UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

β-MANNANASE ON TOP OF EXTRUDED DIETS FOR NILE TILAPIA

Autor: Thaís Pereira da Cruz
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β-MANNANASE IN EXTRUDED DIETS FOR NILE TILAPIA (*Oreochromis niloticus*)

Autora: Thais Pereira da Cruz
Orientador: Prof. Dr. Wilson Massamitu Furuya

TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

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Prof. Dr. Wilson Massamitu Furuya
Orientador

Que a força do medo que tenho, não me impeça de ver o que anseio
Que a morte de tudo em que acredito, não me tape os ouvidos e a boca
Pois metade de mim é o que eu grito; a outra metade é silêncio
Que a música que ouço ao longe, seja linda ainda que tristeza
Que o homem que amo seja pra sempre amado, mesmo que distante
Pois metade de mim é partida, a outra metade é saudade
Que as palavras que falo, não sejam ouvidas como prece, nem repetidas com fervor
Apenas respeitadas, como a única coisa que resta a um homem inundado de sentimentos
Pois metade de mim é o que ouço, a outra metade é o que calo
Que a minha vontade de ir embora, se transforme na calma e paz que mereço
Que a tensão que me corrói por dentro, seja um dia recompensada
Porque metade de mim é o que penso, a outra metade um vulcão
Que o medo da solidão se afaste, e o convívio comigo mesmo se torne ao menos suportável
Que o espelho reflita meu rosto num doce sorriso, que me lembro ter dado na infância
Pois metade de mim é a lembrança do que fui, a outra metade não sei
Que não seja preciso mais do que uma simples alegria, pra me fazer aquietar o espírito
E que o seu silêncio me fale cada vez mais, pois metade de mim é abrigo, a outra metade é cansaço
Que a minha loucura seja perdoada
Pois metade de mim é amor, e a outra metade também

Oswaldo Montenegro

A minha família, John Wesley Nazar da Cruz, Mirian Margarete Pereira Brisola, Diogo Machado, Ester Pereira e Anésia Nazar da Cruz†, João Cipriano†, por todo o amor, carinho, confiança e incentivo que foram fundamentais para que essa importante etapa da minha vida fosse concluída com sucesso. A vocês meu muito obrigado por estarem sempre comigo incentivando, ensinando, confiando e acima de tudo amando. A vocês pertence o meu amor, carinho, admiração e respeito incondicional. Serei eternamente grata por tudo que já fizeram, fazem e sei que ainda irão fazer pelo meu sucesso.

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BIOGRAFIA

THAÍS PEREIRA DA CRUZ, filha de John Wesley Nazar da Cruz e Mirian Margarete Pereira Brisola, nasceu em Ponta Grossa, no estado do Paraná, em 1994.

Em fevereiro de 2012 ingressou no curso de Zootecnia na Universidade Estadual do Ponta Grossa - UEPG, onde desde o dia 06 de fevereiro de 2012 esteve sob orientação do professor Dr. Wilson Massamitu Furuya realizando iniciação científica, e em agosto de 2017 obteve o título de ZOOTECNISTA.

No mês de setembro de 2017 ingressou no programa de Pós-graduação em Zootecnia na Universidade Estadual de Ponta Grossa, em nível de Mestrado, área de produção e nutrição de animais Não Ruminantes, sendo titulada como MESTRE EM ZOOTECNIA em fevereiro de 2020.

Iniciou os estudos em nível de doutorado no mês de março de 2020, no Programa de Pós-Graduação em Zootecnia, área de concentração Produção Animal, tendo como especialidade a área de aquicultura, na Universidade Estadual de Maringá - UEM, na cidade de Maringá, PR, em 22 de setembro de 2022, obteve a qualificação.

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RESUMO

RESUMO: Dois experimentos foram conduzidos para avaliar os efeitos da suplementação de níveis crescentes de β-mananase no desempenho, saúde intestinal, microbioma e coeficientes de digestibilidade aparente (CDA) em juvenis de tilápia do Nilo (*Oreochromis niloticus*) alimentados com dietas à base de ingredientes de origem vegetal. O primeiro experimento teve como objetivo avaliar os efeitos dos níveis crescentes de β-mananase no desempenho, composição corporal, viscosidade e pH da digesta, atividade das enzimas digestivas, parâmetros sanguíneos, teor de ácidos graxos de cadeia curta da digesta, morfologia intestinal e microbioma. Os peixes (n = 504; peso corporal 7,0 ± 0,43 g) foram distribuídos aleatoriamente em 24 aquários de 70 L cada, em sistema de aquicultura de recirculação, em delineamento inteiramente casualizado com seis tratamentos e quatro repetições de 21 peixes por aquário. Os peixes foram alimentados com dietas com níveis crescentes de β-mananase de (0 (controle); 1600; 3200; 4800; 6400; 8000 TMU kg^{-1}) e alimentados manualmente 12 vezes ao dia, durante oito semanas. Peixes alimentados com dieta com 4800 TMU kg^{-1} de β-mananase apresentaram menor viscosidade da digesta (−25,8%) e maior atividade das enzimas amilase (+61,2%), protease (+25,4%) e lipase (+47,7%), e aumentou o ganho de peso (+5,4%) e a taxa de eficiência alimentar (+12,1%) do que os peixes alimentados com a dieta controle. A β-mananase na dieta de 4800 TMU kg^{-1} aumentou o teor de ácido butírico (+63,3%) e baixou o pH intestinal (−8,2%), enquanto aumentou a altura total das vilosidades (+40,4%) dos peixes em relação aos peixes alimentados com a dieta controle.

Peixes alimentados com dieta com 4800 TMU kg^{-1} de β-mananase apresentaram maior abundância de bactérias benéficas, *Proteobacteria*, *Actinobacteria* e *Firmicutes*. Além disso, reduziu significativamente a população de bactérias potencialmente nocivas (*Escherichia*). Concluiu-se que a β-mananase na dieta de 4800 TMU kg^{-1} reduz a viscosidade da digesta, aumenta a atividade das enzimas digestivas e, consequentemente, melhora a digestibilidade e o desempenho, bem como aumenta a produção de ácidos graxos de cadeia curta, a morfologia intestinal e modula positivamente a microbiota intestina. O segundo estudo foi realizado com o objetivo de determinar a viscosidade e o pH das fezes e, posteriormente, os efeitos no CDA de energia e nutrientes, incluindo aminoácidos em juvenis de tilápia do Nilo alimentados com dietas com níveis crescentes de β-mananase na dieta. Os peixes (n = 504; peso corporal 7,0 ± 0,43 g) foram distribuídos aleatoriamente em aquários de 24 com 70 L em sistema de recirculação, em delineamento inteiramente casualizado com seis tratamentos e quatro repetições de 21 peixes por aquário. Os peixes foram alimentados com dietas com níveis crescentes de β-mananase (0; 1600; 3200; 4800; 6400; 8000 TMU kg^{-1}) e alimentados manualmente 12 vezes ao dia durante oito semanas. O óxido de cromo foi usado como um marcador indigerível. Peixes alimentados com a dieta com β-mananase a 4800 TMU kg^{-1} apresentaram redução da viscosidade fecal (−77,1%) e pH (−11,1%), além da otimização da energia bruta CDA (+7,2%), proteína bruta (+3,5%), lípideo bruto (+1,2%), cinzas (+19,7%), aminoácido essencial (+4,0%) e aminoácido não essencial (+3,4%). Além disso, aumentou a energia digestível (+7,23%) e a proteína digestível (+3,54%). A análise dos componentes principais mostra que a viscosidade e o pH das fezes têm uma correlação forte e negativa no CDA de matéria seca, energia bruta, lipídeo bruto, proteína bruta, cinzas, EAA e NEAA. Concluiu-se que a β-mananase no nível de 4800 TMU kg^{-1} na dieta melhora a digestibilidade da energia e nutrientes, incluindo aminoácidos, reduzindo a viscosidade da digesta. No geral, nossos resultados sugerem que a dieta de 4800 TMU kg^{-1} de β-mananase melhora a digestibilidade, o desempenho de crescimento e a saúde intestinal de juvenis de tilápia do Nilo. O uso de β-mananase pode contribuir para a aplicação do conceito de nutrição de precisão mais eficiente, sustentável e econômica.

Palavras-chave: carboidrases, digestibilidade de aminoácidos, saúde intestinal, microbiota, *Oreochromis niloticus*, polissacarídeos não amiláceos, viscosidade da digesta.

ABSTRACT

ABSTRACT: Two experiments were carried out to evaluate the effects of graded β-mannanase supplementation on growth performance, gut health, microbiome, and apparent digestibility coefficients (ADC) in juvenile Nile tilapia (*Oreochromis niloticus*) fed plant-based diets. The first experiment aimed to evaluate the effects of graded β-mannanase levels on growth performance, whole-body composition, digesta viscosity, and pH, the activity of digestive enzymes, blood parameters, digesta short-chain fatty acid content, and gut morphology and microbiome. Fish (n = 504; body weight 7.0 ± 0.43 g) were randomly distributed in 24 aquaria of 70 L each, in a recirculation aquaculture system, in a completely randomized design with six treatments and four replicates of 21 fish per aquarium. Fish were fed diets with graded β-mannanase levels of (0, 1600; 3200; 4800; 6400; 8000 TMU kg^{-1}) and hand-fed 12 times a day for eight weeks. Fish fed diet with a 4800 TMU kg^{-1} β-mannanase showed lower digesta viscosity (−25.8%), and higher activity of amylase (+61.2%), protease (+25.4%) and lipase (+47.7%) enzymes, and increased weight gain (+5.4%) and feed efficiency ratio (+12.1%) than fish fed fed diet control. β-mannanase at 4800 TMU kg^{-1} diet increased butyric acid content (+63.3%) and lowered gut pH (−8.2%), while increased total villus height (+40.4%) of fish relative to that fed diet control. Fish fed diet with 4800 TMU kg^{-1} β-mannanase showed higher abundance of beneficial bacteria, *Proteobacteria*, *Actinobacteria*, and *Firmicutes*. In addition, it significantly reduced the population of potential harmful bacteria (*Escherichia*). It was concluded that β-mannanase at 4800 TMU kg^{-1} diet reduces

viscosity, and activity of digestive enzymes and consequently improves growth digestibility and growth performance, as well increases production of short-chain fatty acids, intestinal morphology and positively modulates the intestinal microbiota population. The second study was carried out with the objective of determining the digesta viscosity and pH and subsequently effects on ADC of energy and nutrientes, including amino acids in juvenile Nile tilapia fed diets with graded levels of β-mannanase in the diet. Fish (n = 504; body weight 7.0 ± 0.43 g) were randomly distributed in 24-70 L aquaria in water recirculation system, in a completely randomized design with six treatments and four replications of 21 fish per aquarium. Fish were fed diets with increasing levels of β-mannanase (0; 1600; 3200; 4800; 6400; 8000 TMU kg^{-1}) and hand-fed 12 times a day for eight weeks. Chromium oxide was used as an indigestible marker. Fish fed a diet with β-mannanase at 4800 TMU kg^{-1} showed reduced fecal viscosity (−77.1%) and pH (−11.1%), while optimized gross energy ADC (+7.2%), crude protein (+3.5%), crude lipid (+1.2%), ash (+19.7%), essential amino acid (+4.0%) and non-essential amino acid (+3.4%). In addition, increased digestible energy (+7.2%) and digestible protein (+ 3.5%). The PCA analysis shows that viscosity and pH of feces have a strong and negative correlation within ADC of dry matter, gross energy, crude lipid, crude protein, ash, and EAA and NEAA. It was concluded that β-mannanase at the level of 4800 TMU kg^{-1} in the diet improves digestibility of energy, and nutrients, including amino acids, by reducing digesta viscosity. Overall, these suggested that β-mannanase 4800 TMU kg^{-1} diet improves digestibility, growth performance and gut health of juvenile Nile tilapia. The use of β-mannanase can lead to contributing to the application of the concept of precision nutrition for more efficient, sustainable, and economical tilapia farming.

Keywords: carbohydrases, amino acid digestibility, gut health, microbiota, *Oreochromis niloticus*, non-starch polysaccharides, digesta viscosity.

I –INTRODUCTION

According to the United Nations Food and Agriculture Organization, the world population is projected to reach 9.7 billion in 2050, representing a 25% increase from 2020 (FAO, 2022). This projected population growth highlights the need for both increased productivity and sustainable practices to meet future food demand (Valenti et al., 2018). In this regard, Nile tilapia (*Oreochromis niloticus*) ranks second in the world's most cultured freshwater fish species, with Brazil being the fourth largest producer (FAO, 2022). One of the significant challenges for all research in this field is that the intensification of tilapia farming raises a critical issue: more meaningful use of plant ingredients, focusing on the use of ingredients that do not compete with human food (Souza et al., 2021; Valenti et al., 2018). This approach has several limitations, as plant-derived ingredients possess a wide range of anti-nutritional factors, including phytin, protease inhibitors, and non-starch polysaccharides (NSPs) (Jiang et al., 2021). Notably, soybean meal, one of the most commonly used vegetable ingredients in aquafeeds, contains substantial amounts of NSPs (Faustino et al., 2021; Khalifa et al., 2018). It is well known that NSPs such as β-mannans cause several adverse effects on nutrient utilization, mainly by increasing digesta viscosity. Thus, tools are needed to minimize such adverse effects (Castillo and Gatlin, 2015; Chen et al., 2016; Sinha et al., 2011).

Exogenous enzymes are promising to improve the sustainability of industrial-scale tilapia culture by reducing the impact of non-retained nutrients, besides the antinutritional factors present in aquafeeds (Nguyen et al., 2020; Staessen et al., 2020). Particularly, β-mannanase may be helpful to elaborate environmentally sustainable diets for fish farming following sustainability principles (Castillo and Gatlin, 2015). Therefore, this study involved evaluations of viscosity and pH of digesta, growth performance, digestibility, digestive enzymes levels, short-chain fatty acids (SCFA) production, gut morphometry, and microbiome modulation by supplementing graded levels effects of β-mannanase on-top of vegetable-based diet fed to Nile tilapia.

1. LITERATURE REVIEW

1.1. Tilapia production

Tilapia is the second most widely farmed fish species globally, thus, it is a subject of particular importance in the production chain (FAO, 2022). According to the Food and Agriculture Organization of the United Nations (FAO, 2022), global tilapia

production increased by 2% in 2021, reaching approximately 6.25 million tons. Additionally, Indonesia produced 1.4 million tons in 2022, followed by Egypt, which for the first time, surpassed 1 million tons. Brazil, in fourth place, produced 534,000 tons in 2021 (FAO, 2022).

Brazilian fish farming increased by 5.93% in 2020 compared to 2019, with tilapia leading such growth (FAO, 2022). There are approximately 70 species of tilapia, but only 10 of them are cultivated around the world (FAO, 2022). Noteworthy, Nile tilapia, Mozambique tilapia (*O. mossambicus*), Blue tilapia (*O. aureus*), Mango tilapia (*Sarotherodon galilaeus galilaeus*), Blackchin tilapia (*S. melanotheron*), Longfin tilapia (*O. macrochir macrochir*), Redbelly tilapia (*Tilapia zilli*), Redbreast tilapia (*Tilapia rendalli*), Sabaki tilapia (*O. spirulus spirulus*) and Three spotted tilapia (*O. andersonii*) are among top ten most cultured tilapia fish species (Zimmermann, Fitzsimmons, 2004). The first fish specimens were brought to Brazil in 1971, and the rapid expansion of aquaculture, was the catalyst for the growth of the tilapia industry around the world (Valenti et al., 2021).

Nile tilapia is the second most farmed freshwater fish species due to its adaptability to a wide range of culture systems and environments, ranging from extensive low-input pond culture to intensive recirculating systems (Carneiro et al., 2022). Alternative feed ingredients at reasonable prices have been proposed to maintain stable costs of fish farming and promote sustainable tilapia aquaculture (Doan et al., 2020; El-Sayed, 2020). Nile tilapia is a fish species that possess omnivorous feeding habit, accepts artificial food from the larval stage, exhibits rapid growth, and lean meat (Schader et al., 2015). This fish species can partially digest soluble carbohydrates and convert them into energy that benefits fish´s growth performance (NRC, 2011; Van Doan et al., 2019). However, further studies are needed to evaluate the potential of exogenous carbohydrases in Nile tilapia diets to promote more economically and environmentally sustainable fish farming.

1.2.Non-starch polysaccharides in fish nutrition

NSPs encompass a wide variety of polysaccharide molecules, excluding α-glucans (starch) (Thitipraphunkul et al., 2003). They are primarily comprise of linked monomers of hexoses and pentoses such as galactose, glucose, arabinose, xylose, and mannose (van Barneveld, 1999). Historically, the classification of NSPs was based on

the methodology used to extract and isolate polysaccharides (Choct, 1997). In 1973, a more precise classification of NSPs into three main groups was proposed: cellulose, non-cellulosic polymers, and polysaccharides. Arabinoxylans, mixed-linked β-glucans, mannans, and xyloglucan fall into the category of non-cellulosic polymers (Bailey and Hunt, 1973), as shown in Table 1.

