Hatten, Fredriksen, Hordvik & Endresen 2001; Zhao, Findly & Dickerson, 2008; Mashoof & Criscitiello, 2016; Ye,, Kaattari, Ma & Kaattari, 2013).

As found in the present study, other studies also reported that the acute stress phase promotes the mobilization of substances and defense cells and the distribution of the different cell types, as necessary (Dhabhar, 2002). However, if stress lasts longer, there will be a reduction in the number of circulating cells, which affects their migration and permanence in the affected organs (Tort, 2011), thus reducing the resistance of fish to diseases and increasing infection by opportunistic pathogens. In this case, dietary supplementation with linseed fiber can be used strategically, giving animals better conditions of defense in stressful situations.

The results also showed that the consumption of linseed fiber, regardless of solubility, had a repressive effect on plasma cortisol levels. It should be noted that plasma glucose did not follow the same cortisol response profile, suggesting that under acute stress conditions, the initial changes in glucose levels are dependent on catecholamine activity and, later, on cortisol activity (Wendelaar Bonga, 1997; Mommsen et al., 1999; Wendelaar Bonga, 2011; Urbinati et al., 2014).

It is not totally clear yet how linseed fiber acts upon the immune system. It is believed that by promoting the development of gut microbiota, immunomodulating products arising from fermentation of fibers (lipopolysaccharides, peptidoglycans and lipoteichoic acids and short-chain fatty acids) are produced in greater quantity and act more intensely on the immune response of animals (Macfarlane & Cummings, 1999; Park & Floch, 2007; Ferreira, 2012). In the digestive tract, fermentation of fiber causes reduction of luminal pH, creating a hostile environment for harmful microorganisms, reducing the pathogenic load of fish and potentiating the immune system, similarly to prebiotics (Burr, Gatlin & Ricke, 2005; Nayak, 2010; Merrifield & Ringø, 2014; Radecki & Yokoyama, 1991). Additionally, linseed fiber is rich in phenolic compounds with antioxidant capacity (flavonoids, tannins and polyphenols)

that assist in maintaining gut integrity (Saura-Calixto, 2011) and act synergistically in some physiological responses. In the fermentation of fiber by digestive microbiota, there is also a gradual release of phenolic compounds in the lumen, which are absorbed by epithelial cells of the intestine, eliminating free radicals (Goñi, 2009). In the case of linseed fiber, phenolic compounds are concentrated mainly in the insoluble fraction, which explains their different concentrations in the diets (Table 1) and suggests best synergetic interactions with other fiber fractions.

The effects of stress on mucosal surfaces of the fish are little known. Research is mostly restricted to studies that evaluate stress caused by water quality, transportation, heavy metal contamination, density, anesthetic agent and air exposure (Vatsos, Kotzamanis, Henry, Angelidis & Alexis, 2010; Tacchi et al., 2015; Guardiola et al., 2015; Guardiola et al., 2016). Furthermore, the majority of studies focused on evaluating only the increased release of mucus but not the differences in mucus composition (Guardiola et al., 2016; Vatsos et al., 2010; Shephard, 1994). Our results show that in situations of acute stress, cutaneous mucus composition is also changed, but it can be modulated beneficially through supply of linseed fiber in diets (1:1, 1:2 and 1:4 S:IF), thus reinforcing the idea of its stress-reducing and health-promoting effects.

Similarly to what was found in the plasma, the increased concentration of total immunoglobulins and increased alkaline phosphatase activity found in cutaneous mucus of fish (1:1, 1:2 and 1:4 S:IF) indicate that linseed fiber stimulates the secretion of immune substances in fish submitted to acute stress because immunoglobulins present in cutaneous mucus act in host defense against superficial infections, and they have bactericidal activity (Beck & Peatman, 2015; Magnadottir, 2006). In turn, alkaline phosphatase has regenerative activity on the skin and acts as an antimicrobial agent, as a result of its hydrolytic capacity (Bates, Akerlund, Mittge & Guillemin, 2007; Beck & Peatman, 2015; Ross et al. 2000).

Similarly to our work, previous studies showed an increase in immunoglobulin levels of cutaneous mucus after administration of immunostimulants in the diet (Sheikhzadeh, Pashaki, Nofouzi, Heidarieh & Tayefi-Nasrabadi, 2012; Sheikhzadeh et al. 2012). In addition to the positive responses in IgT and alkaline phosphatase, the increased mucoprotein production suggests that there was an increase in the thickness of the defense layer, protecting the epithelial cells and preventing the entry of pathogenic agents (Esteban, 2012; Lang, Hansson & Samuelsson, 2007; Roussel & Delmotte, 2004).

As the fish received the experimental diets for a period of 45 days prior to being submitted to stress, their immunity may have been influenced by linseed fiber, hence they

were more able to cope with the situation of stress. It is suggested that while the fish consumed linseed fiber, their immune system was modulated by fermentable substances and by phenolic compounds present in the dietary fiber.

In addition to the previously known antioxidant action that prevents damage to lipids, proteins and nucleic acids, thus preserving fluidity, permeability and cellular integrity (Barrera, 2012; Repetto, Semprine & Boveris, 2012; Zhang, Seeram, Lee, Feng & Heber, 2008), phenolic compounds also have anti-inflammatory activity, which inhibits the production of cytokines, avoiding immunological diseases which arise from inflammation (Liu & Lin, 2013; Veres, 2012).

Considering the above-mentioned findings, it is suggested that the phenolic compounds present in the linseed fiber concentrate have synergistic effects on the immunomodulating response attributed to the fermentative events of NSPs.

It should be noted that the immune system of fish is influenced directly and indirectly by nutrients ingested in food; therefore, the manipulation of the diets by including substances with immunomodulatory potential is extremely important to increase productivity, since it allows better response of animals to stressors present in the course of the productive cycle (Menezes et al., 2006; Pezzato, Barros, Fracalossi, & Cyrino, 2004).

5. Conclusion

Our results showed supplementation with linseed fibers had positive effects on the immune system of silver catfish. When degree of fiber solubility was manipulated, the 1:2 and 1:4 S:IF ratios offered greater stimuli to plasma and immunological indicators of cutaneous mucus.

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Table 1

Dietary formulations and proximate composition of the experimental diets (g/kg)

	Treatments[1]				
Ingredients	1:0.5	1:1	1:2	1:4	Control
Fish meal[2]	582.00	577.00	571.00	567.00	621.00
Maize starch	100.00	100.00	100.00	100.00	100.00
Linseed soluble fiber	93.70	64.80	35.80	13.80	
Linseed insoluble fiber	43.20	73.00	102.80	125.50	
Microcrystalline cellulose					105.70
NaCl	5.00	5.00	5.00	5.00	5.00
Soybean oil	50.00	46.00	42.00	39.00	54.00
Vitamin and mineral mixture[3]	30.00	30.00	30.00	30.00	30.00
BHT[4]	0.10	0.10	0.10	0.10	0.10
Inert[5]	96.00	104.10	113.30	119.60	84.20
Total	1000	1000	1000	1000	1000
Proximate analysis					
Crude protein	381.40	382.80	382.40	383.40	377.80
Digestible energy[6]	3203	3207	3207	3209	3205
Lipids	116.30	115.20	116.50	116.50	119.00
Total dietary fiber	102.90	103.90	103.10	103.30	103.50
Soluble fiber	68.30	51.90	35.00	21.30	02.70
Insoluble fiber	34.60	52.00	68.10	82.00	100.80
Physicochemical properties[7]					
Hydration capacity	2.40	1.79	1.30	1.43	1.51
Fat binding capacity	0.94	0.91	0.97	0.96	1.05
Copper binding capacity	10.80	10.96	10.52	11.02	10.70
Phenolic compounds (mg EAG/g)[8]	55.77	68.80	77.80	86.21	

[1]Ratio soluble: insoluble fiber.
[2]Waste flour tilapia/Copisces-Paraná/ Brazil.
[3]Composition (kg): folic acid 997.50 mg; pantothenic acid 9975.00 mg; biotin 159.60 mg; cobalt 39.90 mg; copper 2800.00 mg; etoxiquin 24.78 g; iron 19.62 g; iodine 120.00 mg; manganese 5200.00 mg; niacin 19.95 g; selenium 119.70 mg; zinc 28.00 g; vit. A 1995000 UI; vit. B1 4987.50 mg; vit. B12 5985,00 mg; vit. B2 4987.50g; vit. B6 4987.50 mg; vit. C 70.00 g; vit. D3 198000.05 UI; vit. E 19950.00 UI; vit. K 997.50 mg.
[4]Butylhydroxytoluene (BHT).
[5]Sand.
[6]Digestibleenergy: calculateddigestibleenergy: [(crudeprotein × 5.65 × 0.85) + (fat × 9.4 × 0.9) + (carbohydrates × 4.15 × 0.7)] (Jobling, 1983).
[7]Hydration capacity: g water/g sample; Fat binding capacity: g fat/g sample; Copper binding: mg Cu/ g sample.
[8]Calculated

