CRISTIANA LEONOR DA SILVA CARNEIRO

Phytase and carbohydrases in plant-based diets for tambaqui juveniles

(*Colossoma macropomum***)**

Thesis submitted to the Animal Biology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Science*.

Adviser: Ana Lúcia Salaro
Co-advisers: Wilson Massamitu Furuya
Daniel Abreu Vasconcelos Campelo
Luis Filipe Costa de Castro

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“All victories hide an abdication”
Simone de Beauvoir

DEDICATION

To my parents, Ozanan Gomes Carneiro (*in memorian*) and Maria Luiza da Silva Carneiro, my safe haven, I deeply thank you for your unconditional love and for fighting to make my dreams come true. Every word of encouragement and gesture of support was essential to go through this journey. You are my constant inspiration.

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RESUMO

CARNEIRO, Cristiana Leonor da Silva, D.S.c., Universidade Federal de Viçosa, março de 2024. **Fitase e carboidrases em dietas vegetais para juvenis de tambaqui (***Colossoma macropomum***)**. Orientadora: Ana Lúcia Salaro. Corientadores: Wilson Massamitu Furuya, Daniel Abreu Vasconcelos Campelo e Luis Filipe Costa de Castro.

Com este estudo objetivamos compreender os efeitos da suplementação dietética com fitase e carboidrases exógenas para juvenis de tambaqui (*Colossoma macropomum*) por meio da avaliação do desempenho produtivo, composição corporal, bioquímica plasmática, atividade das enzimas digestivas, histomorfometria intestinal, microbiota intestinal, e expressão gênica do tecido hepático (RNA-seq). Uma dieta basal extrusada (307,8 g kg^{-1} de proteína bruta e 17,08 MJ kg^{-1} de energia bruta), composta por ingredientes vegetais foi utilizada no preparo de quatro dietas experimentais: CON = dieta controle sem suplementação com enzimas, PHY = dieta suplementada com fitase, XB = dieta suplementada com xilanase e β-glucanase e PHY+XB = dieta suplementada com fitase, xilanase e β-glucanase. Para alcançar os objetivos propostos um ensaio experimental de 8 semanas foi realizado com juvenis de 1.01 ± 0.15 g. A suplementação enzimática não afetou significativamente a sobrevivência, o peso final, o ganho de peso, a taxa de conversão alimentar ou os índices viscerais dos peixes. Entretanto, peixes alimentados PHY+XB apresentaram maior taxa de eficiência proteica e menor índice de gordura visceral em comparação aquales alimentados com CON, indicando melhor utilização da proteína e menor deposição de gordura. Além disso, os peixes alimentados com PHY+XB apresentaram maior teor de proteína corporal e Fe^{3+}. A análise da bioquímica plasmática revelou níveis mais elevados de HDL e níveis mais baixos de proteína plasmática em peixes alimentados com PHY+XB, juntamente com níveis reduzidos de ALP, indicando potenciais benefícios metabólicos. A suplementação enzimática não afetou as atividades das proteases totais, lipase e amilase no intestino dos peixes, nem alterou a estrutura epitelial intestinal. A análise de sequenciamento de alto rendimento revelou que a maioria das leituras derivadas da digesta do tambaqui pertencem aos filos Firmicutes e Proteobacteria. Peixes alimentados com a dieta PHY+XB apresentaram maior número de táxons enriquecidos em comparação aos demais grupos. Todavia, de modo geral, todos os tratamentos foram enriquecidos com bactérias responsáveis pela manipulação e utilização de carboidratos e outros nutrientes, demonstrando uma modulação benéfica para melhor utilização dos nutrientes dietéticos. A análise diferencial do transcriptoma do tambaqui (RNA-seq) revelou um total de 138 genes diferencialmente expressos (DEGs) entre CON *vs.* PHY; 19 genes entre CON *vs.* XB; e 77 genes entre CON *vs.* PH + XB. Através da análise de enriquecimento GO identificamos que as dietas PHY e

PHY+XB modularam a expressão de genes relacionados à homeostase do oxigênio e processos redox. A dieta PHY também afetou o sistema imunológico dos peixes. A dieta XB afetou principalmente o metabolismo dos carboidratos. Em resumo, a suplementação com fitase e xilanase-β-glucanase se mostrou promissora na melhoria de utização da dieta, na composição corporal, na modulação positiva da microbiota intestinal, e na regulação epigenética de processos metabólicos essenciais. Notavelmente, a combinação dessas enzimas revelou ser mais eficaz, ressaltando a importância da sinergia entre os dois compostos para potencializar os benefícios observados.

Palavras-chave: Aditivo alimentar; Ácido fítico; Microbiota; Polissacarídeos não-amiláceos; RNA-seq

ABSTRACT

CARNEIRO, Cristiana Leonor da Silva, D.S.c., Universidade Federal de Viçosa, March, 2024. **Phytase and carbohydrases in plant-based diets for tambaqui juvenile (*Colossoma macropomum*)**. Adviser: Ana Lúcia Salaro. Co-advisers: Wilson Massamitu Furuya, Daniel Abreu Vasconcelos Campelo and Luis Filipe Costa de Castro.

With this study, we aimed to investigate the effects of dietary supplementation with phytase and exogenous carbohydrates on juvenile tambaqui (*Colossoma macropomum*) by evaluating growth performance, body composition, plasma biochemistry, activity of digestive enzymes, intestinal histomorphometry, intestinal microbiota, and gene expression analysis of liver tissue (RNA-seq). An extruded basal diet (307.8 g kg^{-1} of crude protein and 17.08 MJ kg^{-1} of gross energy), composed of vegetable ingredients, was used in the preparation of four experimental diets: CON = control diet without enzyme supplementation, PHY = diet supplemented with phytase, XB = diet supplemented with xylanase and β-glucanase, and PHY+XB = diet supplemented with phytase, xylanase, and β-glucanase. To achieve the proposed objectives, an 8-week experimental trial was conducted with juveniles weighing 1.01 ± 0.15 g. Enzyme supplementation did not significantly affect fish survival, final weight, weight gain, feed conversion rate, or visceral indices. However, fish fed PHY+XB showed a higher protein efficiency rate and lower visceral fat index compared to those fed CON. This suggests better protein utilization and reduced fat deposition. Furthermore, fish fed PHY+XB had higher body protein and Fe^{3+} content. Plasma biochemistry analysis revealed higher levels of HDL and lower levels of plasma protein in fish fed PHY+XB, along with reduced ALP levels, indicating potential metabolic benefits. Enzyme supplementation did not affect the activities of total proteases, lipase, and amylase in the fish intestine, nor did it alter the intestinal epithelial structure. High-throughput sequencing analysis revealed that the majority of reads derived from tambaqui digesta belong to the Firmicutes and Proteobacteria phyla. Fish fed the PHY+XB diet showed a greater number of enriched taxa compared to the other groups. However, in general, all treatments were enriched with bacteria responsible for the manipulation and utilization of carbohydrates and other nutrients, demonstrating a beneficial modulation for improved utilization of dietary nutrients. Differential analysis of the tambaqui transcriptome (RNA-seq) revealed a total of 138 differentially expressed genes (DEGs) between CON vs. PHY, 19 genes between CON vs. XB, and 77 genes between CON vs. PH + XB. Through Gene Ontology (GO) enrichment analysis, we identified that the PHY and PHY+XB diets modulated the expression of genes related to oxygen homeostasis and redox processes. The PHY diet also affected the fish's immune system. The XB diet mainly affects carbohydrate metabolism. In summary,

supplementing with phytase and xylanase-β-glucanase has shown promise in enhancing dietary utilization, body composition, positively influencing the intestinal microbiota, and regulating essential metabolic processes epigenetically. Notably, the combination of these enzymes has proven to be more effective, emphasizing the significance of the synergy between the two compounds in enhancing the observed benefits.

Keywords: Feed additive; Microbiota; Non-starch polysaccharides; Phytic acid; RNA-seq

SUMMARY

GENERAL INTRODUCTION

The tambaqui, *Colossoma macropomum* (Cuvier, 1818), is currently the second most produced fish species in Brazil, with 109 tons produced in 2022 (IBGE, 2022). The significant interest in tambaqui production is primarily attributed to its zootechnical characteristics, including rapid growth (Aride et al., 2004; Saint-Paul, 1986), high efficiency in protein conversion, and good adaptation to confinement conditions (Araujo-Lima & Goulding, 1998; Aride et al., 2004; Gomes et al., 2002; Silva et al., 2007). Furthermore, this species has an omnivorous eating habit (Honda, 1974), which gives it the advantage of being able to consume diets that are based on vegetable protein sources (Martins et al., 2020a).

In Brazil, soybean, corn, and wheat meals are currently the primary plant-based foods used in diets for fish. However, these ingredients contain a significant amount of antinutritional factors, such as phytate and non-starch polysaccharides (NSP), which impair the use of nutrients and directly impact fish performance and health (Cao et al., 2007; Castillo & Gatlin, 2015; Diógenes et al., 2018). This occurs because these components are not adequately digested in the fish's digestive tract of fish, either due to the absence or insufficient production of specific enzymes for these substrates (Castillo & Gatlin, 2015; Papatryphon et al., 1999). In this sense, the main approach currently used to maximize the nutritional value of diets based on plant ingredients is hydrolysis.

Phytate (myo-inositol hexakisphosphate) is an indigestible form of phosphorus found in plant grains commonly used as ingredients in animal feed, including aquatic organisms (Kumar et al., 2012; Lemos & Tacon, 2017; Selle et al., 2010). However, this form of phosphorus is not available to fish and other monogastric animals (Ravindran et al., 1999). Therefore, even with large amounts of phosphorus, diets formulated with vegetable ingredients are routinely supplemented with inorganic sources of this mineral to meet the requirements for this nutrient (Selle et al., 2010). Phytase is the enzyme required to hydrolyze the phytate present in plants and release the phosphorus linked to this molecule (Selle et al., 2010). Its use in animal diets was initially proposed with the aim of reducing the environmental impact caused by large amounts of phosphorus released in effluents (Lemos & Tacon, 2017). However, it was found that in addition to acting on the substrate releasing inorganic phosphorus, phytase also reduces the amounts of myo-inositol phosphate esters that have chelating capabilities (Selle et al., 2010) and

end up reducing the availability of some minerals (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+}, and Fe^{3+}) (Duffus & Duffus, 1991; Nolan et al., 1987). Furthermore, the inclusion of phytase in diets enhances the bioavailability of amino acids and energy for animals (Ravindran et al., 2001; Selle et al., 2010). Supplementation with exogenous phytase has been shown to benefit the growth performance and health of several fish species, such as *Oreochromis niloticus* (Cao et al., 2008; Liebert & Portz, 2005; Maas et al., 2018; Maas et al., 2020), *Pagrus major* (Biswas et al., 2019), *Clarias gariepinus* (Kemigabo et al., 2018), *Cirrhinus mrigala* (Hussaain et al., 2017), and *C. macropomum* (Mendonça et al., 2012; Nwanna et al., 2008).

NSP are complex carbohydrates found in the cell wall structure of cereals and legumes (Kokou & Fountoulaki, 2018). These components have a high-water retention capacity. When in the gastrointestinal tract, they form a mass or gum that increases the viscosity of the digesta. This limits the access of digestive enzymes to the substrate (Castillo & Gatlin, 2015; Sinha et al., 2011). Therefore, they directly interfere with the processes of digestion and absorption of nutrients from diets (Castillo & Gatlin, 2015; Luo et al., 2020; Sinha et al., 2011). Among NSP, arabinoxylans and β-glucans stand out in cereal grains and legumes (Sinha et al., 2011). Arabinoxylans are soluble polymers found mainly in wheat, whose degradation depends on the enzyme xylanase (Berrin & Juge, 2008). However, similar to phytase, this enzyme is not present in the digestive system of most fish (Bogevik, 2015; Maas et al., 2020). Supplementation with exogenous xylanase has been shown to benefit the productive performance and health of several fish species, such as *Cyprinus carpio* (Jiang et al., 2014), *O. niloticus* (Hassaan et al., 2019; Maas et al., 2018, 2020; Saputra et al., 2016), *Larmichthys crocea* (Luo et al., 2020), and *Ctenopharyngodon idella* (Jin et al., 2020). On the other hand, β-glucans are found mainly in cereals such as oats and barley (Saastamoinen et al., 2004; Tiwari & Cummins, 2009). Their degradation depends on the enzyme β-glucanase, which is also absent in most fish (Ebringerová, 2006). This enzyme has been successfully used to supplement fish diets. However, its use is generally associated with xylanase (Brito, 2019; Diógenes et al., 2018).

Given the successful utilization of different types of exogenous enzymes in the diets of fish and other monogastric animals, there has been a recent proposal to use enzyme complexes containing two or more types of enzymes that target different fractions of the diet. Some studies report that these enzymes together exhibit synergistic action, which

can significantly enhance their beneficial effects on fish performance and health (Dalsgaard et al., 2012; Diógenes et al., 2018; Maas et al., 2018, 2020).

Therefore, with this study we aimed to understand the effects of dietary supplementation with phytase and exogenous carbohydrases on tambaqui juveniles (*Colossoma macropomum*) through the evaluation of growth performance, body composition, plasma biochemistry, digestive enzyme activities, intestinal histomorphometry, intestinal microbiota, and hepatic gene expression (RNA-seq).

CHAPTER I

Literature review

1. Overview of Brazilian tambaquiculture

The tambaqui, *Colossoma macropomum* (Cuvier, 1818), is a Neotropical freshwater species belonging to the Serrasalmidae family and native to the Amazon and Orinoco basins (Géry, 1977; Saint-Paul, 1983). Therefore, it is commonly found in countries in South America, such as Brazil, Peru, Colombia, Venezuela, and Bolivia (Araujo-Lima & Goulding, 1997; Gomes et al., 2002). However, due to its attractive zootechnical characteristics, its introduction for aquaculture purposes has been reported in several countries, including Central America, the United States of America, China, Thailand, the Philippines, and even Hungary (FAO, 2024).

Among its zootechnical qualities, we can mention its rapid growth (2–3 kg year^{-1}) (Hilsdorf et al., 2021), with good adaptation to confinement conditions (Araujo-Lima & Goulding, 1998; Gomes et al., 2002; Aride et al., 2004; Silva et al., 2007). Furthermore, it has the advantage of being a rustic species with prolonged resistance to hypoxia (Saint-Paul, 1984; Chagas & Val, 2006; Neves et al., 2020) and broad thermal tolerance (25-34° C) (Araujo-Lima & Goulding, 1998; Gomes et al., 2010).

Regarding feeding habits, the tambaqui is a fish with an omnivorous-frugivorous diet (Honda, 1974). In nature, its diet varies ontogenetically. It can filter phytoplankton and zooplankton throughout its life but prefers fruits and seeds as an adult (Roubach & Saint-Paul, 1994; Aride et al., 2004). These changes in diet also occur due to seasonality and the availability of food in the natural environment. During flood periods, for example, there is a great availability of fruits and seeds, which are rich sources of carbohydrates and lipids (Sandre et al., 2017). In the dry season, the primary food source is zooplankton, which is rich in proteins (Sandre et al., 2017). This dietary plasticity gives tambaqui the advantage of accepting diets based on vegetable protein and lipid sources (Martins et al., 2020a; Paulino et al., 2018; Pereira et al., 2018). Furthermore, it allows these animals to have greater tolerance and ability to utilize digestible carbohydrates as a primary source of energy (Sandre et al., 2017). This is extremely important as it ensures the independence of using sources of animal origin, a resource that has become increasingly scarce and expensive (Tacon & Metian, 2008).

The tambaqui holds significant social and economic importance for the central Amazon region, as it is considered a regional symbol due to its substantial contribution to the fishing industry (Aride et al., 2004). The first records of tambaqui domestication and cultivation date back to the 1930s when Von Ihering, considered the “father of fish farming” in Brazil, highlighted the species' potential for aquaculture (Hernández et al., 1992). In the early 1960s, research institutions in Brazil and countries in the Amazon Basin began to consider tambaqui as a viable candidate for commercial fish farming (Silva et al., 1989, 1999; Guimarães et al., 1999). Despite this, its cultivation only increased in the 1980s in response to an incentive from the government of the state of Amazonas (Aride et al., 2004) and the development of induced reproduction techniques, which allowed the production of larvae and fry. In 1988, some organizations in Latin America dedicated to the development of aquaculture held the first scientific event to discuss and establish cultivation techniques for the species (Hilsdorf et al., 2021). The event was organized by the *Colossoma* Technical-Cultivation working group and took place at the Aquaculture Research and Training Center (CEPTA) in Pirassununga, SP (Hilsdorf et al., 2021). Since then, tambaqui production has grown exponentially over the last few decades and is currently the second most produced species in Brazil, with 109 tons in 2022 (IBGE, 2022).

Tambaquiculture is practiced in all states in the North Brazilian region, as well as in the states of Maranhão, Piauí, Tocantins, Mato Grosso, Goiás, and Minas Gerais (Valenti et al., 2021). The main producing states in Brazil are Rondônia, Roraima, and Maranhão (IBGE, 2022), which rank first, second, and third, respectively. The prominence of these states is due to the work of state governments in promoting economic activity and cooperativism. On the other hand, states such as Amazonas and Pará, which are the main consumers of tambaqui (Gandra, 2010; Pedroza Filho et al., 2016), still require development in their production chain to fully utilize their aquaculture potential. The difficulty that these states face in exploring tambaqui production, is due to a series of problems that hinder the expansion of native fish production. Among these challenges, we can mention the high price of inputs, mainly fry and feed; the shortage of feed mills and fish processing units; the difficulty of ensuring production; and the lack of environmental licensing. However, considering that other states in the North have also been experiencing similar setbacks, it is evident that the absence of public policies to promote the activity remains the primary obstacle.

Another aspect of tambaquiculture is that, although booming, in many fish farms, the activity is still carried out with a low level of technology, generally in extensive and semi-intensive systems in earthen tanks (Farias Lima et al., 2019). Therefore, like other native species, the tambaqui still requires studies on its nutrition, health, production, reproduction, market, and consumption systems. Currently, several research centers are seeking to align tambaqui production with sustainability principles. An example of movement in this direction is the cultivation of tambaqui with other aquatic organisms, a practice also known as multitrophic aquaculture (Flickinger et al., 2019; 2020 a; 2020b).

Regarding to nutrition aspects, in larviculture, tambaqui fry require approximately 32% crude protein (CP) for their nutritional needs (Lima et al., 2016). In frying, fish require around 30% crude protein (CP) (Amancio et al., 2019). The duration of the fattening phase depends on the slaughter weight chosen by the region or property. At this stage, feed containing 28% CP can be used (Gomes et al., 2020). In the wild, tambaqui can reach 30 kg (Hilsdorf et al., 2021). However, when cultivated with unbalanced diets for long periods, it accumulates a large amount of fat in the meat and abdominal cavity, also known as visceral fat (Fernandes et al., 2010). Therefore, a strategy that must be adopted to reduce tambaqui fat is the use of balanced diets that are suitable for the animal's life stage, promoting good growth, survival, feed conversion, and meat quality. Another strategy would be to implement short production cycles (Fernandes et al., 2010), ranging from 8 to 12 months for fish weighing 1.0 to 2.0 kg at slaughter, and from 12 to 18 months for fish weighing over 2.0 kg at slaughter (Corrêa et al., 2018).

With an average conversion rate of 1.81 (kg of feed kg of fish^{-1}) (Feitoza et al., 2018), the average production cost of tambaqui in 2020 was R$3.67 kg^{-1} for fish slaughtered with 1.0-1.2 kg^{-1}, and R$4.42^{-1} kg for fish slaughtered with more than 2 kg (Francisco et al., 2021). There are several factors that influence the cost of production, with feed being one of the most expensive items, representing around 80% of total costs (Martins et al., 2020b). Therefore, it is important to enhance the efficiency of nutrient utilization in diets, minimizing losses as much as possible.

2. Evolving aquaculture: challenges and solutions for a sustainable future

Aquaculture, the cultivation of aquatic organisms in captivity, has experienced exponential growth in recent decades. Fish farming production has increased significantly from 10 million tons to nearly 60 million tons over the past 30 years (FAO, 2022). The

aquaculture industry currently accounts for more than half of the fish consumed in the world, and projections indicate that it will supply 200 million tons of aquatic organisms in 2030 (FAO, 2022). This expansion is driven by the global demand for high-quality animal protein and by the population's need to diversify food sources (FAO, 2022). However, the sustainability of aquaculture production faces a well-known challenge: the availability and high cost of traditional ingredients for formulating diets.

Before the 1970s, when the production of fish with high commercial value, such as salmon and trout, dominated the global aquaculture scenario and caught the interest of nutritionists, the primary focus of the aquafeed industry shifted towards replacing fishmeal with cheaper and ecologically sustainable ingredients (Glencross et al., 2019; Hardy & Kaushik, 2022). Cereal and legume meals emerged as promising candidates due to their relative nutritional quality, abundance, and affordable cost. However, despite being rich in nutrients, the utilization of these foods in diets for fish and other aquatic organisms has proven challenging. This is because vegetable meals have a lower nutritional profile compared to fish meal, presenting deficiencies in amino acids and essential fatty acids, as well as reduced digestibility (Glencross et al., 2012) and presence of antinutritional factors (Kokou & Fountoulaki, 2018). With advances in technology and processing techniques as well as the advent of crystalline amino acids, formulators around the world have been able to partially overcome this obstacle. These new dietary approaches allowed the partial replacement of fishmeal with vegetable meal, such as soybean meal, in the diets of carnivorous species, and also encouraged the growth of another market, that of omnivorous species.

Within the new perspectives in the field of nutrition and production systems, new possibilities have emerged. Over the past 30 years, the production of freshwater species such as *Cyprinus carpio*, *Ictalurus punctatus*, and *Oreochromis niloticus*, which can thrive on plant-based diets, has significantly surpassed the production of marine fish (FAO, 2022). Currently, freshwater species represent almost 90% of global fish production (Hardy & Kaushik, 2022; FAO, 2022). These species consume approximately 63% of all feed produced in the world (Hardy & Kaushik, 2022). As production is expected to continue to increase, the demand for food in this sector is also expected to rise. In light of this, a new paradigm is emerging in global aquaculture. Sustainability now encompasses not only replacing fishmeal but also enhancing nutrient utilization from traditional plant ingredients like soy, corn, and wheat, or substituting them with

alternative vegetable ingredients. Such problems are not just an economic problem, they are also characterized as environmental and social issues of relative urgency, especially considering the scenario of the new globalized world. Given this, it is essential to search for technologies that enable the use of ingredients with low nutritional value, such as co-products from the food industry. In this scenario, several innovative technologies have been proposed, such as the use of exogenous enzymes to reduce antinutritional factors and enhance nutrient digestibility, fermentation to improve the nutritional quality and palatability of ingredients, and bioprocessing to convert biomass waste into valuable feed ingredients.

3. Antinutritional factors and exogenous enzymes in fish feed

The use of commercial feed for aquatic animals in 2017 was estimated at 51.23 million tons and is expected to increase to 73.15 million tons by 2025 (FAO, 2019). However, the increasing economic and environmental pressures placed on animal production require this industry to reduce its dependence on ingredients such as fishmeal and increase the use of local, more accessible, and sustainable raw materials (Tacon, 2020). In this scenario, ingredients of plant origin have been recommended for the preparation of diets for aquatic organisms. This recommendation is primarily due to their low cost, consistent availability, and alignment with the principles of environmental sustainability.

In Brazil, the most commonly used vegetable ingredients in fish formulations are soybean meal, corn meal, wheat bran, rice bran, and their by-products. These foods have relative nutritional quality, but compared to fishmeal, they exhibit nutritional deficiencies, particularly in terms of amino acid and essential fatty acid profiles. Currently, the possibility of supplementing with crystalline amino acids and additional sources of fatty acids has enabled the formulation of balanced diets incorporating these ingredients. However, other obstacles persist and must be overcome. Among them, the presence of significant amounts of antinutritional factors stands out, such as phytate and high levels of non-digestible carbohydrates, also known as non-starch polysaccharides (NSP), which reduce the efficiency of nutrient utilization in these diets (Cao et al., 2007; Castillo & Gatlin III, 2015; Diógenes et al., 2018).

3.1 Phytic acid

Phytic acid (1,2,3,4,5,6-hexakis(dihydrogenphosphate)-myo-inositol, IP6) (Figure 1) and its salts, also known as phytates, were discovered in 1855 by Hartig when he detected the presence of rounded molecules in various plant seeds (Song et al., 2019). This molecule is considered the largest reservoir of phosphorus in vegetables, especially those rich in fiber, which includes most cereal grains and legumes (Song et al., 2019; Wang & Guo, 2021) commonly used as ingredients in feed for terrestrial and aquatic animals (Lemos & Tacon, 2017). Despite being abundant, the phosphorus contained in phytic acid is not readily available to monogastric animals because they have low or no endogenous production of the enzyme that hydrolyzes this molecule, phytase (Ravindran et al., 1999; Jackson et al., 1996; Papatryphon et al., 1999). Therefore, even with large amounts of this mineral, diets formulated exclusively with plant ingredients are routinely supplemented with inorganic sources of phosphorus to meet the animals' requirements for this nutrient (Selle et al., 2010). Therefore, a significant portion of the ingested phosphorus is lost to the environment, leading to eutrophication and a reduction in the carrying capacity of aquatic ecosystems (Liebert & Portz, 2005).

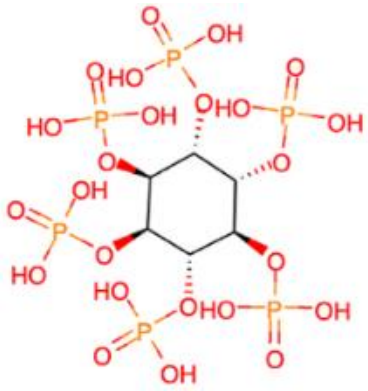

Figure 1. Phytic acid molecule. Extracted from Wang & Guo (2021).

Phytic acid also acts as a chelator, forming complexes (Figure 2) with minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+}, and Fe^{3+} (Nolan et al., 1987; Duffus & Duffus, 1991; Schlemmer et al., 2009; Wang & Guo, 2021), proteins (Spinelli et al., 1983; Richardson et al., 1985; Nolan et al., 1987; Wang & Guo, 2021), and starch (Oatway et al., 2001) (Figure 2). These complexes precipitate or prevent the diffusion of digestive enzymes over the substrate, reducing the bioavailability of these nutrients (Ravindran et al., 1995; Wu & Guo, 2021). Therefore, other undesirable effects of phytate include mineral

deficiency (Singh et al., 2020), increased endogenous losses of amino acids (Papatryphon et al., 1999; Francis et al., 2001; Sugiura et al., 2001), and reduced capacity to utilize dietary carbohydrates (Oatway et al., 2001). However, the growth suppression caused by phytic acid is not only associated with the unavailability of nutrients. This antinutritional factor causes morphological changes in the intestinal mucosa, resulting in enteropathy, and immunological suppression due to changes in the synthesis of immunological components, such as Alpha-1 acid glycoprotein (ACP), lysozyme, complement C3 and C4, antimicrobial peptides, interleukins, and immunoglobulins (Zhong et al., 2019).

In ingredients such as soybean meal, wheat, corn, and rice, phytates can represent 60 to 80% of the phosphorus (Selle et al., 2010; Kumar et al., 2012; Lemos & Tacon, 2017). These organic compounds begin to accumulate during the seed maturation phase, which is likely a plant detoxification strategy, as inorganic phosphorus is toxic to them (Yang et al., 2017). The stored phosphorus is subsequently recycled, serving as a source of energy and antioxidants during the germination process (Loewus, 2002). This makes genetic manipulation to obtain plants with low levels of phytate unfeasible (Oatway et al., 2001; Bregitzer & Raboy, 2006; Dong & Saneoka, 2020). Processes such as grinding, soaking, and cooking can result in a certain degree of phytate removal; however, they have low efficiency. When discarding phytate reservoirs, other nutrients are also sacrificed (Singh & Satyanarayana, 2011). Therefore, enzymatic degradation, facilitated by the enzyme phytase, is the most effective and practical method for hydrolyzing phytates (Wang & Guo, 2021)

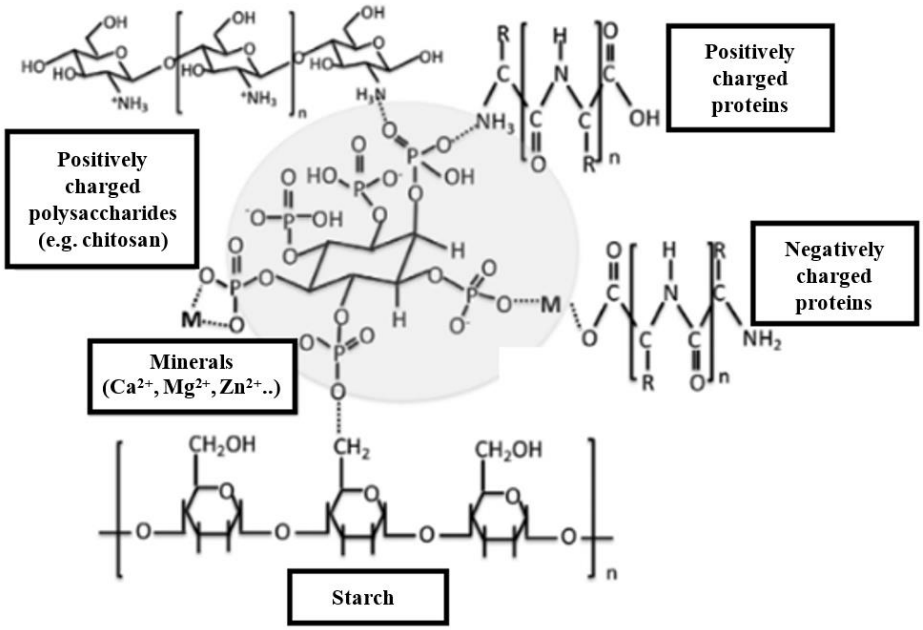

Figure 2. Possible interactions between phytic acid and minerals, proteins, starch polysaccharides. Adapted from Wang & Guo (2021, apud Oatway et al., 2001).

3.2 Phytase

Since the 1980s, phytases (myo-inositol hexakisphosphate phosphohydrolase) have attracted considerable attention in the animal nutrition sector. This is because these enzymes are capable of gradually dephosphorylating phytate (myoinositol (1,2,3,4,5,6) hexakisphosphate), releasing inositol and inorganic phosphorus (Selle et al., 2010; Greiner & Konietzny, 2011; Kumar et al., 2012) (Figure 3). Therefore, the inclusion of adequate amounts of phytase in the diet reduces the need to add phosphorus to the diet of monogastric animals and promotes a reduction of up to 50% in the release of phosphorus into the environment (Greiner & Konietzny, 2011).

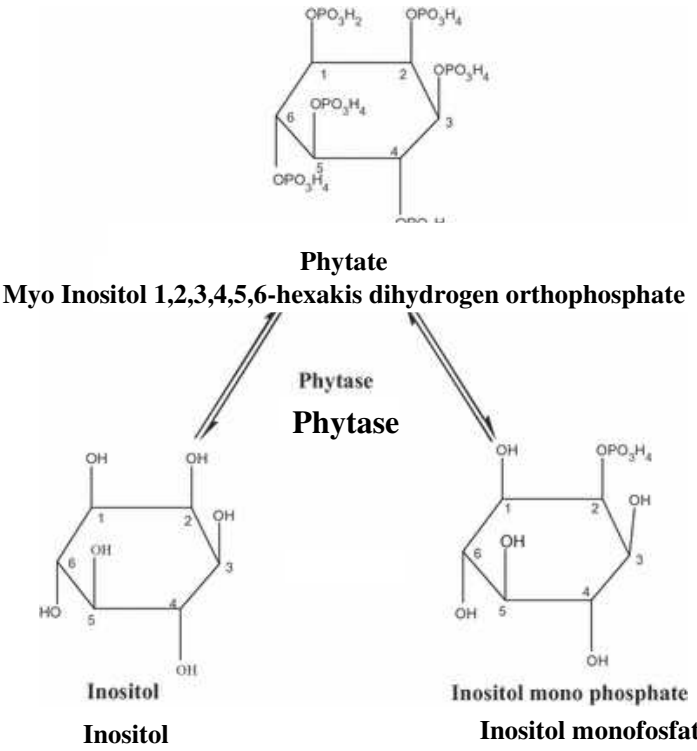

Figure 3. Schematic representation of phytate hydrolysis. Adapted from Kumar et al. (2012).