Table 1. Classification of non-starch polysaccharides.

Category	Monomeric residue	Linkage	Sources
Cellulose	Glucose	β-(1→4)	Most cereals and legumes
Non-cellulosic polymers			
Arabinoxylans	Arabinose and Xylose	β-(1→4)-linked xylose units	Wheat, rye, barley, oat, rice, sorghum
Mixed-linked β-glucans	Glucose	β-(1→3) and β-(1→4)	Oat and barley
Mannans	Mannose	β-(1→4)	Coffee seed
Galactomannans	Galactose and mannans	β-(1→4)-linking mannan chains with α-(1→6)-linked galactosyl side groups	Locust bean gum ad guar gum
Glucomannans	Glucose and mannans	β-(1→4)-linked mannan chain with interspersed glucose residues in the main chain	Sugar-beet pulp, lilies, irises

Adapted: (Sinha et al., 2011).

NSPs are an integrated part of the cell wall of plant ingredients and in a purified soluble form (Liu et al., 2022). In general, NSPs fraction such as β-glucans, β-xylans, and β-mannans remains undigested by fish (Castillo and Gatlin, 2015). The adverse effect is associated with various physiological and morphological factors affecting digesta viscosity, digestibility, growth performance, digestive enzymes activity, blood parameters, SCFA production, gut morphology and intestinal microbiota (Table 2).

Table 2. Factors responsible for anti-nutritive effects of non-starch polysaccharides.

Factors	Effects	References
Changes in digesta viscosity	• Reduced mixing of digestive enzymes and substrates • Hindered effective interaction of digestive enzyme at the intestinal mucosal surface • Increased residence time of the digesta • Impaired nutrient digestion and absorption • Reduced animal performance	(Amirkolaie et al., 2005; Choct et al., 1996; Hossain et al., 2003; Ikegami et al., 1990; Leenhouwers et al., 2007b, 2007a)
Alteration in the gastric emptying and rate of passage	• Reduced rate of gastric emptying • Delayed intestinal absorption of glucose. • Reduced plasma cholesterol and glucose levels	(Angkanaporn et al., 1994; Bach Knudsen, 2001; Choct et al., 1996; Hossain et al., 2003; Leenhouwers et al., 2007b, 2007a; Potkins et al., 1991; Rainbird and Low, 1986; Refstie et al., 1999)
Alteration in the gut morphology	• Decreased size and length of digestive organs. • Reduced concentrations of DNA in jejunum, ileum, and liver, indicating programmed cell death • Reduced villi length. • Increased depth of intestinal crypts in jejunum and ileum	(Iji et al., 2001; Jin et al., 1994; Leenhouwers et al., 2006; Nabuurs, 1998)
Alteration in the native gut microflora	• Enhanced short-chain fatty acids, such as acetic acid, propionic and butyric acids, production • Lower pH of intestinal tract; in long term may disturb the normal microbiota prevailing in gut • Decreased oxygen tension, favoring development of anaerobic microbiota	(Amirkolaie et al., 2006; Leenhouwers et al., 2007a, 2007b)

Adapted: (Sinha et al., 2011).

Of note, NSPs are considered to have low nutritional value for fish because of their low digestibility and anti-nutritional characteristics (Kabir et al., 2020). Previous studies have shown that the type of NSPs can affect fish performance differently (Jiang et al., 2021; Wang et al., 2022). Although previous researches have made much effort,

the underlying mechanisms of NSPs on nutrient digestibility, including amino acids, still not fully understood.

1.3. β-mannans

β-mannans are long-chain NSPs mainly composed of mannose residues found in the most diverse sources, such as vegetables and microorganisms, that remain unchanged after heat treatments, such as drying or roasting grains (Tester and Al-Ghazzewi, 2013). In plants, mannans and heteromannans are essential components of the hemicellulose family and are classified into four subfamilies according to their monosaccharide composition: pure mannans (containing only mannose); glucomannans; galactomannans and galactoglucomannans (Singh et al., 2018), as shown in Figure 1.

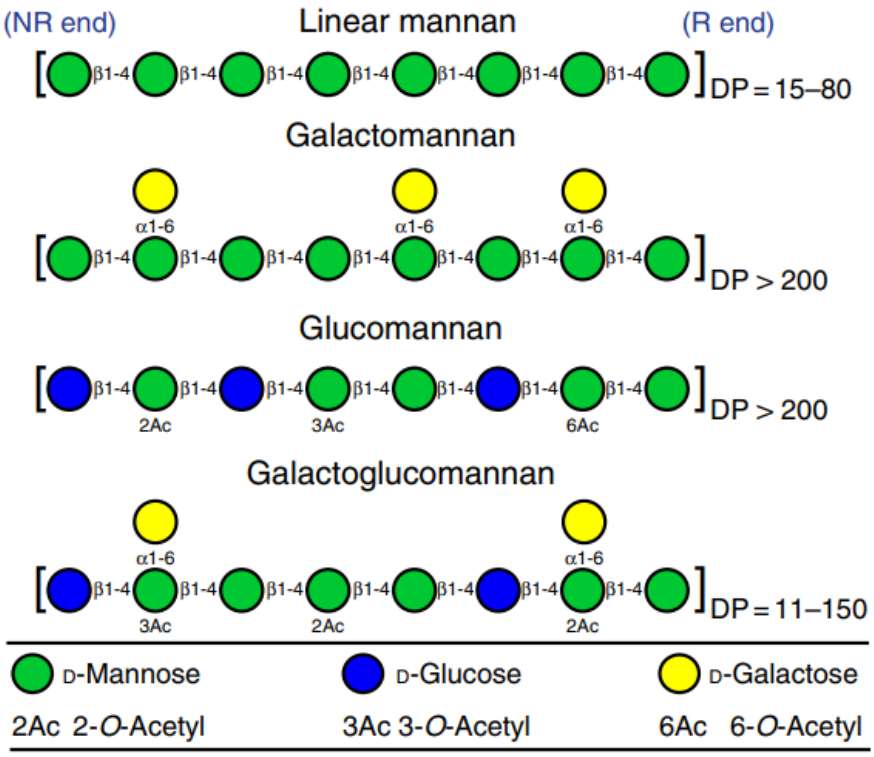

Figure 1. General structure of the main classes of β-mannan (La Rosa et al., 2019).

Galactomannans are composed of mannan chains linked to β-(1,4) with α-(1,6) galactosyl side groups (McCleary, 1986) (Figure 2). Galactomannans are reserve polysaccharides in the endosperm of legume seeds that possess the characteristic of water solubility and the ability to absorb water, thus providing water retention in the grains

(Reid, 1985). The mannose-galactose ratio, which can range from 1 to 5, may affect galactomannans' solubility and viscosity properties (Daas et al., 2000).

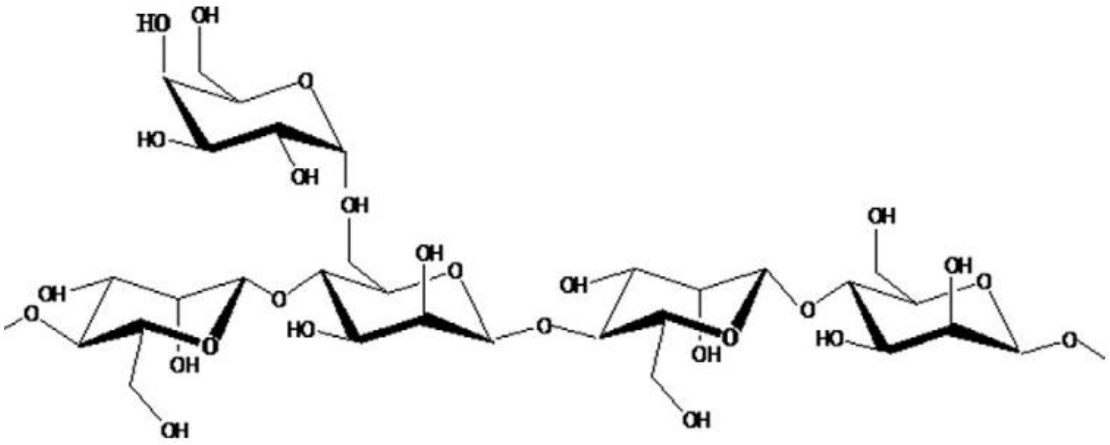

Figure 2. Primary structure of galactomannans (Ebringerová, 2005; Sinha et al., 2011).

Glucomannans are in smaller amounts in cereal grains (Fincher and Stone, 1986) and are polysaccharides found in seeds, mainly of annual cycle plants (Meier and Reid, 1982). Additionally, glucomannans are found in many bulbs, roots, and tubers of many other plants. A previous study has evidenced that galactomannans are soluble in water and are composed of a mannan chain linked to β-(1,4) with glucose residues interspersed in the main chain (Sinha et al., 2011) (Figure 3).

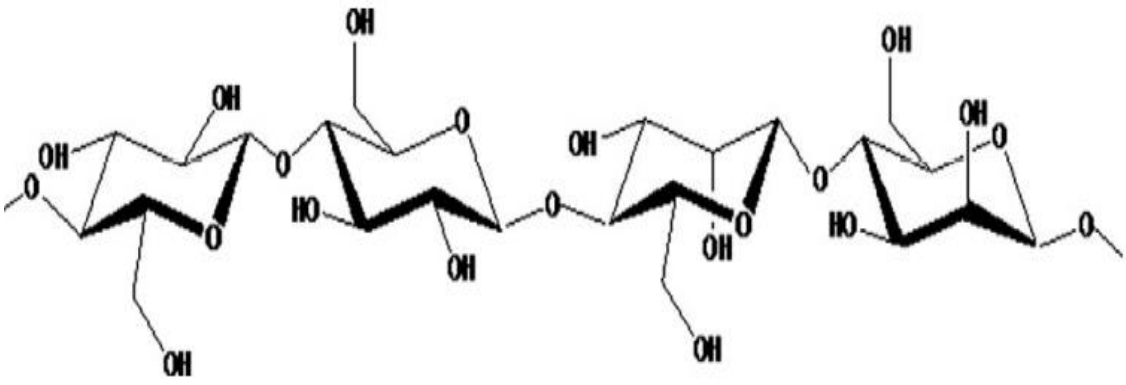

Figure 3. Primary structure of glucomannans (Ebringerová, 2005; Sinha et al., 2011).

The content of soluble β-mannans in different ingredients varies by more than 5% (Faustino et al., 2021). Of note, the β-mannans content is relatively high (in soybean and sunflower meal (~0.6%) and up to 7% in palm kernel meal, as shown in Figure 4.

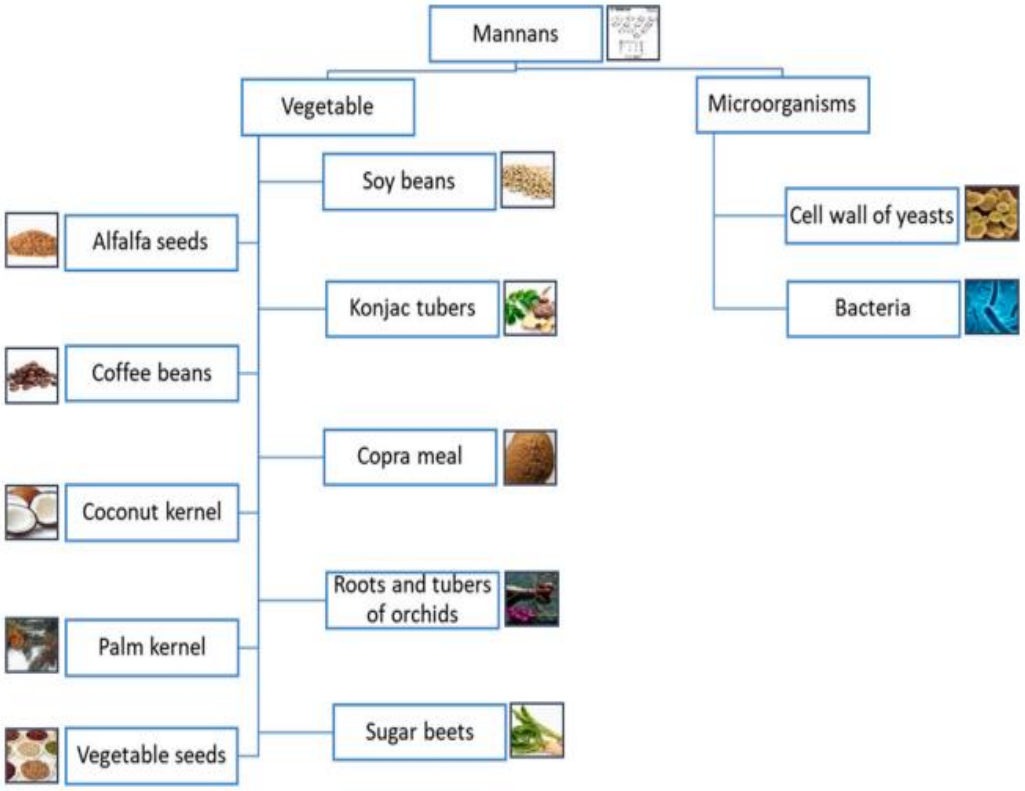

Figure 4. Sources of mannans in vegetable ingredients and microorganisms (Faustino et al., 2021; Olaniyi and Omotere, 2013; Singh et al., 2018).

Previous studies in broilers, pigs, and other monogastric animals have indicated that β-mannan can promote increased digesta viscosity, reduce nutrient digestibility, and negatively impact gut microbiota, SCFAs production, and gut health (Browne et al., 2019; Rainbird et al., 1986). These findings suggest that exogenous β-mannan may not be beneficial for improving digestibility and growth performance in fish.

1.4.Digesta viscosity

Digesta viscosity is influenced by the chemical structure and association of NSPs with cell wall components (Figure 5). The physical effect of high viscosity has deleterious effects on nutrient digestion and absorption (Sternemalm et al., 2008).

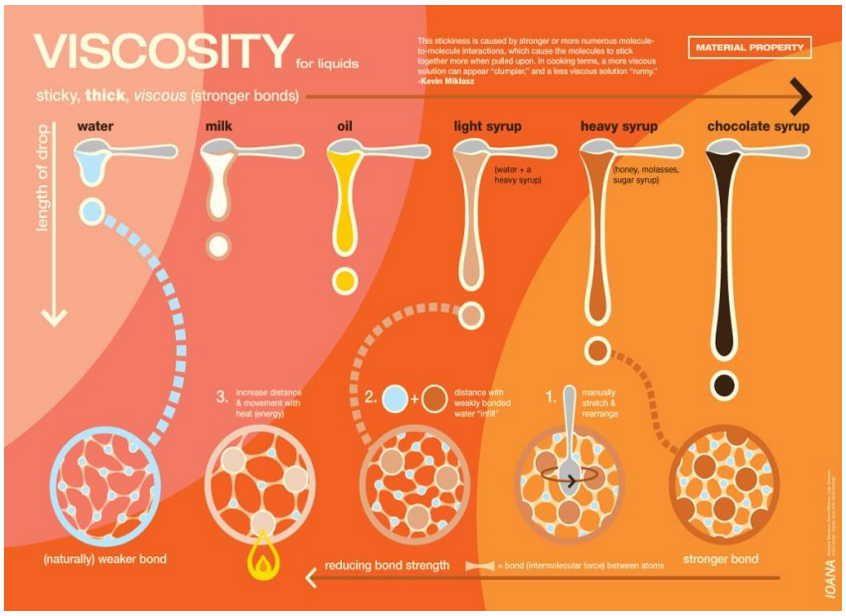

Figure 5. Representation of viscosity dynamics in different liquids (Prasad and Srikant, 2013).

Previous reports confirmed that NSPs from cereals could increase digesta viscosity impair digestibility in Nile tilapia and African catfish (*Clarias gariepinus*) (Leenhouwers et al., 2007b, 2007a). Similarly, early work evidenced that common carp (*Cyprinus carpio*) fed galactomannan-rich diets showed increased gut digesta viscosity, compromising digestion and absorption of nutrients (Hossain et al., 2003).

1.5. Effects of β-mannans on nutrient utilization

1.5.1. Digestibility of nutrients

β-mannans increase digesta viscosity and reduce nutrient digestibility, and the deleterious effects may vary according to fish species, size, and diet composition (Dawood and Shi, 2022; Sinha et al., 2011). Increasing the digesta viscosity of the liquid phase acts as a barrier to the availability of nutrients and increases the rate of passage of digesta through the digestive tract (Bach Knudsen, 2001). Noteworthy, increased digesta viscosity reduces activity of digestive enzymes in a viscous solution and nutrient flux in the mucous layer (Balasubramanian et al., 2018). Increased endogenous nutrient losses and increased thickness of the layer of unstirred water adjacent to the mucosa also lead to decreased digestion and absorption of nutrients (Lange, 2000; Leenhouwers et al., 2007b). These suggested that increased digesta viscosity may reduce nutrient digestion and absorption.