Table 2

Plasma parameters of juvenile *Rhamdia quelen* receiving the experimental diets

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Total proteins	3.95^{b}	3.97^{b}	4.38^{a}	4.42^{a}	3.92^{b}	0.06	0.012
Albumin	0.74	0.71	0.85	0.96	0.69	0.04	0.202
Globulin	2.92^{b}	3.20^{ab}	3.43^{a}	3.35^{a}	3.05^{b}	0.09	0.045
Glucose	57.25	52.75	54.37	48.94	48.62	1.89	0.350
Triglycerides	623.50	788.37	705.87	588.64	623.37	32.78	0.312
Cholesterol	167.50	159.12	175.87	168.00	184.50	6.06	0.748
Alkaline phosphatase	17.48^{b}	18.87^{ab}	20.85^{a}	20.67^{a}	17.27^{b}	0.41	0.030
IgT	2.89^{ab}	2.79^{ab}	3.41^{a}	3.34^{a}	2.63^{b}	0.06	0.002

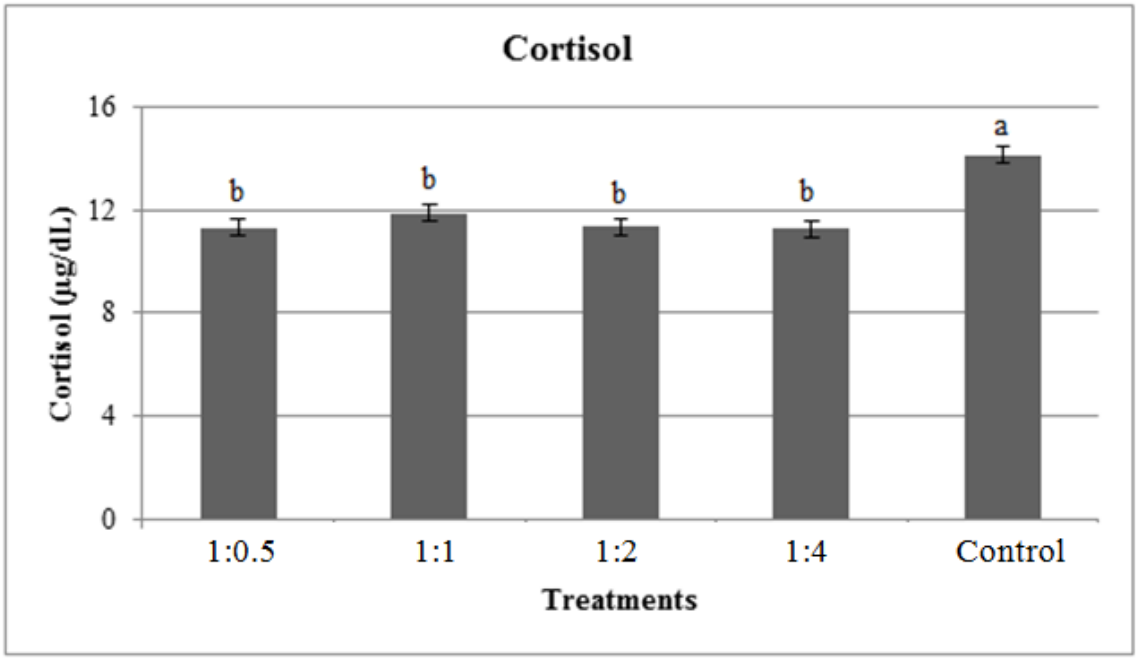
[1]Ratio soluble: insoluble fiber. Total proteins (g/dL); Albumin (g/dL);Globulin (g/dL): total protein−albumin (g/dL); Glucose (mg/gL);Triglycerides (mg/dL); Cholesterol (mg/gL);Alkaline phosphatase (U.I/L); IgT: Total immunoglobulin (mg/dL) and Cortisol (µg/dL).SE: standard error. Different letters on the rows indicate significant difference by the Tukey's test ($P<0.05$).

Table 3

Skin mucus parameters of juvenile *Rhamdia quelen* fed with different ratio soluble: insoluble linseed fiber in the diet

	Treatments[1]						
	1:0.5	1:1	1:2	1:4	Control	SE	P-value
Mucoprotein	3.64^{ab}	3.49^{ab}	3.95^{a}	4.04^{a}	3.18^{b}	0.09	0.007
Protein	58.31	64.32	60.64	65.73	57.33	1.79	0.144
IgT	35.43^{ab}	38.81^{a}	39.06^{a}	42.30^{a}	27.58^{b}	1.81	0.018
pH	6.65	6.70	6.70	6.71	6.69	0.02	0.408
Alkaline phosphatase	31.52^{b}	35.66^{a}	37.61^{a}	35.70^{a}	33.07^{b}	1.24	0.039

[1]Ratio soluble: insoluble fiber. Mucoprotein (mg/dL); Protein (mg protein/g mucus); IgT: Total immunoglobulin (mg protein/g mucus); Alkaline phosphatase (U.I/L);SE: standard error. Different letters on the rows indicate significant difference by the Tukey's test ($P<0.05$).



659

660 **Figure 1-** Plasma cortisol level of *Rhamdia quelen* fed with different ratios of linseed fiber in
661 the diet submitted to acute stress

5 DISCUSSÃO GERAL

A possibilidade de incorporação de diferentes proporções de fibra alimentar concentrada em dietas para peixes é de grande relevância econômica e científica, uma vez que busca a produção sustentável de proteína de alto valor biológico para consumo humano, sem a utilização de antibióticos, os quais podem promover o surgimento de cepas de microrganismos resistentes e deixar resíduos na carne e no ambiente. Para aplicação na nutrição humana, muitos autores sugerem a utilização de técnicas de hidrólise da fibra alimentar, para sua atuação efetiva como prebióticos (CHEN et al., 2013; GÓMEZ et al., 2014; GULLÓN et al., 2011; OLANO-MARTIN et al., 2002).. Segundo estes autores, embora fibras de alto peso molecular expressem atividade prebiótica, as de menor massa molar, como os oligossacarídeos, produzem fermentação intestinal mais seletiva.

Porém, na nutrição de peixes estudos mostram que a utilização de substâncias menos refinadas, como concentrados de fibra alimentar obtidos de distintas fontes, tem capacidade de otimizar o sistema imune dos animais, além de promover o crescimento e deposição nutricional. Os autores destacam ainda, que essas fibras proporcionam efeitos equivalentes ou superiores a prebióticos comerciais consolidados (ADORIAN et al., 2015; ADORIAN et al. 2016; GOULART et al. 2017; MOMBACH, 2015).

Em função disso, as técnicas de concentração da fibra utilizadas nesta tese objetivaram o fracionamento dos nutrientes contidos no grão da linhaça (fibra alimentar, proteína e lipídios) para posterior concentração das frações insolúvel e solúvel de fibra. A eficiência da concentração foi observada pelo valor relativo de fibra alimentar obtido para as frações solúvel (67,56%) e insolúvel (63,07%) (Apêndice A, Tabelas 2). A fração solúvel da fibra de linhaça apresentou em sua composição monossacarídica maior abundância de xilose>glicose>ácido galacturônico>arabinose, enquanto que, a fração insolúvel glicose>xilose>ácido galacturônico, não sendo encontradas quantidades detectáveis de arabinose. É importante destacar que nas amostras analisadas não foram encontradas galactose, ramnose, manose e frutose (Apêndice A, Tabelas 4).

Em relação as propriedades físico-químicas, a fração solúvel da linhaça apresentou capacidade de hidratação 11,5 vezes maior quando comparada a fração insolúvel (Apêndice A, Tabela 5). Este comportamento já era esperado, em função da natureza química da fibra solúvel, que apresenta estrutura altamente ramificada e com grande quantidade de grupos hidrofílicos (STEPHEN; CUMMINGS, 1979; VANDEROOF, 1998). A capacidade de ligação a gordura foi semelhante entre as frações, sendo encontradas 1,30 e 1,65 g óleo/ g na

fração solúvel e na insolúvel, respectivamente (Apêndice A, Tabela 5). A capacidade de ligação ao cobre da fração insolúvel da linhaça foi de 10,58 mg Cu/g de amostra (Apêndice A, Tabela 5), porém na fração solúvel a quantificação não foi possível devido a problemas metodológicos. Os resultados obtidos para os compostos fenólicos demonstram que as técnicas utilizadas para concentração das distintas frações de fibra de linhaça concentram esses compostos na fração insolúvel (Apêndice A, Tabela 5).

Após a analise das características químicas e físico-químicas, as frações de fibra de linhaça foram adicionadas a dietas para juvenis de jundiá, nas proporções 1:0,5, 1:1, 1:2 e 1:4 de fibra solúvel: insolúvel (FS:FI), de modo a fechar a formulação com inclusão de 10% de fibra alimentar total, além de um tratamento controle sem adição de fibra de linhaça. Como consequência das proporções de fibra solúvel: insolúvel, as dietas apresentaram diferenças principalmente quanto a capacidade de hidratação e ao teor de compostos fenólicos (Artigo I, Tabela 1).