This enzyme was first identified in rice bran but can be produced by a wide range of microorganisms, plants, and some animal tissues (Suzuki et al., 1907; Konietzny & Greiner, 2002). Phytases of plant origin have a more restricted pH spectrum of activity, showing decreased stability at pH levels below 4 and above 7.5 (Greiner & Konietzny, 2006). Therefore, they are not recommended for supplementing fish diets (Greiner & Konietzny, 2011), as fish have a gastrointestinal tract pH ranging from 7 to 8 (Morais & Almeida, 2020). On the other hand, microbial phytases are more stable, performing well even at pH values below 3 and above 8 (Greiner & Konietzny, 2006). Furthermore, microbial phytases have better bioefficacy and thermoresistance than those of plant origin (Jongbloed & Kemme, 1990; Pointillart, 1988; Zimmermann et al., 2002).

Phytases can be classified based on their catalytic mechanism into histidine acid phytases (HAPs), β-helical phytases (BPPs), purple acid phytases (PAPs), and cysteine phytases (CPs) (Mullaney & Ullah, 2003; Greiner, 2006; Reddy et al., 2017; Singh et al., 2020). They can also be classified according to their optimal pH of activity in acidic and alkaline phytases (Greiner & Konietzny, 2011; Singh et al., 2020). Most of the known phytases belong to the PAH subfamily and, like other acid phytases, do not require

cofactors to exert their activity (Greiner & Konietzny, 2011). Alkaline phytases, however, exhibit activity that is highly dependent on Ca^{2+} ions (Balaban et al., 2017; Rebello et al., 2017).

Based on the site where dephosphorylation is initiated, phytases can be further classified into 3-phytase (E.C. 3.1.3.8), 6-phytase (E.C.3.1.3.26), or 5-phytase (E.C. 3.1.3.72) (Greiner & Konietzny, 2011; Reddy et al., 2013). The 3-phytases initiate dephosphorylation by the phosphate group located at C3 of the phytate molecule, generating L-myo-inositol-2-phosphate as a product (Reddy et al., 2017; Gessler et al., 2018). These phytases can be obtained from a wide range of fungi and bacteria and therefore represent the majority of phytases available on the market (Reddy et al., 2017; Singh et al., 2020). Examples of 3-phytases are those produced by *Aspergillus niger*, *Neurospora crassa*, *Pseudomonas* sps, *Klebsiella* sps, *Bacillus* sps, *Myceliophthora thermophila*, *Emericella nidulans* and *Thermus thermophilus* (Sajidan et al., 2004; Rao et al., 2009). In contrast, 6-phytases initiate their action through the phosphate group located at C6 of the phytate molecule and result in a complete and more efficient dephosphorylation (Gessler et al., 2018). They produce 6-phytases, some plants, *Escherichia coli*, *Paramecium sp*. and the human lysosome (Reddy et al., 2017). The 5-Phytases were identified more recently and initiate the dephosphorylation of phytate from the phosphate group located at C5 of phytate (Gessler et al., 2018). These are mostly produced by plants, such as *Medicago sativa*, *Phaseolus vulgaris*, *Pisum sativum* and *Lillium* sp. (Rao et al., 2009; Gupta et al., 2015).

The pH and temperature are determining factors for the functioning of enzymes, whether exogenous or endogenous (Lemos & Tacon, 2017). Phytases are less thermotolerant than other enzymes that degrade fiber and, therefore, are more affected by the high temperatures used during feed extrusion processes (Gilbert & Cooney, 2010). Phytases have their activity significantly reduced when temperatures exceed 70° C, while carbohydrases lose activity only if temperatures exceed 80° C (Gill, 1997). In this sense, liquid products, which can be applied via spraying after thermal processing, are recommended (Lemos & Tacon, 2017). Application via spraying adds a step to the production line and requires equipment that is sometimes not available in the animal feed industry (Gilbert & Cooney, 2010). Products in the form of dry powder can be easily added during pre-pelletization/extrusion. However, it is still necessary to find ways to ensure the thermostability of the enzymes (Lemos & Tacon, 2017). Therefore, studies

with various strategies have been developed to create heat-resistant products. These strategies include coating the enzymes, genetic manipulation, and the discovery of new thermotolerant strains (Gilbert & Cooney, 2010).

Phytase supplementation is already a common practice in poultry and swine production. However, its use in fish diets is still incipient. Several studies have been carried out in recent decades, demonstrating the ability of this enzyme to enhance nutrient digestibility, health, and performance of various fish species (Table 1). It is important to remember that the level of supplementation in the diet is given in active phytase units (FTU), a standard unit that can be defined as the amount of enzyme required to liberate 1 mol of inorganic phosphorus per minute from 0.0015 mol L^{-1} of sodium phytate at pH 5.5 at 37° C (Simons et al., 1990). It is observed that this level is variable and depends on various factors, including the composition of the diet, the species studied, and the type or origin of the enzyme used.

Table 1. Beneficial effects of dietary phytase supplementation in fish

Species	Initial weight (g)	Inclusion level (FTU kg^{-1})	Ingredients	Beneficial effects	Reference
Oreochromis niloticus	13	750	Soybean meal, wheat gluten, corn meal and wheat meal	Improvements in growth performance; digestibility and retention of nutrients	Liebert & Portz (2005)
	0.8	1000	Soybean meal, wheat meal, corn gluten and cassava flour	Improvements in growth performance; feed conversion; protein efficiency rate; digestibility and P retention	Cao et al. (2008)
	42	1000	Soybean meal, DDGS, wheat gluten, rapeseed meal, sunflower meal, wheat meal, corn meal, oat meal and rice meal	Improvements in growth; specific growth rate; digestibility of dry matter, crude protein, carbohydrates, energy, ash, P and Ca^{2+}	Mass et al. (2018)
	39	660	Corn, soybean meal, wheat meal, wheat gluten, wheat bran, rapeseed meal, sunflower meal, rice bran, DGGS	Improvements in growth; feed conversion; P availability	Maas et al. (2020)
	25	1500	Soybean meal, wheat meal, wheat flour, corn bran, rice bran, corn gluten	Improvement in the digestibility of energy, protein, amino acids and minerals; decreased P excretion	Pontes et al. (2021)

	57.48	1500 - 2000	Soybean meal, corn meal, rice meal	Improvements in growth performance, and nutrient digestibility	Rodrigues et al. (2022)
	5.40	1500	Maize, corn gluten, wheat middlings, rice bran, soybean meal, fishmeal	Improvements in growth performance, blood parameters, intestinal morphology, body composition and expression of *GHr-1*, *IGF-1*, *FAS* and *LPL* genes	Negm et al. (2024)
Oreochromis aureus x *O. niloticus*	28.9	3000	Menhaden fish meal, poultry by-product meal, soybean meal 48%, DDGS, soy protein concentrate, wheat meal, mill run, blood meal, spray dried poultry, feather meal, hydrolyzed corn, soft white	Improvements on intestinal microbiota	Ray et al. (2024)
Pagrus major	23	2000	Soy protein concentrate, corn gluten and starch	Improvements in weight gain, specific growth rate; feed efficiency; food efficiency; P digestibility; retention of proteins, lipids and energy	Biswas et al. (2019)
Clarias gariepinus	10	1200	Soybean meal, wheat, corn meal, beans, cottonseed cake and cassava flour	Improvements in growth performance; feed conversion; protein efficiency rate, protein and P retention; digestibility of dry matter, protein and P	Kemigabo et al. (2018)

Cirrhinus mrigala	5	1000	Wheat meal, corn gluten, polished rice meal and sunflower meal	Improvements in growth performance; digestibility of crude protein, fat and energy	Hussain et al. (2017)
Sciaenops ocellatus	4.99	5520	Soybean meal, menhaden meal, soy protein concentrate, and red drum meal	Improvements in growth performance and mineral body retention and bone mineralization	Chen & Gatlin III (2024)

3.3 Xylans and β-glucans

Most protein and energy ingredients of plant origin used in fish feed contain fiber. This fraction of the food is composed of several types of complex carbohydrates, also known as non-starch polysaccharides (NSP) (Barletta et al., 2011). Despite being abundant in diets formulated with vegetable ingredients, these carbohydrates are not bioavailable to fish and other monogastric animals. This is because they do not have the endogenous production of enzymes necessary to hydrolyze the β-glycosidic bonds in them in their gastrointestinal tract (Kuz'mina, 1996; Choct, 1997; Stone et al., 2003; Krogdahl, 2005; NRC, 2011; Sinha et al., 2011). Furthermore, soluble NSP have a high-water retention capacity. This property in the gastrointestinal tract contributes to increasing the viscosity of the digesta, which limits the access of digestive enzymes to the substrate (Castillho & Gatlin III, 2015; Sinha et al., 2011). In addition to impairing dietary digestibility, NSP can cause growth retardation by increasing intestinal permeability and the abundance of pathogenic microorganisms, inducing intestinal inflammation, and apoptosis of intestinal epithelial cells (Liu et al., 2022). Therefore, NSP act as antinutrients and directly harm the digestion and absorption processes, as well as the intestinal health of fish (Sinha et al., 2011; Castillo & Gatlin III, 2015).

The main NSP found in cereals and legume grains are in the hemicellulose and cellulose groups (Selvendran et al., 1987; Castillo and Gatlin III, 2015). Hemicellulose is the second most abundant polysaccharide on Earth, representing about 20–35% of the cell wall composition of plant cells (Sticklen, 2008). Among the various types of hemicellulosic polysaccharides, arabinoxylans are the most abundant in plants (Barletta, 2011). These are found in large proportions (60-70%) in the cell wall of endosperm cells and in the aleurone layer of most cereals, with the exception of barley and rice, which contain 20% and 40%, respectively (Fincher & Stone, 1986). In the case of wheat, high levels (64%) of arabinoxylans are also found in non-endospermic tissues of wheat, such as the pericarp and the testa (Barletta, 2011).

The structure of an arabinoxylan is formed by a linear skeleton of xylose units linked through β-(1→4) glycosidic bonds, to which arabinoses are attached as primary side groups (Sinha et al., 2011) (Figure 4). Part of the arabinoxylans present in cereal grains are insoluble in water because they are strongly anchored in the cell wall of cells (Mares & Stone, 1973). However, the vast majority of arabinoxylans can absorb about ten times their weight of water and form highly viscous solutions (Sinha et al., 2011).

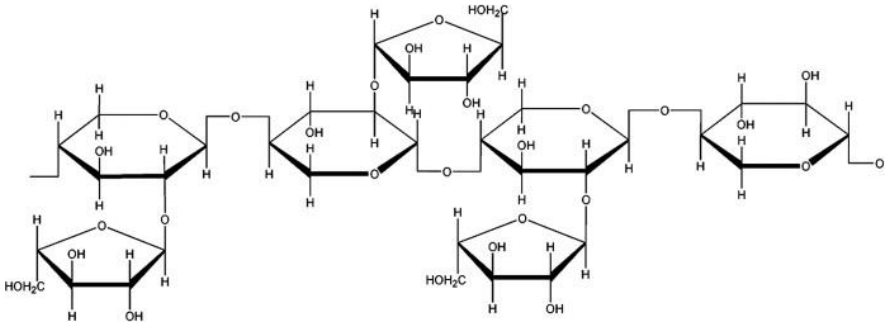

Figure 4. Chemical structure of an arabinoxylan. Extracted from Sinha et al. (2011).

The β-glucans are cellulosic polysaccharides present in significant quantities in plants. In cereal grains, these NSP are mainly concentrated in the aleurone layer and in the walls of endosperm cells (Mishra, 2020). The amount of β-glucans in food varies depending on the vegetable and its growing conditions. Among cereals, barley has the highest β-glucan content (5–11%), followed by oats (3–7%), wheat (0.5-1%), and rye (1.4-2%) (Saastamoinen et al., 2004; Tiwari & Cummins, 2009).

The structure of a mixed-linkage β-glucan consists of a linear chain of glucose units linked by β-(1→3) and β-(1→4) linkages (Bengtsson et al., 1990) (Figure 5). However, it is important to highlight that although these β-glucans and other cellulosic polymers have glucose units linked by β bonds, these NSP have different physical properties. This is because most cellulosic polymers contain only β-(1→4) bonds, which gives them a rigid, crystalline, and insoluble structure (Paloheimo et al., 2010; Sinha et al., 2011). β-glucans also have β-(1→3) bonds that make their structure soluble and flexible (Paloheimo et al., 2010; Sinha et al., 2011). Therefore, like other soluble NSP, β-glucans are viscous in nature and consequently increase the viscosity of intestinal digesta (Sinha et al., 2011).

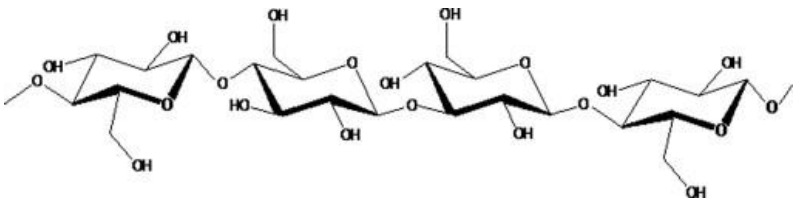

Figure 5. Chemical structure of a β-glucan. Extracted from Sinha et al. (2011).

Both arabinoxylans and β-glucans remain in food even when they are exposed to thermal processing (U.S. Grains Council, 2012; Welker et al., 2014). Furthermore, high temperatures can increase the solubility of these carbohydrates and enhance their water retention capacity in the animals' bodies (Paloheimo et al., 2010). Therefore, the primary approach to enhance the utilization of nutrients from diets abundant in these polysaccharides is by supplementing diets with exogenous enzymes.

3.4 Carbohydrases: xylanase and β-glucanase

Carbohydrases are enzymes that break down carbohydrates into simpler sugars. In animal nutrition, enzymes can be divided into two categories: those that target NSP (β-glycosidic bonds) and those that digest starch (α-glycosidic bonds) (Barletta et al., 2011). The two main enzymes that degrade NSP used in animal feed are xylanase and β-glucanase, which represent almost 80% of the products available on the market (Barletta et al., 2011; Castillo & Gatlin III, 2015). However, other enzymes that degrade fiber have also been used, on a smaller scale, in animal nutrition. They are β-mannanase, pectinase, and α-galactosidase (Barletta et al., 2011).

Xylanases (endo-1,4-β-xylanase, EC 3.2.1.8) hydrolyze the β-(1→4) bonds between the xylose units present in the structure of arabinoxylans (Paloheimo et al., 2010; Hatanaka, 2011; Motta et al., 2013) releasing xylo-oligosaccharides (XOS) and arabinoxylo-oligosaccharides (AXOS) (BASF, 2022; Figure 6). The action of xylanase on the substrate results in a partial hydrolysis of the xylans, which is sufficient to decrease the viscosity of the digesta and enhance the utilization of nutrients by the animals (Paloheimo et al., 2010). For complete hydrolysis, this enzyme must act synergistically with other hemicellulases (Coughlan & Hazlewood, 1993; Paloheimo et al., 2010; Motta et al., 2013).

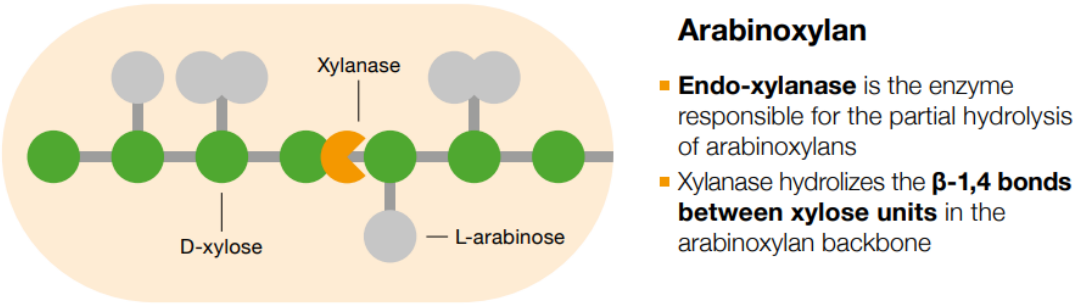

Figure 6. Endo-β-xylanase hydrolyzes arabinoxylans into xylo-oligosaccharides (XOS) and arabinoxylo-oligosaccharides (AXOS) (BASF, 2022).

The catalytic action of xylanases can be inhibited by three classes of inhibitory substances present in cereals. TAXI-type inhibitors (*Triticum aestivum* xylanase inhibitors) are proteins of around 40 kDa, specifically targeting GH11 family xylanases (Paloheimo et al., 2010). XIP-type inhibitors (chitinase-like xylanase inhibitors) are proteins of around 29 kDa capable of inhibiting enzymes from the GH10 and GH11 families (Payan et al., 2004). TL-XI inhibitors (thaumatin-like xylanase inhibitors) have not yet been well-characterized (Paloheimo et al., 2010). However, for feed supplementation, xylanases that do not suffer from this type of inhibition have already been developed (Paloheimo et al., 2010).

The β-glucanases (endo-1,4-β-glucanases EC 3.2.1.4) hydrolyze the β-(1→4) bonds found in β-glucans in cellobiose units, altering the integrity of the endospermic cell wall (Habte-Tsion et al., 2018; Figure 7). This action allows the action of digestive enzymes in the animals' gastrointestinal tract, reducing the viscosity of the digesta (Paloheimo et al., 2010).

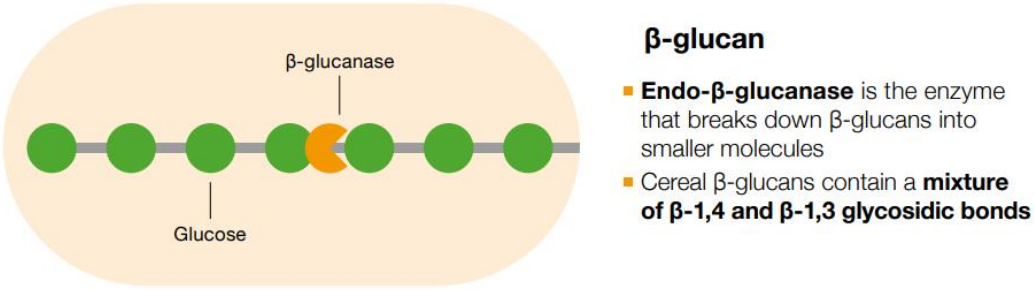

Figure 7. Endo-β-glucanase breaks down β-glucans into smaller molecules (BASF, 2022).

Commercial xylanases and β-glucanases are primarily produced by microorganisms, but they can also be found in marine algae, protozoa, crustaceans, insects, snails, and plant seeds (Sunna & Antranikian, 1997; Motta et al., 2013; Imran et al., 2016). Among the microbial sources of these enzymes, filamentous fungi, such as *Aspergillus sp.*, and *Trichoderma sp.*, that are the most interesting for commercial-scale production. This is because they secrete enzymes into the extracellular environment in higher quantities than yeast and bacteria (Motta et al., 2013). Most microbial xylanases are stable at temperatures between 40-80° C and pH levels ranging from 4.0 to 6.5

(Polizeli et al., 2005). The β-glucanases obtained from these microorganisms are stable at temperatures between 40-70° C and pH of 3.0 to 9.0 (Zhao et al., 2010).

The great diversity of xylans and β-glucans in nature has resulted in various types of xylanases and β-glucanases (Collins et al., 2005; Paloheimo et al., 2010). Therefore, a family classification system (GH), based on substrate specificity, was proposed to distinguish glycosidases (Henrissat, 1991). The main families of xylanases in commercial preparations are the GH10 and GH11 families (Collins et al., 2005). GH11 xylanases have a low molecular weight, high isoelectric point (pI), and a β-sheet structure (Törrönen et al., 1993), while G10 xylanases have a high molecular weight, low pI, and an (α/β)8 structure (Biely et al., 1997). G11 xylanases act exclusively on substrates containing D-xylose, while G10 xylanases are more versatile due to their flexible structure (Biely et al., 1997). Endo-1,4-β-glucanases are classified into around 15 distinct families, however, it is not known for certain which glycoside hydrolases (GHs) contribute to the total activity of this enzyme in commercial preparations (Fernandes et al., 2016).

The main action of xylanases and β-glucanases in the animal organism is to decrease the viscosity of the diets. This allows greater access of digestive enzymes to substrates, thereby improving the utilization of nutrients in the diets (Castillo & Gatlin III, 2015). However, another mechanism that contributes to improving the nutritional value of diets supplemented with these enzymes is the increased release of oligosaccharides that exert a prebiotic effect on the animals' bodies (Choct & Cadogan, 2001).

Xylanases and β-glucanases have demonstrated their ability to enhance the digestibility and growth performance of poultry, pigs, and fish in numerous publications. For poultry and pigs, supplementing diets with these additives has been a common practice for over 30 years (Paloheimo et al., 2010). In fish, although studies began more than 20 years ago, the practice is still in its infancy (Castillo & Gatlin III, 2015). It has been demonstrated that supplementation with xylanases and β-glucanases promotes significant improvements in the utilization of nutrients from diets based on plant ingredients in several fish species (Table 2 and Table 3, respectively).

Given the successful utilization of various exogenous enzymes in the diet of fish and other monogastric animals, some researchers have recently proposed the use of enzyme complexes containing two or more types of enzymes that target different fractions of the substrate. Thus, when these enzymes work together, they can exhibit synergistic

action, significantly enhancing their beneficial effects on fish performance and health. Therefore, the relatively low number of studies evaluating β-glucanase alone is notable, as this enzyme is typically found in enzymatic complexes that also contain xylanase or other enzymes (Table 4).

Table 2. Beneficial effects of dietary xylanase supplementation in fish

Species	Initial weight (g)	Inclusion level (U kg^{-1})	Ingredients	Beneficial effects	Reference
Oreochromis niloticus	1.49 g	-	Soybean meal, wheat bran, rice bran and corn starch	Improvements in growth performance and immune parameters	Saputra et al. (2016)
	42 g	4000	Soybean meal, DDGS, wheat gluten, rapeseed meal, sunflower meal, wheat, corn, oat and rice bran	Improvements in the digestibility of dry matter, crude protein, carbohydrates and energy	Maas et al. (2018)
	1.3g	3750	Sunflower bran, soybean bran, corn bran and oat bran	Improvements in growth performance, activity of digestive enzymes, nutrient digestibility, intestinal and liver morphometry, and nutrient retention	Hassaan et al. (2019)
	39 g	6596	Corn bran, soybean bran, wheat, wheat gluten, wheat bran, rapeseed bran, sunflower bran, rice bran, DGGS	Improvement in the digestibility of dry matter, energy, carbohydrates and NSP	Maas et al. (2020)

	4.09	3000	Fishmeal, casein, soybean meal and corn starch	Improvements on intestinal morphology, serum biochemistry and expression of the *MUC2* gene	Wang et al. (2023)
Cyprinus carpio	8 g	1259	Soybean meal, rice gluten, cottonseed meal, rapeseed and wheat meal	Improvements in growth performance, activity of digestive enzymes, and diversity of the intestinal microbiota	Jiang et al. (2014)
Larmichthys crocea	12 g	600 a 1200	Wheat gluten, wheat meal and soy lecithin	Improvements in growth performance, intestinal morphology, intestinal microbiota diversity, influence on intermediary metabolism, availability and utilization of carbohydrates	Luo et al. (2020)
Ctenopharyngodon idella	232 g	1527 to 1608	Soybean meal, cottonseed meal, rapeseed meal, wheat and starch	Improvements in growth performance, immune parameters and diversity of the intestinal microbiota	Jin et al. (2020)

Table 3. Beneficial effects of dietary supplementation with β-glucanase in fish

Species	Initial weight (g)	Inclusion level (U kg^{-1})	Ingredients	Beneficial effects	Reference
Oncorhynchus mykiss	73 to 110	67 mg kg^{-1}	Soybean meal	Improvement in the digestibility of protein, lipids, ash, phosphorus and dry matter, improvement in energy retention and reduction of nitrogenous waste	Dalsgaard et al. (2012)
Cyprinus carpio	13 g	-	Barley meal	Improvements in hematological and immune parameters	Mohammadbeygi et al. (2012)

Tabela 3. Beneficial effects of dietary supplementation with combination of exogenous enzymes in fish

Species	Initial weight (g)	Enzymes combination	Inclusion level	Ingredients	Beneficial effects	Reference
Oreochromis niloticus	42	Phytase + Xylanase	1000 FTU and 4000 U kg^{-1}, respectively	Soybean meal, DDGS, wheat gluten, rapeseed meal, sunflower meal, wheat bran, corn bran, oat bran and rice bran	Improvements in growth performance and protein retention	Maas et al. (2018)
	1	Xylanase+ β-glucanase	1200 TXU and 500 TGU kg^{-1}, respectively	Wheat flour, soybean meal, corn starch	Improvements in growth performance, intestinal morphology and diversity of intestinal microbiota	Brito et al. (2019)
	39	Phytase + Xylanase	660 FTU and 6596 U kg^{-1}, respectively	Corn, soybean meal, wheat, wheat gluten, wheat bran, rapeseed meal, sunflower meal, rice bran, DGGS	Improvements in growth performance and nutrient digestibility	Maas et al. (2020)
	12	Xylanase+ β-glucanase	1130 TXU kg^{-1} and 510 TGU kg^{-1}, respectively	Corn, soybean meal, wheat bran, wheat flour, soy protein	Improvements in growth performance, blood parameters and intestinal microbiota	Brito et al. (2022)

				concentrate, fish meal and spray-dried blood meal		
	12.09	Xylanase + β-glucanase	1200 TXU and 500 TGU kg^{-1}, respectively	Soybean meal, wheat flour, wheat bran and sorghum DDGS	Improvements in growth performance, activity of digestive enzymes, nutrient digestibility and intestinal microbiota	Macêdo et al. (2023)
Scophthalmus maximus	72	Xylanase+ β-glucanase + residual enzymes	2240 TXU and 1000 TGU kg^{-1}, respectively	DGGS, corn gluten, wheat gluten, soybean meal, wheat bran	Improvements in nutrient digestibility digestive enzyme activity and diversity of the intestinal microbiota	Diógenes et al. (2018)
Oncorhynchus mykiss	10	Xylanase, β-glucanase and protease	208 mg, 67 mg and 228 mg kg^{-1}, respectively	Soybean meal or sunflower meal or rapeseed and wheat meal	Improvements in nutrient digestibility	Dalsgaard et al. (2012)

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CHAPTER II

Growth performance, whole-body composition, plasma biochemistry, activity of digestive enzymes and intestinal histomorphometry of Amazonian tambaqui (*Colossoma macropomum)* fed plant-based diets supplemented with phytase and xylanase-β-glucanase

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ABSTRACT

Fish are unable to efficiently digest certain components found in grains and cereals used in aquatic feeds, such as phytic acid and non-starch polysaccharides (NSP). To solve this problem, exogenous enzymes of microbial origin, such as phytase and xylanase-β-glucanase, have been proposed to increase nutrient utilization in plant-based diets for fish. This study aimed to evaluate the effects of dietary supplementation with these enzymes on the growth performance, body composition, plasma biochemistry, and digestive enzyme activity of tambaqui (*Colossoma macropomum*), a species of Amazonian fish. Juveniles weighing 1.04 ± 0.01 g were subjected to an 8-week feeding trial with four diets: a control diet without enzymes (CON), a phytase diet (PHY), a diet with xylanase-β-glucanase complex (XB), and a diet with both phytase and xylanase-β-glucanase complex (PHY+XB). The results showed that fish fed the PHY+XB diet had a higher protein efficiency rate and lower visceral fat index compared to the CON group, indicating improved nutrient utilization and reduced fat deposition. However, enzyme supplementation did not significantly affect fish survival, final weight, weight gain, feed conversion rate, or visceral indices. Fish fed the XB diet exhibited a lower specific growth rate compared to those fed the CON diet. Furthermore, fish fed PHY+XB had lower body moisture content and higher body protein and Fe^{3+} content. Plasma biochemical analysis revealed higher levels of HDL and lower total plasma protein levels in fish fed PHY+XB, along with reduced plasma ALP levels, indicating potential metabolic benefits. Enzyme supplementation did not affect the activities of total proteases, lipase, and amylase in the fish intestine, nor did it alter the intestinal epithelial structure. Overall, the combined supplementation of phytase and xylanase-β-glucanase favored nutrient utilization. This is evident from the improvements in body composition and plasma biochemistry of tambaqui, indicating the potential to enhance nutrient utilization in aquatic feeds.

KEY-WORDS: absorption, exogenous enzyme, digestion, mineral, phytic acid, non-starch polysaccharide

1. INTRODUCTION

The advent of synthetic amino acids in the 1970s and the possibility of formulating and preparing nutritionally balanced diets from plant-based ingredients represented a major advance in global Aquaculture. Currently, soybean, corn, wheat bran, and their by-products are the main plant-based foods used in fish diets, especially for omnivorous species. However, in addition to the deficiency in essential amino acids, these foods still have disadvantages compared to fishmeal. They contain antinutritional factors and high levels of non-starch polysaccharides (NSP), which can negatively impact intestinal health and the efficiency of fish nutrient utilization.

Both phytic acid and NSP are not satisfactorily digested by fish because they do not produce the specific enzymes needed for their hydrolysis endogenously (Castillo & Gatlin, 2015; Papatryphon et al., 1999). In addition, both compounds remain in significant quantities in food after physical and thermal processing (Singh & Satyanarayana, 2011; U.S. Grains Conciul, 2012; Welker et al., 2014).

Phytic acid complexes 60 to 80% of the phosphorus in grains (Selle et al., 2010; Kumar et al., 2012; Lemos & Tacon, 2017), which makes the supplementation of phosphorus in plant-based diets still necessary (Selle et al., 2010). Furthermore, the phosphorus ingested is released into the environment, leading to eutrophication and a reduction in the carrying capacity of aquatic ecosystems (Liebert & Portz et al., 2005). Phytic acid also acts as a chelator, forming complexes with minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+}, and Fe^{3+} (Nolan et al., 1987; Duffus & Duffus, 1991; Schlemmer et al., 2009; Wang & Guo, 2021), proteins (Spinelli et al., 1983; Richardson et al., 1985; Nolan et al., 1987; Wang & Guo, 2021) and starch (Oatway et al., 2001). These complexes precipitate or inhibit the diffusion of digestive enzymes over the substrate, reducing the bioavailability of these nutrients (Ravindran et al., 1995; Wu & Guo, 2021). Phytase is the enzyme required to hydrolyze the phytate found in plants and release the phosphorus bound to this molecule (Selle et al., 2010). It acts on the substrate by releasing inorganic phosphorus and also reduces the levels of myo-inositol phosphate esters, which have chelating capabilities (Selle et al., 2010), ultimately decreasing the availability of

minerals (Duffus & Duffus, 1991; Nolan et al., 1987). This effect of phytase also contributes to increased bioavailability of amino acids and energy (Ravindran et al., 2001; Selle et al., 2010). Supplementation with exogenous phytase, derived from microbial fermentation, has been shown to benefit the growth performance and health of various fish species, including *Oreochromis niloticus* (Cao et al., 2008; Liebert & Portz, 2005; Maas et al., 2020; Maas et al., 2018), *Pagrus major* (Biswas et al., 2019), *Clarias gariepinus* (Kemigabo et al., 2018), *Cirrhinus mrigala* (Hussain et al., 2017), and *Colossoma macropomum* (Mendonça et al., 2012; Nwanna et al., 2008).

NSP are complex carbohydrates and can represent 60-70% of the cereal cell wall (Fincher & Stone, 1986) and also hinder the availability of other nutrients by increasing the viscosity of digesta and trapping nutrients in complexes that are inaccessible to endogenous fish enzymes (Castilho & Gatlin III, 2015; Sinha et al., 2011). Therefore, both phytic acid and NSP impair the efficiency of nutrient utilization in the diet, consequently preventing fish from reaching their maximum productive potential (Cao et al., 2007; Castillo & Gatlin, 2015; Diógenes et al., 2018). Xylanase and β-glucanase are the two main β-carbohydrases utilized in animal nutrition, constituting nearly 80% of the products available on the market (Barletta et al., 2011; Castillo & Gatlin III, 2015). Xylanase (endo-1,4-β-xylanase, EC 3.2.1.8) hydrolyzes β-(1→4) bonds between xylose units present in the structure of arabinoxylans (Paloheimo et al., 2010; Hatanaka, 2011; Motta et al., 2013), while β-glucanase (endo-1,4-β-glucanase, EC 3.2.1.4) hydrolyzes the β-(1 → 4) bonds of β-glucans into cellobiose units (Habte-Tsion et al., 2018). In this way, both enzymes act by altering the integrity of the fiber, reducing the viscosity of the digesta, allowing the penetration of digestive enzymes into the substrate, and enhancing the utilization of nutrients by animals (Paloheimo et al., 2010). The dietary supplementation with enzyme complexes containing xylanase and β-glucanase, derived from microbial fermentation, has been demonstrated to benefit the health and performance of various fish species, including *Oncorhynchus mykiss* (Dalsgaard et al., 2012), *Scophthalmus maximus* (Diógenes et al., 2018), *Sparus aurata* (Diógenes et al., 2019), and *Oreochromis niloticus* (Brito et al., 2021, 2022; Nakamura et al., 2022; Macêdo et al., 2023; Nascimento et al., 2023).