1.5.2. Effect on glucose metabolism

The presence of β-mannans in the diet of monogastric animals, including fish, has been reported to delay intestinal absorption of glucose (Sinha et al., 2011). For example, African catfish fed diets containing 400 g kg^{-1} rye showed decreased plasma glucose levels than fish fed diets without rye inclusion (Leenhouwers et al., 2007a). Previous work evidenced that inclusion of guar galactomannans and alginates as sources of NSPs, reduced glucose availability in Atlantic salmon (*Salmo salar*) compared to fish diets without guar gum and alginates inclusion (Storebakken et al., 1998).

1.5.3. Effect on protein

The presence of non-starch polysaccharides (NSPs), such as β-mannans, in fish diets has been shown to negatively impact protein and amino acid digestibility. Leenhouwers et al. (2006) investigated the effects of guar gum, an NSPs-rich ingredient, on digestibility in fish. The study found that inclusion of guar gum at levels of 40 and 80 g kg^{-1} diet increased digesta viscosity and a corresponding decrease in the apparent digestibility coefficient of protein. Previous research also observed reduced protein digestibility in trout fed diets containing guar gum, a high-mannan feed ingredient (Morken et al., 2011). Another study reported that African catfish fed a diet containing high-viscosity rye had a more significant protein digestibility reduction than those fed low-viscosity wheat (Leenhouwers et al., 2006). These findings suggest that the viscosity of digesta can directly impact protein and amino acid digestibility. Further research is needed to understand the effects of different levels of NSPs-rich ingredients and different fish species on digestibility of protein and amino acids.

1.5.4. Effect on lipid

In addition to increased intestinal viscosity, β-mannans modify intestinal functions, impairing endogenous secretion of water, proteins, electrolytes, and lipids (Angkanaporn et al., 1994). NSPs can increase bile acid secretion and result in a significant loss of bile acids in the feces (Ikegami et al., 1990). This can result in increased hepatic synthesis of bile acids from cholesterol to restore homeostasis, influencing absorption of lipids and cholesterol in the intestine, thereby dropping blood cholesterol levels (Hossain et al., 2003). Additionally, β-mannans can influence lipid metabolism in the intestine through binding with bile salts, lipids, and cholesterol

(Ouwehand et al., 2009). β-mannans can trap bile salts, thus reducing their efficiency in fat solubilization and, consequently, impairing lipid absorption (Ebihara and Schneeman, 1989). Besides that, the increased digesta viscosity caused by β-mannans negatively affects lipid emulsification and consequently promotes reduced lipolysis (Pasquier et al., 1996).

1.6. Exogenous enzymes in fish nutrition

The use of exogenous enzymes or enzyme complexes in fish nutrition, can improve growth performance, increase digestibility, and contribute to the reduction of nutrient excretion in the aquatic environment, positively impacting the water quality of production systems (Magalhães et al., 2016). Carbohydrases encompass all enzymes that catalyze a reduction in the molecular weight of polymeric carbohydrates, with over 80% of the global carbohydrase market being accounted for by two dominant proteins, xylanase and glucanases (Castillo and Gatlin, 2015). Despite this, the use of carbohydrases in aquaculture has not been widespread, despite its positive effects (Castillo and Gatlin, 2015). Studies that have been conducted with the use of carbohydrates in aquatic species have shown that supplementation of exogenous carbohydrase in fish fed plant-based diets improves nutrient digestibility and reduces nutrient excretion (Kiarie et al., 2021). Although some fish species are generally known for their inefficient metabolism of glucose, the use of carbohydrases can have positive effects not only on carbohydrate digestibility but also on protein and lipid digestibility of plant-based foods (Sinha et al., 2011). Based on promising results and opportunities found in aquaculture fish species, further attention should be devoted to this matter as it could be a tool to increase the use of plant-based feeds and ensure aquaculture sustainability.

1.7. β-mannanase

Endo-1,4-β-mannanase is a crucial carbohydrase for the depolymerization of mannans, glucomannans, galactomannans, and galactoglucomannans. This enzyme catalyzes through the random hydrolysis of β-1,4-mannan bonds in the mannan backbone (Stålbrand et al., 1993). Endo-1,4-β-mannanase releases linear and branched chains of mannan oligosaccharides or mannan oligosaccharides of various lengths, and these are hydrolyzed to β-mannosidase and α-galactosidase monomers. Its action causes

a rapid decrease in the viscosity of polysaccharide solutions, increasing the polymer accessibility with other enzymes (Kremnický and Biely, 1997) (Figure 6).

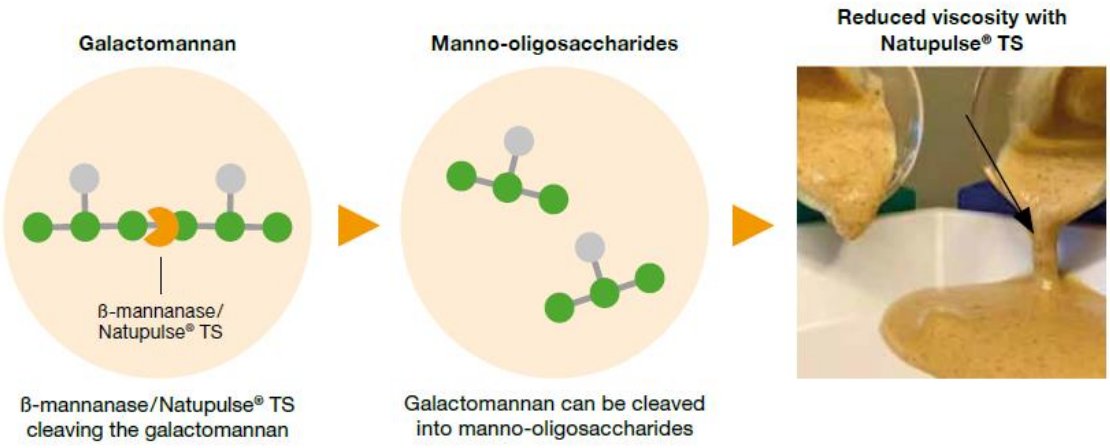

Figure 6. Natupulse® TS is an NSPs enzyme. As an endo-1,4-β-mannanase, it hydrolyzes β-mannans into smaller particles (Choct et al., 2010; Hsiao et al., 2006; Knudsen, 2014; Shastak et al., 2015; Slominski, 2011).

Natupulse® TS is a carbohydrase, more specifically, an endo-1,4-β-mannanase, developed by BASF, and it hydrolyzes β-mannans into smaller particles. This β-mannanase has various effects on viscosity, growth pergormance, digestibility, intestinal microbiota, SCFAs production, and intestinal health in distinct species, such as poultry, turkey, swine, and fish (Kiarie et al., 2021). The main mechanism of action of β-mannanase is:

- ***Reduction of digesta viscosity:***

Studies on monogastric animals have shown that reduced digesta viscosity due to NSPs-degrading enzyme supplementation is the main factor responsible for the observed enhanced performance response on feeding plant materials rich in NSPs (Latham et al., 2015; Leenhouwers et al., 2007a, 2006; Zhang et al., 2021). In this sense, by reducing viscosity, the digesta can flow more easily through the gut, allowing for greater contact between digestive enzymes, thereby increasing nutrient absorption (Sinha et al., 2011). Additionally, reducing viscosity can promote beneficial gut microbes' growth, which are essential for optimal gut health and overall animal

performance (Wang et al., 2022). A schematic representation of digesta viscosity and the access of digestive enzymes is presented in Figure 7.

Figure 7. Natupulse® TS reducing the viscosity of digesta (Choct et al., 2010; Hsiao et al., 2006; Knudsen, 2014; Shastak et al., 2015; Slominski, 2011).

- ***Disruption of cell wall integrity:***

The cell wall in cereals and legumes consists mainly of cellulose, hemicellulose, and arabinoxylan (Ebringerová, 2005). The activity of β-mannanase degrades mannans and creates “holes” in the cell wall (Karina and Garcia, 2018). This allows hydration with water and pancreatic enzyme action, allowing better digestion of nutrients (Jiang et al., 2022). A schematic representation of the increased cell wall permeability caused by the addition of β-mannanase is presented in Figure 8.

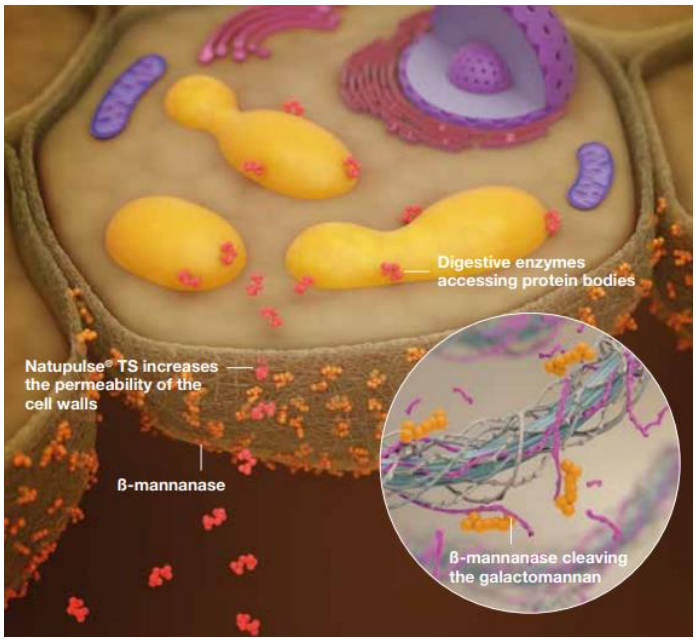

Figure 8. Natupulse® TS supports an increase in permeability of intact soybean cell walls (Choct et al., 2010; Hsiao et al., 2006; Knudsen, 2014; Shastak et al., 2015; Slominski, 2011).

- ***Stimulation of bacterial population:***

β-mannanase breaks down β-mannans and reduces chain length, producing smaller polymers and oligomers (Ma et al., 2022). These fragments further become small enough to act as a substrate for gut microbiota fermentation, modulating the profile of SCFAs production, which reduces gut pH and retro-influence the gut microbiota (Xu et al., 2020). However, care must be taken with the levels of enzyme used, once, overdosed can reduce the size of the oligosaccharides to monosaccharides. If excess monosaccharides are produced, it may result in osmotic diarrhea and/or poor performance (Schutte, 1990). A schematic representation of how microbiota is influenced by β-mannanase addition is presented in Figure 9.

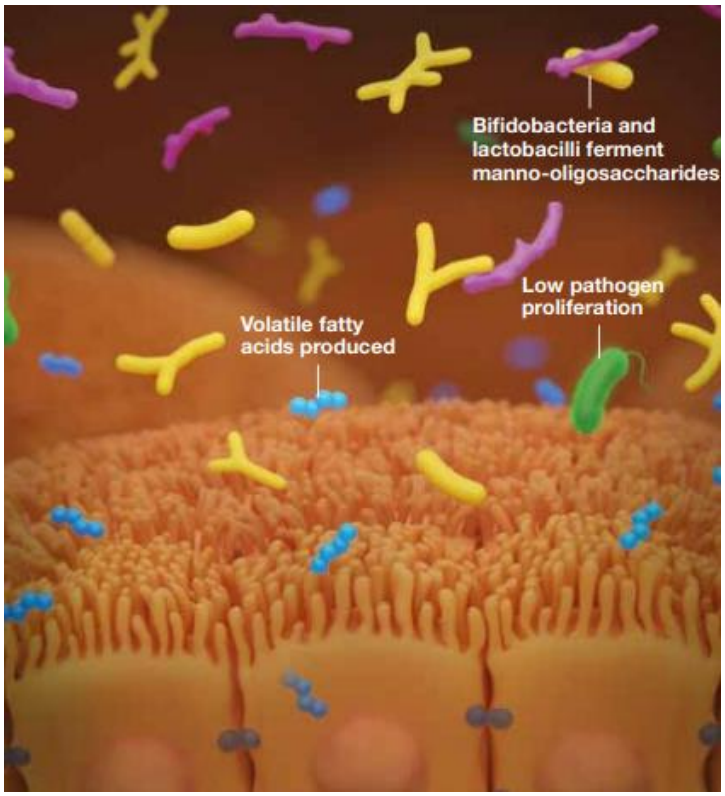

Figure 9. Natupulse® TS cleaves ß-mannans resulting in mannan-oligosaccharides (MOS) (Choct et al., 2010; Hsiao et al., 2006; Knudsen, 2014; Shastak et al., 2015; Slominski, 2011).

The effects of β-mannanase supplementation of β-mannanase in fish feed have been proven to improve feed efficiency and increase growth performance rate, leading to a more cost-effective and sustainable aquaculture industry. Although there is a need for more attention to understanding the effects of β-mannanase in Nile tilapia diets (Castillo and Gatlin, 2015; Chen et al., 2016; Sinha et al., 2011).

1.8. Gut microbiome

Gut microbiota comprises the community of microbes (e.g., Archaea, bacteria, fungi, protozoa, yeast) that live in the gastrointestinal tract. The microbiome, although often used synonymously with the microbiota, represents the genome of the microbiota (Burokas et al., 2015). The function of the microbiota and the physiological responses of the host depend on several intrinsic and extrinsic factors, such as the composition of the microbiota present in the gastrointestinal tract (Figure 10) (Vigneri, 2014). Although there is a significant variation in the composition of the intestinal microbiota of fish between species and individuals, some phyla demonstrate to be dominant, such as

Proteobacteria, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Fusobacteria* (Eichmiller et al., 2016).

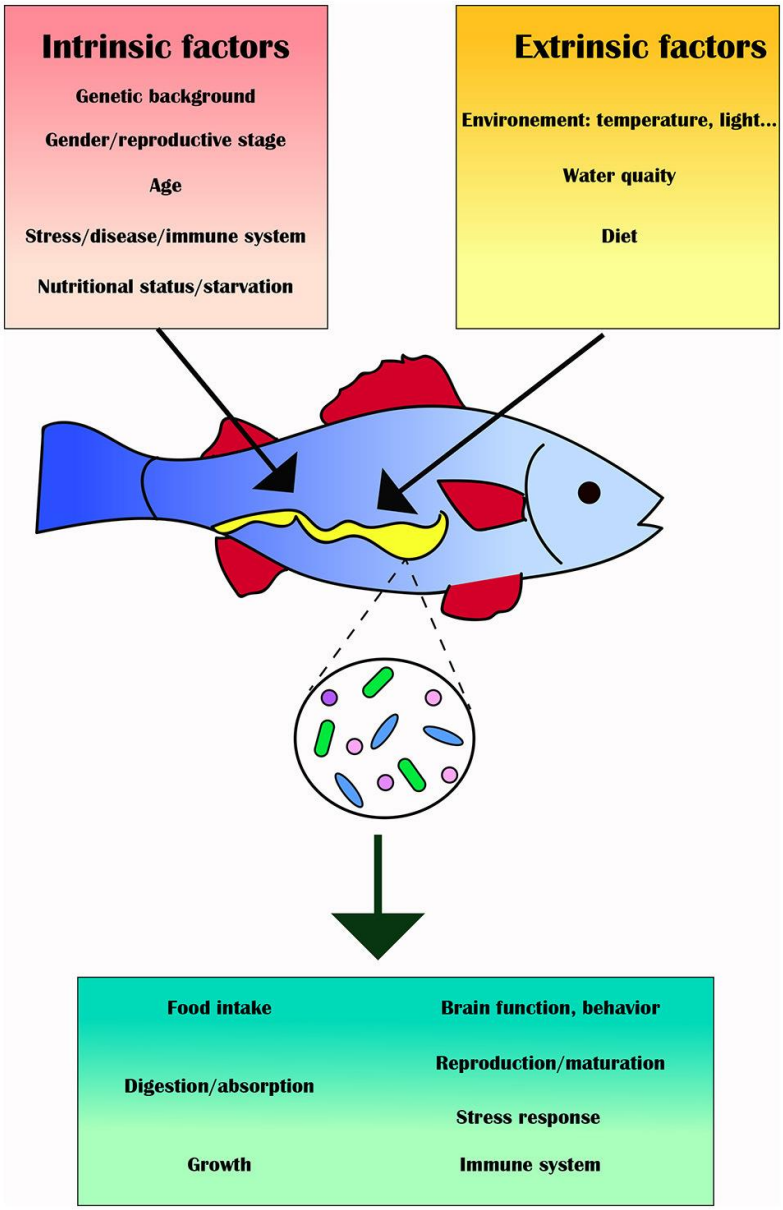

Figure 10. Intrinsic (red box) and extrinsic factors (yellow box) can alter the gut microbiota (green box) and its downstream effects on the fish host (Butt and Volkoff, 2019).

Sequencing data analysis revealed a peculiarly low phylogenetic diversity in fish gut, with *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* representing up to 90% of the intestinal microbiome of fish of distinct species (Ghanbari et al., 2015). *Actinobacter*, *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Lactococcus* and *Pseudomonas* are obligate anaerobic bacteria predominantly found in the intestine of freshwater species, in addition to genera such as *Bacteroidetes*, *Clostridium*, *Fusobacterium* and *Enterobacteriaceae*

(Cahill, 1990). The presence and diversity of gut microbiota are influenced by several factors (Gallo et al., 2020).