Os resultados obtidos no ensaio biológico mostraram que a suplementação das dietas com as proporções 1:2 e 1:4 de FS:FI estimularam o crescimento dos peixes e a deposição de proteína bruta corporal (Artigo I, Tabelas 2 e 3), com impacto positivo sobre diferentes parâmetros imunológicos (Artigo II, Tabelas 2 e 3). Destacamos que o presente estudo traz o primeiro relato sobre parâmetros imunes do muco de jundiás e demonstra que a composição do mesmo responde a inclusão de substâncias com ação prebiótica as dietas. As mesmas dietas são associadas ainda a redução nos níveis de cortisol plasmáticos dos jundiás, do pH da digesta intestinal (Artigo II, Tabela 2 e 3) e na atividade de tripsina (Artigo I, Tabela 5). Essa redução na atividade de tripsina não causou prejuízos para os peixes, visto que não refletiu em alterações no desempenho zootécnico e parâmetros metabólicos (Artigo I, Tabelas 2 e 7). É importante ressaltar que a dieta com 1:0,5 FS:FI, que ocasionou maior atividade de tripsina (Artigo I, Tabela 7), também é a que apresentou a maior capacidade de hidratação (Artigo I, Tabela 1). Possivelmente esta característica da dieta tenha influenciado a viscosidade da digesta, dificultando a interação enzima-substrato e como forma de compensar, o metabolismo digestivo pode ter elevado a secreção e atividade da enzima (EASWOOD, 1992; SINHA et al., 2011).

Independente da proporção na dieta, o consumo de fibra de linhaça pelos jundiás promoveu aumento nas imunoglobulinas totais do plasma e na atividade da fosfatase alcalina do plasma e muco cutâneo (Artigo II, Tabelas 2 e 3), além de refletir em mudanças histológicas intestinais, como maior altura de vilosidade e contagem de células caliciformes e menor espessura da camada muscular (Artigo I, Tabela 6; Artigo II, Tabela 5). Estes

acréscimos são desejáveis, pois alterações na morfologia intestinal, como vilos mais curtos e criptas mais profundas, estão associados à maior susceptibilidade de doenças provocadas por patógenos intestinais (BRUMANO; GATTÁS, 2009; FERREIRA, 2012). Além disso, quanto maior a altura das vilosidades intestinais melhor será a digestão e absorção de nutrientes, refletindo em efeitos positivos sobre desempenho zootécnico, como ocorreu no presente estudo (GOULART et al., 2018). Estes efeitos podem estar atrelados a composição monossacarídica das frações solúvel e insolúvel da fibra de linhaça, que reflete em diferente combinação de monossacarídeos nas dietas. Compostos estes que são responsáveis por promover o crescimento de bactérias benéficas que impactam tanto a nível imunológico, quanto para o crescimento do animal (RINGO et al., 2010).

As alterações a nível intestinal também podem estar relacionadas a presença dos compostos fenólicos associados a fibra, principalmente na fração insolúvel (Apêndice A, Tabela 5). Os compostos fenólicos possuem reconhecida ação antioxidante, e quando estão bioacessíveis na região proximal do intestino, podem ser prontamente absorvidos pela mucosa. Aqueles associados a fibra alimentar passam inalterados pelo trato digestório superior, sendo liberados no intestino em decorrência da fermentação microbiana da fibra, promovendo um ambiente antioxidante a nível intestinal (SAURA-CALIXTO, 2011).

Outro resultado que merece destaque é o perfil de fermentabilidade intestinal dos jundiás. Não há na literatura pesquisas que caracterizem a produção de ácidos graxos de cadeia curta (AGCC) a nível intestinal para a espécie. No presente estudo, foi observado que as diferentes proporções de fibra solúvel: insolúvel consumidas pelos peixes afetam decisivamente as quantidades de AGCC produzidos, apesar de não mudar o perfil de fermentabilidade (acético>propiônico>butírico). A produção de ácido acético foi superior na digesta dos peixes que receberam a dieta com 1:2 de FS:FI, de ácido butírico para os que receberam a dieta com 1:4 de FS:FI, enquanto que a produção de ácido propiônico foi superior na digesta dos peixes que receberam a dieta controle (Artigo II, Tabela 4).

Essas diferenças nas quantidades de AGCC produzidos pelos peixes são reflexos do estímulo que a suplementação de fibras exerce sobre a microbiota intestinal. Como foi observado, as dietas com maior proporção de fibra insolúvel resultaram em maior produção de AGCC, o que pode estar relacionado a maior capacidade das bactérias intestinais em degradar os compostos que formam a matriz insolúvel da parede celular, sendo que a intensidade dessa degradação depende da composição e características físico-químicas da fibra, além de particularidades da microbiota intestinal (VAN SOEST, 1994). Outra possibilidade, é que esta microbiota tenha priorizado a degradação da fração insolúvel da fibra

de linhaça em detrimento a fração solúvel, havendo assim, um excedente de fibra solúvel, que com sua alta capacidade de hidratação acabou tendo efeito negativo sobre a absorção de nutrientes, o que explicaria o desempenho inferior dos peixes alimentados com dietas contendo maiores proporções da fração solúvel de fibra de linhaça. Porém é importante salientar, que mesmo não tendo proporcionado os melhores restados, as dietas com maior proporção de fibra solúvel promoveram respostas semelhantes a dieta controle.

Além dos efeitos mencionados, o ensaio de estresse agudo por hipóxia mostrou que a suplementação de 1:2 e 1:4 de FS:FI de linhaça as dietas possibilita uma melhor resposta ao estresse pelos peixes, com aumento de indicadores imunológicos plasmáticos e do muco cutâneo (Artigo III, Tabelas 2 e 3). Esses resultados confirmam a eficiência das respectivas proporções de fibra e demonstram sua ação mitigadora de estresse para jundiás. É importante destacar que, independente de sua proporção na dieta, a fibra de linhaça proporcionou maiores teores plasmáticos de imunoglobulinas totais e redução dos níveis de cortisol (Artigo III, Tabela 2; Figura 3). Esta observação permite inferir que a fibra de linhaça impulsiona a função imunológica e aumenta a tolerância a condições desfavoráveis de manejo durante o ciclo de cultivo dos jundiás (CASTRO-OSSES et al., 2017; YOKOYAMA et al., 2005).

Sabe-se que o sistema imunológico é influenciado direta e indiretamente pelos nutrientes ingeridos na alimentação, portanto, a adequação de seus níveis na formulação das dietas é de extrema importância, visando um caminho economicamente promissor para o aumento da produtividade em sistemas intensivos de criação de peixes (MENEZES et al., 2006; PEZZATO et al., 2004). Apesar da fibra alimentar não ter uma função verdadeiramente nutricional, nossos resultados mostram que sua presença na dieta, em quantidade e proporções equilibradas, refletem em vários benefícios para o cultivo de jundiás, agindo como promotor de crescimento, imunoestimulante e mitigador de estresse. Embora não se possa afirmar que tais efeitos sejam específicos e duradouros, tem-se como vantagem a possibilidade do seu uso na alimentação como estratégia promotora de saúde e bem estar animal.

6 CONCLUSÃO GERAL

Os resultados deste estudo permitem concluir que a fibra de linhaça tem ação funcional, agindo efetivamente como prebiótico, uma vez que estimula o desempenho, sistema imune e age como mitigadora de estresse para jundiás. Dentre as proporções de fibra testadas, a adição de 1:2 e 1:4 de fibra solúvel: insolúvel as dietas, proporcionaram os melhores resultados para os parâmetros avaliados. Porém, são necessários mais pesquisas a cerca da função da fibra de linhaça, seu modo de ação e análises minuciosas de seus componentes, para orientar sua utilização de forma racional.

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APÊNDICE A – Fracionamento da linhaça e obtenção de ingredientes ricos em proteína e fibra: alternativas para a alimentação animal

RESUMO

O fracionamento da linhaça foi realizado em escala laboratorial com o objetivo de obter frações concentradas em proteína e fibra. Para obtenção do concentrado proteico de linhaça (CPL) foram testados três métodos (pH ácido, alcalino e isoelétrico). O método de pH isoelétrico foi mais eficiente ($P<0,05$) para elevar o conteúdo proteico e também que proporcionou maior rendimento. Nas frações obtidas foi avaliada a composição química, matéria seca, cinzas, lipídios, proteína bruta, fibra alimentar total, solúvel e insolúvel e os minerais cálcio e fósforo. O perfil de aminoácidos foi determinado no farelo de linhaça e no CPL e, nas frações solúvel e insolúvel da fibra e linhaça *in natura*, foi avaliado o perfil de monossacarídeos. Os compostos fenólicos totais e as propriedades físico-químicas (capacidades de hidratação, ligação a gordura e ao cobre) também foram avaliados. Os resultados indicaram que o método de concentração proteica por pH isoelétrico melhorou o perfil aminoacídico e a digestibilidade *in vitro* do CPL em relação ao farelo original. As frações da linhaça apresentaram excelentes propriedades físico-químicas, podendo ser aplicáveis com diferentes finalidades na alimentação animal.