The tambaqui, *Colossoma macropomum* (Cuvier, 1818), is a freshwater fish native to the Amazon and Orinoco basins. It belongs to the Serrasalmidae family and is found in the Neotropical region (Gery, 1977; Saint-Paul, 1983). In South America, interest in

its cultivation began in the 1930s, but it was only in the late 1980s, with scientific advancements in reproduction and larviculture techniques for the species, that it became commercially established (Hilsdorf et al., 2021). Tambaqui has been introduced to other regions and is currently also being cultivated in China and other Asian countries (Amanajás et al., 2018). The interest in the production of this fish is primarily attributed to its rapid growth (2–3 kg $year^{-1}$) (Aride et al., 2004; Saint-Paul, 1985, 1986; Hilsdorf et al., 2021), its good adaptation to high confinement densities (Araujo-Lima & Goulding, 1997; Aride et al., 2004; Gomes et al., 2002; Silva et al., 2007; Hilsdorf et al., 2021), its resistance to low levels of dissolved oxygen (Pedreira & Sipaúba-Tavares, 2001), and the high added value of the cuts (fillet, rib, loin, and stripe) with good market acceptance (Hilsdorf et al., 2021; Dantas Filho et al., 2021). In terms of nutrition, a significant advantage of this animal is its omnivorous-frugivorous eating habit (Honda, 1974; Aride et al., 2004; Roubach & Saint-Paul, 1994), which enables it to thrive on diets primarily composed of plant ingredients (Martins et al., 2020). However, it is still necessary to research feed additives that can enhance feed efficiency. Therefore, with this study, we evaluated how supplementation with phytase and xylanase-β-glucanase enzymes affects the use of nutrients by tambaqui (*Colossoma macropomum*) through growth performance, whole-body composition, biochemical parameters, intestinal digestive enzyme activity and intestinal histomorphometry.

2. MATERIAL AND METHODS

2.1 Ethics statement

This study was carried out at the Laboratory of Fish Nutrition I of the Fish Farm Sector of the Department of Animal Biology at the Federal University of Viçosa (UFV) and was approved by the Ethics Committee on the Use of Production Animals (CEUAP-UFV), protocol 24/2021.

2.2 Basal diet

A basal plant-based diet was formulated based (307.8 g kg^{-1} of crude protein and 17,08 MJ kg^{-1} of gross energy) following the method proposed by Amancio et al. (2019). It was prepared at the Instituto de Pesca (São Paulo, SP, Brazil). All ingredients were ground in an 800 mm sieve in a centrifugal mill (Viera MC 680B, Tatuí, SP, Brazil) before being mixed in a commercial V-blender (MA200; Marconi, Piracicaba, SP, Brazil),

extruded in a single sieve. Experimental feed mill (Model E-62, Ferraz Máquinas e Engenharia LTDA, Ribeirão Preto, SP, Brazil) through a 3 mm diameter die, and dried in a tubular dryer at 55° C (Model E-62, Ferraz Máquinas et Engenharia LTDA, Ribeirão Preto, SP, Brazil).

2.3 Experimental diets

The phytase used was the commercial product Natuphos® E 10000 L (Basf, Ludwigshafen am Rhein, Germany), a liquid solution containing 10000 FTU g^{-1} of hybrid 6-phytase (EC 3.1.3.26), produced from the combination of three bacterial strains (*Hafnia* sp., *Yersinia mollaretii* and *Buttiauxella gaviniae*) and inserted into a genetically modified strain of *Aspergillus niger*. Xylanase and β-glucanase were included using the enzyme complex Natugrain® TS L (Basf, Ludwigshafen am Rhein, Germany), a liquid solution containing 5600 TXU of endo-1,4-β xylanase and 2500 TGU g^{-1} of endo-1,4-β glucanase. Natugrain® TS L is a product obtained from two genetically modified strains of *Aspergillus niger*. According to the manufacturer both commercial products can be mixed without loss of activity and stability.

From the basal diet, four experimental diets were prepared, in which CON: control diet without exogenous enzyme supplementation, PHY: diet supplemented with 1500 FTU kg^{-1} of phytase, XB: diet supplemented with 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase and PHY+XB: diet supplemented with 1500 FTU kg^{-1} of phytase, 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase. To obtain these enzyme concentrations, solutions were prepared by diluting Natuphos® and/or Natugrain® in demineralized water. The incorporation into the diets was carried out by spraying the pellets using a hand sprayer (Guarany, Itu, SP, Brazil). For the preparation of the CON diet, only demineralized water was sprinkled. After application of the enzyme solution, the soybean oil indicated in the formulation was homogeneously sprinkled on the diets, sealing them, and preventing loss of enzymes in the water. Subsequently, the diets were dried in a forced ventilation oven (Marconi, MA035, SP, Brazil) at 55° C for 6 hours. The analyzed chemical composition of the experimental diets were presented in Table 1.

2.4 Fish and culture conditions

Tambaqui juveniles (1.04 ± 0.10 g) from the Aquaculture Laboratory (LAQUA) of the Federal University of Minas Gerais (UFMG) were randomly distributed in 24 polyethylene tanks (100 L) arranged in a water recirculation system (1.5 L min^{-1}), at a density of 16 fish per tank. The tanks were covered with white nylon mesh to prevent fish from escaping. The recirculation system was equipped with constant aeration, controlled temperature, ultraviolet, mechanical and biological filters. During the trial, the laboratory was maintained in a 12-hour photoperiod, with the aid of an analog timer. The fish were manually fed (8:00, 11:00, 14:00 and 17:00 hours) until apparent satiation for 8 weeks.

The water quality parameters were evaluated weekly. Dissolved oxygen was maintained at 8.02 ± 0.9 mg L^{-1} and temperature at 27.35 ± 0.4° C (YSI® 550A multiparameter meter, Florianópolis, SC, Brazil). The pH was maintained at 6.73 ± 0.15 (Combo basic multiparameter meter, AKSO®, São Leopoldo, RS, Brazil), non-ionized ammonia at 0.01 ± 0.005 mg L^{-1} and nitrite at 0.02 ± 0.01 mg L^{-1} (Labcon® analysis kits, Florianópolis, SC, Brazil).

2.5 Sampling collection and preparation

At the end of the experimental period, two fish from each experimental unit (n = 12 per treatment) were captured 2 hours after the first feeding (10:00 hours) and euthanized (0.100mg L^{-1} of benzocaine) to collect the pyloric caeca and intestine. The samples were stored at − 80º C until the analysis to determine the activity of digestive enzymes. Then, fish (n = 60 per treatment) were captured, counted, weighed, and euthanized for blood collection. Blood samples of two fish per tank were obtained through the caudal vein using a heparinized syringe. Blood samples from each tank were pooled (n = 6 per treatment) and centrifuged for 15 minutes at 8578 × g (4° C) to obtain the plasma. After blood collection, the fish were dissected in a tray cooled with ice, obtaining the weight of their viscera, liver, visceral fat and carcass. Intestine samples from two fish per experimental unit (n = 12 per treatment) were collected, fixed in formalin solution (10%) for 12 hours and stored in 70% alcohol until histomorphometric analyses. The carcasses from each experimental unit were pooled (n = 6 per treatment), weighed, freeze-dried (Labconco, Kansas City, MO, USA) and ground in a knife mill (Tecnal, Ourinhos, SP, Brazil) for subsequent chemical analysis.

2.6 Calculations

- Growth performance parameters were calculated as follows:
- Survival rate (%) = (final number of fish / initial number of fish) × 100;
- Weight gain (g) = final weight (g) − initial weight (g);
- Weight uniformity (%) = (number of fish with weight varying ± 20% from the average in each experimental unit/total number of fish per experimental unit) ×100;
- Specific growth rate (% day^{-1}) = [(ln final weight / ln initial weight) / time (days)] × 100;
- Feed intake (% body weight day^{-1}) = (dry feed consumption (g) / final weight (g)) / time (days)] × 100;
- Feed conversion rate = dry feed intake (g) / weight gain (g);
- Protein retention efficiency (%) = [protein gain (g) / protein intake (g)] ×100;
- Hepatossomatic index = [liver weight (g)/ final weight (g)] ×100;
- Viscerossomatic index = [viscera weight (g) /final weight (g)] × 100;
- Visceral fat index = [visceral fat weight (g) / final weight (g)] × 100;

2.7 Chemical analyses

The chemical composition of the experimental diets and whole-body of fish were analyzed according to methods standardized by the AOAC (2006) in terms of dry matter (method 934.01), crude protein (method 981.10), total lipids (method 920.85) and ash (method 942.05). Crude protein was calculated by the percentage of nitrogen multiplied by 6.25. Gross energy was determined using an adiabatic bomb calorimetry (Parr Instruments Co., Moline, USA). To determine the concentrations of minerals (calcium, phosphorus, iron, magnesium and zinc) a mineral solution used was obtained by digesting the sample in nitric acid solution P.A. and perchloric acid P.A. at 200° C for 4 hours. The reading was performed in an atomic absorption spectrophotometer (GBC, Avanta ∑, EVISA®, Europe) at the wavelengths proposed by the official methods of the AOAC (2006). Experimental diets were also analyzed for neutral detergent fiber according to the methodology proposed by Van Soest (1991).

Enzyme activity assays on the experimental diets were performed by CBO Analysis Laboratory (Valinhos, SP, Brazil). Phytase activity of all diets was determined following method established by Engelen et al. (1994). One phytase unit (FTU) was defined as the

amount of phytase that released 1 mmol of inorganic orthophosphate per minute from 0.0051 mol L^{-1} of sodium phytate at a standard pH (5.5) and temperature (37° C) (AOAC, 2000). The activity of xylanase (endo-1,4 xylanase) was determined using 1% oat spelled xylan in 0.05 M sodium acetate buffer (pH 5.3) as substrate. One ml of enzyme dilution was incubated with 1 mL of substrate solution at 50° C for 60 min. Reducing sugars were assayed by adding 3 mL of 3, 5-dinitrosalicylic acid reagent, boiling for 5 min, and measuring the absorbance at 540 nm (Inborr et al., 1993). The thermostable endo-1,4-xylanase unit (TXU) was defined as the amount of enzyme that released five micromoles of reducing sugar per minute, measured as a xylose equivalent, from a solution containing 1 g of arabinoxylan per 100 mL at pH 3.5 and 40° C. Dietary β-glucanase (endo-1,4-β-glucanase) activity was determined by adding 1 mL of phosphatidylcholine buffer into 0.75 g of barley. Thus, the supernatant was recovered to quantify the β-glucanase activity using a commercial kit (Megazyme, Wicklow, LET, Ireland), following the manufacturer's protocol. Additionally, the thermostable endoglucanase unit (TGU) was defined as the amount of enzyme that releases one micromole of reducing sugar per minute, measured as a glucose equivalent, from a solution containing 0.714 g of β-glucan per 100 mL at pH 3.5 and 40° C (Bavaresco et al., 2020).

2.8 Plasma biochemistry

Plasma levels of glucose, total cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), alkaline phosphatase (ALP), phosphorus (P), calcium (Ca), total protein, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). They were measured using colorimetric kits (Bioclin Quibasa, Belo Horizonte, MG, Brazil) in a BS200 automatic biochemical analyzer (Clinical Chemistry Mindray, Shenzhen, China) following the manufacturer's recommendations.

2.9 Digestive enzymes activities

To assess the activity of digestive enzymes, the fish intestine was divided into pyloric ceca, anterior intestine, and middle intestine as illustrated in Figure 2. Subsequently, each fragment was individually macerated in a liquid nitrogen bath and homogenized with 10^{-3} M HCl. The obtained extract was then centrifuged at 10,000 rpm (4° C) for 10 minutes, and the concentration of total proteins and the activities of proteases, amylase, and lipase were measured in the supernatant.

The concentration of total proteins was using to the method described by Bradford with using bovine albumin used albumin as a standard protein. Readings were taken using an ELISA reader at a wavelength of 595 nm.

The activity of proteases was determined using the method described by Tomarelli et al. (1949). To achieve this, the enzyme extract was combined with 2% (w/v) azocasein substrate and 0.1 M Tris-HCl buffer solution (pH 8.0). After 40 minutes of reaction at 37° C, the reaction was halted by adding 10% (w/v) trichloroacetic acid (TCA). The solution mixture was homogenized and centrifuged at 10,000 rpm (25° C) for 5 minutes. The supernatant was then collected and transferred to tubes containing 1M NaOH for reading at 440 nm. The absorbance values obtained were divided by the concentration of total proteins to calculate the specific activity of the proteases, which was expressed in abs^{-1} mg $protein^{-1}$.

Amylase activity was determined using the enzymatic kit (Bioclin® Quibasa - Basic Chemistry, Belo Horizonte, Brazil) following the modified methodology described by Caraway (1959). For this experiment, starch, and a 100 mM phosphate buffer solution (pH 7.0) were added to the enzyme extract. After 7.5 minutes of reaction, 50 mM iodine reagent and distilled water were added. The absorbance reading was taken at 660 nm. Total amylase activity was calculated following the instructions provided by the kit manufacturer. The total amylase activity values were divided by the total protein concentration. Specific amylase activity was expressed as U $dL^{-}1$ mg $protein^{-1}$.

Lipase activity was determined using an enzymatic kit (Bioclin® Quibasa - Basic Chemistry, Belo Horizonte, Brazil), following the methodology outlined by Cherry & Crandall (1932). To achieve this, a 100 mM Tris-HCl buffer solution (pH 8.5), an enzyme inhibitor (8 mM phenylmethyl sulfonyl fluoride), and DTNB color reagent (3 mM dithionitrobenzoic acid) with 100% sodium acetate were added to the enzyme extract. mm. The mixture was homogenized and heated in a water bath at 37° C for two minutes. Afterward, the 20 mM dithiopropanol tributyrate substrate was added. Subsequently, the mixture was homogenized and incubated for 30 minutes at 37° C in a water bath, with acetone added to halt the reaction. The solution was centrifuged at 3,500 rpm (25° C) for five minutes, and the absorbance was measured using a spectrophotometer at 410 nm. Total lipase activity was expressed in U. dL^{-1} and calculated according to the formula provided in the kit's instructions for use. To calculate the specific activity, the obtained

value was divided by the total protein concentration. Specific lipase activity was expressed in UdL^{-1} mg $protein^{-1}$.

2.10 Intestinal histomorphometry

For histomorphometric analysis, 1 cm fragments of the anterior and middle portions of the intestine were obtained as indicated in Figure 2. The fixed fragments were dehydrated in a series of increasing alcohol concentrations before being embedded in glycol methacrylate (Historesin®, Leica, SP, Brazil). Ten semi-serial sections (3 μm thick) were obtained from each fragment using a microtome (Leica RM225, SP, Brazil) and mounted on histological slides. The slides were stained with Alcian blue (AB) in combination with periodic acid-Schiff (PAS) and mounted with Entellan® (Merck). Each section was photo-documented using an optical microscope (Olympus BX53, Tokyo, Japan) coupled to the camera (Olympus DP73, Tokyo, Japan). The heights and widths of five intestinal folds were measured to calculate the average height of the villi in each fish (Figure 3). Based on the height and width measurements, the absorption surface area of each intestinal villus was calculated following the method outlined by Iji et al. (2001). The thickness of the muscle layer was measured at five points on each intestinal sample in order to calculate the average thickness (Figure 3). The number of goblet cells was quantified by counting the magenta-stained dots in the same fivefold. The images were analyzed using Image Pro-Plus software (Media Cybernetics, Rockville, USA).

2.11 Statistical analysis

Data were previously submitted to the Shapiro-Wilk test to verify the normality of errors and to the Bartlett test to verify the homogeneity of variances. The effects of dietary supplementation with exogenous enzymes were evaluated using two-way analysis of variance (ANOVA). When the difference between the treatment means was significant ($p < 0.05$), they were compared using the Tukey test. Statistical analyses were performed using PROC GLM procedure of the SAS® Studio software (SAS Inst. Inc., Cary, USA).

3. RESULTS

3.1 Growth performance

The results of growth performance parameters are shown in Table 2. The survival rate during the experimental period was high (> 98.8%) and was not affected by dietary

treatments. Enzyme supplementation did not have a significant effect ($p > 0.05$) on the final weight, weight gain, weight uniformity, feed conversion rate, and hepatosomatic and viscerosomatic indices of the fish. The effects of enzyme supplementation were observed only on the specific growth rate ($p = 0.04$), protein efficiency rate ($p < 0.01$), and visceral fat index ($p = 0.02$) of the fish. Fish fed the XB diet exhibited a lower specific growth rate compared to fish fed the CON diet. However, there were no differences in the specific growth rates of these fish in relation to the enzymatic treatments. Fish fed the PHY+XB diet exhibited a higher protein efficiency rate compared to fish fed the CON diet or diets containing isolated enzymes such as PHY or XB. In terms of the visceral fat index, fish fed the PHY+XB diet had lower visceral fat levels than fish in the CON group. However, these results did not differ from those of the other groups, PHY and XB.

3.2 Whole-body composition

The chemical composition of the fish carcass is presented in Table 3. Fish fed with the diet containing the enzyme association PHY+XB had lower body moisture content ($p < 0.01$) and higher body protein content ($p = 0.04$) than the other treatments. Fish fed PHY+XB also showed a significant increase in Fe^{3+} content in the carcass ($p < 0.01$) compared to fish fed the CON diet. However, the levels of total lipids, ash, energy, P, Ca^{2+}, Mg^{2+}, and Zn^{2+} were not affected ($p > 0.05$) by enzyme supplementation.

3.3 Plasma biochemistry

Plasma metabolites are presented in Table 4. Fish fed the PHY+XB diet exhibited higher levels of HDL ($p = 0.04$) and lower levels of total plasma protein ($p = 0.01$) compared to fish in other treatment groups. Fish fed PHY+XB also showed a significant decrease in plasma ALP levels ($p < 0.01$) compared to the CON group. However, their ALP levels did not differ from the levels observed in fish fed the PHY and XB diets. The plasma levels of glucose, total cholesterol, triglycerides, LDL, P, Ca^{2+}, AST, and ALT were not affected ($p > 0.05$) by enzyme supplementation.

3.4 Digestive enzymes activities

Digestive enzyme activities in three different segments of the intestine are presented in Figure 4. There were no significant differences ($p > 0.05$) in the activities of total proteases, lipase, and amylase in the pyloric caeca and midgut of fish. Fish fed the

XB diet exhibited higher total protease activity ($p < 0.01$) compared to fish fed the CON or PHY+XB diet, but did not differ from those fed the PHY diet. These fish also exhibited higher lipase activity ($p = 0.01$) compared to fish fed the PHY+XB diet, although there was no significant difference from those fed the CON and PHY diets. Amylase activity in the foregut was also unaffected by enzyme supplementation ($p > 0.05$).

3.5 Intestinal histomorphometry

The results of the histomorphometric analysis are shown in table 5. Supplementation with exogenous enzymes separately or in combination did not result in changes in the structure of the absorptive epithelium of the fish, with no significant differences ($p > 0.05$) in the height and width of the folds, as well as in the thickness of the muscular tunic.

4. DISCUSSION

In this study, we investigated the effects of dietary supplementation with phytase and xylanase-β-glucanase alone or in combination on the growth, metabolism, and intestinal health of tambaqui (*Colossoma macropomum*). The results revealed significant findings, providing valuable insights into the potential of these enzymes to enhance nutrition and health in farmed fish.

Supplementation with isolated phytase and xylanase-β-glucanase in the PHY and XB diets, respectively, did not positively influence the growth performance of the fish. We expected that phytase would enhance the availability of phosphorus, while xylanase-β-glucanase would improve the digestion of complex carbohydrates to increase the availability of these and other nutrients. The basal diet was intentionally formulated to present a nutritional challenge by being deficient in P and containing significant amounts of NSP. However, this improvement did not occur. The basal diet contained approximately 2.6 g kg^{-1} of inorganic (non-phytic) phosphorus, which would not be sufficient to meet the tambaqui's growth requirement of around 3.0 g kg^{-1} (Araújo et al., 2016). This amount of available phosphorus would also not satisfy the requirement of 6.3-7.0 g kg^{-1} for adequate mineralization (Araújo et al., 2016; Menezes et al., 2021). Despite these challenges, the fish grew satisfactorily well without showing any abnormalities, demonstrating the tambaqui's ability to efficiently utilize nutrients from plant sources. It is also possible that with efficient levels of P in the diet, the fish have

absorbed the mineral from the water in the tanks (Menezes et al., 2021). When evaluating inclusion levels between 1000 and 2000 UF kg^{-1} of phytase in the tambaqui diet, Brandão et al. (2016) also found no effects of enzyme supplementation on fish growth. According to Mendonça et al. (2012), supplementation with 1540.62 UFA kg^{-1} of phytase in the diet resulted in greater growth and feed efficiency for fish.

The isolated use of 4000 FTU kg^{-1} in plant-based diets in a 120-day feeding trial for pacu (*Piaractus mesopotamicus*), a species from the same family as tambaqui, also did not promote any improvements in the growth performance and body composition of the fish, with only a slight increase in muscle retention of phosphorus and zinc (Bacchetta et al., 2020). Although these discrepant results can be attributed to variations in fish species, developmental stage, and culture conditions, factors related to diet composition also play a crucial role in the magnitude of responses to phytase supplementation. Aspects such as the source and content of protein and phytate in the diet, levels of dietary calcium and phosphorus, as well as protein digestibility, among others, have been identified as significant influences on phytase activity (Dersjant-Li et al., 2015; Sugiura et al., 2001; Bacchetta et al., 2020).

In this study, the use of the xylanase-β-glucanase complex also did not promote improvements in the growth performance of the fish. These results are contrary to those observed by Brito et al. (2022), who, evaluating the same product at the same dose in juvenile Nile tilapia, observed improvements not only in performance indices but also in body composition, plasma biochemistry, and intestinal histomorphometry of the fish. According to Maas et al. (2020), it is likely that the beneficial effect of carbohydrates is closely correlated with the quality of the diet, and that they only have a satisfactory effect on diets of very low nutritional quality, that is, highly rich in NSP. These authors evaluated the use of xylanase in diets with amounts of NSP similar to those in our study (~170 g kg^{-1}) in comparison with diets with higher levels designated as low-quality diets (~300 g kg^{-1}). They observed that in both diets the enzyme was not able to promote improvements in the performance of juvenile Nile tilapia, but that in the low-quality diet, xylanase improved the digestibility of dry matter, total carbohydrates, and energy. Therefore, it is a fact that the results observed for one species are not easily extrapolated to another with a similar feeding habit and that the diet profile and enzyme dosages can be key factors in their successful use.

When the enzymes, phytase and xylanase-β-glucanase, were provided together in the PHY+XB diet, there was an improvement in the protein efficiency rate and a reduction in visceral fat in the tambaqui. Reducing the accumulation of visceral fat in tambaqui is extremely important as the tendency to accumulate a large amount of abdominal fat, especially when fed with plant-based diets, is a characteristic of this species. Furthermore, the combination of these enzymes demonstrated potential in improving the nutritional value of meat as an increase in protein and Fe^{3+} content was observed in these fish, with a reduction in body moisture content. These results can be attributed to the effect of both enzymes on the availability of amino acids (Brito et al., 2021) with a concomitant increase in body protein and reduction in visceral fat (Khola et al., 1992; Wu, 2013). However, it is likely that in this study, phytase was only able to access these complexed nutrients from the moment that xylanase-β-glucanase opened the gate, promoting the initial hydrolysis of NSP. In a study evaluating phytase and xylanase alone and in combination in Nile tilapia diets, Maas et al. (2018), found that the effect of xylanase was only expressed when in synergism with phytase, and that the use of this mix of enzymes favored the performance and use of nutrients to a greater extent than when using only phytase.

In this study, the improvements of a combined use of phytase and carbohydrases were also observed at a metabolic level, as these fish had higher levels of HDL and lower levels of total plasma protein. Interestingly, these fish showed a decrease in ALP levels in plasma. In the intestine, this enzyme plays an important role in the hydrolysis of organic phosphates in an alkaline medium and probably acts in the dephosphorylation of Myo-inositol monophosphate (IP1) (Yusoff et al., 2011), a product of the action of phytase activity on phytate. Previous studies indicate that both exogenous enzymes (Hassaan et al., 2020; Al-Qahtani et al., 2020) increase ALP activity in the intestine. Therefore, it is likely that the decrease in concentrations of this enzyme in blood plasma in this study occurred due to the greater release of this enzyme into the intestinal lumen. This result is beneficial since high concentrations of ALP and total protein in the blood indicate liver damage (Ryu et al., 2010). Corroborating what was said, in a study evaluating diets with different levels of phytate in Nil tilapia diets, Kumar et al. (2011) observed that higher levels of this antinutritional factor are responsible for increasing blood concentrations of ALP. Similarly, when evaluating phytase dietary supplementation at 1000 FTU kg^{-1}, Adeshina et al. (2023) observed a decrease in ALP in the blood of Nile tilapia. The improvement in Fe^{3+} retention in fish fed the PHY+XB diet may be associated with the

beneficial effect of phytase on the bioavailability of iron from soybean meal and other ingredients rich in phytate since large amounts of this antinutrient impair iron absorption. non-heme due to the presence of phytic acid (Lei & Porres, 2011). All these results suggest physiological improvements, improved lipid metabolism, and liver function.

Regarding digestive enzymes, no effects of phytase and/xylanase- β-glucanase on the activity of total proteases, lipase, and amylase in the pyloric cecum and midgut of fish were observed in this study. In the foregut, however, greater activity of total proteases and lipase was observed in fish fed with the diet fed with the XB diet than in those fed with the CON diet. However, this increase did not result in improvements in growth performance, as these fish showed a lower specific growth rate when compared to fish in the CON group, or PHY and PHY+XB. Xylanase and β-glucanase act by hydrolyzing the long polysaccharide chains of NSP into reduced sugars, so that they can be absorbed or even used by the intestinal microbiota as a prebiotic (Bedford, 2000; Choct & Kocher, 2000). It is possible that, due to tambaqui's adaptation to ingesting high levels of carbohydrates, a significant increase in this nutrient resulted in adverse effects, such as a reduction in specific growth rate.

The intestinal folds are the main site of nutrient absorption, and therefore their size is directly related to the growth performance of fish (Adeshina et al., 2019; Dawood et al., 2019). However, the negative effects of high levels of phytic acid and NSP in the diet are not only associated with nutrient unavailability, these anti-nutritional factors also cause deleterious morphological changes in the intestinal mucosa, leading to impairments in absorptive capacity (Zhong et al., 2019; Liu et al., 2022). Therefore, it was expected that the use of exogenous enzymes would promote improvements in the intestinal mucosa of fish fed diets containing vegetable ingredients. However, in this study, enzyme supplementation did not result in positive changes in intestinal morphology assessed through the evaluation of height and width of the intestinal folds, absorption surface area and thickness of the muscular layer of this organ.

In conclusion, the results of this study indicate that the combined supplementation of phytase and xylanase-β-glucanase can improve nutritional efficiency, providing metabolic benefits to tambaqui that allow a reduction in the amount of visceral fat and improving the nutritional value of fish meat. However, more research is needed to fully elucidate the underlying mechanisms and optimize enzyme supplementation strategies in aquaculture, especially under challenging dietary conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Composition and chemical analysis of the experimental diets

Ingredients (g kg^{-1})	Diets[a]			
	CON	PHY	XB	PHY+XB
Soybean meal 45[b]	450.00	450.00	450.00	450.00
Corn flour[b]	183.00	183.00	183.00	183.00
Wheat meal[b]	150.00	150.00	150.00	150.00
Soybean protein concentrate 85[c]	60.00	60.00	60.00	60.00
Broken rice[b]	60.00	60.00	60.00	60.00
Wheat flour[b]	40.00	40.00	40.00	40.00
Soybean oil[d]	20.00	20.00	20.00	20.00
Calcium cabonate[e]	10.00	10.00	10.00	10.00
Bicalcium phosphate[e]	12.00	12.00	12.00	12.00
Salt	5.00	5.00	5.00	5.00
Mineral and vitaminic mix[f]	5.00	5.00	5.00	5.00
L-Lysine[g]	2.00	2.00	2.00	2.00
DL-Methionine[g]	2.00	2.00	2.00	2.00
Chromium oxide (Cr_2O_3)[h]	1.00	1.00	1.00	1.00
Analyzed composition (as dry matter basis)				
Dry matter	892.51	871.95	884.86	890.289
Energy (kcal kg^{-1})	4080.0	4039.0	4086.0	4096.0
Crude protein	307.80	309.00	305.60	309.90
Total lipids	35.03	36.21	36.89	35.69
Ash	70.02	71.09	70.12	70.52
Total carbohydrates[i]	587.15	583.70	587.39	583.89
Neutral detergente fiber	191.09	190.37	186.78	172.06
Non-fibrous carbohydrate[i]	396.06	393.33	400.63	411.83
Total calcium	11.53	13.04	11.11	10.62
Total phosphorus	6.36	6.67	6.39	6.57
Ca:P ratio	1.81	1.96	1.74	1.62
Iron	0.18	0.29	0.25	0.21
Zinc	0.22	0.21	0.20	0.22
Magnesium	0.05	0.06	0.05	0.05
Phytase (FTU kg^{-1})[j]	ND[l]	1740.0	0.00	1620.0
Xylanase (TXU kg^{-1})[k]	ND[l]	0.00	1496.0	1498.36
β-glucanase (TGU kg^{-1})[k]	ND[l]	0.00	454.0	617.0

[a]CON: control diet without phytase, xylanase and β-glucanase supplementation; PHY: diet supplemented with 1500 FTU kg^{-1} of phytase; XB: diet supplemented with 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase; PHY+XB: diet supplemented

with 1500 FTU kg^{-1} of phytase, 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase;

[b]Guabi Alimentos Ltda, Campinas, SP, Brazil.

[c]Bunge, Ponta Grossa, PR, Brazil.

[d]Cocamar, Maringá, PR, Brazil.

[e]Sarfos, Itumbiara, Goiás, GO, Brazil.

[f]Composition kg^{-1}diet: retinyl acetate (vitamin A. 6,000 IU; cholecalciferol (vitamin D3. 1,000 IU); DL-α-tocopherol (vitamin E. 100 mg); menadione (vitamin K3. 12 mg); thiamine (vitamin B1. 24 mg); riboflavin (vitamin B2. 24 mg); pyridoxine-HC (vitamin B3. 20 mg); cyanocobalamin (vitamin B12. 24 µg; folic acid (vitamin B9. 6 mg); D-pantothenic acid (vitamin B5. 60 mg); ascorbic acid (ROVIMIX® STAY-C®35 (vitamin C. 340 mg); biotin. 0.24 mg; choline chloride (choline. 325 mg); niacin (vitamin B3. 120 mg); ferrous sulfate ($FeSO_4.7H_2O$. 50 mg Fe); copper sulfate ($CuSO_4.5H_2O$. 3 mg Cu); Mg - 20 mg; zinc sulfate ($ZnSO_4 \cdot H_2O$. 30 mg Zn); potassium iodate (KIO_3. 2 mg I); cobalt (II) carbonate ($CoCO_3$. 0.1 mg Co); Sodium selenite (Na_2SeO_3. 0.1 mg se).

[g]Ajinomoto Animal Nutrition Division, São Paulo, SP, Brazil.

[h]Sygma-Aldrich Brazil Ltda, 99.5%, São Paulo, SP, Brazil.

[i]Calculated according to Detmann & Valadares Filho, 2010.

[j]Natuphos® E 10000 L, Basf, Ludwigshafen am Rhein, RP, Germany.

[k]Natugrain® TS (Basf, Ludwigshafen am Rhein, RP, Germany.