1.8.1 Factors affecting the microbiome in the gastrointestinal tract

- *Genetic*

Genetics has already been shown to be a factor that influences the intestinal microbiota, and intra and interspecific variations in the microbiota have been demonstrated (Li et al., 2012). To date, host genetics have been considered the most influential in the formation of the microbiota in fish (Butt and Volkoff, 2019). In contrast, in a study carried out with channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*), reared under the same environmental conditions, they showed similar microbiomes, suggesting that a shared environment can overcome the genetic differences of the host (Bledsoe et al., 2018).

- *Environment and fish size*

Research in zebrafish has revealed distinctions in gut microbiota between juvenile and sexually mature individuals, with juveniles exhibiting greater bacterial diversity in their intestinal microbiota compared to adults. This can be attributed to the variations in circulating hormones present in sexually mature fish and their impact on the microbiota (Cantas et al., 2012). Furthermore, gut-associated lymphoid tissue (GALT) may interact differently with the gut microbiota in juveniles and adults, as this system is not fully developed in juveniles (Figure 11).

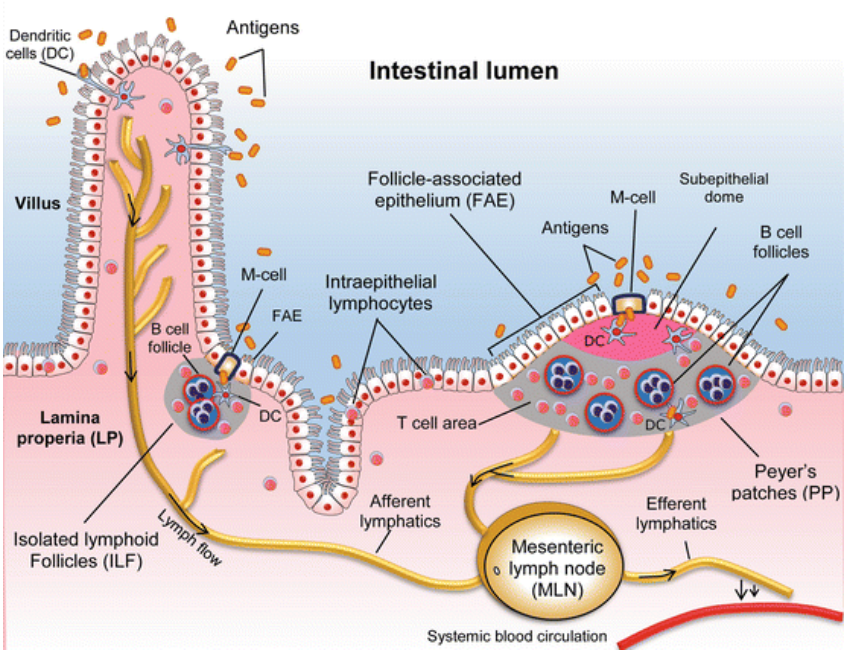

Figure 11. Schematic representation of the gut-associated lymphoid tissue (GALT) (Spahn and Kucharzik, 2004; Zgair et al., 2016).

- *Diet/Eating habits*

Dietary habits can directly influence intestinal microbiota composition (Vatsos, 2017). Intestinal microbiota diversity is lower in carnivorous < omnivores < herbivores fish (Wang et al., 2018). Extreme dietary changes, such as fasting, or ingredient changes, also shape the gut microbiota of fish. Such differences may explain the fact that during extended periods of fasting, morphological changes occur due to reduced nutrient absorption (Bruce et al., 2018).

The effect of food intake on intestinal flora is not restricted to nutritional composition but also to the source of nutrients. A study carried out with common carp shows that an increase in fiber consumption causes an increase in cellulolytic bacteria, such as *Aeromonas*, *Enterobacter*, *Enterococcus*, *Citrobacter*, *Bacillus*, *Raoultella*, *Klebsiella*, *Hydrotalea*, *Pseudomonas*, *Brevibacillus* (Li et al., 2014). Plant-derived proteins have been associated with a significantly reduced diversity of microorganisms, with relative abundances of *Lactobacillales*, *Bacillales*, and *Pseudomonadales*, while animal-derived proteins nourish more *Bacteroidetes*, *Clostridiales*, *Vibrionales*, *Fusobacteriales* and *Alteromonadales* (Michl et al., 2017).

1.8.2. Physiological functions of the intestinal microbiota

Recent studies suggest that the gut microbiota is involved in body homeostasis, consumption, digestive, metabolic, and immune processes (Gonçalves and Gallardo-Escárate, 2017; Mayer et al., 2015). However, the gut microbiota influences the brain-gut axis, affecting both the gut and the brain, thus helping to maintain homeostasis, as exemplified in Figure 12 (Cryan and Dinan, 2012; Vigneri, 2014).

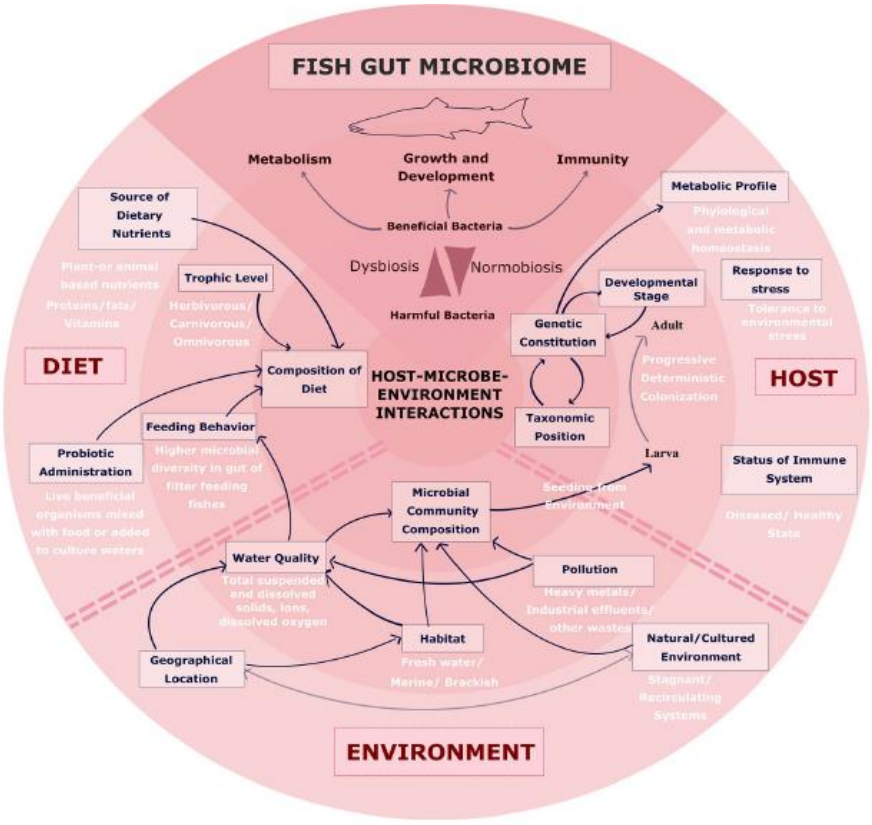

Figure 12. Factors influencing the diversity and function of the gut microbiome of fish (Talwar et al., 2018).

Research with germ-free mice, therefore, without gut microbiota, are leaner than mice with established microbiota, even though they consume fewer calories than germ-free mice (Duca et al., 2012). In addition, such mice have lower appetite-regulating hormones, such as leptin and ghrelin, demonstrating that the intestinal microbiota is directly involved in regulating appetite and metabolism (Figure 13) (Han et al., 2021).

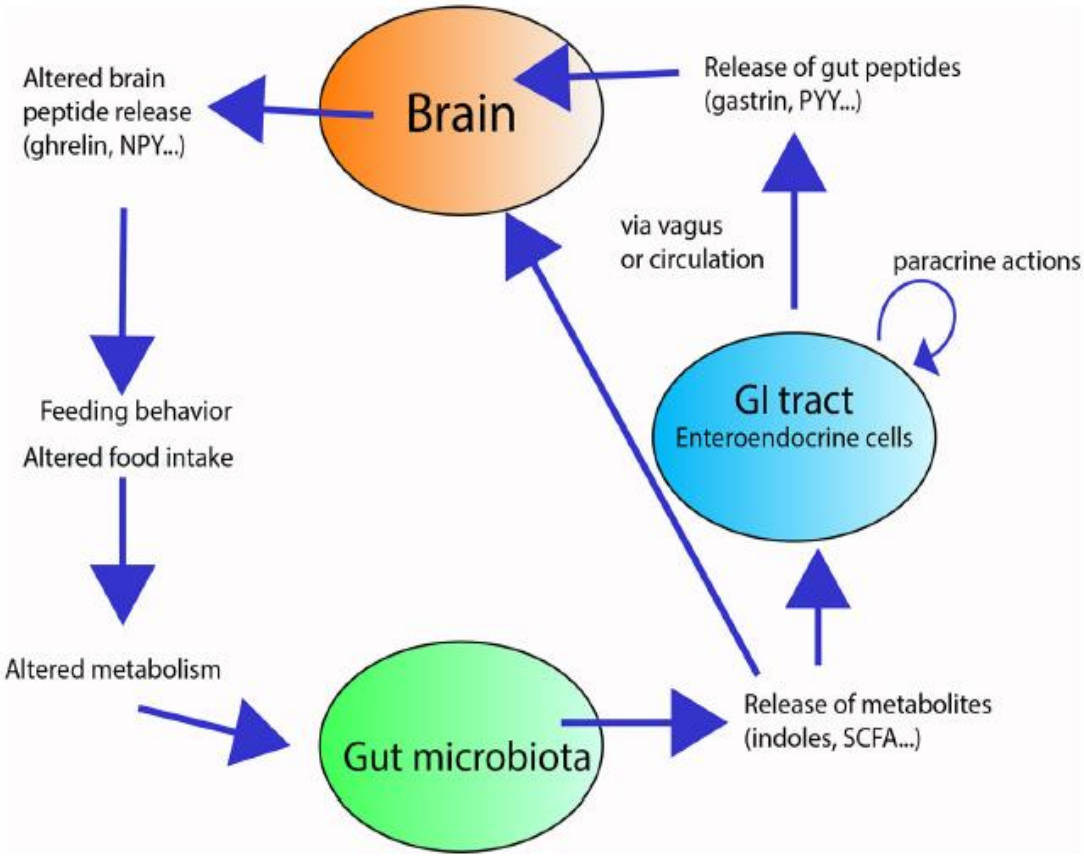

Figure 13. Overview of the gut-microbiota-brain axis in feeding and digestion (Butt and Volkoff, 2019).

Some of the metabolites produced by the intestinal microbiota can act on enterocytes and regulate their intestinal barrier function and nutrient absorption capacity (Ghosh et al., 2021). Also, in enterocytes, intestinal microbiota metabolites can modify the secretory activity of enterocytes, affecting their production of intestinal peptides that modulate intestinal motility and enzyme secretion (Agustí et al., 2018; Franchini et al., 2014; Venkatesh et al., 2014). Once enzyme activity is altered in the intestine, there are significant impacts of its influence on the metabolism of nutrients such as carbohydrates and lipids (Cani and Knauf, 2016; Tolhurst et al., 2012).

Metabolic secretions of microbiota include specific metabolites such as propionic, acetic, and butyric acids, which affect digestive and metabolic processes (Tolhurst et al., 2012). Although there are many other metabolic secretions from the intestinal microbiota, a representative part of such secretions is SCFAs. In addition, it has several more effects, such as effects on pH and gut morphological changes (Zhang and Davies, 2016).

1.9.Short-Chain Fatty Acids

Short-chain fatty acids are carboxylic acids with aliphatic tails and have linear and branched conformations, including acetic, propionic, butyric, valeric, isobutyric, and isovaleric acids (Cook and Sellin, 1998). Among them, acetic acid (C2), propionic acid (C3), and butyric acid (C4) are the most abundant (95%), with an average molar ratio of 60:20:20, respectively (Cummings et al., 1987).

Acetic acid is produced from pyruvate by acetyl-coenzyme A or the Wood-Ljungdahl pathway (Ragsdale and Pierce, 2008). The propionic acid is the primary fermentation metabolite of *Bacteroidetes.* It is generated from the conversion of succinate to methyl malonyl-CoA via the succinate pathway or produced from the acrylate pathway via lactate as a precursor (Hetzel et al., 2003). Also, deoxyhexoses like fucose and rhamnose can be used as substrates for propionic acid synthesis via propanediol (Koh et al., 2016). Butyric acid is the primary *Firmicute*s metabolite and is formed by the condensation of molecules of acetyl-CoA, reduced to butyryl-CoA, and converted to butyric acid by butyric acid phosphotransferase and butyrate kinase (Ait-Belgnaoui et al., 2014; Ragsdale and Pierce, 2008). Butyryl-CoA is also converted to butyrate via acetyl-CoA via transferase, and some microorganisms in the intestine use lactate and acetate to synthesize butyrate, thus preventing lactate accumulation and stabilizing the intestinal environment, as shown in Figure 14 (Ma et al., 2022).

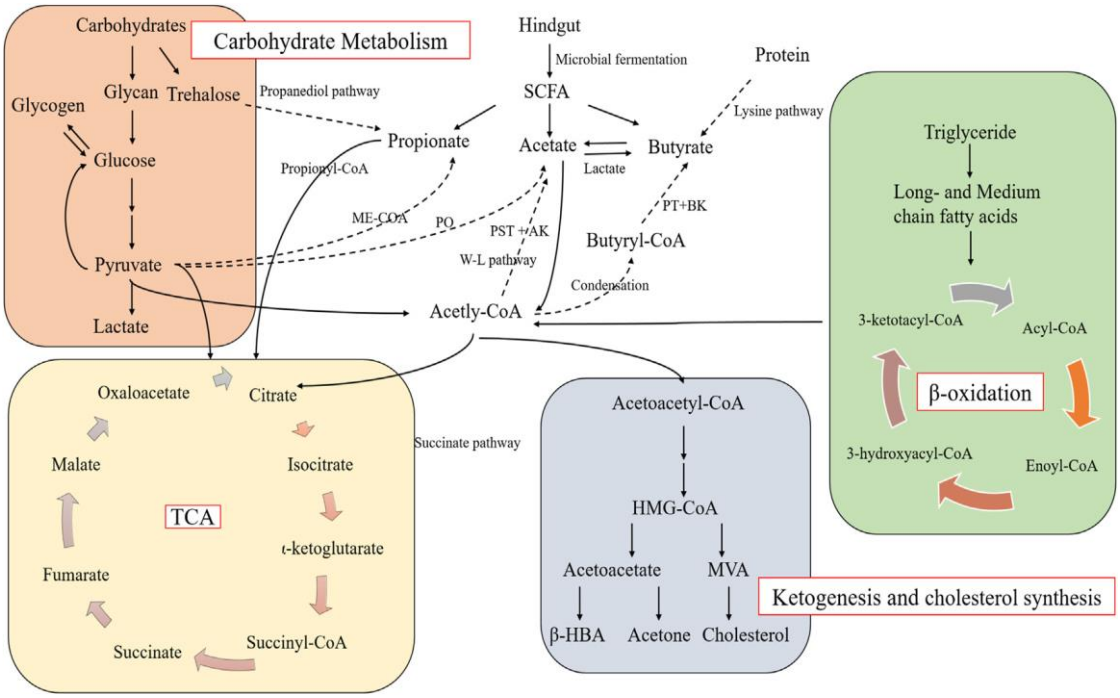

Figure 14. Synthesis pathways of SCFAs and the primary role in carbohydrate and lipid metabolism. PST: phosphotransacetylase; AK: acetokinase; W-L: Wood-Ljungdahl; ME-COA: methyl malonyl-CoA; PO: pyruvic oxidase; PT: phosphotransferase; BK: butyrate kinase; TCA: tricarboxylic acid cycle; MVA: mevalonic acid; β-HBA: β-hydroxybutyric acid; HMG-CoA: β-hydroxy-β-methyl glutaryl-coenzyme A (Hetzel et al., 2003; Ma et al., 2022; Ragsdale and Pierce, 2008).

Studies show that the chemical composition of SCFAs is mainly dictated by the substrate's chemical structure and microbiota activity (Flint et al., 2014). Dietary fiber is the main food component that affects the production of SCFAs, derived mainly from ingredients of plant origin and NSPs fractions. Also, the amount and type of fiber consumed directly influence the type and amount of SCFAs produced by the microbiota (Ríos-Covián et al., 2016). Total SCFAs concentrations in grass carp (*Ctenopharyngodon idella*) gut decreased by almost 50% with dietary changes, and a positive correlation was observed between acetate levels and bacterial counts, thus demonstrating the effects of fiber type on SCFAs composition and production by the microbiota (Flint et al., 2014; Hao et al., 2017a). The pH profoundly influences SCFAs from the lumen to the colonocytes and the growth of SCFAs-producing bacteria (Cook and Sellin, 1998). Other factors such as environment, intestinal morphology and region, rate of passage, and microbiota composition influence SCFAs production (Canfora et al., 2015; Clements et al., 2014; Hao et al., 2017b; Wu et al., 2015). Microbial

metabolism involved in the fermentation of indigestible carbohydrate are shown in Figure 15 (Piazzon et al., 2017).