Palavras chave: Compostos fenólicos. Concentrado proteico. Fibra alimentar. Perfil aminoácidico.

1. INTRODUÇÃO

De acordo com a FAO (2014), até 2050 a demanda mundial de alimentos terá aumento de 70% a fim de atender as necessidades de ingestão básicas de quase dez bilhões de pessoas. Obviamente, o aumento produtivo exponencial das *commodities* agropecuárias alcançados nas últimas décadas não será suficiente para atender satisfatoriamente as demandas futuras, uma vez que o potencial produtivo das distintas espécies é passível de estagnação e a mobilização de terras para a produção é limitada e tem apresentado intensos sinais de degradação ao longo do tempo. Segundo o Diretor Geral da FAO, José Graziano da Silva, uma mudança de paradigma é necessária para substituir o modelo agropecuário dos últimos 40 anos, a fim de tornar os sistemas produtivos mais inteligentes e eficientes (ONUBR, 2015), garantindo a sustentabilidade futura quanto à produção racional de alimentos em larga escala.

Muitas culturas vegetais, embora amplamente adaptadas para cultivo, têm uso restrito na nutrição de animais monogástricos devido aos seus fatores antinutricionais, o que causa subutilização tanto da matéria-prima inicial (grão), como de seus subprodutos de processamento (farelos). Embora com elevados teores de óleos e proteínas, a linhaça (*Linum uistatissimum L.)* e seus subprodutos estão entre os diversos ingredientes de uso restrito para arraçoamento animal (SOLTAN et al., 2008; TOMM, 2006), devido aos seus elevados teores de mucilagem e de compostos fenólicos, que reduzem expressivamente o aproveitamento e desempenho de peixes (BERGAMIN et al., 2011; HASAN et al., 1997) aves e suínos (VRIES, et al., 2012). Contraditoriamente, estes mesmos fatores são apontados como pró-nutricionais para a saúde humana, com ação efetiva na promoção da microbiota intestinal benéfica e com efeito antioxidante a nível celular e metabólico (HALL; TULBEK; XU, 2006). Efeitos estes que também são desejáveis para expressão na criação dos animais e nos produtos derivados, desde que seus fatores desencadeantes não estejam em excesso a ponto de prejudicar o desempenho zootécnico. Neste cenário, podemos sugerir que o problema do uso da linhaça na nutrição animal não está necessariamente relacionado aos seus aspectos qualitativos, mas sim, a escassez de tecnologias racionais e sustentáveis, que garantam a utilização dessa matéria-prima com máxima eficiência nutricional e ambiental.

Sabe-se que a qualidade nutricional dos produtos vegetais pode ser elevada com a aplicação de técnicas químicas, físicas e enzimáticas que melhoram seu valor nutricional e sua digestibilidade (YUE; ZHOU, 2008) podendo dar origem a novos produtos, com ações

nutricional e aditiva potencializadas. No caso da linhaça, é possível aplicar tecnologias para a separação das fibras para uso como agentes prebióticos, bem como, concentrar seu conteúdo proteico, obtendo-se novos ingredientes de aplicação direcionada na nutrição animal e ausentes dos efeitos antinutricionais relatados para a fonte *in natura* (DENG et al., 2006).

Considerando o exposto, o objetivo do presente estudo foi desenvolver e avaliar os produtos, concentrado proteico e fibras solúvel e insolúvel, obtidos a partir do fracionamento da linhaça, a fim de utilizá-los como ingredientes na nutrição animal.

2. MATERIAIS E MÉTODOS

2.1 FRACIONAMENTO DA LINHAÇA

Os grãos de linhaça marrom (*Linum uistatissimum L.)* foram fornecidos pela empresa Giovelli Ltda (Guarani das Missões, RS, Brazil). O fracionamento da matéria-prima foi realizado conforme as etapas descritas na Figura 1. A *fração solúvel* (mucilagem) foi obtida seguindo metodologia descrita por Goulart et al. (2013). Inicialmente, os grãos inteiros foram imersos em água (10%, peso/volume) aquecida (60 a 80ºC), sob agitação constante por 150 minutos. Após, a solução aquosa foi separada dos grãos por filtração (±185 μm) e a fibra solúvel foi precipitada em meio etanólico (75%). Por fim, a fração solúvel obtida e os grãos demucilados foram secos em estufa de circulação de ar (MA035; Marconi, Brasil) (55°C/24 horas), moídos em micro moinho (MA-630; Marconi, Brasil) e acondicionados sob congelamento (-18°C).

O farelo demucilado foi desengordurado com lavagens sucessivas de hexano na proporção de 1:2 (peso/volume) e seco em estufa com circulação de ar (MA035; Marconi, Brasil) (55°C/24 h), para evaporação total do solvente. Este produto (farelo demucilado e desengordurado) foi utilizado para obtenção da fração insolúvel da fibra e do concentrado proteico de linhaça (CPL), através de metodologias propostas por Smith et al. (1946) e Lovatto et al. (2017).

O processo de extração da fibra insolúvel foi realizado através de dispersão do farelo em meio aquoso, utilizando um triturador de facas (LIQ789, Cadence, Brasil) (potência de 400W) por três vezes, a temperatura ambiente, em uma proporção final peso:volume de 1:30, por 3 minutos. Após cada dispersão, a amostra foi filtrada em peneira (140 μm) e a fração sólida resultante da última dispersão, correspondente à *fração insolúvel*, foi seca em estufa

com circulação de ar a 55°C por 24 horas. O sobrenadante foi homogeneizado e utilizado em diferentes métodos de precipitação proteica.

No presente estudo, foram testados três métodos para concentrar a fração proteica do farelo de linhaça, seguindo metodologia e modificações propostas por Lovatto et al. (2017):

- *pH isoelétrico*: a concentração proteica foi realizada com aumento do pH da amostra líquida para 9,0 com NaOH 1N e, após 30 minutos, redução para 4,5 com HCl 1 N (SMITH et al., 1946).
- *pH ácido*: ajustou-se o pH da amostra líquida para 4,5 com HCl 1 N (MODESTI et al., 2007).
- *pH alcalino*: aumento do pH da amostra líquida para 9,0 com NaOH 1N (MODESTI et al., 2007).

As medidas de pH foram realizadas com pHmetro de bancada digital (MPA 210-P, Servilab, Brasil). Após os processos de concentração, as amostras foram deixadas em repouso (overnight) a 8°C, para decantação da fração proteica. Em seguida, o sobrenadante foi descartado, e o precipitado, correspondente ao *concentrado proteico de linhaça*, foi centrifugado a 3500 rpm por 10 minutos, seco a 55°C por 24 horas em estufa com circulação de ar (MA035; Marconi, Brasil), moído em micro moinho (MA-630, Marconi, Brasil) e armazenado sob congelamento (-18°C).

2.2 CONTEÚDOS PROTEICO E LIPÍDICO E RENDIMENTO DE EXTRAÇÃO

Os concentrados proteicos foram analisados quanto ao teor de proteína bruta, através da determinação de nitrogênio total pelo método de Kjeldahl (nº 960.52) (AOAC, 1995) e teor lipídico, seguindo metodologia proposta por Bligh e Dyer (1959). O rendimento (R) foi calculado levando-se em consideração a quantidade obtida, em gramas, de CPL após a secagem em relação à quantidade de amostra inicial, seguindo a equação:

$$R\ (\%) = \frac{\text{massa inicial (g) x massa concentrado proteico após secagem (g)}}{100}$$

2.3 CARACTERIZAÇÃO QUÍMICA

As amostras linhaça *in natura*, farelo de linhaça demucilado e desengordurado, CPL com maior rendimento e teor proteico, fração solúvel e fração insolúvel da fibra, foram avaliadas quanto à composição centesimal: matéria seca (método 925.45b), cinzas (método

923.03), proteína bruta (método 960.52), fibra alimentar total, solúvel e insolúvel (método 991.43) (AOAC, 1995) e lipídios (Bligh e Dyer, 1959). O conteúdo de cálcio e fósforo foi analisado de acordo com metodologia proposta por Tedesco et al. (1995). A análise incluiu as etapas de digestão dos minerais e quantificação por espectrofotometria de absorção atômica (cálcio) e na região visível (fósforo).

O perfil de aminoácidos presentes no farelo de linhaça demucilado e desengordurado e no CPL foi determinado através de cromatografia líquida de alta eficiência (CLAE) em fase reversa com detecção UV a 254 nm (P4000-Thermo Fisher Scientific, Waltham, MA). A extração foi realizada com HCl 6N por 24 horas e a derivatização com fenilisotiocianato (WHITE et al., 1986).

O perfil de monossacarídeos foi analisado nas amostras linhaça *in natura,* fração solúvel e fração insolúvel da fibra, utilizando cromatografia líquida de alta eficiência (CLAE) (Shimadzu), com detector de índice de refração (DIR). A separação foi realizada em coluna AMinexHPX-87H, seguindo metodologia descrita por Sluiter et al. (2008). Para tal, utilizaram-se padrões de ácido galacturônico, arabinose, frutose, galactose, glicose, ramnose e xilose.