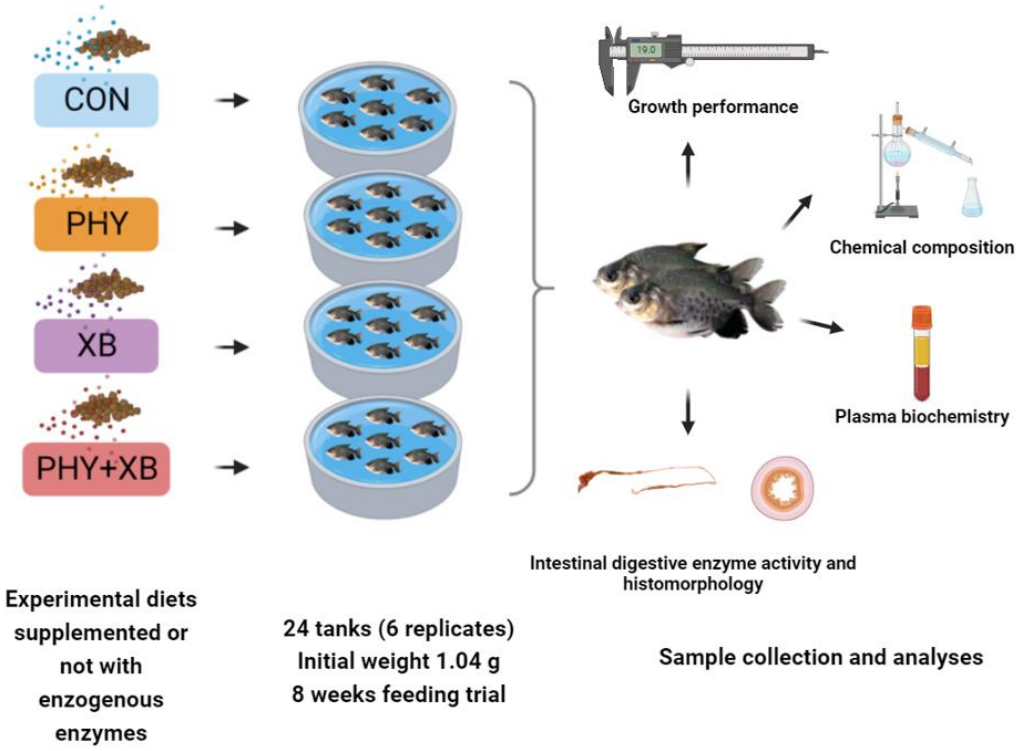

Figure 1. Graphical representation of the 56-day sextuplicate feeding trial and sampling collection carried out with tambaqui (*C. macropomum*) juveniles. The fish were fed with a control diet (CON), a diet containing phytase (PHY), a diet containing xylanase-β-glucanase (XB), or a diet containing phytase + xylanase-β-glucanase (PHY + XB).

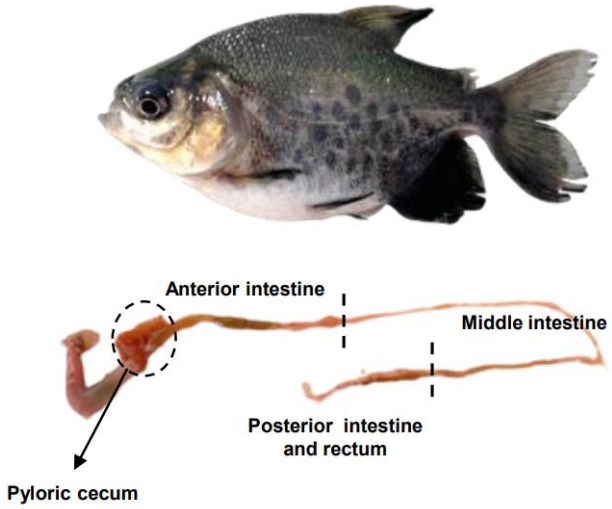

Figure 2. Schematic representation of the different segments of the intestine of *C. macropomum* according to Aguiar et al. (2018).

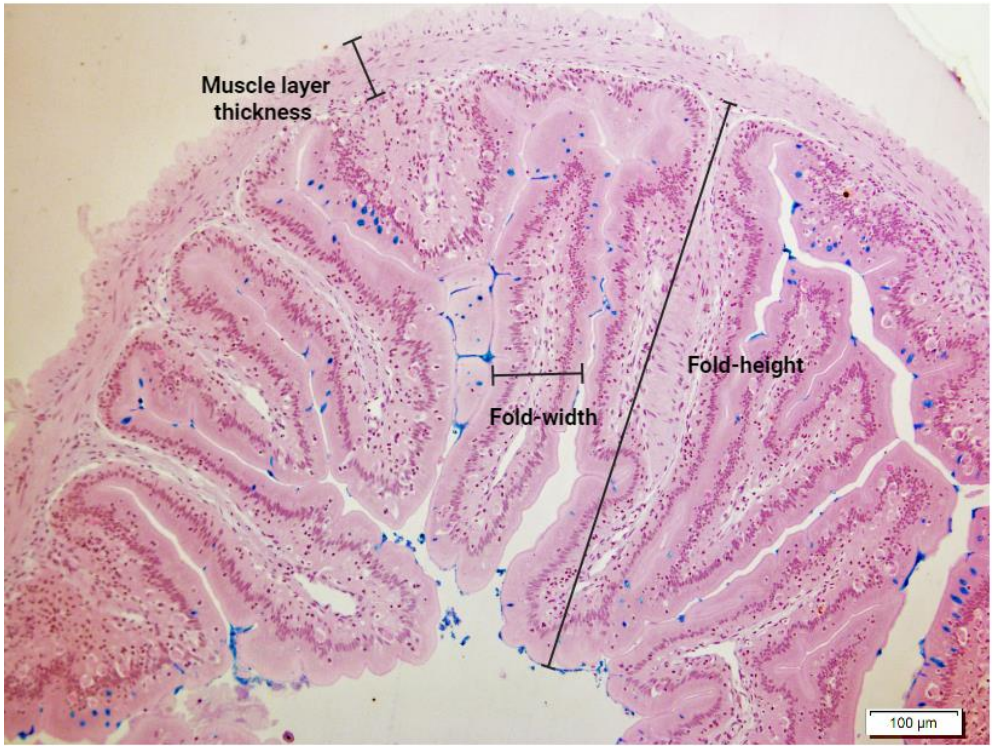

Figure 3. Schematic representation of histological measurements taken from the anterior and midgut of *C. macropomum*.

Table 2. Survival, growth performance and somatic indices (mean ± standard error) of *C. macropomum* juveniles fed the experimental diets for 8 weeks

	CON	PHY	XB	PHY+XB	*P*-value
Survival (%)	98.85 ± 1.04	100.00 ± 0.00	98.85 ± 1.04	100.00 ± 0.00	0.5811
Initial body weight (g)	1.04 ± 0.01	1.04 ± 0.01	1.04 ± 0.01	1.04 ± 0.01	0.9964
Weight gain (g)	38.11 ± 1.96	34.34 ± 1.20	32.96 ± 1.86	35.15 ± 1.81	0.2304
Weight uniformity (%)	44.24 ± 3.64	46.25 ± 4.89	48.95 ± 5.45	58.33± 6.18	0.2506
Specific growth rate (% day^{-1})	6.47 ± 0.09^{a}	6.23 ± 0.07^{ab}	6.047 ± 0.09^{b}	6.28 ± 0.09^{ab}	0.0454
Feed intake (% body weight day^{-1})	1.83 ± 0.03	1.88 ± 0.03	1.87 ± 0.01	1.82 ± 0.06	0.6220
Feed conversion rate	1.09 ± 0.02	1.10 ± 0.01	1.10 ± 0.02	1.03 ± 0.03	0.1707
Protein retention efficiency (%)	45.45 ± 0.61^{b}	45.37± 0.51^{b}	44.62 ± 0.84^{b}	48.89 ± 0.96^{a}	0.0053
Hepatossomatic index (%)	1.69 ± 0.06	1.70 ± 0.07	1.74 ± 0.05	1.80 ± 0.07	0.5970
Viscerossomatic index (%)	6.81 ± 0.26	7.09 ± 0.22	6.93 ± 0.13	7.29 ± 0.26	0.4775
Viscera fat index (%)	1.24 ± 0.02^{a}	1.17 ± 0.03^{ab}	1.19 ± 0.01^{ab}	1.11 ± 0.09^{b}	0.0201

CON (control diet), PHY (diet with phytase), XB (diet with xylanase-β-glucanase) and PHY+XB (diet with phytase, xylanase-β-glucanase); Values with the different superscripts in the same row indicate significant differences determined by Tukey's test ($p < 0.05$) (n = 60).

Table 3. Whole-body composition (mean ± standard error), based on natural matter (%) of *C. macropomum* juveniles fed the experimental diets for 8 weeks

	CON	PHY	XB	PHY+XB	*P*-value
Moisture	73.72 ± 0.10^{a}	74.14 ± 0.19^{a}	73.70 ± 0.24^{a}	72.64 ± 0.32^{b}	0.0012
Crude protein	14.37 ± 0.12^{b}	14.57 ± 0.21^{b}	14.49 ± 0.07^{b}	15.23 ± 0.16^{a}	0.0458
Total lipids	6.86 ± 0.36	6.40 ± 0.37	6.91 ± 0.32	7.11 ± 0.16	0.4523
Ash	3.48 ± 0.15	3.53 ± 0.32	3.46 ± 0.41	3.41 ± 0.13	0.8918
Energy (kJ kg^{-1})	5.28 ± 0.02	5.22 ± 0.03	5.31 ± 0.03	5.25 ± 0.03	0.1339
P	0.51 ± 0.06	0.53 ± 0.03	0.48 ± 0.05	0.51 ± 0.03	0.2582
Ca^{2+}	1.22 ± 0.22	1.22 ± 0.19	1.05 ± 0.18	1.14 ± 0.15	0.4374
Mg^{2+}	0.020 ± 0.002	0.023 ± 0.002	0.020 ± 0.001	0.021 ± 0.001	0.6162
Fe^{3+}	0.001 ± 0.0006^{b}	0.002 ± 0.0002^{ab}	0.003 ± 0.0004^{ab}	0.004 ± 0.0005^{a}	0.0040
Zn^{2+}	0.0007 ± 0.0001	0.0006 ± 0.00011	0.0004 ± 0.00005	0.0006 ± 0.0001	0.4893

Values with the different superscripts in the same row indicate significant differences determined by Tukey's test ($p < 0.05$) (n = 6).

Table 4. Plasma biochemistry (mean ± standard error) of *C. macropomum* juveniles fed the experimental diets for 8 weeks

Parameters	CON	PHY	XB	PHY+XB	*p*-value
Glucose (mg dl^{-1})	71.92 ± 3.77	68.40 ± 1.77	69.40 ± 1.56	65.50 ± 3.52	0.5163
Cholesterol (mg dl^{-1})	129.5 ± 4.84	125.5 ± 3.03	138.2 ± 7.41	141.6 ± 3.95	0.1601
Triglycerides (mg dl^{-1})	245.9 ± 5.15	279.2 ± 19.63	273.0 ± 13.65	265.0 ± 18.43	0.4662
HDL (mg dl^{-1})	4.3 ± 2.11^{b}	4.2 ± 1.35^{b}	4.1 ± 1.92^{b}	9.0 ± 4.65^{a}	0.0445
LDL (mg dl^{-1})	130.67 ± 17.83	118.20 ± 8.87	131.70 ± 15.32	132.50 ± 10.11	0.3415
ALP (µ l^{-1})	97.63 ± 12.85^{a}	71.25 ± 8.82^{b}	69.1 ± 14.66^{b}	67.66 ± 8.52^{b}	0.0052
P (mg dl^{-1})	9.34 ± 1.11	9.96 ± 1.31	10.51 ± 1.59	10.17 ± 1.55	0.5521
Ca (mg dl^{-1})	10.67 ± 1.18	9.86 ± 0.38	9.47 ± 0.91	10.57 ± 0.95	0.1312
Total protein (g dl^{-1})	2.90 ± 0.40^{a}	2.80 ± 0.12^{ab}	2.71 ± 0.22^{ab}	2.38 ± 0.20^{b}	0.0198
AST (µ l^{-1})	68.70 ± 12.82	56.25 ± 5.50	66.88 ± 8.21	67.50 ± 7.11	0.1105
ALT (µ l^{-1})	4.58 ± 0.92	3.75 ± 0.36	5.25 ± 1.44	5.50 ± 0.59	0.4704

CON (control diet), PHY (diet with phytase), XB (diet with xylanase-β-glucanase) and PHY+XB (diet with phytase, xylanase-β-glucanase). Values with the different superscripts in the same row indicate significant differences determined by Tukey's test ($p < 0.05$) (n = 6).

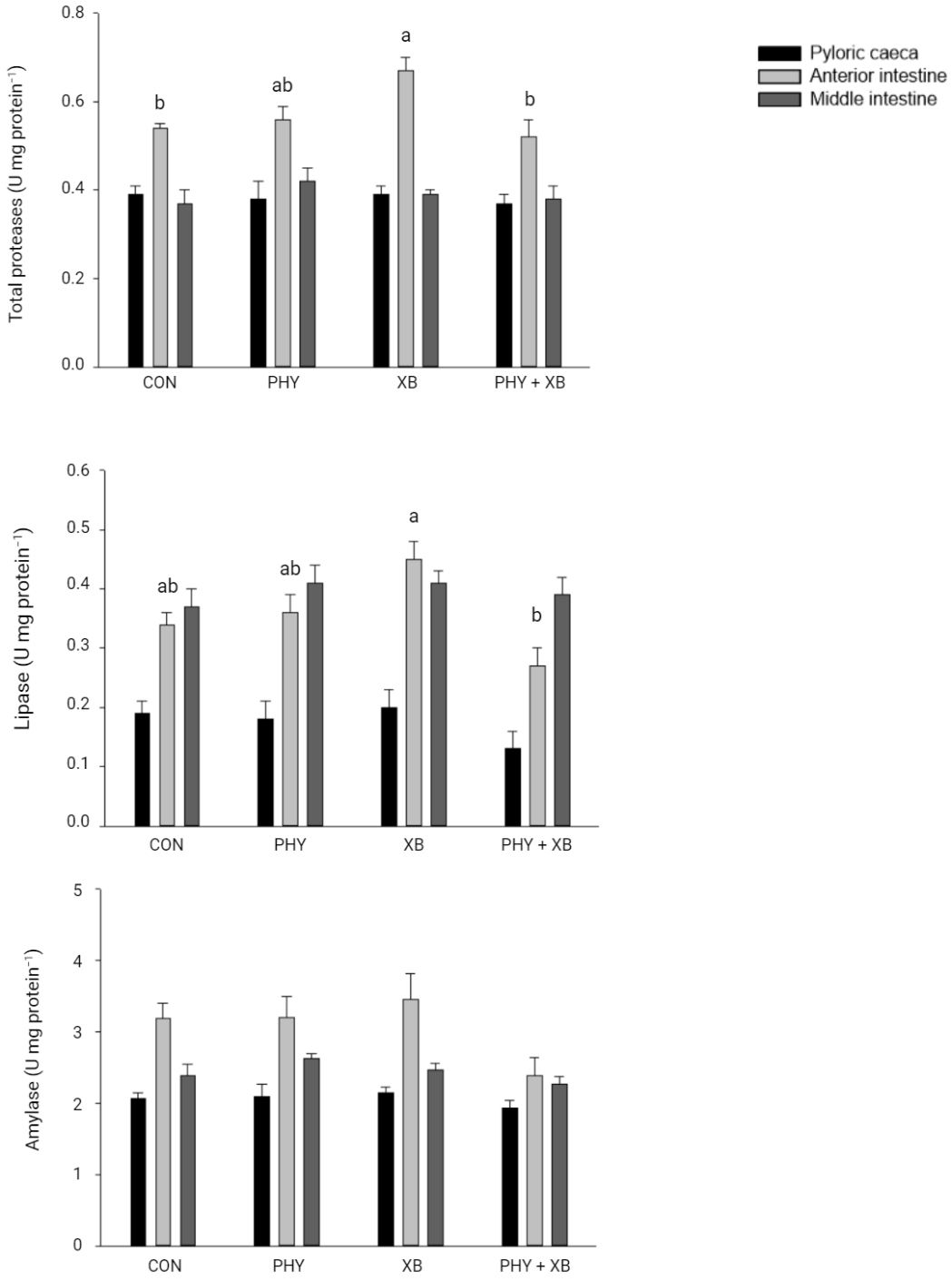

Figure 4. Digestive enzyme activity (mean ± standard error) of *C. macropomum* juveniles fed the experimental diets for 8 weeks. CON (control diet), PHY (diet with phytase), XB (diet with xylanase-β-glucanase) and PHY+XB (diet with phytase and xylanase-β-glucanase). Values with the different superscripts in the same row indicate significant differences determined by Tukey's test ($p < 0.05$) (n =12).

Table 5. Intestinal histomorphometry (mean ± standard error) of *C. macropomum* juveniles fed the experimental diets for 8 weeks

	CON	PHY	XB	PHY+XB	*P*-value
			Anterior intestine		
Fold-height (µm)	613.42 ± 22.44	629.58 ± 16.63	615.63 ± 18.31	625.00 ± 26.02	0.9406
Fold-width (µm)	146.76 ± 4.37	146.25 ± 4.89	148.95 ± 5.45	158.33 ± 6.18	0.3564
Absorption surface area (μm^2)	16.76 ± 0.61	17.31 ± 0.69	16.68 ± 0.92	15.79 ± 0.37	0.4821
Muscle layer thickness (µm)	91.76 ± 2.29	92.31 ± 2.05	91.68 ± 1.45	90.79 ± 2.05	0.9595
			Middle intestine		
Fold-height (µm)	474.16 ± 15.99	512.88 ± 25.62	455.51 ± 16.56	479.66 ± 35.47	0.4423
Fold-width (µm)	100.61 ± 4.38	100.92 ± 4.18	105.76 ± 3.87	108.82 ± 6.14	0.5605
Absorption surface area (μm^2)	19.04 ± 1.10	20.45 ± 1.20	17.44 ± 1.23	17.80 ± 1.27	0.3113
Muscle layer thickness (µm)	65.20 ± 2.10	65.50 ± 2.42	64.34 ± 2.89	64.88 ± 2.01	0.9793

CON (control diet), PHY (diet with phytase), XB (diet with xylanase-β-glucanase) and PHY+XB (diet with phytase and xylanase-β-glucanase). Values with the different superscripts in the same row indicate significant differences determined by Tukey's test ($p < 0.05$) (n =12).

CHAPTER III

Phytase and xylanase-β-glucanase exogenous enzymes impacts on intestinal microbiota of juvenile Amazonian tambaqui (*Colossoma macropomum*)

Written in accordance with the guidelines of the *Applied Microbiology and Biotechnology* journal

ABSTRACT

In this study, our objective was to evaluate the impact of dietary supplementation with phytase and/or xylanase-β-glucanase on the diversity of the intestinal microbiota of juvenile tambaqui (*Colossoma macropomum*) fed plant-based diets. For this purpose, fish (1.04 ± 0.01g) were fed CON (control diet without exogenous enzyme supplementation), PHY (basal diet containing phytase), XB (basal diet containing xylanase-β-glucanase), or PHY+XB (basal diet containing phytase and xylanase-β-glucanase) for 8 weeks. A high-throughput sequencing analysis revealed that the majority of reads derived from tambaqui digesta belong to members of Firmicutes and Proteobacteria. Firmicutes were found to be more abundant in fish fed CON, PHY, and XB diets, while Proteobacteria were more abundant in fish fed the PHY+XB diet. According to Lefse's analysis, fish fed the PHY+XB diet had a greater number of enriched taxa compared to the other groups. However, fish fed XB had the highest number of unique bacterial genera. Although the microbiota of fish fed with PHY was somewhat enriched, it was also modulated. In general, all treatments were enriched with bacteria responsible for the manipulation and utilization of carbohydrates and other nutrients, demonstrating a beneficial modulation for improved utilization of dietary nutrients. In conclusion, the results suggest that the combined action of phytase and xylanase-β-glucanase had a more significant impact on modulating the intestinal microbiota of tambaqui. This indicates that dietary supplementation with this combination of enzymes may be particularly effective in promoting beneficial changes in the intestinal bacterial community.

KEY-WORDS: exogenous enzyme, intestinal health, microbiome, phytic acid, non-starch polysaccharide

1. INTRODUCTION

Aquaculture, as a rapidly growing sector, plays a pivotal role in meeting the increasing global demand for fish protein. Among the myriad of species cultivated, the Tambaqui (*Colossoma macropomum*), a freshwater fish native to the Amazon and Orinoco basins (Gery, 1977; Saint-Paul, 1983), has garnered considerable attention in South America and Asia (Amanajás et al., 2018; Hilsdorf et al., 2021). With its rapid growth (Aride et al., 2004; Saint-Paul, 1985; 1986), adaptability to captivity (Araujo-Lima & Goulding, 1997; Aride et al., 2004; Gomes et al., 2002; Silva et al., 2007), and ability to utilize plant-based diets (Martins et al., 2020), the tambaqui stands as a promising candidate for sustainable aquaculture production.

However, despite its potential, challenges to maximizing the efficiency of tambaqui production still exist. One such challenge lies in optimizing the nutritional quality of plant-based feeds commonly used in tambaqui diets. Although plant-derived ingredients offer cost-effective alternatives to traditional fishmeal, they often contain anti-nutritional factors, such as phytic acid and non-starch polysaccharides (NSP), which can impair nutrient digestion and absorption (Cao et al., 2007; Castillo & Gatlin, 2015). Phytic acid represents the most part of the phosphorus (P) in grains (Selle et al., 2010; Kumar et al., 2012; Lemos & Tacon, 2017), which is not bioavailable to fish, as they do not have the emzimatic aparatus to hydrolyze this molecule (Papatryphon et al., 1999). Phytic acid also complexes and makes minerals and other nutrients unavailable (Nolan et al., 1987; Duffus & Duffus, 1991; Schlemmer et al., 2009; Wang & Guo, 2021). NSP represent 60-70% of the cereal cell wall (Fincher & Stone, 1986) and also hinder the availability of nutrients by increasing the viscosity of digesta and trapping nutrients in complexes that are inaccessible to endogenous fish enzymes (Castilho & Gatlin III, 2015; Sinha et al., 2011). Therefore, both phytic acid and NSP impair the efficiency of nutrient utilization in the diet, consequently preventing fish from reaching their maximum productive potential (Cao et al., 2007; Castillo & Gatlin, 2015; Diógenes et al., 2018).

However, the reason why phytic acid and NSP cause suppression of fish growth does not rely solely on nutritional loss. Intestinal health is fundamental to the health and growth of fish and this depends on an intact mucosa and a balanced and functional intestinal microbiota. High levels of phytic acid and high levels of NSP in the diet lead to morphological damage to the mucosa, impairing the immune function of the intestine (Zhong et al., 2019; Deng et al., 2021; Liu et al., 2022) and also affecting the intestinal

microbiota of fish (Zhou et al., 2017; Liu et al., 2022). Microorganisms residing in the intestinal tract establish a vital connection with the gut-brain axis, influencing not only behaviors, but also the digestive, absorptive, metabolic and immunological processes of fish (Butt & Volkof, 2019). Therefore, any changes in its composition can have significant repercussions on the energy metabolism and health of fish. Given this, it is essential to evaluate the impact of exogenous enzymes on the microbial composition of fish.

The use of exogenous enzymes, such as phytase, xylanase and β-glucanase, presents a promising strategy to alleviate the adverse effects of antinutritional factors in plant-based diets. Phytase hydrolyzes phytate (Selle et al., 2010) and xylanase and β-glucanase different fractions of complex carbohydrates, (Paloheimo et al., 2010; Habte-Tsion et al., 2018) which improves the fish's ability to take advantage of nutrients from plant-based diets. Previous studies have revealed that phytase (Karatas et al., 2023) and xylanase-β-glucanase (Brito et al., 2022; Macêdo et al., 2023) provide improvements in the richness and abundance of beneficial intestinal bacteria in the fish intestine. However, their application and effects on tambaqui remain relatively unexplored. Therefore, elucidating the influence of exogenous enzymes on intestinal microbiota composition in tambaqui holds significant implications for optimizing nutritional interventions.

Therefore, with this study we aim to evaluate the impact of dietary supplementation with phytase and/or xylanase-β-glucanase blend on the intestinal microbiota diversity of tambaqui juveniles fed with plant-based diets. As far as we are aware, this study will be the first to evaluate the intestinal microbiota of tambaqui in the face of the use of feed additives in the diet using the next generation sequence (NSG) approach.

2. MATERIAL AND METHODS

2.1 Ethics statement

This study was conducted at the LaNup – Fish Nutrition Laboratory in the DBA – Department of Animal Biology at the Universidade Federal de Viçosa, as well as at the AGE – Animal Genetics and Evolution Laboratory in the CIIMAR – Interdisciplinary Center for Marine and Environmental Research and at CIBIO – Center for Research in Biodiversity and Genetic Resources, both affiliated with the Universidade do Porto. The study was approved by the Ethics Committee on the Use of Production Animals of the

Universidade Federal de Viçosa, protocol 24/2021. A scheme of the experiment was showed at Figure 1.

2.2 Basal diet

A basal plant-based diet was formulated based (307.8 g kg^{-1} of crude protein and 17.08 MJ kg^{-1} of gross energy) following the method proposed by Amancio et al. (2019), and prepared at the Fisheries Institute (São Paulo, Brazil). All ingredients were ground through an 800 mm sieve in a centrifugal mill (Viera MC 680B, Tatuí, SP, Brazil) before being mixed in a commercial V-shaped blender (MA200; Marconi, Piracicaba, SP, Brazil) and extruded through a single sieve. 1 mm diameter matrix (Model E-62, Ferraz Máquinas e Engenharia LTDA, Ribeirão Preto, SP, Brazil). The processed feed was then dried in a tubular dryer at 55° C (Model E-62, Ferraz Máquinas et Engenharia LTDA, Ribeirão Preto, SP, Brazil). The chemical composition of the basal diet is presented in Table 1.

2.3 Experimental diets

From the basal diet, four experimental diets were prepared: CON (control diet with no exogenous enzyme supplementation), PHY (basal diet containing 1500 FTU kg^{-1} of phytase), XB (basal diet containing 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase) and PHY+XB (basal diet containing 1500 FTU kg^{-1} of phytase, 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase). To obtain these enzyme concentrations, solutions were prepared by diluting Natuphos® and/or Natugrain® in demineralized water. The incorporation into the diets was carried out by spraying the pellets using a hand sprayer (Guarany, Itu, SP, Brazil). For the preparation of the CON diet, only demineralized water was used. After applying the enzyme solution, the soybean oil mentioned in the formulation was evenly distributed on the diets, sealing them and preventing the enzymes from being lost in the water. Subsequently, the diets were dried in a forced ventilation oven (Marconi, MA035, SP, Brazil) at 55° C for 6 hours.

2.4 Fish and culture conditions

Tambaqui juveniles (1.04 ± 0.10 g) from the Aquaculture Laboratory (LAQUA) of the Federal University of Minas Gerais (UFMG) were randomly distributed into 24 polyethylene tanks (100 L) arranged in a water recirculation system (1.5 L min^{-1}), at a

density of 16 fish per tank. The tanks were covered with white nylon mesh to prevent fish from escaping. The recirculation system was equipped with constant aeration, temperature control, ultraviolet, mechanical, and biological filters. During the trial, the laboratory maintained a 12-hour photoperiod with the aid of an analog timer. The fish were manually fed at 8:00, 11:00, 14:00, and 17:00 hours until apparent satiation for 8 weeks.

The water quality parameters were evaluated weekly. Dissolved oxygen was maintained at 8.02 ± 0.9 mg L^{-1} and the temperature at 27.35 ± 0.4 °C (YSI® 550A multiparameter meter, Florianópolis, SC, Brazil). The pH was maintained at 6.73 ± 0.15 (Combo basic multiparameter meter, AKSO®, São Leopoldo, RS, Brazil), non-ionized ammonia level was 0.01 ± 0.005 mg L^{-1} and nitrite level was 0.02 ± 0.01 mg L^{-1} (Labcon® analysis kits, Florianópolis, SC, Brazil).

2.5 Intestinal microbiota

2.5.1 Sample collection and DNA extraction

At the end of the experiment, two fish from each experimental unit were captured 2 hours after feeding and euthanized using a benzocaine solution with a concentration of 0.1 mg L^{-1}. The purpose was to collect the intestine with digesta, including the pyloric cecum. The samples were conditioned in liquid nitrogen and then transferred to a −80°C freezer for storage until extraction. Intestine samples were previously homogenized. DNA extraction was performed using the QIAamp® PowerFecal® DNA Kit (Qiagen, Hilden, NRW, Germany). A blank was included for each extraction batch to check for possible contamination.

2.5.2 16S rRNA gene amplification, library preparation and sequencing

The hypervariable region V3-V4 of the ribosomal 16S rRNA gene was amplified using universal primers 341F 805R (CCCTACACGACGCTCTTCCGATCTG) and 805-R (GACTGGAGTTCCTTGGCACCCGAGAATTCCA). DNA was amplifed in triplicate in a volume of 14.0 µl, including 5 µl of extracted DNA, 5 µl of Taq PCR Master Mix Kit (Qiagen, Hilden, Germany), 0.4 µl of each primer, and 3.2 µl of ultrapure water. The PCR process was then carried out by a T100™ Thermal Cyeler System (BIO-RAD, USA) under the following conditions: 95° C for 15 min, 40 cycles of 95 °C for 30 s, 55° C for 60 s, and 72° C for 30 s, followed by 60° C for 5 min. PCR amplicons were checked by

electrophoresis on a 2% agarose gel stained with GelRed™ and visualized using GelDoc™ (Bio-Rad Laboratories, California, USA). The metagenomic library was constructed from the amplifiers using the Illumina® "Nextera DNA Library Preparation Kit," a commercial kit. The amplifiers were pooled and subsequently, sequenced, using the Illumina® "MiSeq" sequencer (Degnan & Ochman, 2012).

2.5.3 Data Processing

Bioinformatic procedures were carried out in the RStudio (v4.3.2) environment. The readings obtained in the sequencing were subjected to pre-processing to remove adapter sequences, primers, and reorient reads using the Cutadapt package (v1.15). The filtering, trimming, denoising, dereplication, sample inference, chimera identification, and merging of paired-end reads were performed using the Devisive Amplicon Denoising Algorithm (DADA2, v1.30.0) according to pipeline described by Callahan et al. (2016). The taxonomic identities were established utilizing the SILVA (v132) database in which Amplicon Sequence Variants (OTUs) were clustered by 99% homology. A phylogenetic tree of Amplicon Sequence Variants (ASVs) was constructed using DECIPHER package (v2.26.0) and FastTree. Subsequently, the Phyloseq R package (v1.42.0) was employed to create a OTU table, taxonomic assignments, phylogenetic tree, and metadata.

2.6 Statistical analysis

2.6.1 Microbial Diversity Analyses

The Alpha Diversity was estimated for each sample by calculating the Chao, Simpson and Shannon diversity indices. For microbiome comparison between samples, Beta Diversity was performed by NMDS (Non-Metric Multidimensional Scaling) multidimensional analysis based on the presence and absence of OTUs (Operating Taxonomic Units) using the Jaccard distance. The bar graphs were constructed to show the most abundant taxa. The ten most abundant phyla and the fifty most abundant families and genera are presented. For the Veen diagram, the OTUs were present in at least three of six replicates were considered present in a sample and the diagram was designed using jvenn online tool (Bardou et al., 2014). The construction of the graphs, multivariate analyses, and statistics were done using the RStudio software, with package ggplot2 (Wickham & Chang, 2008) and software GraphPad Prism 8.0.2. Initially, data distribution analysis was performed using the Kolmogorov-Smirnov test. After confirming the

absence of the Gaussian distribution profile of the data, the diversity index was compared with Kruskal-Walli's test, followed by correction by Dunn's test with α = 5%, comparing treatments in relation to control.

2.6.2 Bacteria Enrichment Analysis

The differential analysis of the type Linear Discriminant Analysis Effect Size (LEfSe) was used to identify biomarker between the treatments. This analysis was carried out in RStudio software, using the package 'microbiomeMarker', using 'CPM' as a normalization method and LDA score greater than 2. Non-parametric statistical analyses were performed using White's t-test for two groups comparison, and ANOVA and Kruskal-Wallis test for multiple groups comparisons (Segata et al. 2011).