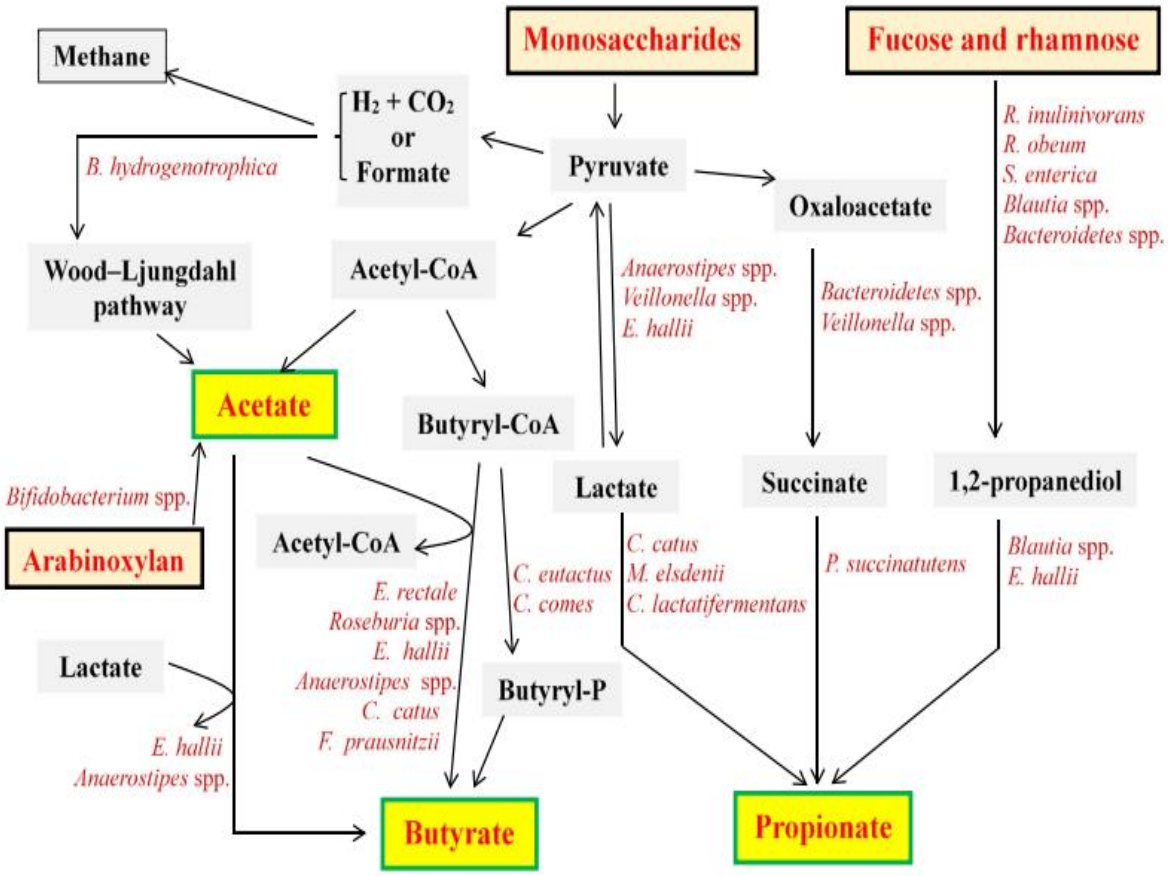

Figure 15. Overview of the production of acetate, propionate and butyrate by microbial fermentation in the intestine (Louis et al., 2014; Reichardt et al., 2018; Tran et al., 2020).

About 95 to 99% of SCFAs produced in the intestine are rapidly absorbed in the hindgut in monogastric animals (Den Besten et al., 2013). In tilapia, SCFAs absorption is driven mainly by anion exchange with bicarbonate (ratio of 1:4) between intestinal lumen and the blood (Titus and Ahearn, 1992). Once absorbed, colonocytes use about 98% of the butyrate, and the remainder of the SCFAs is transported to the liver (Den Besten et al., 2013; Morrison and Preston, 2016; Ríos-Covián et al., 2016). The remaining butyrate and acetate are destined for lipogenesis, while propionic acid is used for hepatic gluconeogenesis are excreted (Morrison and Preston, 2016; Ríos-Covián et al., 2016). The effects of SCFAs on host metabolism have been evaluated with several fish species, including performance improvements, feed efficiency, immune response, survival, glucose metabolism, lipid metabolism (Byrne et al., 2015;

Corrêa-Oliveira et al., 2016; Hoseinifar et al., 2017; Koh et al., 2016; Louis et al., 2014). Additionally, a special attention is driven to effects to butiric acid, Butyrate, salt of butyric acid, is considered an important nutrient for integrity of the epithelium along the gastrointestinal tract, where it has several effects in cells, influencing their maturation and differentiation, promoting an increase in cell proliferation and helping to maintain intestinal integrity (Morrison and Preston, 2016; Natarajan and Pluznick, 2014; Tan et al., 2014).

1.10. Intestinal morphology

Intestinal morphology represents a barrier of the organism against pathogens, including mechanical, chemical, immune and microbial barriers (Dawood, 2021). Disruption of the integrity of any of these barriers would lead to metabolic dysfunction of the body and affect the health of the intestine, which in turn results in a compromising of animal production performance and health (Camilleri et al., 2012).

Several histological dynamics in the intestine, such as crypt cell proliferation, cell migration along the crypt-villus axis, and cell extrusion from the apex of the villus via apoptosis, are all part of the control of cell desquamation and a dynamic renewal process in small intestine cell (Rombout Jan et al., 2011). The high viscosity of digesta in the lumen can increase the rate of villous cell loss, leading to villous atrophy, a phenomenon associated with increased production of crypt cells and, generally, with an increased crypt depth (Montagne et al., 2003).

Additionally, between the effects of β-mannanase in the diets, are the releasing of mannan oligosaccharides (MOS). The MOS are non-digestible oligosaccharides derived via partial hydrolysis of the mannans polysaccharide (Tester and Al-Ghazzewi, 2013). It is a prebiotic widely used in aquaculture due to its positive effects on growth, which can be generally divided into two main groups: α- and β-MOS (Lu et al., 2019). While α-MOS are obtained by cleavage of α-(1,6) bonds from yeast cell wall mannans, β-MOS are commonly obtained from mannans-rich plants through cleavage of β-(1,4)-glycosidic bonds (Yamabhai et al., 2016). Dietary MOS supplementation improved fish growth performance and gut health, and the results showed that appropriate dietary MOS supplementation could improve intestinal microbiota and increase the concentration of propionic acid and butyrate, suggesting that dietary MOS supplements were beneficial for fish gut health (Figure 16) (Liu and Huang, 2018).

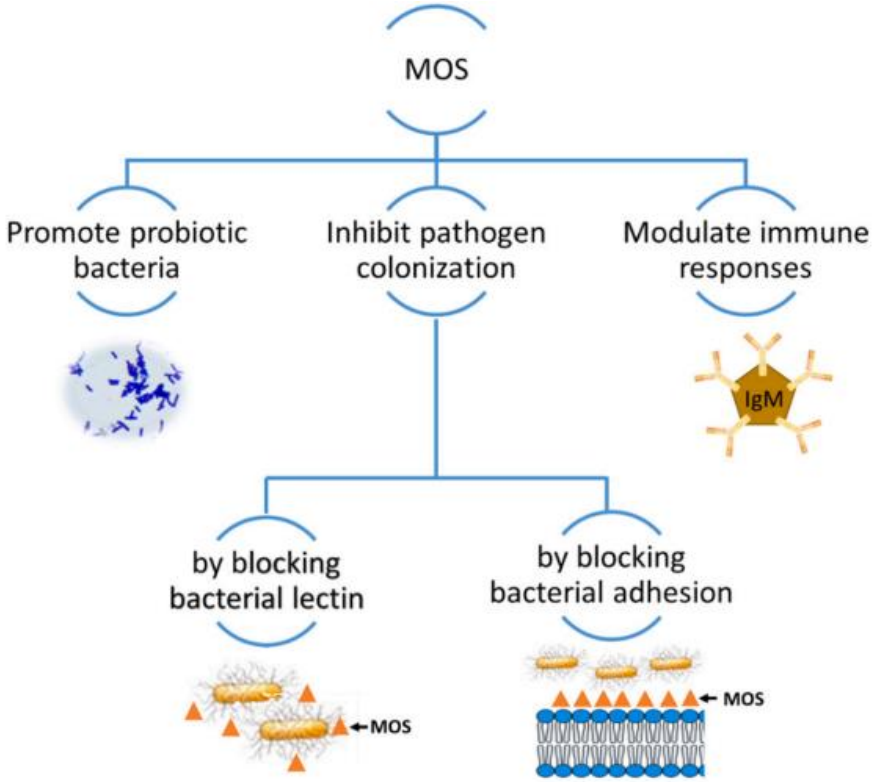

Figure 16. Mechanisms action of mannan oligosaccharides pathogen colonization inhibition (Faustino et al., 2021).

The MOS protected the intestinal morphology, which may be due to some aspects, such as the inhibition of the colonization of pathogenic bacteria on the intestinal surface. In addition to reducing excess reactive oxygen species, which can cause stomach injuries (Liu and Huang, 2018). Although some effects of MOS in fish organisms are known, more studies are still needed to evaluate the effects of the addition of β-mannanase on the release and use of MOS.

1.11. Considerations

The use of plant ingredients in aquafeeds has been increasing due to their relative availability and lower cost. However, plant-based diets also increase NSPs contents, particularly mannans, in fish feeds. Currently, there needs to be more information on the effects of mannans on fish physiology and nutrition. β-mannans are known to affect the viscosity of fish digesta, delaying gastric emptying and decreasing nutrient availability, which can negatively impact fish growth performance. The impact of mannans on the intestinal ecosystem is also unclear, with some studies suggesting that they may have adverse effects on fish growth and health. Supplementing fish diets

with exogenous enzymes, such as carbohydrases, can help improve nutrient utilization in fish fed plant-based diets. More research is needed to fully understand the effects of mannans, including their potential use as immunostimulants in Nile tilapia, and to create environmentally sustainable diets for fish farming that comply with sustainability principles. Furthermore, it is important to consider the economic dimension of the aquaculture system when assessing the use of enzymes such as β-mannanase. These suggest that β-mannanase is responsible for breaking down β-mannan, a complex carbohydrate found in plant-based feed ingredients.

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2. OBJECTIVES

To evaluate the effects of graded β-mannanase levels on viscosity of digesta and feces, pH of digesta and feces, growth performance, digestibility, digestive enzymes activity, blood parameters, SCFA production, gut health, and microbiome modulation in juveniles of Nile tilapia.

2.1. Specifc objectives

- Evaluate how increasing levels of β-mannanase affect growth performance, body composition and blood parametters of juvenile Nile tilapia;
- To assess the effects of β-mannanase on digesta viscosity, activity of digestive enzymes and nutrient digestibility;
- Determine the effects of liquid β-mannanase on short-chain fatty acids, gut morphology and microbiome responses;
- Evaluate the effects of increasing levels of β-mannanase on digesta viscosity and apparent digestibility coefficients of energy and nutrients, including amino acids in juvenile Nile tilapia;
- To establish a comprehensive correlation between fecal viscosity and nutrient digestibility in Nile tilapia fed a fiber-rich diet, using multivariate analysis.

CHAPTER II

Article I - Effect of dietary β-mannanase on growth performance, digesta viscosity, short-chain fatty acid, gut morphology, and microbiome of juvenile Nile tilapia fed plant-based diet

ABSTRACT: This study aimed to evaluate graded levels of dietary β-mannanase supplementation on growth performance, digesta viscosity, activity of digestive enzymes, short-chain fatty acid production (SCFAs), gut morphology, and microbiome of juvenile Nile tilapia fed plant-based diets. Fish (n = 504; body weight 7.0 ± 0.43 g) were randomly distributed in 24 aquaria of 70 L each in a recirculation aquaculture system in a completely randomized design with six treatments and four replicates of 21 fish in each aquarium. Fish were fed diets with graded levels of β-mannanase at 0 (control), 1600, 3200, 4800, 6400, and 8000 TMU kg^{-1}, and hand-fed 12 times a day until apparent satiety for eight weeks. Fish fed diet with β-mannanase at 4800 TMU kg^{-1} showed reduced digesta viscosity (−25.8%), body weight gain (+5.4%) and feed efficiency ratio (+12.1%), higher activity of amylase (+61.2%), protease (+25.4%) and lipase (+47.7%) enzymes, than fish fed control diet. Dietary β-mannanase at 4800 TMU kg^{-1} increased butyric acid content (+63.3%), reduced gut pH (−8.2%), and increased total villus height (+40.4%) in relative to fish fed control diet. Analysis of the "core microbiota" revealed that dietary β-mannanase modulated gut microbiota of juvenile Nile tilapia, and fish fed the diet with 4800 TMU kg^{-1} dietary β-mannanase showed higher abundance of beneficial bacteria, *Proteobacteria*, *Actinobacteria, Firmicutes,* and reduced population of potential harmful bacteria (*Escheiria* sp.). Overall, it concluded that β-mannanase at level 4800 TMU kg^{-1} in the diet enhances the growth performance of juvenile Nile tilapia by reducing digesta viscosity, enhancing digestive enzyme activity and short-chain fatty acid production, improving gut morphometry, by modulating gut microbiome. The use of liquid carbohydrates in diets based on alternative foods for tilapias emerges as an innovative

tool to improve the productive performance of fish sustainably and at a lower production cost.

Keywords: β-mannans, β-mannanase, carbohydrase, *Oreochromis niloticus*, microbiome, non-starch polysaccharides

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is the second most reared freshwater fish species worldwide, with omnivorous eating habits, consequently able to take advantage of ingredients of plant origin (FAO, 2022). These characteristics are valuable when it arises to not competing with human food, which is seen as a great advantage, given the growing demand for quality and sustainable food (Schader et al., 2015). However, plant-origin ingredients contain several anti-nutritional factors, such as non-starch polysaccharides (NSPs), that limit their inclusion in aquafeeds (Castillo and Gatlin, 2015; Sinha et al., 2011). Soybean meal is a particularly NSP-rich feed ingredient, containing 1.3-2.7% of β-mannans, which are primarily composed of galactomannans and glucomannans, and β-mannans that are not digested by fish (Tester and Al-Ghazzewi, 2013; Tiwari et al., 2020). β-mannans cause an increase in digesta viscosity, causing impairments in the diffusion and contact of digestive enzymes with their respective substrates (Chen et al., 2016; Liu et al., 2022; Siti-Norita et al., 2015). Such impairments reduce the digestibility and absorption of nutrients, thereby decreasing fish's growth performance (Dawood and Shi, 2022). Thus, using β-mannanase effectively reduces the anti-nutritional effects caused by NSPs (Castillo and Gatlin, 2015; Chen et al., 2016; Dawood et al., 2020).

The β-mannanase enzyme can hydrolyze the mannan bonds present in food. Its primary mode of action is the reduction of digesta viscosity, allowing for greater diffusion and access of digestive enzymes to substrates. Thus, β-mannanase increases digestibility and utilization of nutrients, thereby improving growth performance in monogastric animals (Chen et al., 2016; Sallam et al., 2020; Yilmaz et al., 2007). Furthermore, the release of additional nutrients and mannans for digestion by the enzyme leads to increased carbohydrate fermentation by the intestinal microbiota, which has been shown to

positively modulate the intestinal microbiota, stimulating beneficial bacteria production for the host's health and well-being (Guan et al., 2021; Tiwari et al., 2020). This modulation may also lead to a reduction in pathogenic organisms through changes in the short-chain fatty acid (SCFAs) production pattern, responsible for a decrease in intestinal pH (Dawood et al., 2022; Louis et al., 2014). SCFAs, a by-product of carbohydrate fermentation by intestinal microorganisms, can be used as an energy source and have been shown to stimulate intestinal health through an increase in the height and width of villi, as certain SCFAs, such as butyric acid, are utilized almost exclusively for the nutrition of intestinal absorptive cells (He et al., 2020; Kasubuchi et al., 2015; Tran et al., 2020). However, whether the β-mannanase regulates the underlying mechanism of gut health of Nile tilapia is largely unknown. Thus, the current research aimed to explore the effects of graded levels of exogenous β-mannanase supplementation on growth performance, blood parameters, digestive enzyme activity, SCFAs production, gut morphology, and microbiome composition in juvenile Nile tilapia fed extruded vegetable-based diets.

2. Material and methods

2.1. Ethics statement

All fish procedures were performed following the Guidelines for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of the State University of Ponta Grossa (Protocol: 22.000024303-4).