2.4 DIGESTIBILIDADE *IN VITRO*

A digestibilidade *in vitro* do farelo de linhaça demucilado e desengordurado e do CPL foi determinada de acordo com metodologia descrita por Mauron (1973), com modificações propostas por Dias et al. (2010). A digestão das amostras foi realizada com adição das enzimas pepsina (1: 10 000, Nuclear) e pancreatina (Sigma, São Paulo, Brasil). A digestibilidade resulta das interações do nitrogênio total presente na amostra, do nitrogênio digerido, do nitrogênio produzido pela autodigestão das enzimas e do nitrogênio solúvel originalmente contido na amostra.

2.5 COMPOSTOS FENÓLICOS E PROPRIEDADES FÍSICO-QUÍMICAS

As amostras foram submetidas à extração, sequencial, com solução metanólica acidificada (50:50, volume/volume, pH 2,0) e acetônica (70:30, volume/volume). A quantificação dos compostos fenólicos totais foi realizada através do método de Folin–Ciocalteu (WATERHOUSE, 2003), sendo os resultados expressos em mg equivalentes de ácido gálico (EAG) por 100 g de amostra.

As capacidades de hidratação e ligação a gordura foram determinadas de acordo com Wang e Kinsella (1976). Às amostras foram adicionados água ou óleo e, após homogeneização, permaneceram em repouso, a temperatura ambiente, por 24 horas. Em seguida, foram centrifugadas (1300 x g/ 20 min), sendo o sobrenadante descartado. Os resultados foram expressos em g de água/óleo absorvidos em um grama de amostra seca. A capacidade de ligação ao cobre foi estimada de acordo com a metodologia de McBurney et al. (1983).

2.5 DELINEAMENTO EXPERIMENTAL E ANÁLISE ESTATÍSTICA

Para os dados obtidos nos três métodos de concentração proteica utilizou-se um delineamento experimental casualizado. Os resultados foram submetidos à análise de variância (ANOVA) e as médias foram comparadas pelo teste de Tukey a 5% de significância. Para os demais dados são apresentadas as médias ± desvio padrão.

3. RESULTADOS

3.1 MÉTODOS DE CONCENTRAÇÃO PROTEICA

O método de pH isoelétrico foi mais eficiente ($P<0,05$) para elevar o teor proteico e reduzir o conteúdo lipídico, bem como, proporcionou maior rentabilidade extrativa numérica que os demais métodos testados (Tabela 1). O método de concentração por pH ácido mostrou-se pouco eficiente, devido ao baixo rendimento de extração. A concentração por pH alcalino revelou-se ineficiente para concentração e rentabilidade proteica.

3.2 COMPOSIÇÃO QUÍMICA

A composição química das frações obtidas a partir da linhaça está apresentada na Tabela 2. O fracionamento em fibras solúvel e insolúvel proporcionou notória redução da proteína bruta, 42% e 29,4%, respectivamente, em comparação com a linhaça *in natura*. A fração solúvel, rica neste constituinte, apresentou reduzido teor proteico (13,20%). Na fração insolúvel, o conteúdo proteico encontrado foi de 16,07%. Já no CPL, houve aumento de 60% no teor de proteínas em relação ao farelo demucilado e desengordurado, utilizado para sua obtenção.

Houve drástica redução (97,51%) no teor lipídico da fração solúvel em comparação com a linhaça *in natura*, que passou de 34,11% para 0,85%. A fração insolúvel também teve expressiva redução de lipídios (79,27%). Da mesma forma, o processo utilizado para obtenção do CPL permitiu reduzir 31% do conteúdo lipídico (Tabela 2).

A matéria seca da linhaça *in natura* foi superior às demais frações analisadas, enquanto que a matéria mineral foi superior na fração insolúvel (Tabela 2). Os teores de cálcio das frações solúvel e insolúvel apresentaram-se iguais (1,58%). Entretanto, o CPL teve menor concentração deste mineral (Tabela 2). As frações linhaça *in natura*, farelo de linhaça e CPL apresentaram teores equivalentes de fósforo total (0,58, 0,59 e 0,57%, respectivamente). Na fração insolúvel foram obtidos valores superiores à fração solúvel (Tabela 2).

O fracionamento da fibra da linhaça mostrou-se extremamente eficiente, visto que a fibra solúvel da fração solúvel correspondeu a 67,56%. Na amostra inicial (linhaça *in natura*), esta era de 17,3%, indicando incremento de 290,52% (Tabela 2). Da mesma forma, observou-se aumento (220,21%) no teor de fibra insolúvel da fração insolúvel, em comparação à linhaça *in natura*, de 19,1% para 63,07%. No CPL constatou-se redução no teor de fibra alimentar total, de 50,6% (farelo de linhaça) para 29,1% (Tabela 2).

Os resultados do aminograma (Tabela 3) do farelo de linhaça demucilado e desengordurado e do CPL revelaram a superioridade do concentrado em relação aos níveis dos aminoácidos analisados. Para a nutrição de animais monogástricos, os aminoácidos lisina e metionina+cistina são limitantes. Desta forma, nossos resultados mostraram-se relevantes, visto que houve aumento na concentração dos respectivos aminoácidos, de 60% e 83%, no CPL em relação ao farelo. Este acréscimo na composição aminoacídica e a redução no teor de fibra alimentar total (Tabela 2) culminaram na melhora da qualidade proteica, refletida na maior digestibilidade *in vitro* da proteína do CPL (88,98%) em relação ao farelo de linhaça (75,69%).

Nas amostras analisadas, não foram encontradas quantidades observáveis de galactose, ramnose, manose e frutose. Na linhaça *in natura* observou-se maior concentração de glicose, seguida de xilose, ácido galacturônico e arabinose. Os mesmos monossacarídeos foram identificados na fração insolúvel, com exceção da arabinose. Na fração solúvel foram encontrados maiores teores de xilose, seguido por glicose, ácido galacturônico e arabinose. O percentual total de monossacarídeos foi maior na fração solúvel (82,60%) do que na fração insolúvel (64,24%).

3.3 COMPOSTOS FENÓLICOS E PROPRIEDADES FÍSICO-QUÍMICAS

A separação da fibra da linhaça concentrou os compostos fenólicos na fração insolúvel (Tabela 5). Nesta porção, o teor encontrado foi de 654,7 mg EAG/100g, enquanto que na fração solúvel foi 293,4 mg EAG/100g, conteúdo inferior ao contido na linhaça *in natura.* Além disso, observou-se que o método de concentração proteica por pH isoelétrico foi eficaz na redução do conteúdo de compostos fenólicos em, aproximadamente, 24% em relação ao farelo (Tabela 5).

Em relação às propriedades físico-químicas, houve uma redução de 49% na capacidade de hidratação do farelo de linhaça demucilado e desengordurado, comparando-se com a linhaça *in natura*. A concentração proteica proporcionou aumento de 13% nesta propriedade em relação ao farelo (Tabela 5). A capacidade de hidratação da fração solúvel da fibra de linhaça foi de 43,53g água/g, o que representa 11,48 vezes a mais do que a quantidade encontrada na fração insolúvel (3,79 g água/g) (Tabela 5).

Os resultados obtidos para capacidade de ligação a gordura variaram de 1,16 g óleo/ g na linhaça *in natura*, para 1,30 e 1,65 g óleo/ g na fração solúvel e na fração insolúvel, respectivamente (Tabela 5). A menor capacidade de ligação a gordura foi observada no CPL (0,74 g óleo/ g), que apresentou redução de 59% em relação ao farelo (Tabela 5).

Quanto à capacidade de ligação ao cobre, esta passou de 10,28 mg Cu/g na linhaça *in natura* para 10,58mg Cu/g na fração insolúvel. Não foi possível realizar a análise na fração solúvel, devido a problemas metodológicos possivelmente causados por reações químicas entre os regentes utilizados e o excesso de hidratação da mesma. A concentração proteica provocou acréscimo de 13% na propriedade de ligação ao cobre, que passou de 10,10 mg Cu/g (farelo de linhaça) para 11,39 mg Cu/g (CPL).

4. DISCUSSÃO

4.1 CARACTERIZAÇÃO QUÍMICA

A linhaça (*Linum usitatissimum* L.) é uma das culturas mais antigas produzidas no mundo. Nativa do Oeste Asiático e do Mediterrâneo, ela é cultivada há cerca de 4000 anos, sendo utilizada como fonte de óleo, linho e alimento. Para o consumo humano, destaca-se pelo seu alto conteúdo de ômega-3 (533 mg/g), conferindo-lhe propriedades funcionais (SHIM et al., 2014; TURNER et al., 2014; MARTIN et al., 2006). Além disso, tem sido

adicionada à alimentação animal, na forma de farelo e óleo, para melhorar o desempenho produtivo e saúde dos animais, assim como para proporcionar o enriquecimento nutricional de leite, ovos e carne (TURATTI, 2001). Porém, os nutrientes e compostos encontrados na linhaça ainda são pouco explorados na nutrição animal, fazendo dela uma matéria-prima subutilizada.