3. RESULTS

According to Chao's α diversity index (Figure 2a), dietary supplementation with phytase and xylanase-β-glucanase, separately (PHY and XB) or in combination (PHY+XB), significantly increased the richness of bacterial taxa in the fish intestine compared to fish fed an enzyme-free diet (CON). However, no significant differences were observed in the bacterial communities ($p > 0.05$) by Shannon (Figure 2b) and the Simpson indexes (Figure 2c). This indicates that the distribution and uniformity of species are similar between groups, and that the difference in the Chao index may be linked to the presence of unique taxa in the intestines of fish fed diets containing exogenous enzymes.

In the analysis of beta diversity conducted using the non-metric multidimensional scaling (NMDS) method, it was observed that the intestinal microbiota of fish subjected to the same dietary treatment did not exhibit a normal distribution along the NMDS1 and NMDS2 axes (Figure 3). Despite the core microbiota being similar among all fish, this observation suggests a notable variability in the composition of the microbiota among fish that received the same diet, indicating the complexity in the structuring of the tambaqui intestinal microbiota.

Regarding relative abundance, among the 79 bacterial OTUs identified in the samples, the phyla Firmicutes, Proteobacteria, Verrucomicrobia, Actinobacteria, and Bacteroidetes were the most abundant in the fish core microbiota (Figure 4a). However, it is possible to observe that the relative abundance differed between the groups. Fish fed the PHY+XB diet exhibited a higher abundance of the phylum Proteobacteria, while in

fish from the CON, PHY, and XB treatments, the phylum Firmicutes was more abundant (Figure 4a). It was also possible to observe that fish that received diets supplemented with the enzyme phytase (PHY and PHY+XB) exhibited a high abundance of the phylum Bacteroidetes, while fish that received the control diet (CON) or the diet containing only xylanase-β-glucanase (XB) showed a reduced abundance of this phylum.

At the family level, *Clostridiaceae_1*, *Enterobacteriaceae*, and *Terrimicrobiaceae* were abundant in fish from all treatments (Figure 4b). However, fish-fed diets containing enzymes (PHY, XB, or PHY+XB) exhibited a higher abundance of specific bacterial families (e.g., *Amoebophilacea„ Ruminococcaceae*, *Bacillaceae*, *Rhizobiales*, and *Leuconostocaceae*) compared to fish fed the CON diet (Figure 4b). This suggests that enzyme supplementation may influence the composition of the intestinal microbiota, promoting the enrichment of certain bacterial families. It was also observed that fish fed the PHY+XB diet showed a more significant abundance of Enterobacteriaceae than fish that received the other diets (Figure 4b). At the genus level, *Candidatus_Arthromitus* sp., *Plesiomonas* sp., *Terrimicrobium* sp., and *Ruminoclostridium* sp. were some of the groups that appeared in all treatments (Figure 4c). However, only fish fed diets containing phytase (PHY and PHY+XB) showed a relative abundance of bacteria from the genus *Candidatus_Cardinum* sp. (Figure 4c). Additionally, in fish fed the PHY+XB diet, there was a more pronounced abundance of bacteria from the genus *Plesiomonas* sp. compared to the other diets (Figure 4c).

The LEfSe analysis identified differentially abundant taxa in the intestines of fish that were fed diets containing exogenous enzymes (PHY, XB, and PHY+XB) compared to fish in the control group (CON) (Figure 5). The fish that were fed the PHY+XB diet had the highest number of enriched taxa, with 27 of them, compared to the fish fed the CON diet (Table S1). The most enriched genera in this group were *Plesiomonas* sp., *Alsobacter* sp., *Legionella* sp., *Rhodopirullela* sp., *Gemmobacter* sp., alphaI_cluster (Beijerinckiaceae), and *Fimbriiglobus* sp. (Figure 5). Fish fed the XB diet were found to have been fed with 10 enriched genera (Table S1), and the most enriched genera in this group were *Streptococcus* sp., *Bradyrhizobium* sp., and *Alsobacter* sp. (Figure 5). Finally, the fish fed the PHY diet had a total of 7 enriched genera (Table S1), including *Clostridium_sensu_stricto_8* sp., *Paracoccus* sp., and *Alsobacter* sp. (Figure 5).

Regarding the number of shared bacterial operational taxonomic units (OTUs) observed, it was found that 21 bacterial genera were common in the intestines of fish from

all treatments (Figure 6). It was also possible to evaluate which bacterial genera were found exclusively in each group. Fish fed the CON and PHY diets each had only one unique genus: *Brevibacterium* sp. and *Nocardia* sp., respectively (Figure 6, Table S2). Fish fed with the XB diet exhibited the highest richness, with 24 exclusive genera, of which we can highlight *Enterobacter* sp., *Solibacillus* sp., *Pelagibacterium* sp., and *Roseomonas* sp. (Figure 6, Table S2). Finally, fish fed with the PHY+XB diet presented 6 exclusive genera: *Crenobacter* sp., *Bdellovibrio* sp., *Microbacterium* sp., *Romboustia* sp., *Lachnoclostridium_5* sp., and *Anaerovorax* sp. (Figure 6, Table S2).

4. DISCUSSION

To our knowledge, this study represents the first description of the intestinal microbiota of *Colossoma macropomum* for evaluating dietary effects using Next-Generation Sequencing (NGS) technology. The results of this study demonstrate that supplementing plant-based diets with exogenous enzymes (phytase and xylanase-β-glucanase) affects the composition of the intestinal microbiota of tambaqui juveniles. Although dietary supplementation with phytase and/or xylanase-β-glucanase did not modify the composition of the fish core microbiota, it was found that the use of enzymes led to the enrichment of certain taxa and the emergence of unique microorganisms in the samples. This suggests that the enzymes added to the diet may have promoted the growth or colonization of additional bacterial taxa in the fish gut.

The core intestinal microbiota of tambaqui juveniles was dominated by the phyla Firmicutes and Proteobacteria. The dominance of these phyla in the microbiota has been verified in several species of freshwater fish (Wu et al., 2012; Kim et al., 2021; Luo et al., 2022). However, we observed that the relative abundance of these microorganisms changes depending on the combination of enzymes used in the diet. While fish fed CON, PHY, and XB diets showed dominance of the genus Firmicutes, fish fed the PHY+XB diet exhibited a microbiota dominated by bacteria from the phylum Proteobacteria. Hypotheses have already been formulated regarding the factors that determine the relative abundance of these phyla in the gastrointestinal tract of fish. According to Rawls et al. (2006), the higher abundance of one phylum compared to the other is primarily attributed to the physiological differences between these groups.

The main difference between these groups is due to the structure of the cell wall, with Firmicutes being Gram-positive bacteria and Proteobacteria being Gram-negative

bacteria. But another characteristic that can differentiate these two phyla is their tolerance to oxygen. Members of the phylum Firmicutes are anaerobic, while members of the phylum Proteobacteria are oxygen-tolerant (Epsey, 2013). It is known that nutrient intake can trigger dynamic changes in intestinal oxygenation. Myo-inositol trispirophosphate (IP3), a product of phytic acid hydrolysis by the action of the enzyme phytase (Li et al., 2017), enhances the release of dioxygen from hemoglobin and improves oxygen levels under hypoxic conditions in vivo (Kieda et al., 2006). Therefore, it is likely that phytase favored the increase in Proteobacteria due to this mechanism of action, but it only had consequences for the microbiota in combination with xylanase.

The genus *Alsobacter* sp. belonging to the phylum Proteobacteria was enriched in the intestines of all fish that received diets supplemented with enzymes. *Alsobacter* sp. is a recently described by Bao et al. (2014). These nitrogen-fixing bacteria are commonly found in soils but can also inhabit adjacent aquatic environments (Deng et al., 2023) and colonize the intestines of fish (Deng et al., 2021; Wang et al., 2023). Despite the limited information on the colonization by *Alsobacter* sp. in the intestines of fish, it is known that bacteria from the *Rhizobiaceae* family play a role in cellulose degradation (McDonald et al., 2012, 2015), which is beneficial to fish organisms fed diets rich in NSP.

Fish fed with the PHY+XB diet showed the highest enrichment in bacterial abundance. In addition to *Alsobacter* sp., genera of Proteobacteria such as *Gemmobacter sp.* (*Rhodobacteriaceae*), *alphaI_cluster* (*Beijerinckiaceae*), *Plesiomonas sp.* (*Enterobacteriaceae*), and *Legionella sp.* (*Legionellaceae*) were prominently enriched in these fish. Despite the lack of information about the Beijerinckiaceae family in the intestines of fish, it is known that, like other members of the Rhizobiales order, these microorganisms are also nitrogen fixers (Marín et al., 2014; Zhang et al., 2024). It is also known that in *Litopenaeus vannamei* shrimp, the presence of these bacteria in the midgut was responsible for the production of reduced nitrogen, highlighting the role of nitrogen fixation in animals (Garibay-Valdez et al., 2021). *Gemmobacter sp.* has been reported at times as dominant in the intestines of omnivorous fish such as *Oreochromis niloticus* (Deng et al., 2022) and *Cyprinus carpio* (Zhang et al., 2021). It is known for its ability to utilize carbohydrates as an energy source (Hao et al., 2016). In a study evaluating transition diets for *Megalobrama amblycephala*, Wei et al. (2018) found an increase in *Gemmobacter sp*. abundance, in a positive correlation with the increase in vegetable items in the diet compared to animal-origin items. It is also believed that members of the

Rhodobacteriaceae family, such as *Gemmobacter sp*. may be related to lipid metabolism, exerting hypocholesterolemic effects, as they inhibit micelle formation by assisting in the binding of bile acids to dietary cholesterol (Afrose et al., 2010; Sheu et al., 2013; Zhang et al., 2014; Koo et al., 2017; Williams et al., 2024). Still discussing fish fed a diet containing only an enzyme association, PHY+XB, we observed an enrichment of the genera *Rhodopirullela sp*. and *Fimbriiglobus sp*. belonging to the phylum Planctomycetes, whose members are known for their ability to ferment carbohydrates (Schlesner & Stackebrandt, 1986; Ward et al., 2015; Hao et al., 2016). Given the ability of these organisms to utilize carbohydrates from plants, it is likely that the release of oligosaccharides by the action of xylanase-β-glucanase on arabinoxylans and β-glucans provided oligosaccharides that can be fermented by these bacteria, favoring their growth (Geraylou et al., 2014) favoring its growth.

Despite being considered pathogenic (She et al., 2017; Martins et al., 2013), several studies highlight that *Pleisomonas sp.* and *Legionella sp.* (*Legionellaceae*) natural inhabitants in the intestines of healthy fish (Sugita et al., 1988; Wu et al., 2010; Navarrete et al., 2010), and in many cases, they are even the dominant genus (Donkeng et al., 2011; Silva et al., 2005). *Pleisomonas* sp. has been identified in species such as *Morone saxatilis* (Nedoluha & Westhoff, 1997), *Lepomis macrochirus*, *Micropterus salmoides*, *Ictalurus punctatus* (Larsen et al., 2014), *Prochilodus argenteus* (Silva et al., 2005), *Oreochoromis niloticus* (Sugita et al., 1987, 1988; Donkeng et al., 2011), and *Oncorhynchus mykiss* (Pond et al., 2006). *Legionella* sp. has already occurred in *O. mykiss* (Navarrete et al., 2010) and *Salmo salar* (Butgen et al., 2018). Since the fish in this study were obtained from laboratory breeding, cultivated in the same recirculation system, and the presence of these organisms occurred independently of the treatment, we believe that the higher abundances in fish that received the PHY+XB diet are due to the greater availability of specific nutrients in the intestines of these fish. *Pleisomonas* sp. grow well in medium containing inositol (Jeppesen et al., 1995), a product of the action of phytase on the phytic acid molecule (Reddy et al., 2017; Gessler et al., 2018). However, the more significant effect in the PHY+XB treatment, in relation to that containing only phytase, is probably due to the favoring of the action of phytase by the action of xylanase-β-glucanase.

Among the unique genera observed in fish fed with the PHY+XB, *Bdellovibrio* sp. is recognized as an effective predator of pathogenic bacteria, playing the role of a 'living antibiotic'. For this reason, it has been widely used as a probiotic in aquaculture (Chu &

Zhu, 2010; Cao et al., 2012; Waso et al., 2021). Furthermore, it contributes to the elimination of free radicals, improving the activity of the host's antioxidant enzymes (Liu et al., 2022). Its presence, therefore, can contribute to the maintenance of intestinal health (Cui et al., 2021). Ray et al. (2024) observed an increase in *Bdellovibrio* sp. in *Oreochromis aureus* x *O. niloticus* fed diets without phosphorus and supplemented with phytase. It turns the *Bdellovibrio* sp. abundance similar to that of fish fed diets supplemented with phosphorus but without the enzyme.

Another unique genus found in fish fed PHY+XB diet, *Anaerovorax* sp., is known to maintain the stability of the microbial system as a keystone species (Jia et al., 2022). Studies indicate that this bacterium is a biohydrogenating bacterium (Huws et al., 2011). They ferment nitrogenous organic substances such as putrescine (Matthies et al., 2000) and chitin (Dai et al., 2016), producing acetate and butyrate (Matthies et al., 2000). Therefore, it is likely that these microorganisms are also closely linked to the cycling of proteins and amino acids (Kong et al., 2022). Therefore, it appears that the diet supplemented with combined digestive enzymes promoted a higher enrichment in intestinal bacterial abundance. This effect is likely attributed to the synergistic action of enzymes, which favored the growth of bacteria actively involved in the breakdown of complex carbohydrates and other nutrients. As consequence, this process can enhance the utilization of nutrients by these animals. However, the emergence of pathogenic strains must be investigated more thoroughly so that appropriate formulations can be developed.

In addition to *Alsobacter* sp. the microbiota of fish fed with the XB diet was also enriched with Proteobacteria of the genus *Bradyrhizobium* sp. This genus also plays a role in the carbon and nitrogen cycles in freshwater ecosystems (Magalhães et al., 2022), which highlights the impact of these carbohydrases on the *Rhizobiaceae* community. In addition to the increased abundance of these microorganisms, fish fed with the XB were the ones that presented the greater number of exclusive taxa. Among the exclusive genera we highlight *Enterobacter sp.*, *Solibacillus* sp. and *Pelagibacterium* sp. Members of *Enterobacter sp.* and *Sollibacillus* sp. are cellulose degraders (Li et al., 2016; Ramya et al., 2023) while *Pelagibacterium* sp., is a bacterium whose enrichment can be favored in media containing degradation products of oligosaccharides from arabinoxylans and β-glucans (Cutts, 2022). These results lead us to believe that xylanase-β-glucanase was responsible for the greater diversity of species, both in fish fed with XB and in those fed with PHY+XB. This more significant effect of xylanase-β-glucanase on microbial

richness may be related to the fact that the hydrolysis of NSP in the diet leads to the production of oligosaccharides like that can act as probiotics (Geraylou et al., 2012; Bedford, 2019). Arabinoxylan-oligosaccharides (AXOS), for example, represent the product of xylanase action on arabinoxylans, and have a proven prebiotic function in fish (Geraylou et al., 2012; Gericke et al., 2019). The fermentation of AXOS by intestinal microorganisms leads to the production of SCFA and therefore alters the microbiota through changes in intestinal pH (Geraylou et al., 2014).

In addition to Proteobacteria of the genus *Alsobacter* sp., fish fed a diet supplemented with phytase (PHY) showed a higher abundance of *Clostridium* sp. compared to the control group. These microorganisms are known as fermenters, degrading complex polysaccharides and producing SCFA that serve as a readily available energy source for enterocytes (Fuller et al., 2005; Vinolo et al., 2011) and as anti-inflammatory factors (Atarashi et al., 2013). Similarly, in our study, dietary supplementation with 1000 U kg^{-1} of phytase resulted in an increase in the abundance of *Clostridum* sp. in *O. niloticus* (Hu et al., 2016) and in pigs (Metzler-Zebeli et al., 2010). There was also enrichment of the genus *Paracoccus* sp. whose members are responsible for producing several bioactive compounds, including β-galactosidases (Wierzbicka-Woś et al., 2011). They also have high contents of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in their composition and can even be used as probiotics in fish diets (Wanka et al., 2018). Dietary supplementation with *Paracoccus marcusii* enhanced the growth performance, intestinal antioxidant activity, and suppressed intestinal inflammation in *Ctenopharyngodon idellus* (Xue et al., 2020). Fish fed with PHY did not present many unique taxa, such as *Nocardia* sp. was the only one observed. This genus of filamentous bacteria is the causative agent of nocardiosis, a granulomatous disease that can affect the skin, muscles, and internal organs of fish. However, as the fish remained healthy throughout the experiment, we believe that this event was occasional and not related to the dietary treatment itself. The insignificant effect of phytase alone (PHY) on microbial enrichment, when compared to its combined use with xylanase-β-glucanase (PHY+XB), can be explained by the fact that the action of phytase was enhanced by the prior action of xylanase-β-glucanase in the initial hydrolysis of NSPs.

In addition to the phyla mentioned above, fish fed diets supplemented with enzymes were enriched with bacteria from Bacteroidetes phyla. However, this effect was more pronounced in fish fed diets containing phytase, PHY and PHY+XB. The phylum

Bacteroidetes is known for its ability to degrade and use a wide range of polysaccharides (McKee et al., 2021). This is because these bacteria have special genetic structures, called polysaccharide utilization loci (PULs), which are characterized by being structures that encode proteins involved in the processes of detection, binding, deconstruction, import and, therefore, use of glycans (McKee et al., 2021). In a study evaluating dietary supplementation with increasing levels of xylanase (600-1200 U kg^{-1}) for *Larimichthys crocea*, Luo et al. (2020), found an increase in bacteria from this phylum compared to the control group. However, in our study, these bacteria were more abundant in fish fed the PHY and PHY+XB diets, which leads us to believe that phytase had a greater influence on this result. We believe that the primary action of phytase, which acts at a slightly more acidic pH than carbohydrases, has promoted a favorable environment for the emergence of fiber-degrading bacteria, which in themselves play the role of carbohydrases in the hydrolysis of xylans and β-glucans. Thus, the abundance of these bacteria seems to be more related to access to the substrate (complex polysaccharides) than to the product (oligosaccharides).

In short, we found that supplementation with phytase and/or xylanase-β-glucanase affected the composition of the intestinal microbiota of tambaqui juveniles. The combination of the two enzymes (PHY+XB) had the most significant effect on the microbiota, enriching the abundance of bacteria and increasing species richness. This is probably due to the potentiation of the action of phytase by the action of xylanase-β-glucanase in the hydrolysis of NSP. Phytase (PHY) and xylanase-β-glucanase (XB) alone also altered the composition of the microbiota, but to a lesser extent than the combination of the two enzymes. In general, enzymatic treatments promoted the enrichment of bacteria involved in the metabolism of polysaccharides and their digestion products, which may enhance their utilization. Therefore, supplementing with phytase and associated xylanase-β-glucanase may be an effective strategy to beneficially modulate the intestinal microbiota of tambaqui juveniles. However, more studies are needed to better understand the mechanisms by which enzymes affect the microbiota and to determine the long-term effects of supplementation on fish health.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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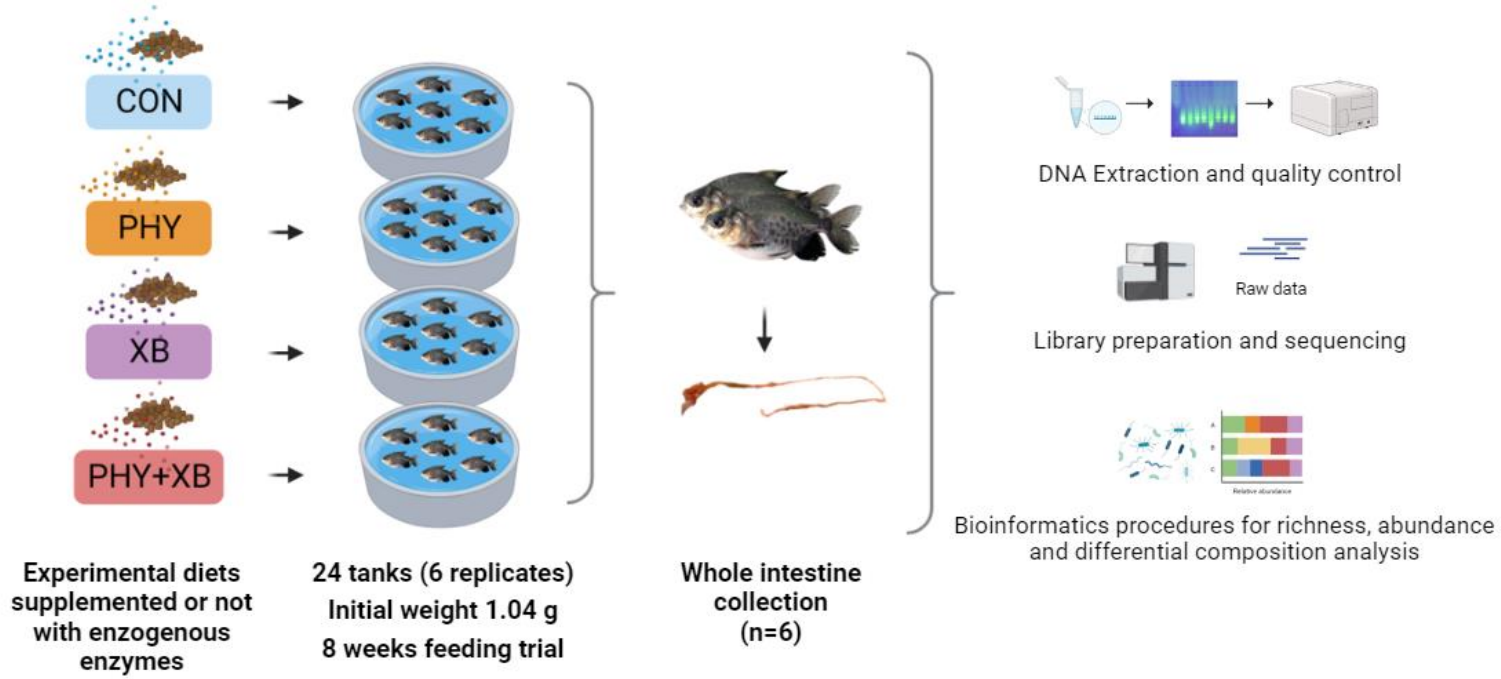

Figure 1. Graphical representation of the 8-week sextuplicate feeding trial carried out with tambaqui (*C. macropomum*) juveniles. The fish were fed with a control diet (CON), a diet containing phytase (PHY), a diet containing xylanase-β-glucanase (XB), or a diet containing phytase + xylanase-β-glucanase (PHY + XB). After the test, the intestines of one fish per tank were collected for DNA extraction, sequencing and microbiota analyses (n = 6).

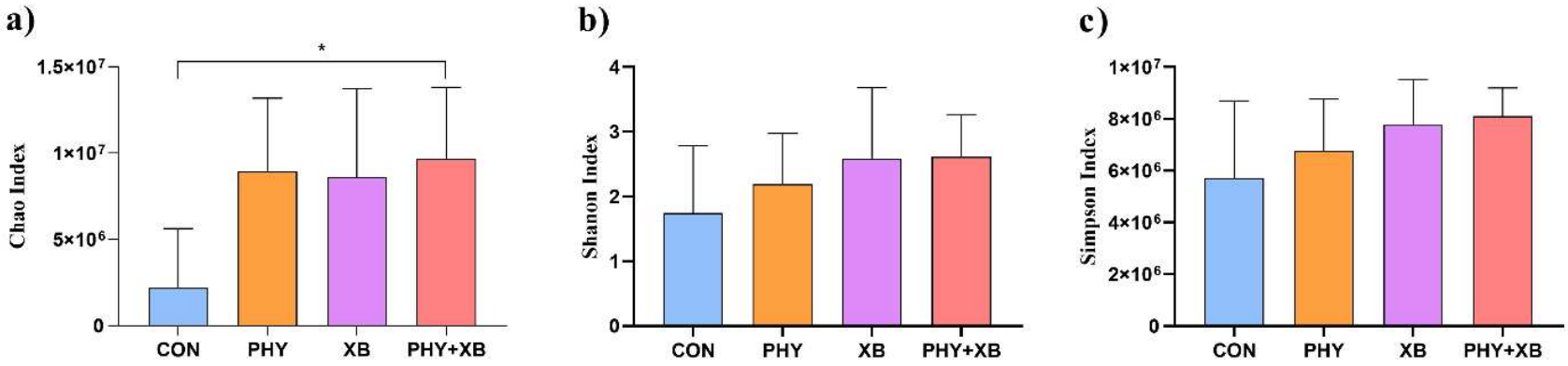

Figure 2. Phylogenetic α-diversity of intestinal bacterial communities of juvenile tambaqui (*C. macropomum*) fed a control diet (CON), and phytase (PHY), xylanase-β-glucanase (XB), or phytase + xylanase-β-glucanase (PHY + XB) supplemented diets. a) Chao Index. b) Shannon Index. c) Simpson Index. Asterisks (*) indicate that indexes were significantly different by the t-test ($p<0.05$).

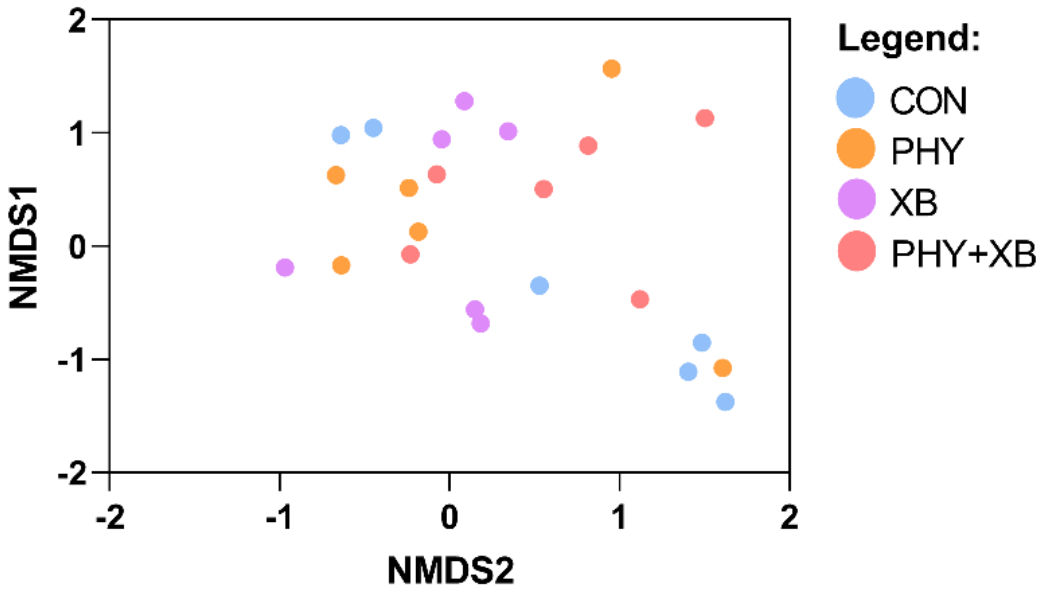

Figure 3. Phylogenetic β-diversity of the intestinal bacterial communities of juvenile tambaqui (*C. macropomum*) fed a control diet (CON), and phytase (PHY), xylanase-β-glucanase (XB), or phytase + xylanase-β-glucanase (PHY+XB) supplemented diets, using the Nonmetric multidimensional scaling (NMDS).

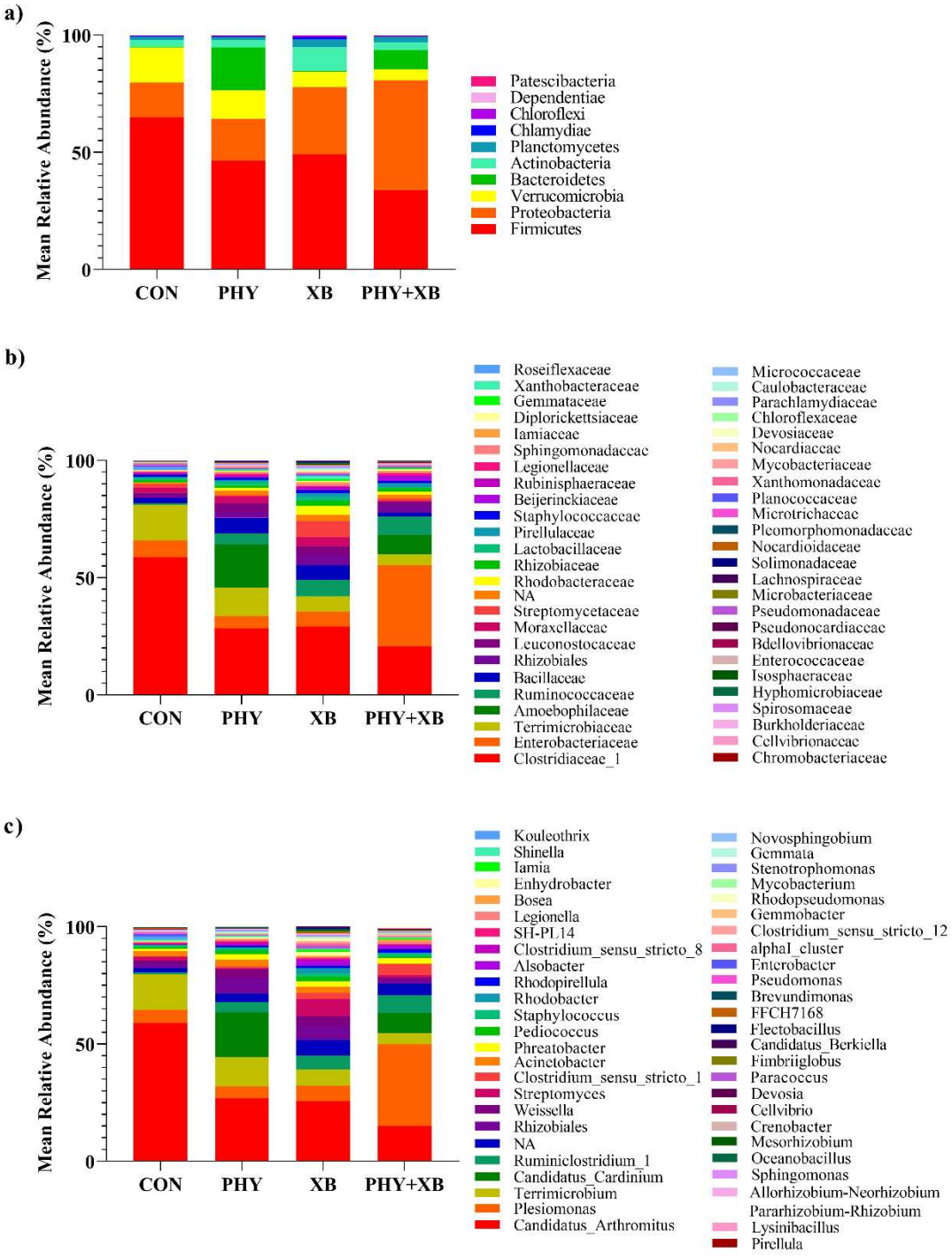

Figure 4. Taxonomic distribution of bacterial OUT's. (a) 10 most prevalent bacterial phylum (b) 50 most prevalent bacterial family (c) 50 most prevalent bacterial genera in the intestine communities of juvenile tambaqui (*C. macropomum*) fed a control diet (CON), and phytase (PHY), xylanase-β-glucanase (XB), or phytase + xylanase-β-glucanase (PHY + XB) supplemented diets.