2.2. Diets

A basal diet contained 311.2 g kg^{-1} of crude protein and 18.98 MJ kg^{-1} of gross energy, without β-mannanase supplementation (control) was formulated based on

soybean meal, broken rice, wheat bran, corn, and poultry by-product meal as primary food ingredients, and formulated to meet the dietary requirements of Nile tilapia (NRC, 2011). From the basal diet, five other diets were elaborated by supplementing 1600, 3200, 4800, 6400 and 8000 TMU kg^{-1} diet of β-mannanase. Exogenous β-mannanase enzyme inclusion replaced an equal silica amount, as shown in Tables 1 and 2.

Table 1. Ingredients composition of the experimental diets (g kg^{-1} diet).

Ingredients	g kg^{-1} (as-fed basis)
Broken rice[a]	80
Soybean meal[b]	440
Poultry by-product meal[c]	150
Wheat bran[b]	100
Corn[b]	165
Soybean oil[d]	20
Corn starch[e]	20
DL-methionine 99[f]	2
L-lysine[f]	3
Dicalcium phosphate[g]	10
Mineral and vitamin mix[h]	8
Inert (Silica)[i]	1
Cr_2O_3[j]	1

[a] Armazém São Vito, São Paulo, SP, Brazil.
[b] Bunge, Ponta Grossa, PR, Brazil.
[c] BRF, Toledo, PR, Brazil.
[d] Coamo, PR, Brazil.
[e] Yoki, São Bernardo do Campo, São Paulo, Brazil.
[f] Ajinomoto Animal Nutrition Division, SP, Brazil.
[g] Sarfos, Goiás, Brazil.
[h] Customized premix (Composition per kilogram of feed (IU or mg kg^{-1} of diet): vitamin A (retinyl acetate), 6,000 IU; vitamin D_3, (cholecalciferol), 1,000 IU; vitamin E (DL-α-tocopheryl acetate), 60 mg; vitamin K_3 (menadione Na-bisulphate), 12 mg; vitamin B_1 (thiamine HCl), 24 mg; vitamin B_2 (riboflavin), 24 mg; vitamin B_6 (pyridoxine HCl), 20 mg; vitamin B_{12} (cyanocobalamin), 0.05 mg; folic acid, 6 mg; D-calcium pantothenate, 60 mg; ascorbic acid (ascorbyl polyphosphate), 350 mg; D-biotin, 0.24 mg; choline chloride, 800 mg; niacin, 120 mg; ferrous sulfate ($FeSO_4.H_2O.7H_2O$), 50 mg; copper sulfate ($CuSO_4.7H_2O$), 3 mg; manganese sulfate ($MnSO_4.H_2O$), 20 mg; zinc sulfate ($ZnSO_4.7H_2O$), 30 mg; potassium iodide (KI), 0.4 mg, cobalt sulfate ($CoSO_4.4H_2O$), 0.25 mg; sodium selenite (Na_2SeO_3), = 0.1 mg, BHT, 200 mg; calcium propionate, 1000mg.
[i] Merck Company, Germany.
[j] Sygma-Aldrich Brazil Ltda, 99.5%, São Paulo, SP, Brazil.

All diets were ground through a 0.8-mm screen in a centrifugal mill (Viera MC 680B, Tatuí, SP, Brazil). The extrusion process was performed through a 1.5-mm die diameter in a single screen extruder with die temperature set at 92°C (Exteec EX30, Ribeirão Preto, SP, Brazil), obtaining pellets with 2.5-mm of diameter and floatability rate higher than 99%. After that, the pellets were dried in a drying drum with rotary drier at 55ºC (pellet temperature) for 10 min (Model E-62, Ferraz Máquinas e Engenharia LTDA, Ribeirão Preto, SP, Brazil).

Table 2. Analyzed composition of the basal diet (g kg^{-1} dry matter basis).

Item	g kg^{-1} (as-dry matter basis)
Dry matter	932.1
Gross energy (MJ kg^{-1})	18.98
Crude protein	311.2
Crude fiber	38.24
Crude lipid	31.40
Ash	64.3
Amino acid	
Essential amino acid	
Arginine	1.910
Histidine	0.811
Isoleucine	1.149
Leucine	2.536
Lysine	1.796
Methionine	0.582
Phenylalanine	1.620
Threonine	1.409
Tryptophan	0.366
Valine	1.687
Non-essential amino acid	
Alanine	1.722
Aspartic acid	2.829
Cysteine	0.511
Glutamic acid	4.756
Glycine	1.892
Proline	0.000
Serine	1.807
Tyrosine	0.944

Liquid β-mannanase (Natupulse® TS, BASF, Ludwigshafen am Rhein, Germany; 8000 TMU g^{-1}) were top-sprayed onto each kilogram of diet to supply 1600;

3200; 4800; 6400 and, 8000 TMU kg^{-1} diet of endo-1,4-β-mannanase, being applied 0.2; 0.4; 0.6; 0.8 and 1.0 g kg^{-1} of Natupulse. The same procedure was applied to unsupplemented diet to receive the same treatment, but without the commercial β-mannanase inclusion in soybean oil.

2.3. Fish and Experimental Design

The experiment was conducted at the Aquaculture Laboratory of the State University of Ponta Grossa, Ponta Grossa, PR, Brazil. All-male masculinized Nile tilapia fingerlings (*n* = 1500; 3.0 ± 0.5 g; Premium Aquabel strain) were obtained from Aquabel Fish Farm (Rolândia, PR, Brazil). Fish were acclimated for a 4-week period in a circular tank (500 L), with temperature and dissolved oxygen set at 28°C and 6 mg L^{-1}, respectively. Fish were hand-fed a commercial extruded diet (Supra, 1.0 mm Ø; Alisul Alimentos, Maringá, PR, Brazil), with 460 g kg^{-1} of crude protein, six times daily for 21 days. Afterward, fish (*n* = 504; 7.0 ± 0.43 g; mean ± SD) were grouped-weighed and randomly distributed into 24 plastic aquaria (70 L each) equipped with a recirculating system composed of a decanter to remove solids, a mechanical filter with bio-balls, heater (3000W) and a central UV-light disinfection system (55W). The aeration system was comprised of a centrifugal 0.5-HP blower (Sulpesca, Toledo, PR, Brazil) fitted with silicone airline tubing, with a porous stone in each experimental aquarium. Each aquarium was siphoned daily to keep a renovation of 10% of the water volume and remove fish metabolites. Temperature was set at 28 ± 0.5°C, dissolved oxygen was kept at 6.2 ± 0.2 mg L^{-1}, and water flow was kept at 1.2 L min^{-1} per aquarium throughout the trial. Data of individual aquarium temperature and dissolved oxygen were monitored daily using YSI Multi-Parameter Water Quality Meter (YSI Incorporated, Ohio, USA). Water quality parameters were monitored weekly with a pH-meter (TEC-2, Piracicaba, SP, Brazil) and

kept at 7.0 using calcium carbonate and phosphoric acid; ammonia, nitrite, and nitrate analysis were performed using commercial kits (Alfakit, Florianópolis, SC, Brazil), and were kept at 0.01; 0.02 and 0.01 mg L^{-1}, respectively. Fish were hand-fed from 8:00 to 18:00 h; 12 times daily until apparent satiety for 60 days.

2.4. Sample collection

At the beginning of the feeding trial, 50 fish with a 24-h period of fasting were randomly sampled for initial whole-body composition analysis. On day 59 of the experimental trial, all fish were staggered fed, and after 4 h, four fish from each aquarium were randomly sampled and euthanized with overdose of tricaine methanesulphonate (MS-222;800 mg L^{-1}), individually weighed, and samples of intestine and gut digesta activity of pH, digestive enzymes SCFAs and microbiome analysis. For histology analysis, the middle part of the intestine (1 cm) of four fish from each aquarium (16 fish per treatment) was collected and fixed in 10% buffered formalin for 24 h. Further, the mid-intestine content was aseptically collected for microbiome analysis. For this, the gut was gently squeezed, and 750 mg of digesta from each fish was collected with a 1,000 mL micropipette, pooled in a 2-mL cryogenic tube, snap-frozen in liquid nitrogen, and stored at −80°C. On day 60, fish were fasted for 24h and bulk weighed, and six fish from each aquarium were randomly sampled and euthanized with an overdose of MS-222 (Sigma-Aldrich; 800 mg L^{-1} water) for whole-body proximate composition analysis. In parallel, four fish from each aquarium were randomly collected, anesthetized for blood collection, and euthanized for liver and visceral fat weight measurements. A pooled 3 mL blood aliquot was collected from the caudal vein for biochemical analysis. Plasma was obtained by centrifugation at 3000 rpm for 10 min (Kasvi – SKU K14-1215, São José dos Pinhais, PR, Brazil). Feces samples were collected daily from days 50 to 58 of the feeding

trial, 30 min. after feeding, kept in falcon tubes (50 mL), and frozen at −20°C for viscosity analysis.

2.5 Chemical analysis

The proximate composition of diets and whole-body fish samples were performed according to standard methods of Association of Official Analytical Chemists (AOAC, 2002). Moisture analysis was determined by oven-drying at 105°C until constant weight, while crude lipid analysis was performed by ether-extraction method (Folch et al., 1957). Crude protein (N × 6.25) analysis was performed using the macro Kjeldahl method (Tecnal, MA-036, Piracicaba, SP, Brazil) after acid hydrolysis. The analysis of ash was achieved by overnight combustion in a muffle furnace at 550°C (Tecnal, 2000B, Belo Horizonte, MG, Brazil). The crude fiber analysis was performed according to loss on ignition of dried lipid-free residues following digestion with 1.25% H_2SO_4 and 1.25% NaOH. The profile of dietary amino acids were determined by High Performance Liquid Chromatography (HPCL) (Hitachi, Tokyo, Japan), at the Laboratory of Ajinomoto do Brasil Indústria e Comércio de Alimentos Ltda, Division of Animal Nutrition (São Paulo, SP, Brazil) (Rayner, 1985). Tryptophan was determined after alkaline hydroxylation of the sample with lithium hydroxide.

2.6 Calculations

Growth performance parameters were calculated as follows:

- Body weight gain (%) = [(final weight (g) − initial weight (g)) / (initial weight (g))] × 100.
- Feed intake (% of body weight per day^{-1}) = [dry feed intake (g) / average fish weigh (g) / days fed] × 100.

- Feed efficiency ratio = [weight gain dry feed consumed (g) / dry feed consumed (g)].
- Protein efficiency ratio = (%) = [(protein gain (g) x protein intake (%))] x 100.
- Energy retention efficiency (%) = [(energy gain (MJ) / energy intake (MJ))] × 100.
- Hepatosomatic index (%) = [(liver weight (g) / body weight (g))] × 100.
- Visceral fat ratio (%) = [(visceral fat weight (g) / visceral fat (g)] × 100.
- Survival (%) = number of fish at the end of the experimental trial / number of fish at the beginning of the experimental trial x 100.

2.7. Digesta pH and viscosity

Digesta pH was measured using a pH-meter (Kasvi – ATC-K39-0014PA, São José dos Pinhais, PR, Brazil), placed directly in the gut digesta. Feces samples were centrifuged at 3000 rpm x g for 10 min (Kasvi – SKU K14-1215, São José dos Pinhais, PR, Brazil) to obtain the liquid phase. The supernatant obtained was placed in the viscometer (Brookfield Digital Viscometer, Model DV-II Version 2.0, Brookfield Engineering Laboratories Inc., Stoughton, MA), set at 28°C. The viscosity measurement was the average 50.0/s shear rate, and the viscosity values were recorded as apparent viscosity in centipoise (cP).

2.8. Activity of digestive enzymes

Intestinal tissues were homogenized in buffer (10 mM phosphate / 20 mM Tris-pH 7.0) for 1 minute (4°C) using Potter Dounce homogenizer. Then the samples were centrifuged at 5000 rpm for 5 minutes, and the supernatants were collected for enzymatic assays. Amylase and lipase activity were estimated using commercial kits

(Kit Bioclin). The total non-specific proteolytic activity was measured using the casein hydrolysis method (Kunitz, 1946) with minor modifications (Walter, 1984). The enzymatic reaction consisted of 1% casein in water (0.25 mL), 0.1 M Tris HCl pH 7 (0.25 mL) and enzyme sample (0.1 mL), being incubated for 1 h at 3°C. The reaction was stopped by adding 0.6 ml of 8% trichloroacetic acid. After holding for 1 h at 2°C, samples were centrifuged at 1800 × g for 10 min, and absorbance of the supernatant was recorded at 340 nm. Tyrosine was used as a standard, and one unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 µg of tyrosine per minute.

2.9. Blood parameters

Blood parameters were analyzed by spectrometry in a semi-automatic biochemical analyzer (BIO-2000 IL, Barueri, SP, Brazil) using commercial kits (Bioclin – Quibasa, Belo Horizonte, MG, Brazil) to determine total protein (Cat. 90.019.00), triglycerides (Cat. 90.022.00), cholesterol (Cat. 90.021.00), and glucose (Cat. 90.017.00) contents. Additionally, the blood parameters of alanine aminotransferase (Cat. 90.013.00) and aspartate aminotransferase (Cat. 90.015.00) enzymes were analyzed using commercial kits.

2.10. Short Chain Fatty Acids

The concentrations of acetic, propionic and butyric acids in the samples were determined by gas chromatography using Shimadzu© GC-2010 Plus chromatograph equipped with AOC-20i automatic injector, Stabilwax-DA™ capillary column (30m, 0.25mm ID, 0.25µm df, Restek©) and flame ionization detector (FID), after acidification

with 1 M o-phosphoric acid p.a. (Ref. 100573, Merck©) and fortification with a mixture of free volatile acids (Ref. 46975, Supelco©).

An aliquot of 1μL of each sample was injected with a split ratio of 40:1, using helium as carrier gas with a linear velocity of 42 $cm.s^{-1}$, obtaining the separation of the analytes in a chromatographic run of 11.5 minutes. The injector and detector temperatures were 250ºC and 300ºC, respectively, and the initial column temperature was 40ºC. The column temperature ramp started with a gradient from 40 to 120ºC at the rate of $40^{o}C.min^{-1}$, followed by a gradient from 120 to 180ºC at the rate of $10^{o}C.min^{-1}$ and from 180 to 240ºC at the rate of $120^{o}C.min^{-1}$, keeping the temperature at 240ºC for another 3 min. For the quantification of analytes, a calibration of the method was performed with dilutions of WSFA-2 standard (Ref. 47056, Supelco®) and glacial acetic acid (Ref. 33209, Sigma-Aldrich®) analyzed under the conditions described above. The determination and integration of peaks were performed using the software GCsolution v. 2.42.00 (Shimadzu©).

2.11. Intestinal histology

The middle part of the intestines of four fish from each aquarium (16 fish per treatment) was sampled (1cm) and fixed in buffered formalin (10%) for 24 hours. Intestinal fragments were embedded in paraffin blocks (Prophet et al., 1992), using semi-serial 5 μm cross-sectioned, and finally stained with hematoxylin-eosin (HE), according to previously described methodology (Dimitroglou et al., 2010). For the villous height measurement, 100 intact villi were measured per fish, totaling 1600 measures per treatment. The histological sections were examined under an optical microscope attached to a camera (Pro-Series from Media Cybertechniques, Olympus, Japan) to capture images. The total villus height (TVH), villus width (VW), and villus

epithelium thickness (VET) were measured using the Image-Pro Plus software (Image Pro Plus - version 5.2- Cyber Media).

2.12. Microbiome

Commercial kit GenElute™ Soil DNA Isolation Kit (Sigma Aldrich®) was used to extract the DNA from the samples, following the protocol recommended by the manufacturer. The extracted DNA was quantified by spectrophotometry at 260nm. The integrity of the extracted DNA was checked by electrophoresis on a 1% agarose gel, stained with a 1% ethidium bromide solution, and visualized with ultraviolet light. A 250-base segment of the hypervariable region V4 of the ribosomal 16S rRNA gene was amplified using universal primers 515F and 806R and the following PCR conditions: 94°C for 3 min, 18 cycles of 94°C for 45 sec, 50°C for 30 sec and 68°C for 60 sec, followed by 72°C for 10 min. The amplifiers were pooled and sequenced in Illumina® "MiSeq" sequencer (Degnan and Ochman, 2012). A summary of the sequences used in the taxonomic classification is furnished in Table 3.

Table 3. Summary of the sequences used in taxonomic classification.

Sample count / summary	
Number of samples	24
Number of genera	23
Number of readings	3,230,149
Minimum number of readings per sample	32,924
Maximum number of readings per sample	337,408

Readings obtained in the sequencer were analyzed using the QIIME (Quantitative Insights Into Microbial Ecology) platform, followed by a workflow of removal of low-quality sequences and chimeras and taxonomic classification (Caporaso et al., 2011). The identity (> 97%) between the sequences was considered against a

database. An average of 140.441 readings per sample were used to generate the classification of bacterial communities, normalizing the data and not comparing samples with different readings, thus avoiding a taxonomy bias.