Em média, a linhaça apresenta 20% de proteína bruta, 41% de lipídios, 28% de fibra alimentar, 92,3% de matéria seca e 3,4% de matéria mineral (SHIM et al., 2014). Composição semelhante à encontrada no presente estudo para a linhaça *in natura*, que apresentou 22,76% de proteína bruta, 34,11% de lipídios, 34,6% de fibra alimentar total (17,3% solúvel e 19,1% insolúvel), 97,29% de matéria seca e 3,09% de matéria mineral. Segundo Shim et al. (2014), essas variações na composição são decorrentes da cultivar de linhaça, de características geográficas como o tipo de solo, características climáticas, entre outros.

Nossos resultados demonstraram que as técnicas adotadas para o fracionamento da linhaça foram adequadas para a obtenção de um concentrado proteico e das frações solúvel e insolúvel da fibra. O que fica claro ao observarmos que a proteína bruta da linhaça *in natura* (22,76%) foi efetivamente concentrada no CPL (53,24%), restando baixas concentrações deste nutriente nas frações solúvel e insolúvel da fibra (13,20% e 16,15%, respectivamente). Os maiores teores de matéria seca encontrados para a linhaça *in natura* , provavelmente esteja relacionado ao seu teor lipídico (34,11%), visto que esta amostra não foi submetida a nenhum tipo de processamento. É importante destacar que para a obtenção do farelo de linhaça, foram realizadas lavagens com hexano, a fim de reduzir o teor lipídico desse ingrediente. Assim, consequentemente, houve redução deste componente nas frações obtidas a partir do farelo, sendo esta mais pronunciada na fração solúvel da fibra, que apresentou 0,85% de lipídios. A matéria mineral apresentou pequena variação (2,44-4,16%) entre as frações provenientes da linhaça, reflexo tanto dos níveis de cálcio e fósforo das mesmas, quanto de outros minerais presentes (não avaliados).

As diferenças encontradas na eficiência da extração proteica podem ser decorrentes das propriedades dos radicais das estruturas químicas primárias dos aminoácidos que compõem os ingredientes utilizados na concentração proteica (LOVATTO et al., 2017). O método que baseou-se no pH isoelétrico foi mais eficaz para extrair e concentrar a proteína da linhaça devido a maioria dos aminoácidos presentes possuírem pontos isoelétricos entre 4,5 e 6,5 (SGARBIERI, 1996), tornando-a apropriada para esta finalidade. O aumento no conteúdo lipídico promovido pelo método de concentração por pH alcalino se deve as interações

lipídico-proteicas e formação de lipoproteínas hidrofóbicas (ARAÚJO, 2008; LOVATTO et al., 2017).

A composição aminoacídica das fontes vegetais pode variar de acordo como tipo de cultivar, procedimentos de controle das culturas, pragas e processos industriais (ANDRIGUETTO, 1988; TAVERNARI, 2010). Segundo Linden e Lorient (1996) as técnicas para obtenção de concentrados proteicos podem modificar o perfil aminoacídico da matéria-prima, e diminuir a concentração de antinutrientes. Os teores de aminoácidos aumentaram no CPL, em parte devido à redução no teor de fibras deste produto proveniente do fracionamento. As fontes vegetais são normalmente deficientes em lisina e metionina+cistina, dois aminoácidos limitantes na alimentação de animais monogástricos. O aumento nos níveis desses aminoácidos no CPL confirmam que o método pH isoelétrico foi eficiente, não apenas para aumentar o conteúdo proteico, mas principalmente para melhorar a qualidade proteica do farelo de linhaça.

A maior digestibilidade in vitro observada no CPL destaca a superioridade deste ingrediente em relação à fonte original. Essas características qualitativas são fundamentais para elevar a inclusão de fontes vegetais nas dietas de animais monogástricos visto que os farelos vegetais apresentam desvantagens, como menor concentração proteica, presença de elementos antinutricionais e carboidratos de estrutura complexa que reduzem a digestibilidade do alimento (GUILLAUME et al., 2001).

De forma geral, o excesso de fibra na dieta é considerado um ponto negativo, pois, diminui a digestibilidade dos nutrientes e aumenta a produção de resíduo fecal, contribuindo para a poluição do ambiente (NRC, 1993). Entretanto, ingredientes vegetais íntegros (como farelos e tortas) constituem fontes de fibras, proteínas e lipídios e podem ou não ter sucesso na nutrição animal. De acordo com Fedeniuk e Biliaderis (1994), a fibra solúvel encontrada na linhaça, além de proporcionar aumento no tempo de retenção do alimento no estômago, diminui o seu consumo. Uma vez que apresenta ótima capacidade de retenção de água, provoca aumento da viscosidade e consequentemente, reduz a digestibilidade dos nutrientes. Entretanto, Adorian et al. (2015; 2016) relataram ação funcional a nível imunológico e produtivo, em peixes alimentados com dietas contendo concentrado de fibra alimentar de linhaça (fração solúvel+insolúvel). Resultado semelhante ao que foi relatado por Goulart et al. (2017) ao suplementar apenas a fração solúvel da linhaça em dietas para jundiás (*Rhamdia quelen*). Esses relatos permitem questionar se são as fibras que apresentam efeito adverso no desempenho animal ou se o prejudicial é sua adição em excesso nas dietas.

Os teores de fibra alimentar encontrados neste estudo confirmam a eficiência das técnicas adotadas, visto que houve redução no CPL (29,1%) e concentração nas frações solúvel e insolúvel (73,65% e 71,61%, respectivamente). É possível observar que da fibra alimentar remanescente no CPL, 21,2% constituiu-se da porção solúvel. Esta, possivelmente seja oriunda da parte interna do grão ou residual da extração da fibra solúvel do grão de linhaça, apontando alguma falha no processamento. Independente de sua origem, esta continua presente no CPL provavelmente em virtude da técnica utilizada para sua concentração, que inclui a dispersão do farelo em água. Porém, este fato não prejudicou a concentração das frações solúvel (FS) e insolúvel (FI) da fibra de linhaça, sendo que ambas foram superiores a 60% (67,56% FS e 63,07% FI, respectivamente).

Para análise de monossacarídeos foram selecionadas apenas amostras de linhaça *in natura* e as frações solúvel e insolúvel da fibra. Estas amostras apresentaram baixa variabilidade de monossacarídeos, com predominância de glicose, xilose, ácido galacturônico, e arabinose. Como a fibra alimentar total da linhaça *in natura* mostrou-se inferior às frações solúvel e insolúvel, por consequência, apresentou menor percentual de monossacarídeos totais (47,10%), tendo em maior abundância glicose (24,28%). Na fração solúvel observou-se um percentual elevado de monossacarídeos totais (82,60%), com predomínio de xilose (41,17%). Já na fração insolúvel o teor de monossacarídios totais foi de 64,24%, com maior percentual de glicose (42,60%) e ausência de arabinose.

Estudos avaliando a composição de monossacarídeos da fibra solúvel da linhaça foram realizados por diversos autores, que relataram maior variabilidade em sua composição, com presença de ácido urônico (11,1%), arabinose (15,5-20,0%), frutose (8,4%), fucose (3%), galactose (11,7-17,1%), glicose (6,9%), ramnose (11-25,3%) e xilose (29,1-35,4%) (GOULART et al., 2017; RAY et al., 2013; SHIM et al., 2014;). Dentre os monossacarídeos presentes na fibra solúvel da linhaça, oligômeros de xilose são encontrados em maiores quantidades (GOULART et al., 2017). Até o momento, não foram encontrados na literatura estudos avaliando a composição monossacarídica da fibra insolúvel da linhaça. Desta forma, os resultados apresentados em nosso estudo são inéditos. De acordo com Ringo et al. (2010) monossacarídeos como os presentes em ambas as frações, são responsáveis por promover o crescimento de bifidobactérias benéficas que contribuem para o aumento do crescimento do animal. Além disso, há a possibilidade de atuação direta sobre algumas populações de bactérias patogênicas, por meio de exclusão competitiva (FREITAS et al., 2014). Deste modo, é possível utilizar ambas as frações de fibra da linhaça como prebiótico em dietas para

monogástricos. Porém, estudos in vivo devem ser conduzidos para estabelecer as quantidades a serem adicionadas nas dietas das diferentes espécies.