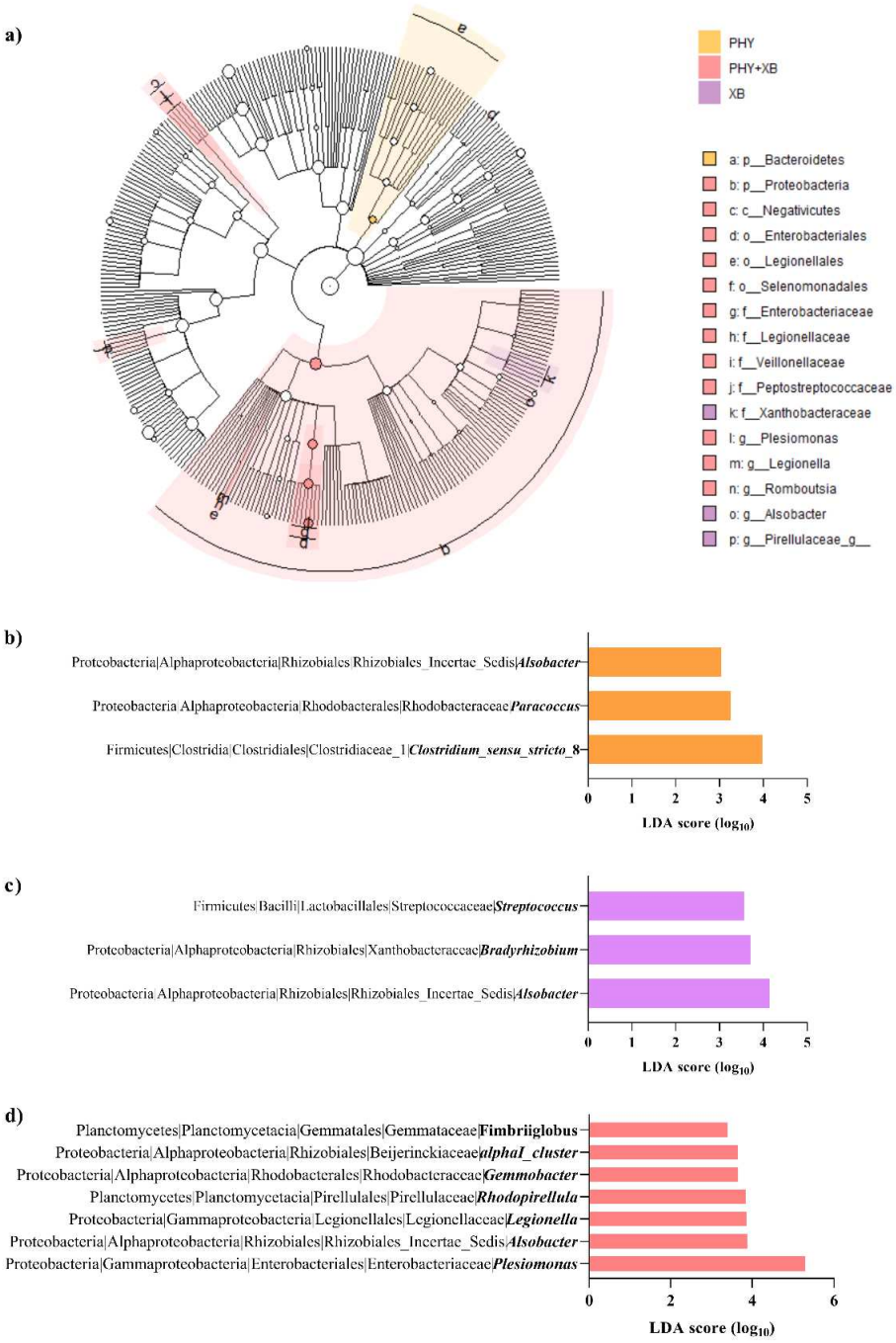

Figure 5. Bacterial taxa differentially enriched in PHY, XB and PHY+XB groups, using Linear Discriminant Analysis Effect Size (LEfSe). a) Taxa at all taxonomic levels were differentially enriched when comparing all treatments and the CON group. b) Taxa at the genus taxonomic level were differentially enriched when comparing CON *vs.* PHY treatment. c) taxa at the genus taxonomic level were differentially enriched when comparing CON *vs.* XB treatment. d) taxa at the genus taxonomic level were differentially enriched when comparing CON *vs.* PHY+XB treatment. Abbreviations: CON: control

diet, PHY: diet with phytase, XB: diet with xylanase-β-glucanase, PHY+XB: diet with phytase and xylanase-β-glucanase.

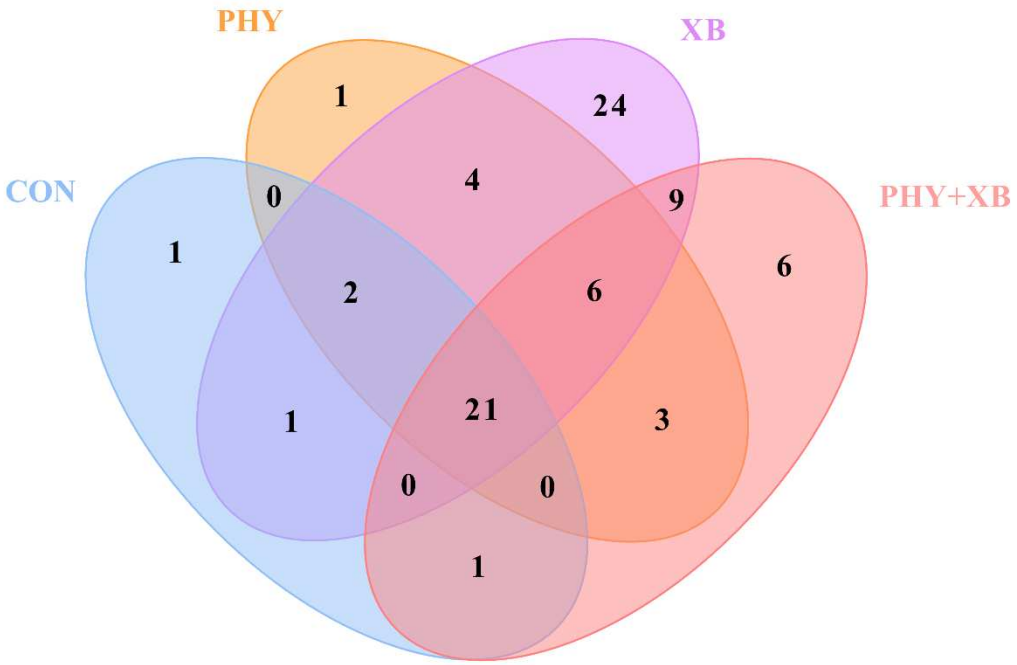

Figure 6. Total of unique and shared bacterial OTU between juvenile tambaqui (*C. macropomum*) fed a control diet (CON), and phytase (PHY), xylanase-β-glucanase (XB), or phytase + xylanase-β-glucanase (PHY + XB) supplemented diets.

Supplementary File

Phytase and Xylanase-B-glucanase exogenous enzymes impacts on intestinal microbiota of juvenile Amazonian tambaqui (*Colossoma macropomum*)

Supplementary Material and Methods

Experimental diets – exogenous enzymes

The phytase used was the commercial product Natuphos® E 10000 L (Basf, Ludwigshafen am Rhein, Germany), a liquid solution containing 10000 FTU g^{-1} of hybrid 6-phytase (EC 3.1.3.26), produced from the combination of three bacterial strains (*Hafnia* sp., *Yersinia mollaretii* and *Buttiauxella gaviniae*) and inserted into a genetically modified strain of *Aspergillus niger*. Xylanase and β-glucanase were included using the enzyme blend Natugrain® TS L (Basf, Ludwigshafen am Rhein, Germany), a liquid solution containing 5600 TXU of endo-1,4-β xylanase and 2500 TGU g^{-1} of endo-1,4-β glucanase. Natugrain® TS L is a product obtained from two genetically modified strains of *Aspergillus niger*. According to the manufacturer, both commercial products can be mixed without any loss of activity or stability.

Library preparation procedures

Barcodes were assigned to each sample. PCR products were previously diluted, and triplicates were pooled. The barcoding PCR contained 3 µl of PCR product, 7.5 µl of 2× Kapa HiFi Hot Start (Roche, Wilmington, MA, USA), 1.5 µl of each barcode, and 3 µl of distilled water. The thermocycling conditions were as follows: 95 °C for 3 min, followed by 10 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 5 min, followed by a final step of 72 °C for 5 min. These PCR products were also checked by electrophoresis on a 2% agarose gel stained with GelRed™ and visualized using the GelDoc™ (Bio-Rad Laboratories, California, CA, USA). PCR-barcoded products were cleaned with 0.8X AMPure XP beads (Beckman Coulter, Brea, CA, USA), followed by two washes with 80% ethanol. Afterward, the products were resuspended in EB buffer (Qiagen, Hilden, NRW, Germany). Individual libraries were quantified using a BioteKTM EpochTM microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples were pooled before sequencing.

Supplementary Results

Table S1. All taxa at all taxonomic levels differentially enriched in pairwise comparisons

Enrich Group in the comparison of the control group (CON) and the PHY treatment	**LDA Score (log_{10})**	***p*-value**
k__Bacteria|p__Bacteroidetes	5.158509	0.003884907
k__Bacteria|p__Bacteroidetes|c__Bacteroidia	5.155326	0.036048708
k__Bacteria|p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae_1|g__Clostridium_sensu_stricto_8	3.975304	0.030888518
k__Bacteria|p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiales_f__	3.445159	0.049510167
k__Bacteria|p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiales_f__|g__Clostridiales_f___g__	3.443314	0.049510167
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhodobacterales|f__Rhodobacteraceae|g__Paracoccus	3.257448	0.022229618
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Rhizobiales_Incertae_Sedis|g__Alsobacter	3.041617	0.049336176
Enrich Group in the comparison of the control group (CON) and the XB treatment	**LDA Score (log_{10})**	***p*-value**
k__Bacteria|p__Actinobacteria	5.344739	0.01040562
k__Bacteria|p__Actinobacteria|c__Actinobacteria	5.325377	0.01040562
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Rhizobiales_Incertae_Sedis|g__Alsobacter	4.142449	0.04951017
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Xanthobacteraceae	4.116189	0.02248668
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Xanthobacteraceae|g__Bradyrhizobium	3.704477	0.03408693
k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae	3.598461	0.01319510

k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae|g__Streptococcus	3.561117	0.01319510
k__Bacteria|p__Bacteroidetes|c__Bacteroidia	3.531314	0.03704073
k__Bacteria|p__Bacteroidetes	3.531314	0.03704073
k__Bacteria|p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae	3.291183	0.04951017
Enrich Group in the comparison of the control group (CON) and the PHY+XB treatment	**LDA Score (log_{10})**	***p*-value**
k__Bacteria|p__Proteobacteria	5.371810	0.016309172
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacteriales|f__Enterobacteriaceae|g__Plesiomonas	5.300944	0.016309172
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacteriales	5.286345	0.024974679
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacteriales|f__Enterobacteriaceae	5.286345	0.024974679
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria	5.278886	0.037372988
k__Bacteria|p__Bacteroidetes	4.720929	0.037040731
k__Bacteria|p__Bacteroidetes|c__Bacteroidia	4.720929	0.037040731
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Beijerinckiaceae	3.998982	0.024974679
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhodobacterales|f__Rhodobacteraceae	3.937432	0.022486683
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhodobacterales	3.937352	0.022486683
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Rhizobiales_Incertae_Sedis|g__Alsobacter	3.875488	0.002801666
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Legionellales	3.859868	0.006117484
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Legionellales|f__Legionellaceae	3.859816	0.006117484
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Legionellales|f__Legionellaceae|g__Legionella	3.859572	0.006117484

k__Bacteria|p__Planctomycetes|c__Planctomycetacia|o__Pirellulales|f__Pirellulaceae|g__Rhodopirellula	3.837193	0.022486683
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Rhizobiaceae|g__Rhizobiaceae_g__	3.728944	0.046319841
k__Bacteria|p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiales_f__	3.714812	0.049510167
k__Bacteria|p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiales_f__|g__Clostridiales_f___g__	3.714642	0.049510167
k__Bacteria|p__Planctomycetes|c__Planctomycetacia|o__Gemmatales|f__Gemmataceae	3.672527	0.036048708
k__Bacteria|p__Planctomycetes|c__Planctomycetacia|o__Gemmatales	3.670653	0.036048708
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhodobacterales|f__Rhodobacteraceae|g__Gemmobacter	3.652777	0.046319841
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Beijerinckiaceae|g__alphaI_cluster	3.641915	0.020096311
k__Bacteria|p__Planctomycetes|c__Planctomycetacia|o__Gemmatales|f__Gemmataceae|g__Fimbriiglobus	3.400664	0.022229618
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Microbacteriaceae	3.280004	0.022229618
k__Bacteria|p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptostreptococcaceae	3.267052	0.032630283
k__Bacteria|p__Firmicutes|c__Negativicutes|o__Selenomonadales|f__Veillonellaceae	3.217881	0.007397125
k__Bacteria|p__Firmicutes|c__Negativicutes	3.214644	0.007397125
k__Bacteria|p__Firmicutes|c__Negativicutes|o__Selenomonadales	3.214136	0.007397125

Table S2. Bacterial genera used to construct the Venn Diagram

Shared between all treatments	**Exclusive of CON**	**Exclusive of PHY**	**Exclusive of XB**	**Exclusive of PHY+XB**
Candidatus_Arthromitus	*Brevibacterium*	*Nocardia*	*Enterobacter*	*Crenobacter*
Plesiomonas			*Pirellula*	*Bdellovibrio*
Terrimicrobium			*Brevundimonas*	*Microbacterium*
Ruminiclostridium_1			*Flectobacillus*	*Romboutsia*
NA			*Devosia*	*Lachnoclostridium_5*
Bacillus			*Clostridium_sensu_stricto_5*	*Anaerovorax*
Weissella			*Chthonobacter*	
Streptomyces			*Solibacillus*	
Clostridium_sensu_stricto_1			*Aeromicrobium*	
Acinetobacter			*Ensifer*	
Phreatobacter			*Pelagibacterium*	
Pediococcus			*Dermacoccus*	
Staphylococcus			*Roseomonas*	
Clostridium_sensu_stricto_8			*Kocuria*	
SH-PL14			*Amycolatopsis*	
Legionella			*Rheinheimera*	
Bosea			*Clostridium_sensu_stricto_3*	

Iamia			*Luteolibacter*	
Shinella			*Sphingobacterium*	
Mycobacterium			*Aureimonas*	
Gemmata			*Hydrogenispora*	
			Schlesneria	
			Rubrobacter	
			Hyphomicrobium	

CHAPTER IV

Differential liver gene expression in the liver of Amazonian tambaqui (*Colossoma macropomum*) fed plant-based diets supplemented with phytase and/or xylanase-β-glucanase using RNA-seq approach

Written in accordance with the guidelines of the *Gene* journal

ABSTRACT

In this study, we conducted a differential analysis to assess how dietary supplementation with phytase and/or xylanase-β-glucanases affects the liver gene expression of the Amazonian fish tambaqui (*Colossoma macropomum*). Juveniles weighing 1.04 ± 0.01g were distributed among 24 tanks and fed four experimental diets: CON (control diet without enzymes), PHY (diet with phytase), XB (diet with xylanase-β-glucanase complex), and PHY+XB (diet with phytase and xylanase-β-glucanase complex) for 8 weeks. Liver RNA-Seq was used to investigate the hepatic transcriptome response to different diets. A total of 526,072 genes were identified. Differential analysis revealed a total of 139 differentially expressed genes (DGEs): 54 up-regulated and 84 down-regulated genes between CON *vs.* PHY; 19 genes (6 up-regulated and 13 down-regulated) between CON *vs.* XB; and 77 genes (10 up-regulated and 67 down-regulated) between CON *vs.* PHY+XB. Gene Ontology (GO) functional enrichment and KEGG pathway analysis were conducted for each comparison of differentially expressed genes. Through GO enrichment analysis, we found 15 entries significantly enriched in PHY, 9 in XB, and 22 in PHY+XB. The PHY and PHY+XB diets appeared to primarily impact oxygen homeostasis and redox processes. The PHY diet also affected the activity of the fish's immune system. The XB diet mainly affects carbohydrate metabolism. It is concluded that exogenous enzymes contribute to improving the energy homeostasis of fish by modulating genes through the availability of nutrients.

KEY-WORDS: exogenous enzyme, non-starch polysaccharide, RNA-seq, phytic acid, transcriptome

1. INTRODUCTION

For some time now, the main concern in the aquafeed industry has been the replacement of fishmeal with alternative ingredients, mainly from vegetable sources. In respect of the production of Neotropical omnivorous fish, this issue does not seem to be a problem, since supplementation with synthetic amino acids has made the use of diets composed of grains and cereals meals (e.g., soy, corn, wheat and their by-products) a reality. However, even for these species, some details still compromise the efficiency and sustainability of these diets. One of these factors would be the presence of indigestible fractions, which remain even when subjected to previous heat treatments (Singh & Satyanarayana, 2011; U.S. Grains Council, 2012; Welker et al., 2014).

As an example, we can mention phytic acid, which molecule corresponds to the main reservoir of phosphorus (P) in cereal grains and legumes (Song et al., 2019; Wang & Guo, 2021) commonly used in fish diets. Unfortunately, the P contained in phytic acid is unavailable to fish, since they do not synthesize phytases endogenously (Ravindran et al., 1999; Jackson et al., 1996; Papatryphon et al., 1999). Phytic acid can also complex other minerals (Nolan et al., 1987; Duffus & Duffus, 1991; Schlemmer et al., 2009; Wang & Guo, 2021), proteins (Spinelli et al., 1983; Richardson et al., 1985; Nolan et al., 1987, Wang & Guo, 2021) and starch carbohydrates (Oatway et al., 2001), forming the salt known as phytate. Another indigestible fraction of vegetables corresponds to the structural components of the cell wall known as non-starch polysaccharides (NSP) (Barletta et al., 2011). Soluble NSP, like arabinoxylan and β-glucans, retain a large volume of water and increase the viscosity of the digesta, limiting the access of digestive enzymes to the substrate (Castillo & Gatlin III, 2015; Sinha et al., 2011), impairing directly intake, and digestion processes (Sinha et al., 2011; Castillo & Gatlin III, 2015). All these antinutritional factors do not cause growth retardation only due to the unavailability of nutrients. When at high levels in the diet, they cause inflammation and morphological damage and changes in the intestinal microbial community, leading to immunological suppression, which can directly affect the absorptive capacity of the nutrients of fish and the way they metabolize nutrients (Zhong et al., 2019, Liu et al., 2022).

In this sense, the main approach that has been used to improve flexibility in the formulation of plant-based diets is the use of exogenous enzymes (e.g., phytase, carbohydrases). Phytase is an enzyme that performs the gradual dephosphorylation of

phytate, releasing inositol and inorganic phosphorus for absorption (Selle et al., 2010; Greiner & Konietzny, 2011; Kumar et al., 2012). Xylanase and β-glucanase are the two main NSP-degrading enzymes used in animal feed, corresponding to almost 80% of the products available on the market (Barletta et al., 2011; Castillo & Gatlin III, 2015). The action of xylanase on the substrate results in a partial hydrolysis of xylans, which significantly reduces the viscosity of the digesta and improves the access of endogenous enzymes to the substrate and, consequently, the use of nutrients by the animals (Paloheimo et al., 2010). However, for complete hydrolysis, this enzyme must act synergistically with other hemicellulases (Coughlan & Hazlewood, 1993; Paloheimo et al., 2010; Motta et al., 2013), such as β-glucanases. β-glucanases hydrolyze β-glucans into cellobiose units, altering the integrity of the endospermic cell wall (Habte-Tsion et al., 2018). The combined use of phytase and NSP-degrading enzymes in plant-based fish diets is recent but has been shown to be efficient in improving nutrient digestibility, growth performance, and intestinal health of fish (Dalsgaard et al., 2012; Diógenes et al., 2018; Maas et al., 2018; 2020).

The efficiency in the use of dietary nutrients depends not only on their structure and digestion, but also on the coordinated absorption and distribution of these nutrients through metabolism, which can be verified by the expression and regulation of multiple genes. The RNA-seq technique, a powerful tool in molecular biology, offers a comprehensive, high-throughput approach to analyze gene expression profiles in a given tissue or organism. By employing RNA-seq, researchers can "blindly" identify genes differentially expressed under different conditions or treatments, providing valuable information about the molecular mechanisms underlying physiological responses.

The tambaqui, *Colossoma macropomum* (Cuvier, 1818), is an Amazonian omnivorous fish (Honda, 1974) known for its adaptability to plant-based diets (Martins et al., 2020). This species, characterized by rapid growth (Aride et al., 2004; Saint-Paul, 1986), adaptability to confinement (Araujo-Lima & Goulding, 1997; Aride et al., 2004; Gomes et al., 2002; Silva et al., 2007), and desirable meat flavor (Sousa et al., 2020), has attracted interest from producers worldwide. Previous studies have demonstrated that phytase supplementation in diets partially composed of vegetable ingredients enhances the growth performance of tambaqui (Mendonça et al., 2012). However, to date, no studies have evaluated the efficacy of new-generation phytases and other enzymes, such as xylanase and β-glucanase, in tambaqui diets. Therefore, our study aims to assess,

through differential analysis, how dietary supplementation with phytase and/or xylanase-β-glucanase affects the liver gene expression of tambaqui, providing insights into the metabolic responses of this Amazonian fish.

2. MATERIAL AND METHODS

2.1 Basal diet

A basal plant-based diet was formulated based (307.8 g kg^{-1} of crude protein and 17.082 MJ kg^{-1} of gross energy) following the method proposed by Amancio et al. (2019), and prepared at Instituto de Pesca (São Paulo, SP, Brazil). All ingredients were ground in an 800 mm sieve in a centrifugal mill (Viera MC 680B, Tatuí, SP, Brazil) before being mixed in a commercial V-blender (MA200; Marconi, Piracicaba, SP, Brazil), extruded in a single sieve. Experimental feed mill (Model E-62, Ferraz Máquinas e Engenharia LTDA, Ribeirão Preto, SP, Brazil) through a 3 mm diameter die, and dried in a tubular dryer at 55° C (Model E-62, Ferraz Máquinas et Engenharia LTDA, Ribeirão Preto, SP, Brazil).

2.2 Experimental diets

The phytase used was the commercial product Natuphos® E 10000 L (Basf, Ludwigshafen am Rhein, Germany), a liquid solution containing 10000 FTU g^{-1} of hybrid 6-phytase (EC 3.1.3.26), produced from the combination of three bacterial strains (*Hafnia* sp., *Yersinia mollaretii* and *Buttiauxella gaviniae*) and inserted into a genetically modified strain of *Aspergillus niger*. Xylanase and β-glucanase were included using the enzyme complex Natugrain® TS L (Basf, Ludwigshafen am Rhein, Germany), a liquid solution containing 5600 TXU of endo-1,4-β xylanase and 2500 TGU g^{-1} of endo-1,4-β glucanase. Natugrain® TS L is a product obtained from two genetically modified strains of *Aspergillus niger*. According to the manufacturer both commercial products can be mixed without loss of activity and stability.

From the basal diet, four experimental diets were prepared, in which CON: control diet without exogenous enzyme supplementation, PHY: diet supplemented with 1500 FTU kg^{-1} of phytase, XB: diet supplemented with 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase and PHY+XB: diet supplemented with 1500 FTU kg^{-1} of phytase, 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase. To obtain these

enzyme concentrations, solutions were prepared by diluting Natuphos® and/or Natugrain® in demineralized water. The incorporation into the diets was carried out by spraying the pellets using a hand sprayer (Guarany, Itu, SP, Brazil). For the preparation of the CON diet, only demineralized water was sprinkled. After application of the enzyme solution, the soybean oil indicated in the formulation was homogeneously sprinkled on the diets, sealing them and preventing loss of enzymes in the water. Subsequently, the diets were dried in a forced ventilation oven (Marconi, MA035, SP, Brazil) at 55 °C for 6 hours. The analyzed chemical composition of the experimental diets were presented in Table 1.

2.3 Fish and culture conditions

Tambaqui juveniles (1.04 ± 0.10 g) from the Aquaculture Laboratory (LAQUA) of the Federal University of Minas Gerais (UFMG) were randomly distributed in 24 polyethylene tanks (100 L) arranged in a water recirculation system (1.5 L min^{-1}), at a density of 16 fish per tank. The tanks were covered with white nylon mesh to prevent fish from escaping. The recirculation system was equipped with constant aeration, controlled temperature, ultraviolet, mechanical, and biological filters. During the trial, the laboratory was maintained in a 12-hour photoperiod, with the aid of an analog timer. The fish were manually fed (8:00, 11:00, 14:00 and 17:00 hours) until apparent satiation for 8 weeks.

The water quality parameters were evaluated weekly. Dissolved oxygen was maintained at 8.02 ± 0.9 mg L^{-1} and temperature at 27.35 ± 0.4° C (YSI® 550A multiparameter meter, Florianopolis, SC, Brazil). The pH was maintained at 6.73 ± 0.15 (Combo basic multiparameter meter, AKSO®, São Leopoldo, RS, Brazil), non-ionized ammonia at 0.01 ± 0.005 mg L^{-1} and nitrite at 0.02 ± 0.01 mg L^{-1} (Labcon® analysis kits, Florianopolis, SC, Brazil).

2.4 Sampling collection and preparation

At the end of the experiment, fish were fasted for 24 hours. Then, fish were captured, counted, anesthetized, weighed, and euthanized sampling. Liver samples from one fish of three tanks per treatment (n = 3) were collected and submerged in RNA *later* solution (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) at 4° C for 24 h and then, stored at – 80° C until RNA extraction.

2.5 RNA sequencing and differential gene expression

The workflow from liver collection to data analysis is shown in Figure 1.

2.5.1 RNA extraction and sequencing

For total RNA extraction, ~ 60 mg of liver tissue was homogenized in 1 ml of trizol (NZY Tech Lda., Lisbon, Portugal) using a Precellys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, IDF, France). The homogenate was incubated at room temperature for 5 minutes for the addition of 200µl of chloroform and subsequent centrifugation for 15 minutes at 12000 × g at 4° C. In the resulting aqueous solution, the RNA was extracted using the NZY Total RNA Isolation kit (NZY Tech Lda., Lisbon, Portugal), following the manufacturer's recommendations. Total RNA concentrations and quality were evaluated using a microplate spectrophotometer (EPOCH, BioTek Instruments, Winooski, VT, USA) and the integrity was observed in a 2% agarose gel. The best samples (n = 3 per treatment) were selected and shipped to Macrogen Inc. (Seoul, South Korea) where mRNA Poli(A)$^+$ was isolated and reverse transcribed to produce the cDNA. Sequencing was carried out in Illumina HiSeq-4000 platform (150 × 2bp, paired-end, 25 million sequencing reads).

2.5.2 Quality control and mapping

RNA-Seq quality profile of each sample was assessed with FastQC (v.0.11.8) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic (v.0.38) (Bolger et al., 2014) was used to trim and drop reads with quality scores below 5, at leading and trailing ends, with an average quality score below 20 in a 4 bp sliding window and with less than 36 bases length. Trimmed reads foram mapeadas contra o genoma de referência do tambaqui (GAC_904425465.1) usando HISAT2 (v2.1.0) (Kim & Salzberg, 2015). A anotação de genes e contagem de transcritos mapeados foram realizadas com StringTie (v1.3.4) (Pertea et al., 2015).

2.5.3 Differential gene expression analysis and functional annotation analyses

The counts generated during the filtering steps were used to perform DGE analyses on the DEApp platform (https://yanli.shinyapps.io/DEApp/). Genes with less than 1 CPM (count per million mapped reads) in at least 3 samples were excluded from the dataset. To visualize the dispersion and grouping of samples, multidimensional mapping was

carried out using the MDS analysis technique. The MDS chart was built using the Degust tool on the Edger platform. DEseq2 and edgeR negative binomial generalized linear models were both used to identify differentially expressed genes between groups. Genes were considered DEGs if false discovery rate (FDR)-corrected $p < 0.05$ and $\log2|\text{fold change}| \geq 2$. The heatmap was generated in the Heatmapper Expression tool (http://heatmapper.ca/expression/) using the "Average Linkage" clustering method and the "Pearson" distance measurement method. Venn diagrams were used to show the similarities/dissimilarities of DEGs transcripts between different groups.

To examine the functional enrichment of transcripts of identified differentially expressed genes, we performed a homology analysis between the tambaqui genome and the zebrafish (*Danio rerio*) genome using the BLASTP tool (Basic Local Alignment Search Tool for Protein) available on the platform from NCBI (National Center for Biotechnology Information). The similarity significance criterion was defined as an Expect (E) value less than 1×10^{-5}. From these alignment analyses, we identified homologous genes between the two species. For functional enrichment analysis, gene IDs relative to zebrafish genes were annotated in g: GOSt, available in the g: Profiler web tool (Raudvere et al., 2019). We utilize Gene Ontology (GO) annotations in the three domains: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF), and KEGG pathways to determine the function and biological significance of the genes. We used the Benjamini-Hochberg false discovery rate (FDR) method at a 0.05 level of significance.

3. RESULTS

To gain insights into global changes in gene expression in tambaqui fed diets containing different combinations of enzymes, we performed whole transcriptome sequencing of liver tissue. The Multidimensional Scaling plot (MDS) showing the similarities and dissimilarities of the hepatic gene expression profiles of fish fed the CON, PHY, XB, and PHY+XB diets is presented in Figure 2. The normalization plots and the number of DEGs genes obtained in DEApp using the edgeR and DESeq2 methods are shown in Figure S1. Based on liver transcriptome analysis and considering all DGE obtained using edgeR and DESeq2, there were a total of 138 DEGs (54 up-regulated and 84 down-regulated genes) between CON *vs*. PHY (Table S1, Figure 3a and 3b); 19 DEGs (6 up-regulated and 13 down-regulated) between CON *vs.* XB (Table S2, Figure 3a and

3b); and 77 DEGs (10 up-regulated and 67 down-regulated) between CON *vs.* PHY+XB (Table S3; Figure 3a and 3b). A total of five DGEs were shared among all treatments. Among these, three genes were down-regulated (*keg*, *src*, *asgrl2*), while two were up-regulated (*cd22* and *rps6kc1*). A total of 18 DGEs were shared between the phytase-containing treatments (PHY and PHY+XB), with 17 being down-regulated and one being up-regulated. Among the treatments containing xylanase (XB and PHY+XB), three down-regulated and one up-regulated DGE were shared. Only one up-regulated gene was shared between treatments with isolated enzymes (PHY and XB). Heatmaps and a hierarchical clustering tree representing the DEGs related to the comparisons CON *vs.* PHY, CON *vs.* XB, and CON *vs.* PHY+XB were presented in Figure 4a, 4b, and 4c, respectively.

All significantly altered GO terms are listed in Table 2. The GO analysis showed that the PHY diet influenced the regulation of genes involved in essential processes such as oxidation-reduction reactions, oxygen transport, immune system regulation, and metabolism. of carbohydrates (Table 2, Figure 5). The XB diet, on the other hand, affected the regulation of genes that seem to be involved in essential processes like carbohydrate metabolism (Table 2, Figure 5). The PHY+XB diet influenced the regulation of genes related to oxygen binding, hemoglobin binding, and tetrapyrrole binding, thereby altering biological processes such as oxygen transport. The same diet also affected fish lipid metabolism. (Table 2, Figure 5). No KEGG pathway enrichment was observed in tambaqui fed either diet.

4. DISCUSSION

To date, little is known about the effects of exogenous enzymes on the fish transcriptome and, most importantly, how potential changes can impact the efficiency of nutrient utilization and their health. This study aims to fill this gap by potentially being the first to evaluate the effects of phytase and xylanase-β-glucanase on the tambaqui transcriptome. We chose to evaluate the liver tissue transcriptome because this organ is considered an indicator of the metabolic and physiological state of fish.

We hypothesized that improving nutrient availability, especially phosphorus (P) and fermentable oligosaccharides for short-chain fatty acids (SCFA), could trigger epigenetic changes in the fish transcriptome. Surprisingly, we found that diets containing phytase, PHY, and PHY+XB primarily enhanced the regulation of genes related to

oxidation-reduction processes, hemoglobin biosynthesis, and hematopoiesis. Due to its classical effect, we expected that phytase would influence the expression of phosphorus metabolism genes. However, adjacent effects can be obtained from the release of other compounds in the gastrointestinal tract, such as myo-inositol. Myo-inositol phosphates play important roles in biological systems, functioning as messengers in important cell signaling pathways (Koumbis et al., 2011). This degradation product of phytic acid can significantly increase in the blood and liver after consuming diets with phytase (Whitfield et al., 2022), potentially offering benefits that extend beyond enhancing the availability of P and other minerals. In broiler chickens, myo-inositol reduces the severity of woody breast myopathy (WB), a disease that occurs as a consequence of the accelerated growth and large breast volume of current poultry strains (Greene et al., 2019; Whitfield et al., 2022). The accelerated growth of muscle fibers creates a hypoxic environment in the tissue, which can lead to fibrosis (Greene et al., 2019). This is because myo-inositol phosphates act as allosteric effectors of hemoglobin (Hb), a protein that transports oxygen through tissues (Benesch & Benesch, 1967; Koumbis et al., 2011). In this study, phytase promoted the down-regulation of the hemoglobin genes, *hbaa1* and *hbaa2*. It also promoted a reduction in the expression of the heme oxygenase-2 gene, *hao2*, the enzyme responsible for the breakdown of the heme group, and the release of iron, carbon monoxide, and bilirubin/biliverdin within tissue cells (Kim & Doré, 2005). Previous studies have shown that these genes are strongly activated in fish subjected to hypoxic conditions (Gracey et al., 2001; Wawrowski et al., 2011; Pan et al., 2017; Cadiz et al., 2017). The expression of these and other genes involved in oxygen homeostasis is regulated by the activation of the hypoxia-inducible factor (HIF) (Gassmann & Wenger, 2002). In this study, phytase also promoted the down-regulation of the egg-laying defect genes, *egln1* and *egln3*, which are essential for suppressing HIF (Dengler et al., 2021). In a study with *Hypophthalmichthys molitrix*, Li et al. (2022) observed that HIF and the genes it regulates, including *egln1* and *egln3*, were activated under hypoxic conditions in the organism's attempt to maintain active biological functions during the stressful situation. Therefore, it is likely that phytase promotes beneficial effects on oxygen transport and homeostasis by releasing myo-inositol, which benefits the fish in the stressful conditions of the cultivation environment.