2.13. Statistical analysis

All results were described as least square means and pooled standard error of means (SEM). All data were tested for normality using Kolmogorov–Smirnov test, and homogeneity was tested using Levene's test. Data were analyzed as a two-way ANOVA using the General Linear Model (GLM) procedure. The dose-response effect of supplemental β-mannanase was determined using an orthogonal polynomial contrast for linear and quadratic effects (SAS, version 9.2). In addition, Dunnett's test procedure was used to compare data from each β-mannanase supplementation level with the non-supplemented diet (control). The Welch test ($P < 0.05$) was applied for microbiome analysis, followed by the Bonferroni correction test. The analyses were performed using the statistical metagenomics program STAMP for statistical analysis of metagenomic profiles (Parks et al., 2014). The averages for biodiversity between treatments were compared using the number of observed OTUs and the Chao1 index by the Kruskal Wallis test ($P < 0.05$) once a non-parametric distribution was detected by the Shapiro-Wilk test. Multivariate analysis was employed to conduct principal component (PC) analysis, and the score and loading plot were utilized to ascertain the correlation among individual variables of the first two eigenvalues (PC 1 and 2). All data were analyzed according to the Proc GLM of the Statistical Analysis System (Version 9.0), and values were presented as mean ± standard error.

3. **Results**

3.1. Growth performance

The effects of dietary β-mannanase supplementation on the growth performance of juvenile Nile tilapia are presented in Table 4. The final body weight ($P < 0.001$; $R^2 = 0.508$; $Y_{max.} = 4480$ TMU kg^{-1} β-mannanase), body weight gain ($P < 0.018$; $R^2 = 0.248$; $Y_{max.} = 4320$ TMU kg^{-1} β-mannanase), protein retention efficiency ($P < 0.001$; $R^2 = 0.752$; $Y_{max.} = 5360$ TMU kg^{-1} β-mannanase) and energy retention efficiency ($P < 0.001$; $R^2 = 0.752$; $Y_{max.} = 5440$ TMU kg^{-1} β-mannanase) tended to increase, while feed intake ($P < 0.001$; $R^2 = 0.752$; $Y_{max.} = 5520$ TMU kg^{-1} β-mannanase) tended to decrease in a quadratic pattern by the polynomial regression analysis in fish fed graded levels of β-mannanase.

Table 4. Growth performance of juvenile Nile tilapia fed the experimental diets[1].

	β-mannanase [2] (TMU kg^{-1} diet)							*P-value*		
Parameter	0	1600	3200	4800	6400	8000	SEM[3]	L[4]	Q[4]	Dunnet[5]
Initial body weight (g)	7.16	7.13	7.24	7.13	7.14	7.26	0.034	0.619	0.726	0.938
Final body weight (g)	118.21	119.02	121.57	123.94*	124.11*	118.09	0.509	0.185	<0.001	<0.001
Body weight gain (%)	1552.4	1570.4	1580.6	1637.0*	1638.3*	1530.1	9.024	0.583	0.018	0.049
Feed intake (% body weight day^{-1})	2.97	2.72*	2.67*	2.66*	2.60*	2.67*	0.020	<0.001	<0.001	<0.001
Feed efficiency ratio	0.99	1.08*	1.10*	1.11*	1.14*	1.10*	0.011	<0.001	<0.001	<0.001
Protein retention efficiency (%)	38.48	43.69*	46.12*	46.82*	46.05*	45.45*	0.52	<0.001	<0.001	<0.001
Energy retention efficiency (%)	36.25	41.04*	42.42*	42.85*	42.03*	40.66*	0.36	<0.001	<0.001	<0.001
Hepatosomatic index (%)	2.95	3.23	3.19	3.31	3.37	3.20	0.060	0.289	0.311	0.785
Visceral fat ratio (%)	2.14	1.83	1.67	1.99	2.18	1.95	0.044	0.720	0.173	0.067

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1}) by Dunnet´s test ($P < 0.05$).

The feed efficiency ratio ($P < 0.001$; $R^2 = 0.745$; $Y_{min.} = 5440$ TMU kg^{-1} β-mannanase) increased quadratically in fish fed graded levels of dietary β-mannanase (Figure 1).

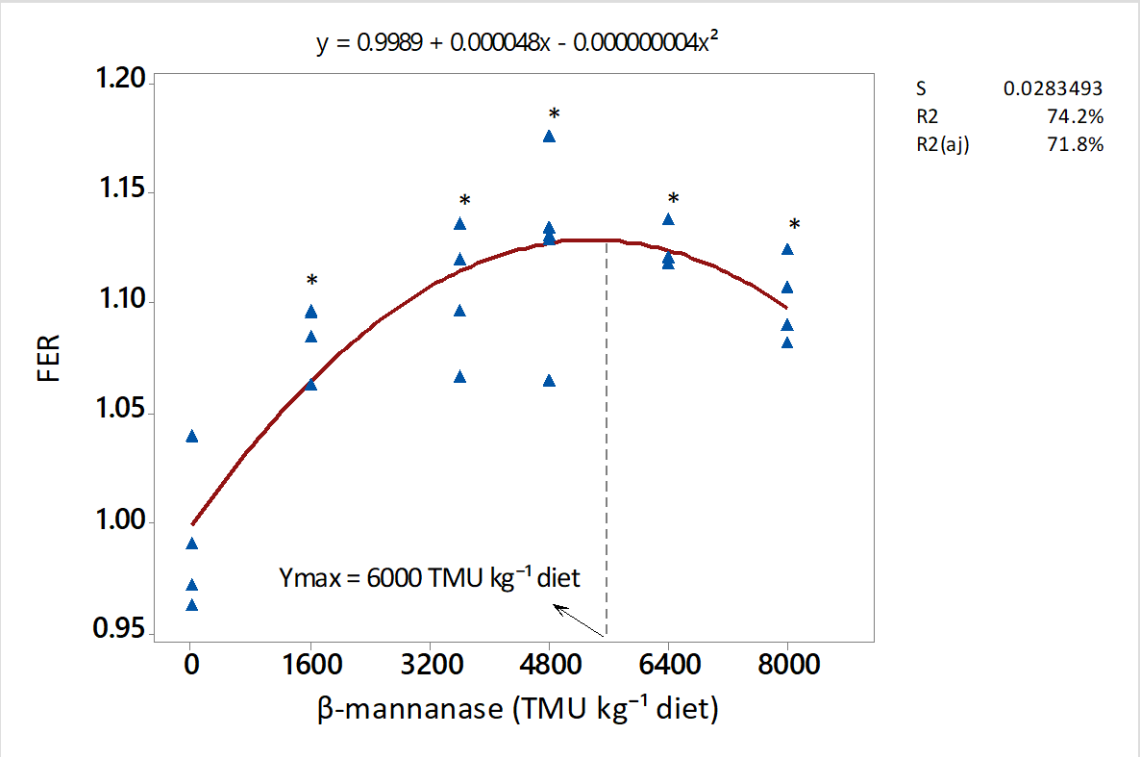

Figure 1. Feed efficiency ratio of juvenile Nile tilapia fed diets with graded levels of β-mannanase. Each dot triangle represents mean value of 21 fish as replicate aquarium. Orthogonal polynomials were used to evaluate quadratic responses to the levels of β-mannanase. Means with asterisks superscripts differ significantly from control diet (β-mannanase = 0 TMU kg^{-1} diet) by Dunnet´s test ($P < 0.05$).

Based on Dunnet's test, final body weight ($P < 0.001$) and body weight gain ($P < 0.049$) were significantly higher in fish fed 4800 and 6400 TMU kg^{-1} diet of β-mannanase than those fish fed control diet. Additionally, feed conversion ratio ($P < 0.001$), protein retention efficiency ($P < 0.001$), and energy retention efficiency ($P < 0.001$) were significantly higher in fish β-mannanase-supplemented diets than fish fed control diet. There were no significant differences in hepatosomatic index, and the visceral fat ratio of fish fed experimental diets ($P > 0.05$), and no fish mortality was recorded during the feeding trial.

3.2. Whole-body composition

The effects of β-mannanase supplementation on whole-body composition of juvenile Nile tilapia are presented in Table 5. Whole-body crude protein ($P = 0.007$), crude lipids ($P < 0.001$), and ash ($P = 0.008$) contents increased linearly with increasing levels of dietary β-mannanase.

Table 5. Whole-body composition (g kg^{-1}) of juvenile Nile tilapia fed the experimental diets[1].

	β-mannanase [2] (TMU kg^{-1} diet)							*P-value*		
Parameter	0	1600	3200	4800	6400	8000	SEM[3]	L[4]	Q[4]	Dunnet[5]
Moisture	73.38	73.11	72.86	73.31	72.06	73.28	0.123	0.330	0.331	0.110
Crude protein	12.55	12.98	13.42*	13.18*	13.51	13.32	0.080	0.007	0.065	0.039
Crude lipid	9.75	10.39	10.58*	10.41	10.61*	12.17*	0.126	<0.001	0.074	<0.001
Ash	3.33	3.22	3.45	3.45	3.41	3.76	0.039	0.008	0.240	0.054
Gross energy (MJ kg^{-1})	6.48	6.54	6.61	6.66	6.74	6.55	0.039	0.347	0.289	0.745

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1}) by Dunnet´s test ($P < 0.05$).

Additionally, Dunnet's test showed significant increases in whole-body crude protein, and crude lipid in fish fed diets with 3200 to 8000 TMU kg^{-1} dietary β-mannanase than fish fed control diet. However, whole-body moisture and gross energy contents were not affected by adding different β-mannanase levels in the diet ($P > 0.05$).

3.3. Activity of digestive enzymes

The effects of dietary β-mannanase on the activity of digestive enzymes of Nile tilapia juveniles are summarized in Table 6. The amylase activity increased linearly ($P < 0.001$) according to increasing β-mannanase levels in the diets.

Table 6. Digestive enzymes in the gut of juvenile Nile tilapia fed the experimental diets[1].

	β-mannanase [2] (TMU kg^{-1} diet)							*P-value*		
Parameter	0	1600	3200	4800	6400	8000	SEM[3]	L[4]	Q[4]	Dunnet[5]
Amylase (μm g^{-1})	20.72	25.52*	33.86*	33.40*	40.41*	43.82*	1.356	<0.001	0.152	<0.001
Protease (μm g^{-1})	26.94	28.83*	33.44*	33.79*	33.61*	33.85*	0.525	<0.001	<0.001	<0.001
Lipase (μm g^{-1})	3.25	3.78*	4.37*	4.80*	4.42*	3.90*	0.091	0.009	<0.001	<0.001

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1}) by Dunnet′s test ($P < 0.05$).

Differently, protease ($P < 0.001$; $R^2 = 0.908$; $Y_{max.} = 6320$ TMU kg^{-1} β-mannanase) and lipase ($P < 0.001$; $R^2 = 0.861$; $Y_{min.} = 4800$ TMU kg^{-1} β-mannanase) activity tended to increase in a quadratic manner ($P < 0.001$) according to graded β-mannanase levels. According to Dunnet's test, the addition of β-mannanase promoted higher activity ($P < 0.001$) of digestive enzymes compared to fish fed control diet.

3.4. Blood parameters

The effects of dietary β-mannanase on blood parameters of juvenile Nile tilapia are shown in Table 7. Alanine aminotransferase activity ($P < 0.001$; $R^2 = 0.763$; $Y_{max.} = 4720$ TMU kg^{-1} β-mannanase) and triglycerides contents ($P < 0.001$; $R^2 = 0.438$; $Y_{min.} = 4480$ TMU kg^{-1} β-mannanase), tended to decrease in a quadratic manner ($P < 0.001$) according to dietary β-mannanase increased in the diet. In contrast, blood glucose levels decreased linearly ($P < 0.013$) as dietary β-mannanase increased.

Table 7. Blood parameters of juvenile Nile tilapia fed the experimental diets[1].

Parameter	β-mannanase [2] (TMU kg^{-1} diet)						SEM[3]	*P-value*		
	0	1600	3200	4800	6400	8000		L[4]	Q[4]	Dunnet[5]
Aspartate aminotransferase (IU L^{-1})	32.30	32.30	39.29	36.09	33.18	37.98	1.345	0.452	0.705	0.817
Alanine aminotransferase (IU L^{-1})	65.60	54.24	22.88*	28.09*	34.65*	47.30*	2.796	0.031	<0.001	<0.001
Triglycerides (mg dl^{-1})	222.59	242.45	308.65*	311.09*	319.32*	230.47	9.154	0.263	0.001	0.010
Glucose (mg dl^{-1})	62.77	65.49	58.02	57.66	57.47	50.96	1.364	0.013	0.625	0.194
Total protein (g dl^{-1})	2.42	2.27	1.94	2.35	2.31	2.30	0.056	0.951	0.333	0.653
Cholesterol (mg dl^{-1})	118.38	105.56	108.53	117.02	121.01	118.50	2.487	0.459	0.502	0.806

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1}) by Dunnet´s test ($P < 0.05$).

Based on Dunnet's test, alanine aminotransferase activity was significantly lower ($P < 0.001$) in fish fed diets with 3200 to 8000 TMU kg^{-1} dietary β-mannanase than fish fed control diet. In addition, blood triglycerides were significantly ($P < 0.010$) higher in fish fed diets with 3200 to 6400 TMU kg^{-1} dietary β-mannanase than fish fed control diet. Nevertheless, there were no significant differences ($P > 0.05$) in aspartate aminotransferase, total protein, and cholesterol content in plasma.

3.5. Short chain fatty acids, viscosity, and pH of feces

The effects of dietary β-mannanase addition on gut SCFAs production, pH, and viscosity values are shown in Table 8. Gut acetic acid ($P < 0.001$; $R^2 = 0.593$; $Y_{max.} = 4160$ TMU kg^{-1} β-mannanase diet), propionic acid ($P < 0.001$; $R^2 = 0.637$; $Y_{max.} = 2880$ TMU kg^{-1} β-mannanase) and butyric acid ($P < 0.001$; $R^2 = 0.632$; $Y_{max.} = 3920$ TMU kg^{-1} β-mannanase), increased in a quadratic manner with the inclusion of increasing levels of dietary β-mannanase. However, the gut viscosity and pH decreased linearly ($P < 0.001$) as the β-mannanase supplementation increased in the diet.

Table 8. Short-chain fatty acids, digesta viscosity and pH of juvenile Nile tilapia fed the experimental diets[1].

	β-mannanase [2] (TMU kg^{-1} diet)							*P-value*		
Parameter	0	1600	3200	4800	6400	8000	SEM[3]	L[4]	Q[4]	Dunnet[5]
Acetic acid	11.62	12.61	13.61*	14.43*	13.20	11.53	0.229	0.705	<0.001	<0.001
Propionic acid	0.97	0.92	1.03	1.03	0.88	0.57*	0.030	0.005	<0.001	<0.001
Butyric acid	0.49	0.69*	0.78*	0.80*	0.58	0.51	0.025	0.727	<0.001	<0.001
Viscosity (cP)	3.41	2.89*	2.89*	2.53*	2.17*	1.76*	0.095	<0.001	0.178	<0.001
pH	7.93	7.38*	7.29*	7.28*	7.28*	7.25*	0.040	<0.001	0.001	<0.001

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1}) by Dunnet´s test ($P < 0.05$).

Based on Dunnet's test, acetic acid content ($P < 0.001$) was higher in fish fed diets with 4800 and 6400 TMU kg^{-1} diet of β-mannanase compared to fish fed control diet, whereas the propionic acid production ($P < 0.001$) was higher in the fish fed diet with 8000 TMU kg^{-1} diet of β-mannanase than fish fed control diet. The butyric acid production ($P < 0.001$) was higher in fish fed diets with 1600, 3200, and 4800 TMU kg^{-1} diet of β-mannanase than fish fed control diet. The viscosity ($P < 0.001$) and digesta pH ($P < 0.001$) was lower in fish fed β-mannanase supplemented diet relative to that fed control diet.

3.6. Gut morphometry

The effects of dietary β-mannanase on gut morphometry are shown in Table 9. The total villus height ($P = 0.003$; $R^2 = 0.495$; $Y_{min.} = 5040$ TMU kg^{-1} β-mannanase) and the villus width ($P = 0.031$; $R^2 = 0.239$; $Y_{min.} = 4560$ TMU kg^{-1} β-mannanase) increased in a quadratic manner in fish fed graded levels of dietary β-mannanase.

Table 9. Intestinal morphology of juvenile Nile tilapia fed the experimental diets[1].

Parameter	β-mannanase [2] (TMU kg^{-1} diet)						SEM[3]	*P-value*		
	0	1600	3200	4800	6400	8000		L[4]	Q[4]	Dunnet[5]
Total villus height (µm)	341.4	368.7	474.7*	479.4*	615.5*	383.9	20.789	0.021	0.003	<0.001
Villus width (µm)	138.3	143.2	158.0	167.5	168.1	140.7	4.612	0.320	0.031	0.194
Villus height: villus width	2.5	2.6	3.0	2.9	3.7*	2.8	0.106	0.038	0.101	0.011

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1}) by Dunnet´s test ($P < 0.05$).