4.2 COMPOSTOS FENÓLICOS E PROPRIEDADES FÍSICO-QUÍMICAS

A linhaça é sabidamente rica em compostos fenólicos, grupo que inclui várias substâncias com capacidade antioxidante, como flavonoides e taninos (GALVÃO et al., 2008; GOÑI et al., 2009). Porém, não há relatos na literatura dos teores presentes nas diferentes frações deste ingrediente. Deste modo, nossos resultados são originais, pois mostram que o fracionamento da linhaça através das técnicas utilizadas permite produzir um CPL (459,30 mg EAG/100g) e uma fração insolúvel de fibra (654,70 mg EAG/100g) ricos em compostos fenólicos, agregando valor aos produtos obtidos. Estes compostos possuem diferentes pesos moleculares e podem estar livres ou ligados à parede celular. É importante destacar que a fibra alimentar e os compostos fenólicos ligados a ela seguem processos fisiológicos comuns, produzindo efeito sinérgico no trato gastrintestinal (GOÑI et al., 2009). Evidências científicas apontam que os compostos fenólicos associados à fibra constituem em torno de 50% dos antioxidantes dietéticos totais (MACAGNAN et al., 2016).

A presença de compostos fenólicos associados à fibra alimentar exercem efeitos sobre as suas propriedades físico-químicas e fisiológicas. Um exemplo é a sua ação na manutenção da integridade intestinal, visto que alguns compostos são bioacessíveis na região proximal do intestino, podendo ser prontamente absorvidos pela mucosa intestinal (SAURA-CALIXTO, 2011). Outros, por sua vez, passam inalterados através do trato gastrintestinal superior em associação com as fibras, atingindo o cólon, onde podem ser fermentados por ação das enzimas bacterianas. Estes últimos tornam-se substrato fermentável para a microflora bacteriana, promovendo um ambiente antioxidante a nível intestinal (SAURA-CALIXTO, 2011). Devido ao potencial antioxidante os compostos fenólicos também podem ser importantes no processo de armazenamento de ingredientes e rações, principalmente em produtos com alto teor lipídico (SILVA; SILVA, 1999).

Alguns compostos fenólicos, como os taninos, principalmente os condensados, são considerados fatores antinutricionais na alimentação animal, pois podem combinar-se com proteínas e formar complexos que inibem proteases digestivas e enzimas amilolíticas e lipolíticas reduzindo a digestibilidade proteica de leguminosas e cereais (FRANCIS et al., 2001; SILVA e SILVA, 1999). Fato este que não foi observado em nosso estudo, já que a digestibilidade do CPL foi superior a da matéria-prima inicial (Tabela 2). Provavelmente, no

processo de fracionamento da linhaça a maior proporção de taninos condensados (pouco digestíveis) permaneceu ligada a fibra alimentar, não interferindo na digestibilidade proteica do CPL.

As propriedades físico-químicas da linhaça e suas frações demonstraram que a capacidade de hidratação da linhaça *in natura* (5,31 g água/g) foi reduzida no farelo de linhaça (2,69 g água/g), no CPL (3,04 g água/g) e na fração insolúvel da fibra (3,79 g água/g), enquanto na fração solúvel foi elevada, atingindo 43,53 g água/g. A maior porcentagem dos aminoácidos polares (lisina, arginina, histidina, serina, treonina, ácido aspártico e glutâmico) encontradas no CPL (28,7%) em comparação ao farelo (17,5%) pode explicar o aumento da propriedade de hidratação deste ingrediente. Esse resultado corrobora com os achados de Lovatto et al. (2017), que utilizando o pH isoelétrico também observaram maior capacidade de hidratação no concentrado proteico em relação ao farelo original.

Vários pesquisadores têm mostrado interesse em estudar a capacidade de hidratação de ingredientes utilizados na alimentação animal, devido a sua correlação com o aumento da viscosidade do alimento a nível de trato gastrointestinal, influenciando tempo de trânsito intestinal, desenvolvimento de órgãos, consumo de alimento e sensação de saciedade (ARROYO et al., 2012; GIGER-REVERDIN, 2000; JIMÉNEZ-MORENO et al., 2011; SERENA; BACH KNUDSEN, 2007). Brachet et al. (2015) estudaram a capacidade de hidratação de vinte e quatro matérias-primas utilizadas em dietas para não ruminantes, entre elas trigo, milho, cevada, farelo de soja e farelo de trigo. Os resultados obtidos pelos autores supracitados variaram de 0,54-5,60 g água/g amostra, sendo similar à variabilidade encontrada no presente estudo para as frações de linhaça, com exceção da fração solúvel da fibra. Os autores destacaram a baixa capacidade de hidratação dos cereais e a alta capacidade dos subprodutos (BRACHET et al., 2015).

É importante destacar que uma capacidade de hidratação excessivamente alta origina reduções na digestão e absorção de aminoácidos, carboidratos, minerais e outros nutrientes, com consequente queda na produtividade (TEJEDOR et al., 2001). Em função disso, autores têm proposto tal parâmetro como ferramenta para melhorar os modelos de caracterização de alimentos, devendo inclusive ser utilizado como um novo critério na formulação de dietas (BRACHET et al., 2015; GOUS, 2014). Glencross et al. (2007) destacam que as características físico-químicas dos ingredientes influenciam a qualidade tecnológica e digestibilidade dos alimentos para organismos aquáticos.

A capacidade de ligação a gordura (CLG) apresentou pouca variabilidade entre as amostras estudadas (0,74-1,81 g óleo/g), sendo a maior capacidade observada no farelo de

linhaça e a menor no CPL. A menor CLG do CPL pode ser explicada pelo fato da absorção de óleo variar conforme o número de grupos hidrofóbicos (aminoácidos apolares) expostos na proteína (DENCH et al., 1981), os quais estão geralmente localizados internamente, dificultando a capacidade de ligarem-se com a gordura (LOVATTO et al., 2017). Ingredientes com alta capacidade de ligação a gordura podem causar redução na absorção lipídica a nível intestinal, impactando negativamente no desenvolvimento animal, principalmente nas fases iniciais. Por outro lado, para animais em manutenção, este parâmetro pode ter impacto positivo, visto que evita o acúmulo de gordura e o aumento dos níveis plasmáticos de colesterol e triglicerídeos. Assim, considerar a capacidade de ligação a gordura dos ingredientes utilizados na formulação das dietas, auxilia no ajuste das mesmas a diferentes fases. Além disso, as capacidades de hidratação e ligação a gordura das rações têm ação sobre a dureza, estabilidade na água, flutuabilidade e tempo de armazenamento (DRAGANOVIC et al. 2011; LOVATTO et al., 2017).

Quanto à capacidade de ligação ao cobre, esta foi maior no CPL, possivelmente em decorrência de ligações químicas entre os grupamentos proteicos reativos e os íons de cobre. No entanto, a concentração proteica foi realizada no ponto isoelétrico, predominando a igualdade entre cargas positivas e negativas da proteína. Ingredientes alimentares com alta capacidade de ligação ao cobre normalmente possuem forte capacidade de ligação iônica com demais elementos minerais, fazendo com que as dietas interfiram negativamente a sua absorção (ARRUDA et al., 2003). É importante destacar que a fração solúvel da fibra de linhaça não teve a propriedade de ligação ao cobre mensurada, mesmo após várias tentativas de adaptação da técnica. Este problema metodológico possivelmente seja reflexo da alta capacidade de hidratação desta fração, que em contato com os reagentes utilizados na técnica, criaram uma solução extremamente viscosa.

As propriedades físico-químicas de ingredientes são pouco exploradas na nutrição animal, porém podem impactar a nível metabólico e fisiológico no organismo, podendo refletir positiva ou negativamente na produção animal. A carência destas informações para os ingredientes convencionalmente utilizados na formulação de dietas para monogástricos limita sua utilização. No entanto, quando se trata do desenvolvimento de novos produtos estas análises se tornam imprescindíveis.

Com este estudo foi possível obter três distintas frações a partir da linhaça: uma composta majoritariamente por proteína (CPL) e duas compostas principalmente por fibras (frações solúvel e insolúvel). A composição química do CPL revelou características satisfatórias em relação à proteína, devido ao maior aporte proteico, perfil de aminoácidos e

digestibilidade em comparação ao farelo de linhaça, possibilitando o seu uso em substituição a fontes proteicas de origem animal e vegetais utilizadas na nutrição animal. Além disso, sua concentração de compostos fenólicos deve ser melhor estudada e caracterizada, uma vez que podem apresentar capacidade antioxidante. As frações de fibra solúvel e insolúvel podem ser utilizadas como ingredientes com ação funcional, especialmente prebiótica e antioxidante. A inclusão destes produtos com diferentes finalidades na alimentação animal ainda precisa ser avaliada a fim de determinar níveis e aceitação, principalmente com relação à fração solúvel, a fim de evitar efeitos negativos ao desempenho animal.