The PHY diet also influences genes associated with the fish's immune system. An example of this is that the *tlr5a* gene was negatively regulated in these individuals. Toll-

like receptors (TLRs) are a type of pattern recognition receptors (PRRs) that play a role in identifying pathogen-associated molecular patterns (PAMPs), thereby regulating innate and specific immune responses to pathogen infections (Creagh & O'Neil, 2006; Gong et al., 2017). *Aeromonas hydrophila* infection activated the expression of the *tlr5a* and *tlr5b* genes in *Ctenopharyngodon idella* (Zhan et al., 2023). Therefore, the down-regulation of this gene in fish fed a diet containing phytase may be linked to an improvement in the intestinal health of these fish. The same was observed in relation to the *cxcl12a* gene. The CXCL12 protein, which belongs to the CXC chemokine family, binds to its receptor CXCR4 to regulate hematopoietic cells and attract leukocytes to infected tissue (Zhou et al., 1998). Therefore, the infection activates these genes in a similar manner. For example, *Streptococcus agalactiae* infection activated this gene in *Oreochromis niloticus* (Gao et al., 2020). Furthermore, the down-regulation of the expression of other genes related to pathogen recognition, such as *cfbl* (Jalili et al., 2020), *c1r* (Boshra & Sunyer, 2006), *zbtb33* (Kjærner-Semb et al., 2016), and *cish* (Maehr et al., 2014), highlights the role of phytase in improving the health and intestinal microbiota of fish.

In this study, fish fed diets containing PHY+XB also showed enrichment of pathways related to lipid metabolism. These fish mainly exhibited lower expression of the *lipin1* gene, which is closely associated with the adipogenesis process. Lipins play a crucial role in the synthesis and storage of lipids by regulating the activity of phosphatidate phosphatase (PAP) (Csaki et al., 2014). PAP is the enzyme responsible for catalyzing the conversion of phosphatidate into diacylglycerol, an important intermediate in the synthesis of triglycerides and phospholipids (Pascual & Craman, 2013). Therefore, excess *lipin1* promotes obesity and insulin sensitivity (Phan & Reue, 2005). Therefore, reduced *lipin1* expression leads to decreased PAP activity, which may result in lower lipid production and, consequently, reduced adipogenesis. Another gene related to lipid metabolism that was also regulated by the PHY+XB diet in this study is the *rxraa* gene. The RXRA (Retinoid X Receptor Alpha) genes are nuclear receptors that form heterodimers with retinoic acid receptors (RARs) and fatty acid receptors (FXRs) and, therefore, influence the synthesis, oxidation, transport, and storage of lipids (Dawson & Xia, 2012; Wang & Tontonoz, 2018). In a study evaluating the inhibition of Stearyl coenzyme A desaturase (SCD), an enzyme that catalyzes limiting steps in the production of monounsaturated fatty acids, Xu et al. (2023) found that the *rxraa* gene was less

expressed in the liver. This indicates that in this study, the PHY+XB diet likely enhanced metabolization in tambaqui liver tissue. In summary, the reduced expression of lipogenic genes in fish fed diets containing PHY+XB may be an important mechanism for regulating adipogenesis.

Regarding to fish fed the XB diet, genes involved in lipid and carbohydrate metabolism were regulated. For example, there was a decrease in the expression of the acss1 gene. Short-chain acetyl-CoA synthetases (ACSS) are key enzymes in metabolizing acetate to acetyl-CoA in mitochondria (Castro et al., 2012; Zhou et al., 2023). Thus, acetate, along with other short-chain fatty acids (SCFAs) when available, are primary sources of energy that play a role in cell growth processes. In normal situations, intracellular acetate is mainly derived from the metabolism of amino acids, glucose, and fatty acids (Shi & Tu, 2015). However, it is known that one of the effects of the action of xylanase and β-glucanase carbohydrases is the formation of SCFA-fermentable oligosaccharides in the fish intestine (Geraylou et al., 2014). In the intestine, acetate produced by the intestinal microbiota is absorbed and sent to the liver via the hepatic portal system, where it is metabolized (Tremaroli & Bäckhed, 2012). Therefore, it is likely that the suppression of *assc1* occurred in response to high levels of this short-chain fatty acid (SCFA) with a simultaneous increase in acetyl-CoA in the fish liver. In agreement with our findings, Zhou et al. (2023) found that when evaluating dietary supplementation with sodium acetate in *O. niloticus*, although ACSS proteins and Acetyl-CoA content increased in the liver, there was a subsequent reduction in the expression of *acss* genes in this tissue.

As expected, the XB diet also promoted the regulation of genes involved in carbohydrate metabolism. In this study, inhibition of the g6pca.1 gene was observed in fish fed with XB. Glucose-6-phosphatase (G6Pase) plays a crucial role in glucose homeostasis. It catalyzes the hydrolysis of glucose-6-phosphate into glucose and inorganic phosphate, which is the final biochemical step common to both gluconeogenesis and glycogenolysis (Foster & Nordlie, 2002; Hutton & O'Brien, 2009). The regulation of this enzyme's activity is controlled by the g6pc gene group (Argaud et al., 1996). The activation of other carbohydrate metabolism genes can be influenced by the availability of dietary nutrients through the regulation of DNA methylation levels (Marandel et al., 2016). Zhang et al. (2021) reported that high levels of carbohydrates in the diet of *Siniperca chuatsi* increased DNA methylation in the promoter regions of

g6pca, inhibiting its expression and the release of glucose into the blood via gluconeogenesis. *S. chuatsi* is a carnivorous fish; however, similar results in *g6p6a* expression were observed in *Cyprinus carpio* (Panserat et al., 2002) and *O. niloticus* (Chen et al., 2017). Carbohydrases, such as xylanase and β-glucanase, are known to facilitate the hydrolysis of complex carbohydrates, leading to an elevation in blood glucose levels. Therefore, we believe that xylanase-β-glucanase promotes an enhancement in the utilization of dietary carbohydrates and its inhibited gluconeogenic pathways.

In conclusion, we confirmed through transcriptome analysis that exogenous enzymes can modulate the expression of nutrient metabolism genes in tambaqui. We also found that phytase has the potential to improve the oxidative and immune status of fish, while xylanase- β-glucanase appears to have positive effects on energy metabolism.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical statement

This study was carried out at the Laboratory of Fish Nutrition I of the Fish Farm Sector of the Department of Animal Biology at the Federal University of Viçosa (UFV)

and at the Animal Genetics and Evolution Laboratory of the Interdisciplinary Center for Marine and Environmental Research (CIIMAR) at the University of Porto (UP). The study was approved by the Ethics Committee on the Use of Production Animals (CEUAP-UFV), protocol 24/2021.

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Table 1. Composition and chemical analysis of the experimental diets

Ingredients (g kg^{-1})	Diets[a]			
	CON	PHY	XB	PHY+XB
Soybean meal 45[b]	450.00	450.00	450.00	450.00
Corn flour[b]	183.00	183.00	183.00	183.00
Wheat meal[b]	150.00	150.00	150.00	150.00
Soybean protein concentrate 85[c]	60.00	60.00	60.00	60.00
Broken rice[b]	60.00	60.00	60.00	60.00
Wheat flour[b]	40.00	40.00	40.00	40.00
Soybean oil[d]	20.00	20.00	20.00	20.00
Calcium cabonate[e]	10.00	10.00	10.00	10.00
Bicalcium phosphate[e]	12.00	12.00	12.00	12.00
Salt	5.00	5.00	5.00	5.00
Mineral and vitaminic mix[f]	5.00	5.00	5.00	5.00
L-Lysine[g]	2.00	2.00	2.00	2.00
DL-Methionine[g]	2.00	2.00	2.00	2.00
Chromium oxide (Cr_2O_3)[h]	1.00	1.00	1.00	1.00
Analyzed composition (as dry matter basis)				
Dry matter	892.51	871.95	884.86	890.289
Energy (kcal kg^{-1})	4080.0	4039.0	4086.0	4096.0
Crude protein	307.80	309.00	305.60	309.90
Total lipids	35.03	36.21	36.89	35.69
Ash	70.02	71.09	70.12	70.52
Total carbohydrates[i]	587.15	583.70	587.39	583.89
Neutral detergente fiber	191.09	190.37	186.78	172.06
Non-fibrous carbohydrate[i]	396.06	393.33	400.63	411.83
Total calcium	11.53	13.04	11.11	10.62
Total phosphorus	6.36	6.67	6.39	6.57
Ca:P ratio	1.81	1.96	1.74	1.62
Iron	0.18	0.29	0.25	0.21
Zinc	0.22	0.21	0.20	0.22
Magnesium	0.05	0.06	0.05	0.05

Phytase (FTU kg^{-1})[j]	ND[l]	1740.0	0.00	1620.0
Xylanase (TXU kg^{-1})[k]	ND[l]	0.00	1496.0	1498.36
β-glucanase (TGU kg^{-1})[k]	ND[l]	0.00	454.0	617.0

[a]CON: control diet without phytase, xylanase and β-glucanase supplementation; PHY: diet supplemented with 1500 FTU kg^{-1} of phytase; XB: diet supplemented with 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase; PHY+XB: diet supplemented with 1500 FTU kg^{-1} of phytase, 1120 TXU kg^{-1} of xylanase and 500 TGU kg^{-1} of β-glucanase;

[b]Guabi Alimentos Ltda, Campinas, SP, Brazil.

[c]Bunge, Ponta Grossa, PR, Brazil.

[d]Cocamar, Maringá, PR, Brazil.

[e]Sarfos, Itumbiara, Goiás, GO, Brazil.

[f]Composition kg^{-1}diet: retinyl acetate (vitamin A. 6,000 IU; cholecalciferol (vitamin D3. 1,000 IU); DL-α-tocopherol (vitamin E. 100 mg); menadione (vitamin K3. 12 mg); thiamine (vitamin B1. 24 mg); riboflavin (vitamin B2. 24 mg); pyridoxine-HC (vitamin B3. 20 mg); cyanocobalamin (vitamin B12. 24 µg; folic acid (vitamin B9. 6 mg); D-pantothenic acid (vitamin B5. 60 mg); ascorbic acid (ROVIMIX® STAY-C®35 (vitamin C. 340 mg); biotin. 0.24 mg; choline chloride (choline. 325 mg); niacin (vitamin B3. 120 mg); ferrous sulfate ($FeSO_4.7H_2O$. 50 mg Fe); copper sulfate ($CuSO_4.5H_2O$. 3 mg Cu); Mg - 20 mg; zinc sulfate ($ZnSO_4 \cdot H_2O$. 30 mg Zn); potassium iodate (KIO_3. 2 mg I); cobalt (II) carbonate ($CoCO_3$. 0.1 mg Co); Sodium selenite (Na_2SeO_3. 0.1 mg se).

[g]Ajinomoto Animal Nutrition Division, São Paulo, SP, Brazil.

[h]Sygma-Aldrich Brazil Ltda, 99.5%, São Paulo, SP, Brazil.

[i]Calculated according to Detmann & Valadares Filho, 2010.

[j]Natuphos® E 10000 L, Basf, Ludwigshafen am Rhein, RP, Germany.

[k]Natugrain® TS (Basf, Ludwigshafen am Rhein, RP, Germany.

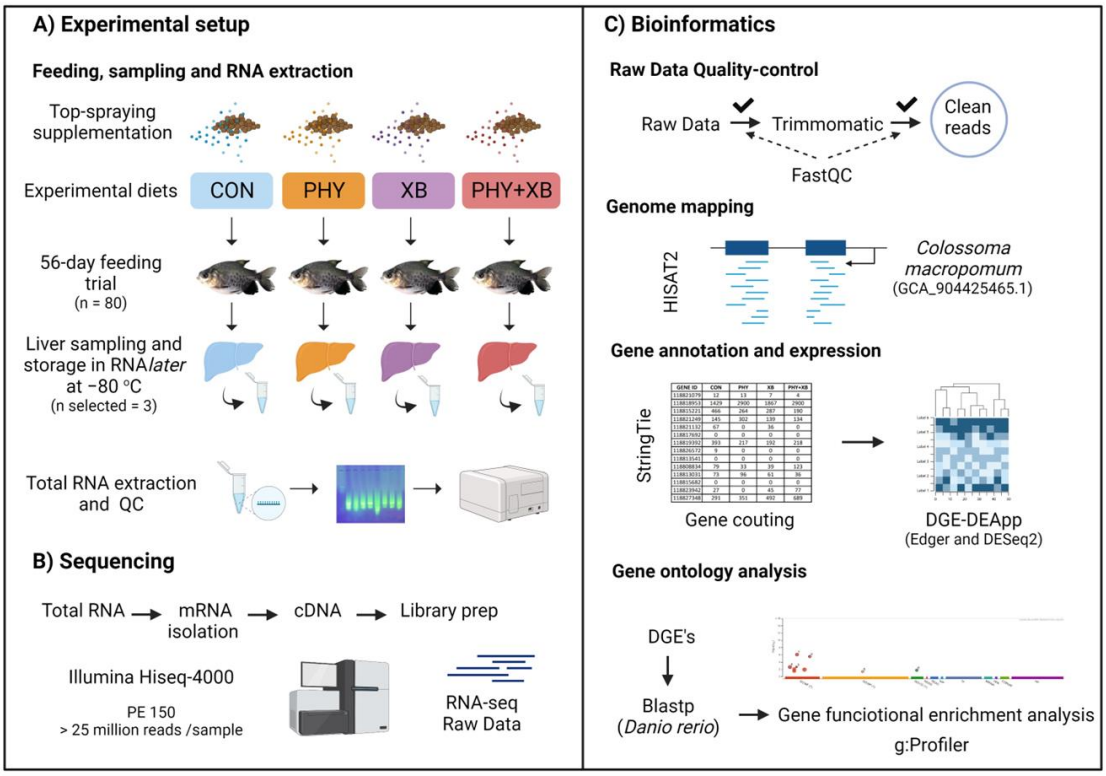

Figure 1. General workflow of the RNA-seq and differential gene expression analysis of the present study. A) *Colossoma macropomum* juvenile were fed with the diets CON (control diet), PHY (diet with phytase), XB (diet with xylanase-β-glucanase) and PHY+XB (diet with phytase and xylanase-β-glucanase) for 8 weeks and liver samples were collected to Total RNA extraction. B) RNA-sequencing was performed using Illumina Hiseq-4000 and C) clean reads were mapped and annotated to differential gene expression analysis.

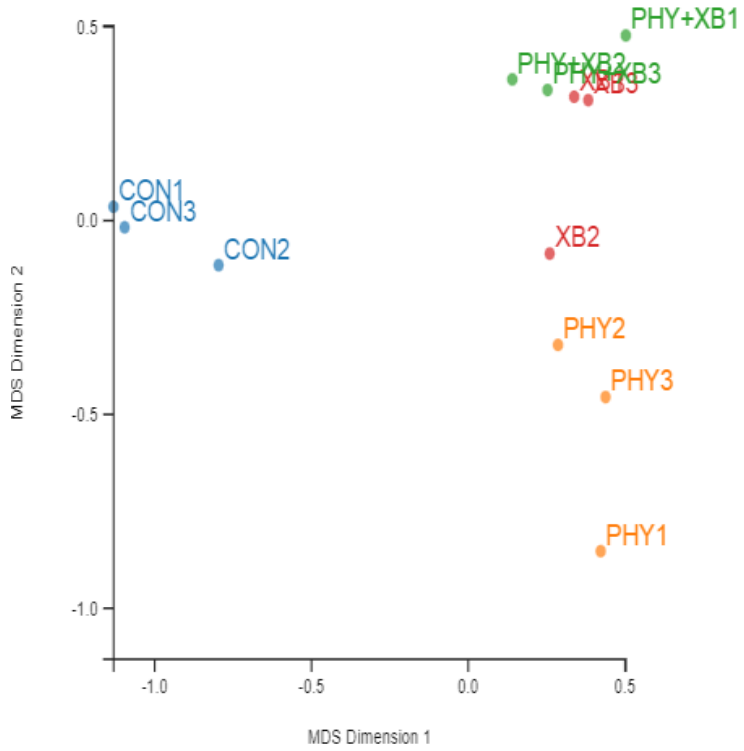

Figure 2. Transcriptome analysis of fish liver in response to dietary enzyme supplementation. MDS plot (based on log2 fold gene expression changes) generated DEApp showing the separation of fish fed CON, PHY, XB and PHY+XB diets. Abbreviations: CON: control diet, PHY: diet with phytase, XB: diet with xylanase-β-glucanase, and PHY+XB: diet with phytase and xylanase-β-glucanase.

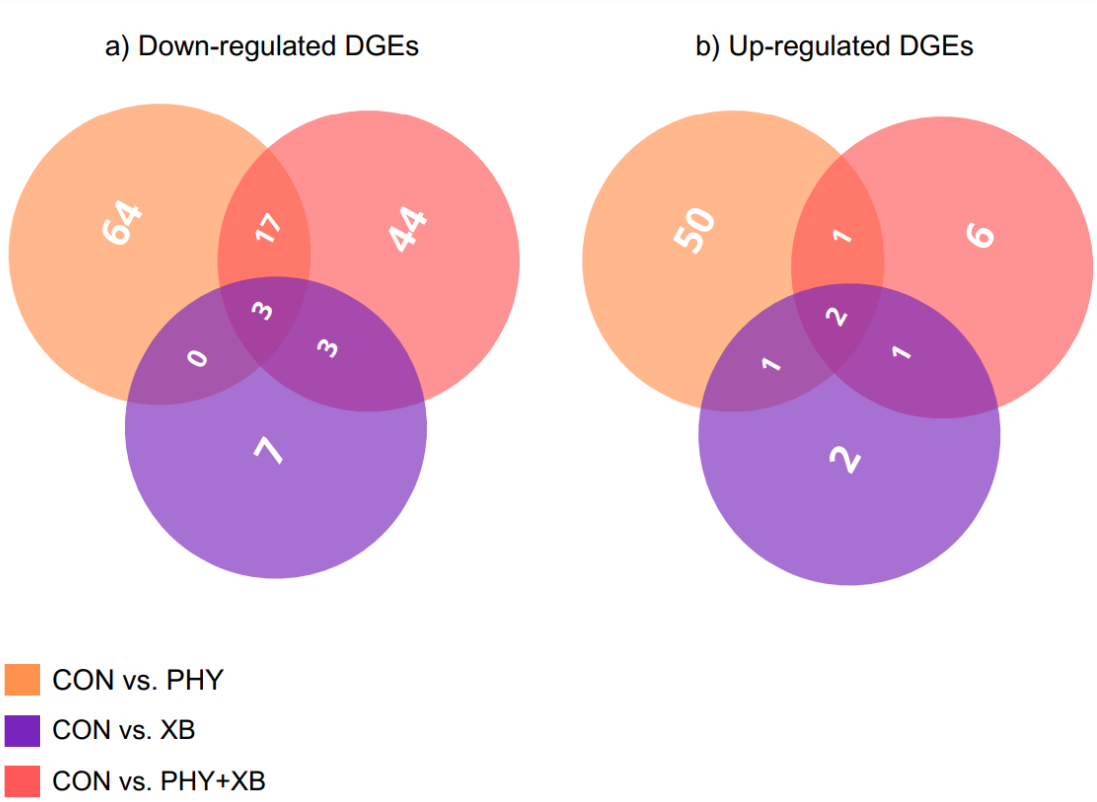

Figure 3. Number of unique and shared a) down-regulated DGEs and b) up-regulated DGEs, in the three comparisons: CON *vs.* PHY, CON *vs.* XB, and CON *vs.* PHY+XB. Abbreviations: CON: control diet, PHY: diet with phytase, XB: diet with xylanase-β-glucanase, and PHY+XB: diet with phytase and xylanase-β-glucanase.

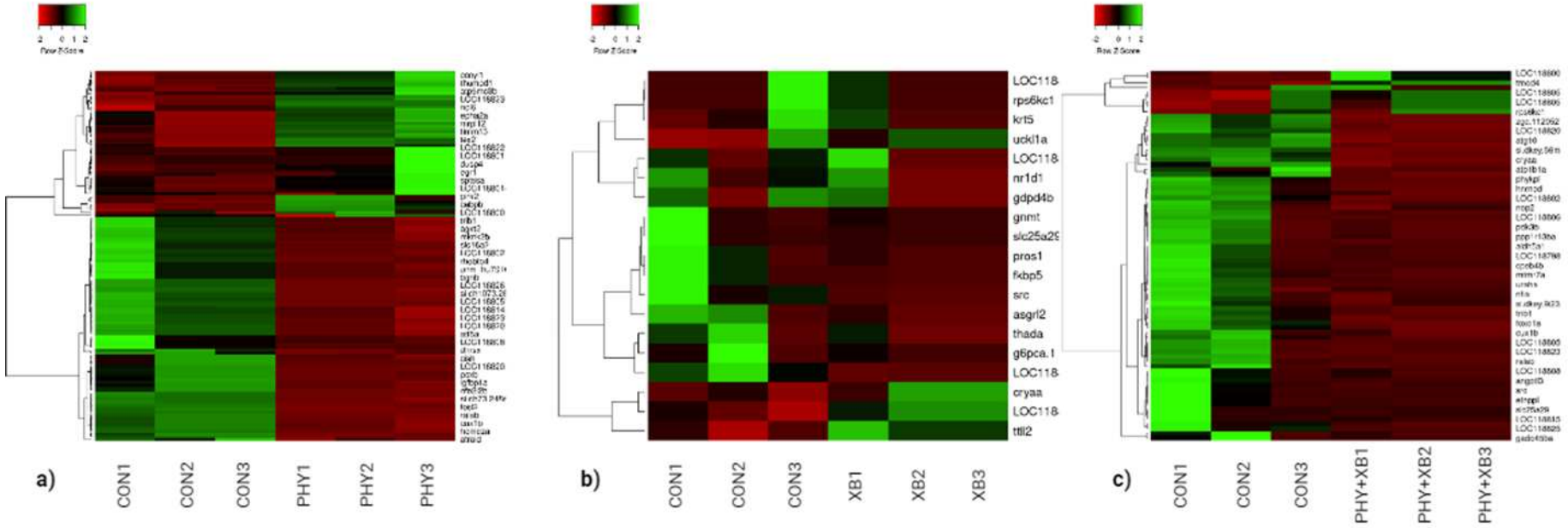

Figure 4. Heat meaps of the up and down-regulated DGE's in the three comparisons: a) CON *vs.* PHY, b) CON *vs.* XB, and c) CON *vs.* PHY+XB. Abbreviations: CON: control diet, PHY: diet with phytase, XB: diet with xylanase-β-glucanase, and PHY+XB: diet with phytase and xylanase-β-glucanase. Green: up-regulated DGE's and Red: down-regulated DGE's.

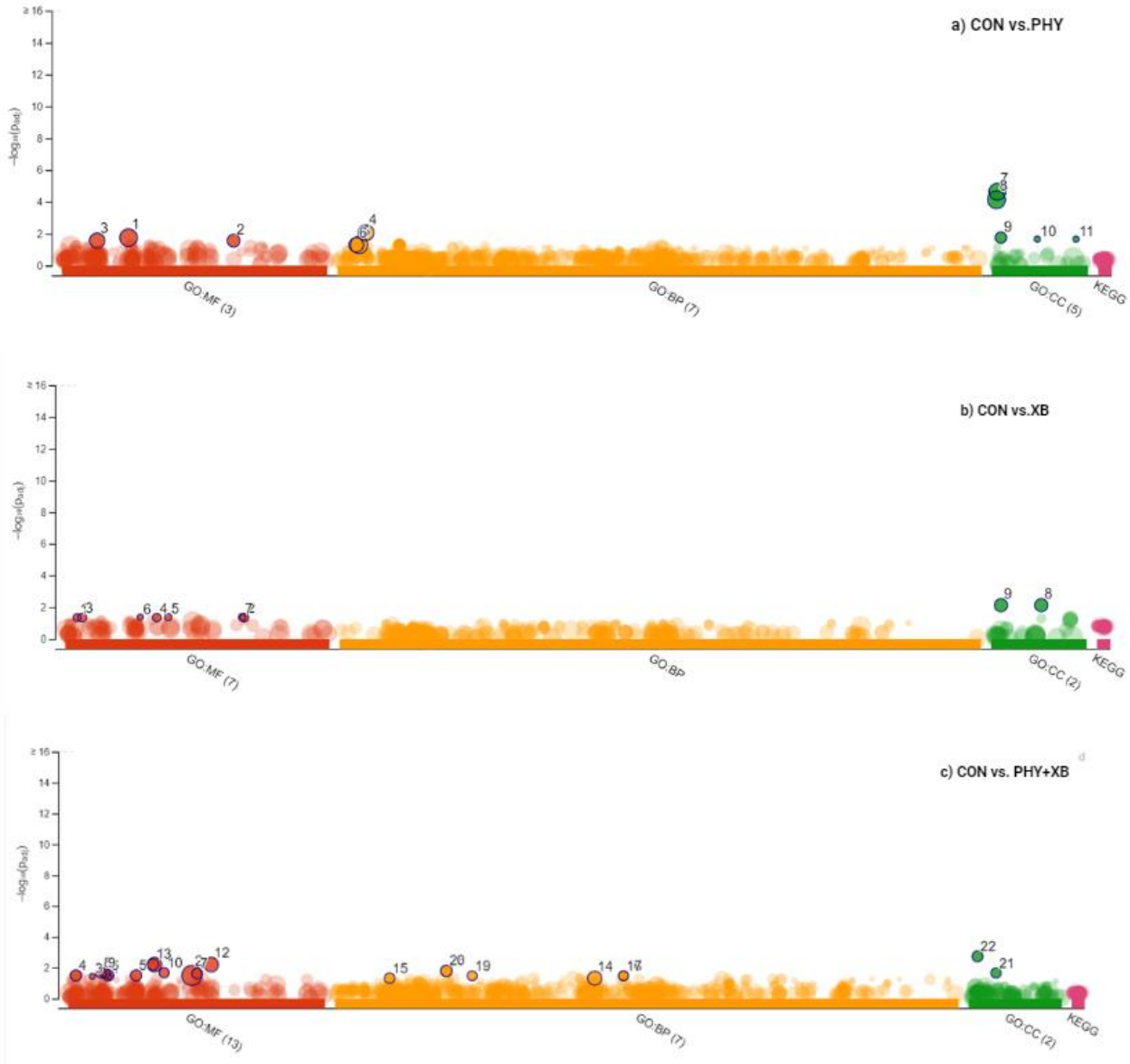

Figure 5. Manhattan GOSt multiquery graph of the transcriptomes of all DGE's (Deseq and Edger) using g: Profiler. Significant results (FDR < 0.05) are highlighted. The Y-axis represents Benjamini-corrected P-values (log10) and the X-axis represents functional terms grouped and color-coded by data source. The position of terms in the frames is fixed and terms from the same branch (Gene Ontology-GO) are close to each other. Abbreviations: CON: control diet, PHY: diet with phytase, XB: diet with xylanase-β-glucanase, and PHY+XB: diet with phytase and xylanase-β-glucanase, GO:MF: GO terms for molecular function, GO:BP: GO terms for biological process, GO:CC: GO terms for cellular component, KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: false discovery rate, G: OSt: gene ontology statistics.

Table 2. Significant GO terms (p<0.05) observed between contrasts CON *vs.* PHY, CON *vs.* XB and CON *vs.* PHY+XB and the genes* involved in each pathway

CON *vs.* PHY					
Source	**GO:ID**	**Term name**	***p*-value**	**Down-regulated genes**	**Up-regulated genes**
GO:MF	GO:0016491	oxidoreductase activity	0.01758	*hao2, dhrsx, si:ch211-5k11.8, cyp2x9, haao, sqor, bco1l, egln1a, egln3, unm_hu7310*	
GO:MF	GO:0048029	monosaccharide binding	0.02698	*asgrl2, egln1a, egln3*	
GO:MF	GO:0005506	iron ion binding	0.02698	*hbaa1, cyp2x9, haao, egln1a, egln3*	
GO:BP	GO:0002682	regulation of immune system process	0.00842	*si:ch1073-280e3.1, zbtb33, cish, cfbl, c1r, tlr5a*	
GO:BP	GO:0002376	immune system process	0.04982	*si:ch1073-280e3.1 , Ita, zbtb33, cish, cxcl12a, cfbl, c1r, tlr5a*	
GO:BP	GO:0002253	activation of immune response	0.04982	*si:ch1073-280e3.1, cfbl, c1r, tlr5a*	
GO:BP	GO:0006959	humoral immune response	0.04982	*si:ch1073-280e3.1, cfbl, c1r*	
GO:BP	GO:0006958	complement activation classical pathway	0.04982	*si:ch1073-280e3.1, cfbl, c1r*	
GO:BP	GO:0002455	humoral immune response mediated by circulation hemoglobin	0.04982	*si:ch1073-280e3.1, c1r*	
GO:BP	GO:0006956	complemente activation	0.04982	*si:ch1073-280e3.1, cfbl, c1r*	
GO:CC	GO:0005615	extracellular space	0.00002	*si:ch1073-280e3.1, asgrl2, ctsf, cxcl12a, igfbp1a, igf2a, c1r, si:ch1073-416d2.3, smoc1, c5*	
GO:CC	GO:0005576	extracellular region	0.00007	*si:ch1073-280e3.1, Ita, angptl6, ctsf, cxcl12a,*	

				igfbp1a, igf2a, c1r, si:ch1073-416d2.3, smoc1, c5	
GO:CC	GO:0005833	hemoglobin complex	0.01735	*hbba1, si:ch211-5k11.8*	
GO:CC	GO:0043540	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase complex	0.02172	*pfkfb1*	
GO:CC	GO:0140494	migrasome	0.02172	*cxcl12a*	
CON vs. XB					
Source	**GO:ID**	**Term name**	***p*-value**	**Down-regulated genes**	**Up-regulated genes**
GO:MF	GO:0050218	propionate-CoA ligase activity	0.04204	*acss1*	
GO:MF	GO:0017174	glycine N-methyltransferase activity	0.04204	*gnmt*	
GO:MF	GO:0031955	short-chain fatty acid-CoA ligase activity	0.04204	*acss1*	
GO:MF	GO:0030280	structural constituent of skin epidermis	0.04499		*krt5*
GO:MF	GO:0050309	sugar-terminal-phosphatase activity	0.04499	*g6pca.1*	
GO:MF	GO:0004346	glucose-6-phosphatase activity	0.04499	*g6pca.1*	
GO:MF	GO:0003987	acetate-CoA ligase activity	0.04499	*acss1*	
GO:CC	GO:0005882	intermediate filament	0.00745	*cyt1l*	*krt5*
GO:CC	GO:0045111	intermediate filament cytoskeleton	0.00745	*cyt1l*	*krt5*
CON vs. PHY+XB					
Source	**GO:ID**	**Term name**	***p*-value**	**Genes involved**	
GO:MF	GO:0019825	oxygen binding	0.00629	*hbaa2, hbba1*	
GO:MF	GO:0046906	tetrapyrrole binding	0.00629	*cyp2x9, cyp2f2, hbaa2, hbba1*	
GO:MF	GO:0020037	heme binding	0.00629	*cyp2x9, cyp2f2, hbaa2, hbba1*	
GO:MF	GO:0031720	haptoglobin binding	0.02132	*hbaa2*	
GO:MF	GO:0008195	phosphatidate phosphatase activity	0.02176	*lpin1*	
GO:MF	GO:0005344	oxygen carrier activity	0.02176	*hbaa2*	
GO:MF	GO:0042577	lipid phosphatase activity	0.02371	*lpin1*	
GO:MF	GO:0008483	transaminase activity	0.03166	*phykpl, etnppl*	
GO:MF	GO:0016769	transferase activity, transferring nitrogenous groups	0.03166	*phykpl, etnppl*	

GO:MF	GO:0003707	nuclear steroid receptor activity	0.03166	*rxraa, nr3c1*
GO:MF	GO:0036094	small molecule binding	0.03166	*ralaa, cryaa, phykpl, asgrl2, arap1a, ece2b, trib1, cyp2x9, myh10, CR855320.3, cyp2f2, ftr97, pdk3b, rxraa, nr3c1, src, clec14a, etnppl, rhobtb4, hbaa2, hbba1, rps6kc1, uckl1a*
GO:MF	GO:0004777	succinate-semialdehyde dehydrogenase (NAD^+) activity	0.03643	*aldh5a1*
GO:MF	GO:0008523	sodium-dependent multivitamin transmembrane transporter activity	0.03643	*slc5a6b*
GO:BP	GO:0015669	gas transport	0.01589	*hbaa2, hbba1*
GO:BP	GO:0015671	oxygen transport	0.01589	*hbaa2, hbba1*
GO:BP	GO:0019432	triglyceride biosynthetic process	0.03303	*lpin1*
GO:BP	GO:0046463	acylglycerol biosynthetic process	0.03303	*lpin1*
GO:BP	GO:0046460	neutral lipid biosynthetic process	0.03303	*lpin1*
GO:BP	GO:0006641	triglyceride metabolic process	0.04740	*lpin1*
GO:BP	GO:0044282	small molecule catabolic process	0.04740	*aldh5a1, uraha, lpin1*
GO:CC	GO:0005833	hemoglobin complex	0.00184	*hbaa2, hbba1*
GO:CC	GO:0031838	haptoglobin-hemoglobin complex	0.02207	*hbba2*

*The genes are homologous to *Danio rerio*. Abbreviations: CON: control diet, PHY: diet with phytase, XB: diet with xylanase-β-glucanase, and PHY+XB: diet with phytase and xylanase-β-glucanase.