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Figura 1 – Etapas do fracionamento da linhaça para obtenção do Concentrado Proteico de Linhaça (CPL) e das frações solúvel e insolúvel

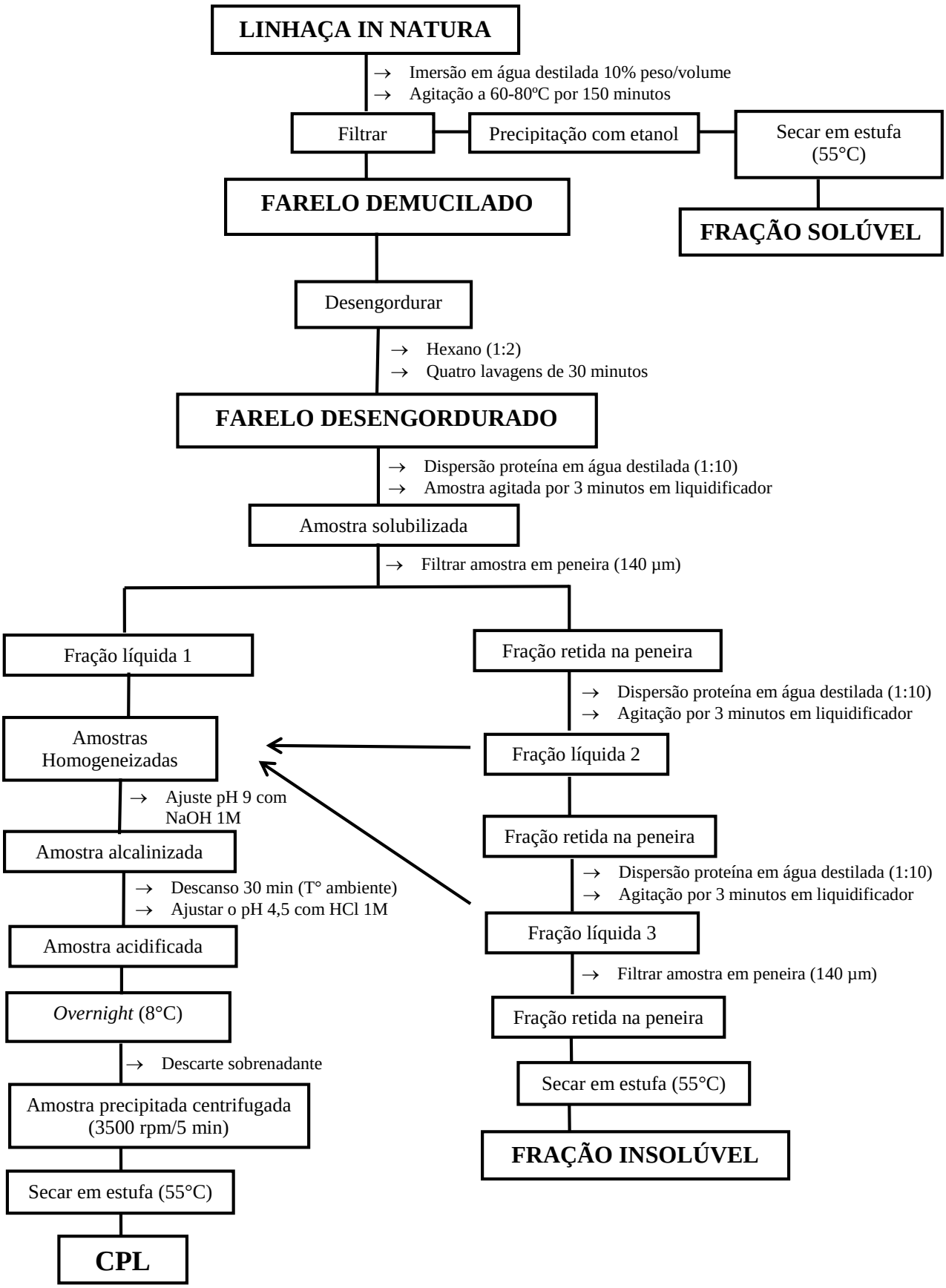

Tabela 1- Composição proteica, lipídica e rendimento de extração do concentrado proteico de linhaça, utilizando diferentes métodos de concentração: pH isoelétrico, pH ácido e pH alcalino

Conteúdo	Método de concentração proteica		
	pH isoelétrico	*pH ácido*	*pH alcalino*
	% da matéria in natura		
Proteína Bruta	53,24±0,29[a]	42,85 ± 3,72[ab]	33,13±0,175[c]
Lipídios	12,75±0,11[b]	25,54±0,48[a]	13,06±0,15[b]
Rendimento	44,71	37,00	18,70

Fonte: Elaborada pelas autoras. Médias ± desvio padrão. Letras distintas na linha diferem estatisticamente pelo teste de Tukey ($P < 0,05$).

Tabela 2- Composição nutricional da linhaça *in natura*, farelo de linhaça[1], concentrado proteico de linhaça e frações solúvel e insolúvel de fibra

Conteúdo	Linhaça *in natura*	Farelo de linhaça[1]	CPL	Fração solúvel	Fração insolúvel
			% da matéria in natura		
Proteína bruta	22,76±0,37	33,24±0,06	53,24±0,58	13,20±0,80	16,15±1,14
Lipídios	34,11±0,41	18,45±0,13	12,75±0,18	0,85±0,02	7,07±0,13
Fibra alimentar total	36,40±2,71	50,60±3,78	29,10±5,43	73,65±3,18	71,61±0,76
Solúvel	17,30±0,14	22,30±2,12	21,20±5,25	67,56±16,33	8,54±3,92
Insolúvel	19,10±1,28	28,30±4,24	7,88±0,17	6,09±3,25	63,07±6,16
Matéria seca	97,29±0,01	93,68±0,10	93,62±0,04	91,25±0,13	93,62±0,31
Matéria mineral	3,09±0,01	3,89±0,02	2,44±0,04	2,48±0,04	4,16±0,05
Cálcio[2]	1,80	1,48	1,32	1,58	1,58
Fósforo	0,58±0,02	0,59±0,03	0,57±0,07	0,20±0,01	0,41±0,03
Digestibilidade proteica *in vitro*	NA	75,69±3,54	88,98±1,65	NA	NA

Fonte: Elaborada pelas autoras. [1]demucilado e desengordurado. CPL: concentrado proteico de linhaça. NA: não analisado. [2]Sem desvio padrão.

Tabela 3- Composição aminoacídica do farelo de linhaça (demucilado e desengordurado) e do concentrado proteico de linhaça

Aminoácidos (%)[1]	Farelo de linhaça[1]	CPL
Essenciais		
Arginina	3,45	5,90
Fenilalanina	1,70	2,97
Histidina	0,50	1,00
Isoleucina	1,54	2,63
Leucina	1,98	3,34
Lisina	1,41	2,25
Metionina+cistina	0,54	0,99
Treonina	1,11	1,87
Triptofano[2]	NA	NA
Valina	1,74	3,02
Não essenciais		
Ácido aspártico	2,96	4,84
Ácido glutâmico	6,51	10,33
Alanina	1,47	2,52
Glicina	1,80	2,83
Prolina	1,20	1,98
Serina	1,52	2,51
Tirosina	0,92	1,34

Fonte: Elaborada pelas autoras. [1]Determinados por Cromatografia Líquida de Alta Eficiência (HPLC) no Laboratório de Fontes Proteicas (LaFoP) da UNICAMP, Campinas, SP. CPL: concentrado proteico de linhaça.[2]NA: não analisado.

Tabela 4 - Composição monossacarídica (%) da linhaça *in natura* e frações solúvel e insolúvel da fibra

	Monossacarídeos (%)		
	Linhaça *in natura*	Fração solúvel	Fração insolúvel
Ácido Galacturônico	10,51	14,96	8,56
Arabinose	2,87	8,05	0
Glicose	24,28	18,5	42,60
Xilose	12,31	41,17	13,09
Total	47,10	82,68	64,24

Fonte: Elaborada pelas autoras. Não foram encontradas quantidades observáveis, em nenhuma das amostras, de galactose, ramnose, manose e frutose.

Tabela 5 – Propriedades físico-químicas e compostos fenólicos das frações da linhaça

Propriedades físico-químicas					
	Linhaça *in natura*	Farelo linhaça[1]	CPL	Fração solúvel	Fração insolúvel
CH (g água/g)	5,31±0,12	2,69±0,11	3,04±0,08	43,53±2,35	3,79±0,16
CLG (g óleo/g)	1,16±0,06	1,81±0,09	0,74±0,02	1,30±0,05	1,65±0,01
CLC (mg Cu/g)	10,28±1,06	10,10±1,41	11,39±0,02	-	10,58±0,41
Compostos fenólicos (mg de EAG/100 g)					
	375,5±12,24	600,30±159,31	459,3±9,87	293,4±128,01	654,7±177,40

Fonte: Elaborada pelas autoras. [1]demucilado e desengordurado. CPL: concentrado proteico de linhaça. CH: capacidade de hidratação. CLG: capacidade de ligação a gordura. CLC: capacidade de ligação ao cobre. EAG: equivalentes de ácido gálico.

ANEXO A – Normas da revista Animal Feed Science and Technology

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1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communications
4. Book Reviews

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.
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A *Short Communication* is a concise but complete description of a limited investigation, which will not be included in a later paper. Short Communications should be as completely documented, both by reference to the literature and description of the experimental procedures employed, as a regular paper. They should not occupy more than six printed pages (about 12 manuscript pages, including figures, tables and references).
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