Supplementary files

Differential gene expression of Amazonian tambaqui (*Colossoma macropomum*) fed plant-based diets supplemented with phytase and xylanase-β-glucanase

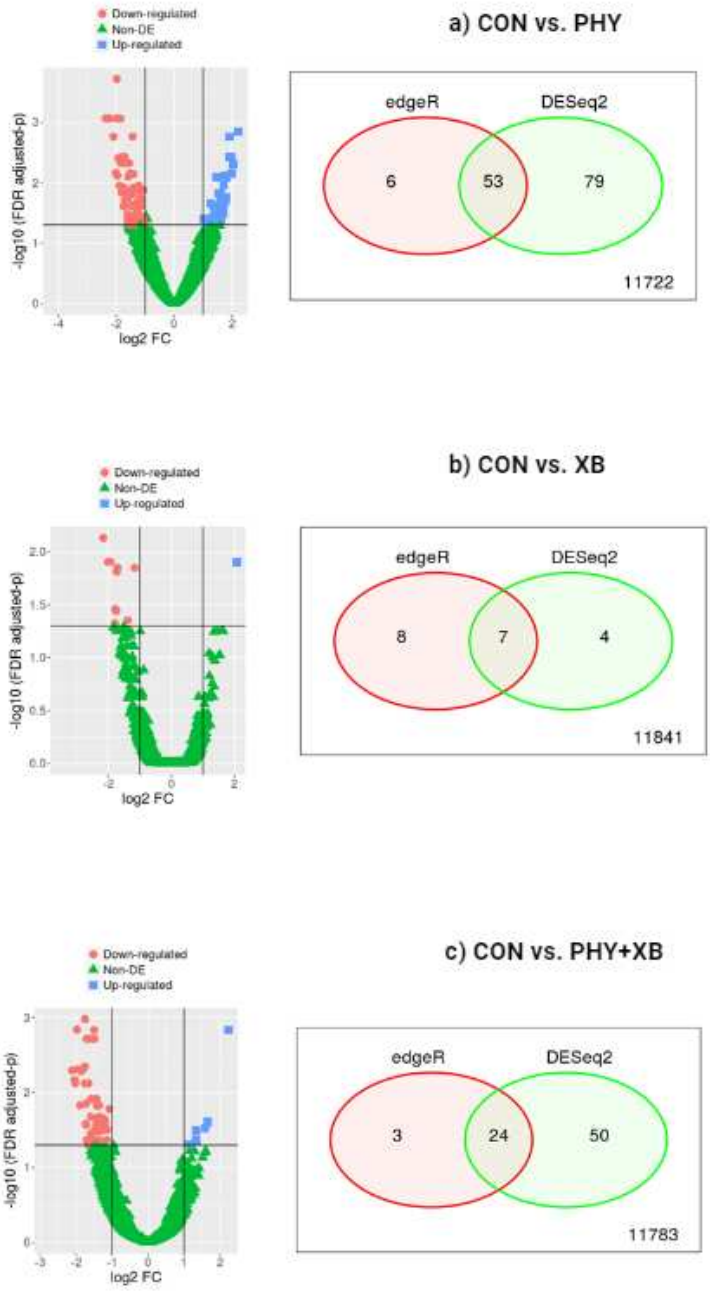

Figure S1. Normalization graphs of counts and number of DGEs obtained in the three comparisons: a) CON *vs.* PHY, b) CON vs. XB, and c) CON *vs.* PHY+XB. using the edgeR and DEseq methods on the DEApp platform. Abbreviations: CON: control diet,

PHY: diet with phytase, XB: diet with xylanase-β-glucanase, and PHY+XB: diet with phytase and xylanase-β-glucanase.

Table S1. All differentially expressed genes (DGEs) between fish fed the CON diet (control) and fish fed the PHY diet (phytase)

CON *vs.* PHY							
edgeR and Deseq2 overlap	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
ifit8	118810341	−4.904186366	6.36E^{-06}	0.007539132	−2.369326146	3.17E^{-07}	0.000874349
hbbe2	118805793	−4.778965212	7.16E^{-06}	0.007724589	−1.889345973	3.32E^{-05}	0.011276593
ppp1r13ba	118823572	−3.580736689	5.81E^{-05}	0.022231299	−2.014951123	1.20E^{-05}	0.006942735
tlr5b	118820884	−3.349646378	6.13E^{-06}	0.007539132	−2.237838375	3.64E^{-07}	0.000874349
hbaa1	118805798	−3.34812049	0.000204584	0.045152079	−1.954751777	1.74E^{-05}	0.007715656
bco1	118818629	−3.185330158	4.24E^{-06}	0.006984899	−2.098985243	1.50E^{-06}	0.001735929
LOC118801818	118801818	−3.026114134	5.35E^{-05}	0.021892525	−1.815137935	5.26E^{-05}	0.014490964
hif1al2	118819818	−2.715786355	1.40E^{-05}	0.012813088	−1.922069026	5.22E^{-06}	0.004024373
LOC118805991	118805991	−2.68146001	0.000137237	0.034962235	−1.739021479	5.59E^{-05}	0.014978419
igfbp1b	118825612	−2.676998889	0.000139191	0.034962235	−1.774878086	4.05E^{-05}	0.012315046
arl6ip6	118807764	−2.607684444	4.25E^{-06}	0.006984899	−1.871467478	4.32E^{-06}	0.003811909
igf2a	118817533	−2.575685795	4.17E^{-06}	0.006984899	−1.986533514	3.78E^{-07}	0.000874349
clec	118824387	−2.532930239	5.11E^{-05}	0.02162744	−1.847258158	7.12E^{-06}	0.004780872
nlrp3l	118826817	−2.518395983	0.00016919	0.040131762	−1.697458204	9.83E^{-05}	0.019593438
src	118822154	−2.503068271	2.19E^{-06}	0.006506109	−1.994334498	2.02E^{-07}	0.000874349
nfe2l2b	118807814	−2.439808296	6.32E^{-05}	0.022876086	−1.737007885	3.71E^{-05}	0.012129178
raraa	118809677	−2.390710099	7.15E^{-08}	0.000424054	−1.976948748	1.69E^{-08}	0.000194872
si:ch211−195b13.1	118805276	−2.349396739	8.01E^{-07}	0.003168494	−1.865357817	4.57E^{-07}	0.000880386
zbtb33	118807157	−2.288911351	5.59E^{-05}	0.022112071	−1.618515291	8.19E^{-05}	0.017866928
rhobtb4	118826937	−2.143044742	1.65E^{-05}	0.012951995	−1.699894835	6.94E^{-06}	0.004780872
pcxb	118826304	−2.107436402	0.000118878	0.032042997	−1.588577762	6.62E^{-05}	0.016276117
mknk2b	118813913	−2.105543802	1.18E^{-05}	0.011644899	−1.703796225	3.59E^{-06}	0.003775885
ifi44b	118808604	−2.068554144	0.000147743	0.035759913	−1.588777113	7.99E^{-05}	0.017756604
trib1	118805469	−1.975317475	3.26E^{-05}	0.018404308	−1.588030552	1.96E^{-05}	0.008083526
gig2p	118814287	−1.872600357	0.0001415	0.034962235	−1.452682038	0.000130217	0.0233612

sqor	118811699	−1.872254045	2.23E^{-05}	0.014697437	−1.563333294	7.44E^{-06}	0.004780872
akap9	118805453	−1.866805046	3.68E^{-05}	0.019024382	−1.535549974	1.28E^{-05}	0.007050939
bgnb	118822102	−1.82194013	8.05E^{-05}	0.024755073	−1.458295822	6.33E^{-05}	0.015903696
slc16a2	118821018	−1.780631957	0.000106281	0.030011736	−1.466440939	4.90E^{-05}	0.013827881
arap1a	118802270	−1.683999035	7.17E^{-05}	0.024305135	−1.414731469	3.88E^{-05}	0.012129178
cyp2f2	118806691	−1.661108016	6.37E^{-05}	0.022876086	−1.423404874	1.42E^{-05}	0.007160255
cfbl	118819932	−1.625346132	4.45E^{-05}	0.021162446	−1.436775415	1.44E^{-06}	0.001735929
cd302	118806281	−1.624241423	7.58E^{-05}	0.024755073	−1.401715914	1.51E^{-05}	0.007265293
unm_hu7910	118825690	−1.380004459	0.00024041	0.049147983	−1.226341328	3.17E^{-05}	0.011112184
mettl2a	118821079	1.669027092	0.000183654	0.042708619	1.367466142	0.000141432	0.024404362
timm13	118818953	1.670875245	0.000232518	0.048380061	1.359634749	0.000146984	0.024753436
cog8	118817692	1.739465847	4.84E^{-05}	0.02124553	1.459102736	2.19E^{-05}	0.008180447
rtca	118819392	1.766699544	0.000220849	0.046772702	1.412030453	0.000153115	0.024753436
ubiad1	118813541	1.860400703	4.49E^{-05}	0.021162446	1.528186568	2.12E^{-05}	0.008180447
ccnyl1	118808834	1.982616958	0.000138635	0.034962235	1.549142487	5.89E^{-05}	0.015122839
chac1	118815682	2.114399103	0.000244497	0.049147983	1.609655493	5.70E^{-05}	0.014978419
trmt61b	118823942	2.132668855	0.000116467	0.032042997	1.581640858	9.15E^{-05}	0.018882321
nol6	118827348	2.291179266	8.14E^{-05}	0.024755073	1.705841825	3.17E^{-05}	0.011112184
ttll2	118815733	2.437748351	2.92E^{-05}	0.018249056	1.656913548	7.54E^{-05}	0.017401997
pim2	118813798	2.439850699	6.84E^{-05}	0.023854785	1.772267977	1.72E^{-05}	0.007715656
dnaJ homolog subfamily B member 1-like	118801139	2.548331892	4.71E^{-06}	0.006984899	1.916423226	1.46E^{-06}	0.001735929
mitoregulin	118823670	2.637969158	4.64E^{-05}	0.021162446	1.829438732	1.83E^{-05}	0.0078475
nipa2	118808227	2.66686626	0.000104415	0.030011736	1.83034762	2.19E^{-05}	0.008180447
dusp4	118820305	2.71148599	2.10E^{-05}	0.014663242	1.919211213	4.62E^{-06}	0.003811909
ier2a	118802494	2.776802796	3.69E^{-05}	0.019024382	1.965874119	4.23E^{-06}	0.003811909
cd22	118800122	2.896031038	9.12E^{-05}	0.027046803	1.54496797	0.000519249	0.048805228
myca	118799079	3.483217339	1.75E^{-05}	0.012951995	2.225191223	8.73E^{-07}	0.001442535
egr1	118807052	3.643744933	7.84E^{-05}	0.024755073	2.042099494	8.24E^{-06}	0.005015279

edgeR only	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
LOC118801662	118801662	−8.375014803	$1.67E^{-05}$	0.012951995	–	–	–
keg	118815655	−6.319507083	$3.27E^{-13}$	$3.88E^{-09}$	–	–	–
LOC118800988	118800988	3.126164333	0.00020939	0.045152079	–	–	–
LOC118801065	118801065	3.127881358	0.000207516	0.045152079	–	–	–
LOC118815145	118815145	3.160858505	0.000201047	0.045152079	–	–	–
rps6kc1	118815259	7.792618568	$3.13E^{-05}$	0.018404308	–	–	–
Deseq2 only	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
lta	118803816	–	–	–	−1.752406179	0.000156301	0.024753436
igfbp1a	118815472	–	–	–	−1.611394649	0.000234613	0.033077527
cish	118808348	–	–	–	−1.605149787	0.000310866	0.039716596
fosl2	118823528	–	–	–	−1.602135018	0.000504305	0.048709479
sh3glb2a	118813495	–	–	–	−1.568925903	0.0004114	0.044514987
angptl6	118806145	–	–	–	−1.531865634	0.000307239	0.039716596
chia.2	118800527	–	–	–	−1.525914443	0.000478794	0.04813338
egln3	118823274	–	–	–	−1.488580197	0.000139349	0.024404362
cdh2	118817327	–	–	–	−1.48329907	0.000539946	0.049542186
homeza	118826209	–	–	–	−1.461874698	0.000559878	0.04969618
LOC118823972	118823972	–	–	–	−1.456753982	0.000301384	0.039594377
rab40b	118809335	–	–	–	−1.449947189	0.000333624	0.039720569
irs1	118798903	–	–	–	−1.442994118	0.000417472	0.044514987
dhrsx	118803054	–	–	–	−1.442228943	0.000472447	0.048099017
cux1b	118797261	–	–	–	−1.421928586	0.00048651	0.048458648
pde4d	118819278	–	–	–	−1.414864292	0.000331929	0.039720569
pfkfb1	118822062	–	–	–	−1.413816427	0.000338136	0.039720569
SE-cephalotoxin-like	118824004	–	–	–	−1.375159778	0.000561004	0.04969618
ralab	118796823	–	–	–	−1.350975274	0.000413449	0.044514987
atraid	118815517	–	–	–	−1.334761442	0.000274053	0.036841001
agxt2	118820452	–	–	–	−1.32612672	0.000231325	0.03301663

mtmr7a	118806948	−	−	−	−1.309681689	0.000509804	0.048709479
itih3l	118799317	−	−	−	−1.296324588	0.000114159	0.021635948
ctsf	118806838	−	−	−	−1.291122441	$3.86E^{-05}$	0.012129178
nxpe3	118820907	−	−	−	−1.290202838	0.00019005	0.028943215
cxcl12a	118810424	−	−	−	−1.286507423	0.000335425	0.039720569
bnip3	118809090	−	−	−	−1.285991342	0.000190268	0.028943215
LOC118805986	118805986	−	−	−	−1.262355904	0.000206653	0.031027463
si:ch211−175m2.5	118802624	−	−	−	−1.24686443	0.000131345	0.0233612
LOC118805987	118805987	−	−	−	−1.246148836	$4.43E^{-05}$	0.013144259
dlg1l	118796740	−	−	−	−1.240684224	0.000374406	0.042024362
si:ch1073−280e3.1	118802624	−	−	−	−1.231588706	0.000123575	0.022677049
nkt1	118798964	−	−	−	−1.22189383	0.000494604	0.048458648
hif1an	118810449	−	−	−	−1.198218851	0.000330464	0.039720569
c1sl1	118820650	−	−	−	−1.193775745	0.000363285	0.041583509
cfhl	118818710	−	−	−	−1.183610209	$8.42E^{-05}$	0.018009037
hhip	118810845	−	−	−	−1.162442038	0.000109674	0.021132411
c5	118826942	−	−	−	−1.153937798	0.000153803	0.024753436
LOC118805984	118805984	−	−	−	−1.146527657	$8.57E^{-05}$	0.018009037
si:ch1073−416d2.3	118823705	−	−	−	−1.145134201	0.000259182	0.036101275
foxa3	118817996	−	−	−	−1.119202404	0.000527216	0.049154422
haao	118810212	−	−	−	−1.095951671	0.000517759	0.048805228
smoc1	118823737	−	−	−	−1.08425773	0.00050666	0.048709479
acy1l	118799443	−	−	−	−1.077202485	0.000556522	0.04969618
aqp9b	118817575	−	−	−	−1.073638132	0.000340138	0.039720569
hao2	118800172	−	−	−	−1.067790645	0.000312621	0.039716596
asgrl2	118802057	−	−	−	−1.067041228	$4.59E^{-05}$	0.013272414
atf5a	118814733	−	−	−	−1.024643738	0.00044068	0.046315492
slc39a8	118821174	−	−	−	1.028596031	0.000344522	0.039830191
atp5mc3b	118807817	−	−	−	1.111202237	0.000445039	0.046352171

mrpl12	118806101	–	–	–	1.116668815	0.000532375	0.0492383
mpc2b	118816047	–	–	–	1.196506754	0.000391341	0.043088515
uqcrfs1b	118818156	–	–	–	1.262619003	0.000118705	0.02213473
tusc2b	118799776	–	–	–	1.276343891	0.000419698	0.044514987
thumpd1	118798310	–	–	–	1.28343695	0.000457728	0.047248141
usp3	118811666	–	–	–	1.320345507	0.000290485	0.038601159
slc35b3	118800436	–	–	–	1.341261797	0.000338737	0.039720569
tbcc	118809361	–	–	–	1.353682961	0.000490926	0.048458648
fam136a	118825236	–	–	–	1.356435311	0.000474292	0.048099017
tex2	118821182	–	–	–	1.358140089	0.000269715	0.036684401
epha2a	118808468	–	–	–	1.387040067	0.000553716	0.04969618
srsf10l	118812806	–	–	–	1.413047685	0.000373069	0.042024362
prps1b	118807365	–	–	–	1.427642509	0.000577466	0.049821558
cebpb	118813419	–	–	–	1.428954928	0.000182387	0.028494259
hpdl	118808788	–	–	–	1.444109989	0.000227938	0.032939867
si:ch211-214j24.10	118812208	–	–	–	1.484662587	0.000219388	0.032277358
ing2	118810805	–	–	–	1.495178944	0.00057707	0.049821558
sptssa	118824027	–	–	–	1.532284725	0.000563117	0.04969618
slc35e1	118818894	–	–	–	1.538063033	0.000575759	0.049821558
golt1bb	118811613	–	–	–	1.61821945	0.000386885	0.043007486
nup50	118812405	–	–	–	1.636140045	0.000151998	0.024753436
flvcr1	118815221	–	–	–	1.692346075	0.000220561	0.032277358
axin2	118821249	–	–	–	1.696366303	0.000154965	0.024753436
ddx5	118821132	–	–	–	1.711094104	$7.51E^{-05}$	0.017401997
abcf2a	118826572	–	–	–	1.772957193	$9.67E^{-05}$	0.019593438
smim11a	118798405	–	–	–	1.787473979	0.000103201	0.020222091
sgk1	118822004	–	–	–	1.789484855	$7.68E^{-05}$	0.017401997
LOC118801435	118801435	–	–	–	1.813067426	$7.31E^{-05}$	0.017401997
lipg	118813031	–	–	–	1.994050118	$1.42E^{-05}$	0.007160255

Table S2. Differentially expressed genes (DGEs) between fish fed the CON diet (control) and fish fed the XB diet (xylanase-β-glucanase)

CON *vs.* XB							
edgeR and Deseq2 overlap	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
fkbp5	118808338	−3.207705449	9.92E^{-06}	0.0236651	−2.155187699	7.78E^{-07}	0.007394133
slc25a29	118815601	−2.975205121	5.60E^{-05}	0.048008961	−2.003120917	5.28E^{-06}	0.012526556
nr1d1	118821148	−2.758488191	1.27E^{-05}	0.025144768	−1.933365241	4.03E^{-06}	0.012526556
acas	118802910	−2.46755224	6.09E^{-05}	0.04816677	−1.744177236	3.41E^{-05}	0.036027335
thada	118810216	−2.276633088	3.93E^{-05}	0.048008961	−1.729031727	1.13E^{-05}	0.01537267
src	118822154	−2.127050682	4.42E^{-05}	0.048008961	−1.705793815	8.66E^{-06}	0.014154511
cd22	118800122	3.652447507	1.27E^{-06}	0.007513814	2.066222253	3.32E^{-06}	0.012526556
edgeR only	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
gdpd4b	118820389	−8.5047	5.41E^{-05}	0.048009	–	–	–
g6pca.1	118809163	−5.0793	1.97E^{-06}	0.007802	–	–	–
keg	118815655	−5.0389	6.19E^{-10}	7.34E^{-06}	–	–	–
ttll2	118815733	2.36914	4.54E^{-05}	0.048009	–	–	–
krt5	118821990	2.6286	5.67E^{-05}	0.048009	–	–	–
uckl1a	118813153	5.58881	2.73E^{-05}	0.046235	–	–	–
rps6kc1	118815259	8.33857	9.98E^{-06}	0.023665	–	–	–
krt13	118805184	9.79346	5.15E^{-05}	0.048009	−1.752406179	0.000156301	0.024753436
Deseq2 only	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
cryaa	118797904	–	–	–	−1.778476025	2.93E^{-05}	0.034779
gnmt	118823180	–	–	–	−1.777450616	5.50E^{-05}	0.047464
pros1	118797881	–	–	–	−1.380220172	4.67E^{-05}	0.044382
asgrl2	118802057	–	–	–	−1.162107737	8.94E^{-06}	0.014155

Table S3. Differentially expressed genes (DGEs) between fish fed the CON diet (control) and fish fed the PHY+XB diet (phytase + xylanase-β-glucanase)

CON *vs.* PHY+XB							
edgeR and Deseq2 overlap	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
trim39	118825457	−4.904824467	5.33E^{-05}	0.026319638	−2.018225529	1.23E^{-05}	0.007512149
lta	118803816	−4.44747306	2.92E^{-05}	0.019400667	−2.111703245	5.22E^{-06}	0.005040012
cryaa	118797904	−2.797699452	1.63E^{-05}	0.013839282	−1.950983955	4.57E^{-06}	0.004893504
dr1	118823519	−2.608296768	9.97E^{-06}	0.013135681	−1.867610375	6.58E^{-06}	0.005174246
src	118822154	−2.470024338	2.84E^{-06}	0.008431091	−1.979293158	2.46E^{-07}	0.001449533
zgc:112052	118818158	−2.391119581	2.41E^{-05}	0.019057711	−1.823148699	5.99E^{-06}	0.005040012
ifi44b	118808604	−2.3851374	1.47E^{-05}	0.013378913	−1.825120181	5.70E^{-06}	0.005040012
LOC118823972	118805987	−2.312917416	3.27E^{-05}	0.019400667	−1.758451064	1.27E^{-05}	0.007512149
popdc2	118799750	−2.251592203	5.83E^{-06}	0.009891531	−1.756111197	3.82E^{-06}	0.004504978
zfp585a	118812818	−2.0945762	4.78E^{-05}	0.024640841	−1.587668731	5.17E^{-05}	0.020997993
rhobtb4	118826937	−2.059161503	3.14E^{-05}	0.019400667	−1.651109659	1.22E^{-05}	0.007512149
mtmr7a	118806948	−2.052994241	2.66E^{-05}	0.019400667	−1.65664312	1.10E^{-05}	0.007512149
nktr	118798964	−2.047403548	4.11E^{-06}	0.009749027	−1.704313753	1.19E^{-06}	0.001917945
cyp2f2	118806691	−2.038914765	1.21E^{-06}	0.004802409	−1.753231525	8.87E^{-08}	0.001045221
trib1	118805469	−1.940133483	3.96E^{-05}	0.022339434	−1.573295057	2.17E^{-05}	0.012135715
eppk1	118806199	−1.93796545	1.24E^{-05}	0.013378913	−1.649391806	1.43E^{-06}	0.001917945
LOC118802431	118802431	−1.76852773	5.84E^{-06}	0.009891531	−1.533489831	1.46E^{-06}	0.001917945
smoc1	118808604	−1.719690511	1.38E^{-05}	0.013378913	−1.504379699	1.40E^{-06}	0.001917945
LOC118805987	118805987	−1.686451071	3.06E^{-05}	0.019400667	−1.488316402	1.07E^{-06}	0.001917945
uraha	118817889	−1.682579155	1.32E^{-05}	0.013378913	−1.49587046	4.97E^{-07}	0.001465571
myh10	118808147	−1.681599802	6.33E^{-05}	0.030020097	−1.432194208	2.26E^{-05}	0.012135715
arap1a	118802270	−1.680034434	6.99E^{-05}	0.031862924	−1.42362782	3.31E^{-05}	0.015028628
spty2d1	118811533	−1.582459628	7.61E^{-05}	0.033441475	−1.363889429	3.13E^{-05}	0.014753391
cd22	118800122	3.911022058	2.66E^{-07}	0.00176677	2.238905579	4.62E^{-07}	0.001465571

edgeR only	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
keg	118815655	−3.9599	2.98E^{-07}	0.001767	–	–	–
uckl1a	118813153	5.38252	4.75E^{-05}	0.024641	–	–	–
rps6kc1	118815259	8.42139	8.39E^{-06}	0.012443	–	–	–
Deseq2 only	gene_id	edgeR_logFC	edgeR_pvalue	edgeR_FDR	DESeq2_log2FC	DESeq2_pvalue	DESeq2_padj
lpin1	118823353	–	–	–	−2.038724761	9.17E^{-06}	0.006753
LOC118823714	118823714	–	–	–	−1.894892617	3.06E^{-05}	0.014753
slc25a29	118815601	–	–	–	−1.731324129	8.26E^{-05}	0.026086
entpd2b	118826936	–	–	–	−1.72758096	2.73E^{-05}	0.014012
tlr5a	118820884	–	–	–	−1.720359719	8.94E^{-05}	0.027036
cyp2j13	118815406	–	–	–	−1.70127183	0.000219371	0.043099
klhl30	118808045	–	–	–	−1.670875291	0.000307784	0.049154
gadd45ba	118818878	–	–	–	−1.666271995	0.00027544	0.048461
ppp1r13ba	118823572	–	–	–	−1.658104753	0.000308567	0.049154
foxo1a	118802946	–	–	–	−1.596749229	0.000233572	0.043704
si:dkey-9i23.6	118810538	–	–	–	−1.59483932	0.00016835	0.035438
clec14a	118823421	–	–	–	−1.575742489	0.000216996	0.043099
cux1b	118797261	–	–	–	−1.533270647	0.000162701	0.034871
magi3a	118822346	–	–	–	−1.500186767	0.00021868	0.043099
nop2	118817716	–	–	–	−1.474961363	0.000307082	0.049154
ralab	118796823	–	–	–	−1.471092544	0.000111071	0.030449
atp1b1a	118808012	–	–	–	−1.464873946	0.000122693	0.031514
gpr146	118805766	–	–	–	−1.445594301	0.0002873	0.048635
rxraa	118819959	–	–	–	−1.439438351	0.000130996	0.031514
pla2g3	118824426	–	–	–	−1.433234314	8.27E^{-05}	0.026086
sun1	118820933	–	–	–	−1.424615003	0.00014108	0.032706
cpeb4b	118820264	–	–	–	−1.415643514	5.88E^{-05}	0.022401
c18orf63	118816054	–	–	–	−1.398819221	0.000145285	0.032706
atg10	118824565	–	–	–	−1.381768956	0.000285348	0.048635

angptl3	118807968	−	−	−	−1.370321854	0.000147047	0.032706
gja12.1	118815758	−	−	−	−1.353491851	5.00E^{-05}	0.020998
slc5a6b	118823723	−	−	−	−1.352702275	0.000227286	0.043387
tspan5b	118816983	−	−	−	−1.318587751	0.000292934	0.048635
nfia	118819555	−	−	−	−1.31751574	8.41E^{-05}	0.026086
tchhl1	118804524	−	−	−	−1.297431403	0.000150198	0.032788
gp1	118817646	−	−	−	−1.296395434	0.000107175	0.03008
fndc3a	118802869	−	−	−	−1.28524108	0.000248118	0.0457
etnppl	118826549	−	−	−	−1.268817453	0.000252327	0.045761
hnrnpd	118824369	−	−	−	−1.253039033	0.000128323	0.031514
phykpl	118800825	−	−	−	−1.225841393	6.47E^{-05}	0.023819
LOC118805988	118805988	−	−	−	−1.219198272	5.89E^{-05}	0.022401
ece2b	118802640	−	−	−	−1.21615601	0.000256476	0.045808
nr3c1	118820212	−	−	−	−1.207276688	9.44E^{-05}	0.027815
pdk3b	118819870	−	−	−	−1.200479561	7.00E^{-05}	0.024278
cfhl	118818710	−	−	−	−1.151934254	0.000129576	0.031514
aldh5a1	118800372	−	−	−	−1.146599231	0.000210733	0.043099
cfbl	118819932	−	−	−	−1.143556252	0.000125059	0.031514
asgrl2	118802057	−	−	−	−1.077109738	3.80E^{-05}	0.016596
slc30a4	118803971	−	−	−	1.140033538	0.000289755	0.048635
hbbe2	118805788	−	−	−	1.325725925	0.000145278	0.032706
hbbe2	118805792	−	−	−	1.340467462	0.000125151	0.031514
tmod4	118817539	−	−	−	1.341332534	0.000228195	0.043387
hbae1	118805784	−	−	−	1.587044806	0.000103344	0.029713
hbbe1	118805783	−	−	−	1.656628073	6.89E^{-05}	0.024278
dusp4	118819870	−	−	−	1.659302661	7.45E^{-05}	0.025094

ANEXO I

UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
CEUAP/UFV

Campus Universitário – Viçosa, MG – 36570-900 – Telefone:(31) 3899.3275 – e-mail: ceuap@ufv.br – site: www.ceuap.ufv.br

Viçosa, 09 de Jun. de 2021

CERTIFICADO

Certificamos que o projeto intitulado **"Suplementação dietética com carboidrases e fitase exógenas para o tambaqui (Colossoma macropomum): efeitos sobre crescimento, microbiota intestinal e metabolismo de carboidratos"**, protocolo **nº 024/2021**, sob a responsabilidade de **Ana Lucia Salaro** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi apreciado pela Comissão de Ética no Uso de Animais de Produção da Universidade Federal de Viçosa (CEUAP-UFV) em reunião de **19 de Maio de 2021.**

Finalidade: **(x)Pesquisa** **( )Ensino** Vigência do Projeto: de **10 de Out. de 2021** a **10 de Out. 2024**
Espécie/linhagem: ***Tambaqui (Colossoma macropomum)*** Nº de animais: **384**
Peso: 1 g Idade: 1 mês Sexo: Origem: Universidade Federal de Minas Gerais Cnpj/CPF 17.217.985/0001-04 Endereço: Av. Antônio Carlos, 6627 – CEP: 31270-901 Responsável:Prof. Dr. Ronald Kennedy Luz

CERTIFICATE

We certify that the project entitled **"Dietary supplementation with exogenous carbohydrases and phytase for tambaqui (Colossoma macropomum): effects on growth, intestinal microbiota and carbohydrate metabolism",** protocol **nº 024/2021**, under the responsibility of **Ana Lucia Salaro** - which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **May, 19th of 2021**.

Finality: **(x)Research** **( )Education**Duration of the Project: from **Oct. 10th, of 2021** to **Oct. 10th, of 2024**. Species / strain: **Tambaqui (Colossoma macropomum)** Nº of animals: **384**
Weight: 1 g Age: 1 month Sex: Source: Universidade Federal de Minas Gerais Cnpj/CPF 17.217.985/0001-04 Endereço: Av. Antônio Carlos, 6627 – CEP: 31270-901 Responsável:Prof. Dr. Ronald Kennedy Luz

Luciana Navajas Rennó
Coordenadora da CEUAP/UFV