However, the villus height:width ratio increased linearly (P = 0.038), with increased dietary levels of β-mannanase. According to Dunnet's test, the total villus height was higher (P < 0.001) in fish fed diets with 3200 and 6400 TMU kg^{-1} diet of β-mannanase than fish fed control diet (Figure 2).

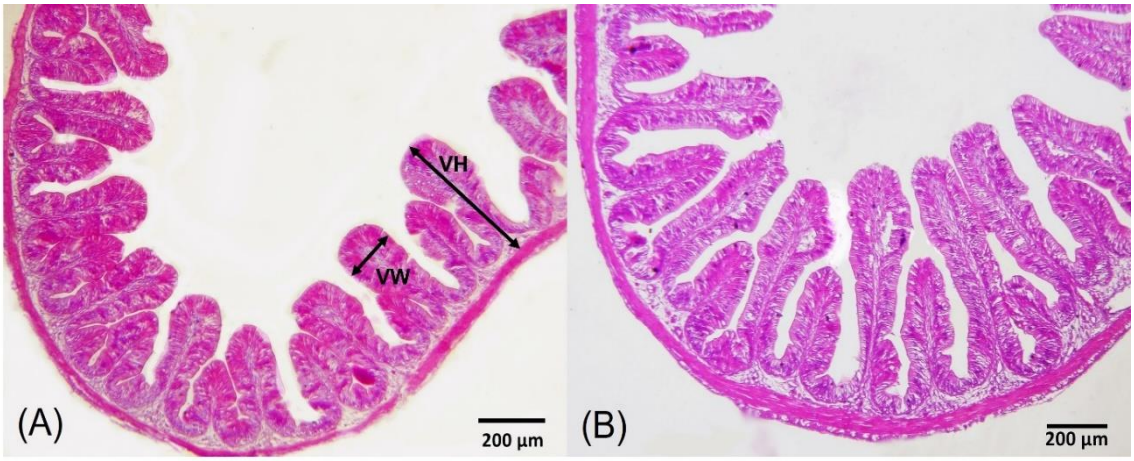

Figure 2. Villus height (VH), villus width (VW), and villus:width relation (VWR) of the medium intestine wall of juvenile Nile tilapia fed either diet control, not supplemented (control) or supplemented with β-mannanase 4800 TMU kg^{-1}. Objective: 40×. Staining: Hematoxylin-eosin.

Additionally, the villus height:width ratio was higher (P = 0.011) in fish fed diet with 6400 TMU kg^{-1} of dietary β-mannanase than fish fed control diet. Notably, fish fed diet with 6400 TMU kg^{-1} diet β-mannanase showed higher villus height with similar villus width, in which resulted in higher villus height:width ratio relative to fish fed control diet.

3.7. Gut microbiota population characteristics

The alpha diversity index showed higher (P < 0.05) bacterial diversity in fish fed the β-mannanase supplemented diets. The taxonomic composition of bacterial communities at the phylum level is presented in Figure 3.

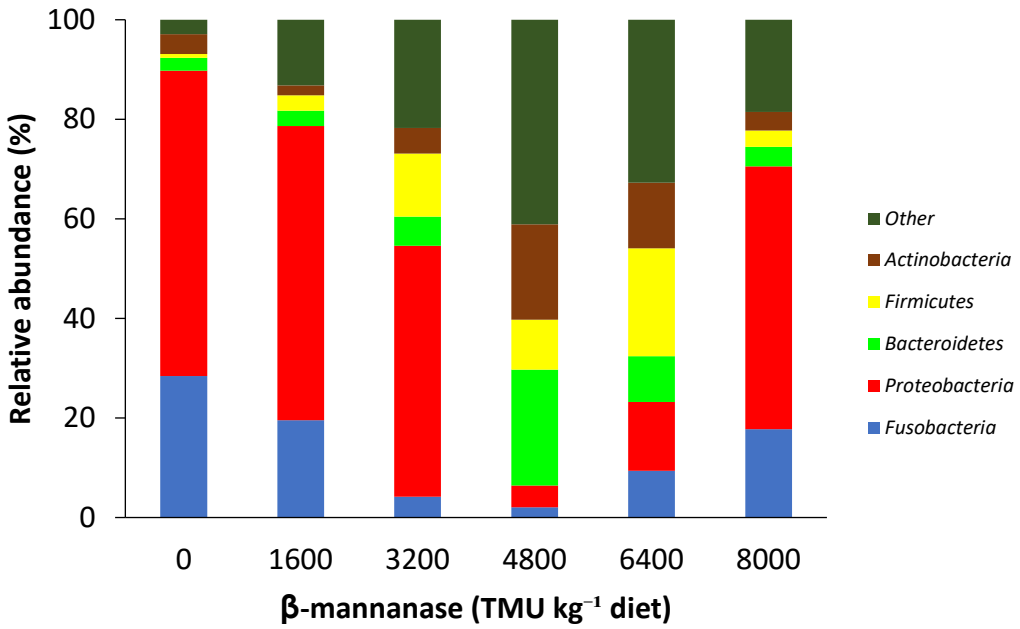

Figure 3. General view of the taxonomic composition of the bacterial community of juvenile Nile tilapia fed diets with graded levels of β-mannanase at the phylum level using a stacked plot. Data represent the means of four replicate cages of 21 fish each.

A clear grouping of samples by principal component analysis (PCA) was observed, suggesting differentiation of the bacterial communities due to the dietary treatments. Principal component analysis shows that the PC1 axis represents 81.9% of the observed microbiota modulation responses, primarily represented by dietary β-mannanase levels of 3200, 4800 and, 6400 TMU kg^{-1}, composed of the phyla *Firmicutes*, *Bacteroidetes* and, *Actinobacteria* (Figure 4A). Figure 4B shows that fish fed diets without and with 1600 and 8000 TMU kg^{-1} dietary β-mannanase are negatively correlated with the microbiota of fish that received 3200 to 6400 TMU kg^{-1} dietary β-mannanase, being mainly represented by phylum *Fusobacteria* and *Proteobacteria*.

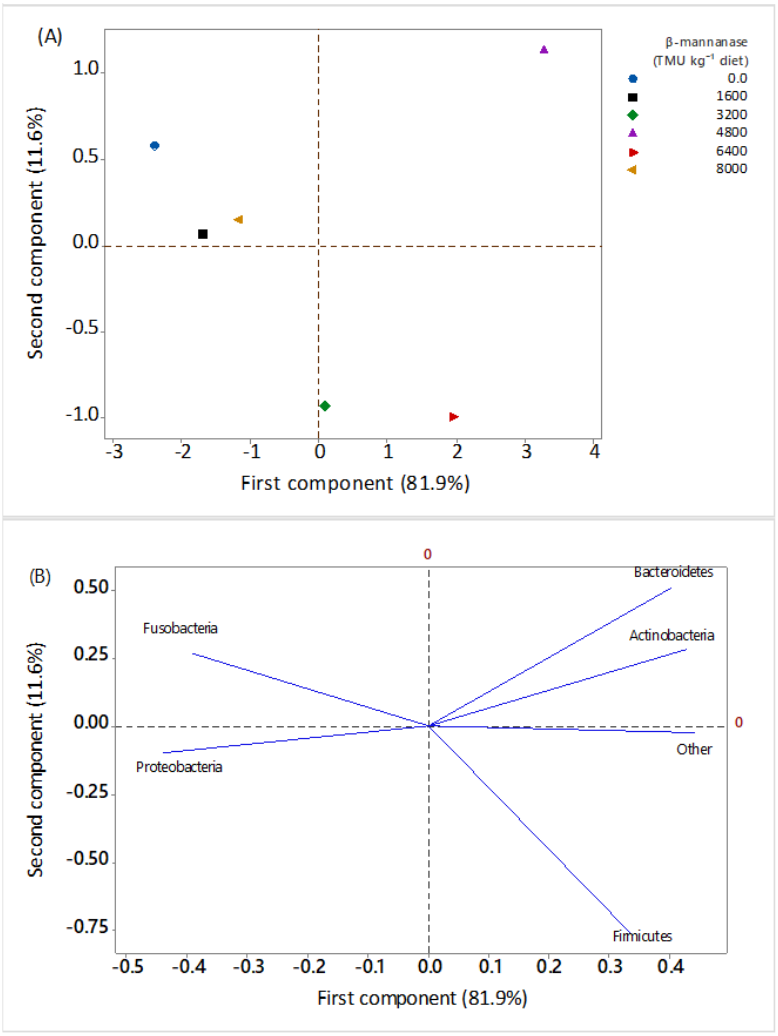

Figure 4. Principal component analysis (PCA) of the phylum of the gut bacterial communities of Nile tilapia diets with graded levels of β-mannanase. The figure was constructed using the Bray-Curtis distance method and represents the phylogenetic distance between samples, a summary of the bacterial composition. Each point represents the mean of entire microbiota of four replicate aquaria. Distant points indicate more different microbiota.

Figure 5 shows a box plot of the individual components of the significant phyla *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidete* and *Fusobacteria* identified in the gut microbiome of juvenile Nile tilapia fed graded dietary β-mannanase levels.

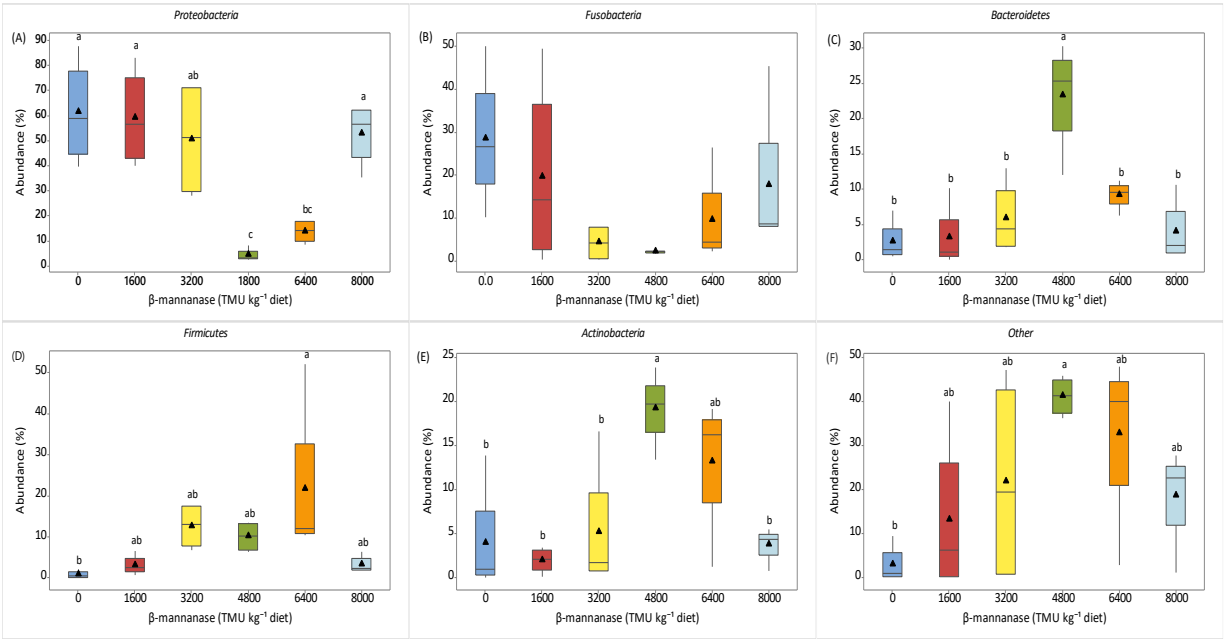

Figure 5. Box plot comparing the differences between the main phylum observed in the gut of juvenile Nile tilapia fed diets with graded levels of β-mannanase. (A) *Proteobacteria*; (B) *Fusobacteria*; (C) *Bacteroidetes*; (D) *Firmicutes*; (E) *Actinobacteria*; (F) Others phylum. The black lozenge dot on the boxplot's right side indicate each treatment's mean values, while the boxplots show the lower, median, and upper quartiles. Means not sharing a common letter differs significantly by Kruskall-Wallis test complemented by Shapiro-Wilk test ($P < 0.05$).

Analyzing each phylum individually, *Proteobacteria* was higher in fish fed control diet, and 1600, 3200, and 8000 TMU kg^{-1} dietary β-mannanase, which significantly ($P < 0.05$) differed from fish fed diets with 4800 and 6400 TMU kg^{-1} dietary β-mannanase. The *Bacteroidete* phylum was significantly ($P < 0.05$) higher in fish fed diet with 4800 TMU kg^{-1} dietary β-mannanase than those fed other diets. For the *Firmicutes* phylum, fish fed 6400 TMU kg^{-1} β-mannanase showed a significant ($P < 0.05$) increase relative to that fed diet control. The *Actinobacteria* phylum was significantly ($P < 0.05$) higher in fish fed diet with 4800 TMU kg^{-1} treatment compared to those fed diet control and diets with 1600, 3200, and 8000 TMU kg^{-1} of β-mannanase. Fish fed the diet containing 4800 TMU kg^{-1} of β-mannanase showed a higher diversity ($P < 0.05$) of phyla than fish fed diet control. The top twenty bacterial genera abundance of juvenile Nile tilapia fed diets with graded levels of β-mannanase are presented in Table 10.

Table 10. Top 20 bacterial genera abundance of juvenile Nile tilapia fed the experimental diets[1].

Genus	β-mannanase [1] (TMU kg^{-1} diet)						
	0	1600	3200	4800	6400	8000	*P-value*
Cetobacterium	13.35±1.63	26.14±1.43	16.42±2.00	28.22±3.10	32.24±2.65	23.54±1.24	0.879
Novosphingobium	50.87±2.08^{a}	29.21±1.60^{ab}	6.13±0.67^{b}	3.72±0.23^{b}	5.02±0.41^{b}	32.38±2.17^{ab}	0.003
Pelomonas	2.67±0.38	5.39±0.71	0.55±0.08	1.23±0.20	1.37±0.20	0.00±0.00	0.471
Phocaeicola	1.19±0.18	0.17±0.01	0.12±0.01	0.10±0.01	1.78±0.10	1.56±0.27	0.469
Alistipes	0.05±0.01	2.56±0.25	0.85±0.08	0.23±0.03	0.53±0.02	0.13±0.01	0.075
Escherichia	0.88±0.05^{a}	0.17±0.01^{bc}	0.04±0.01^{c}	0.05±0.01^{c}	0.11±0.01^{bc}	0.82±0.05^{ac}	0.002
Lactobacillus	0.06±0.00	0.16±0.01	0.34±0.02	0.53±0.06	0.52±0.03	0.29±0.03	0.440
Streptococcus	0.25±0.02^{b}	0.21±0.02^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.11±0.01^{b}	1.29±0.08^{a}	0.001
Rhodopseudomonas	0.42±0.06	0.53±0.05	0.06±0.01	0.08±0.01	0.08±0.01	0.35±0.02	0.390
Leucobacter	0.24±0.02	0.33±0.04	0.08±0.00	0.37±0.06	0.05±0.01	0.30±0.05	0.815
Mediterraneibacter	0.24±0.04	0.00±0.00	0.01±0.00	0.00±0.00	0.72±0.10	0.40±0.07	0.576
Clostridium	0.22±0.01	0.17±0.01	0.04±0.00	0.01±0.00	0.06±0.00	0.82±0.10	0.160
Bifidobacterium	0.01±0.00^{b}	0.02±0.00^{b}	0.10±0.01^{b}	1.43±0.22^{ab}	1.66±0.09^{a}	0.99±0.01^{ab}	0.007
Flavobacterium	0.00±0.00	0.01±0.00	1.25±0.17	0.00±0.00	0.00±0.00	0.00±0.00	0.163
Staphylococcus	0.50±0.05	0.23±0.03	0.05±0.01	0.02±0.00	0.26±0.03	0.03±0.00	0.273
Prevotella	0.19±0.02	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.86±0.14	0.424
Comamonas	0.27±0.02	0.38±0.02	0.32±0.05	0.01±0.00	0.02±0.00	0.05±0.01	0.302
Coprenecus	0.29±0.04	0.11±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.53±0.09	0.556
Bosea	0.26±0.02	0.25±0.02	0.01±0.00	0.15±0.02	0.07±0.01	0.09±0.01	0.472
Lactococcus	0.02±0.00	0.03±0.00	0.00±0.00	0.01±0.00	0.00±0.00	0.77±0.14	0.462

[1] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[a-b] Mean values with different superscripts lowercase letters in the same row indicate significant differences by Welch-test.

The occurrence of *Novosphingobium* bacteria genera was lower (P = 0.003) in fish fed diets 3200, 4800 and 6400 TMU kg^{-1} of β-mannanase, differing from fish fed control diet. Conversely, the abundance of *Escherichia* bacteria was higher (P = 0.002) in fish fed the control diet compared to those fed diets containing 1600, 3200, 4800, and 6400 TMU kg^{-1} β-mannanase. Additionally, the abundance of *Streptococcus* bacteria in fish fed 8000 TMU kg^{-1} β-mannanase was higher (P = 0.001) than in those fed other diets containing β-mannanase. The abundance of *Bifidobacterium* was lower (P = 0.007) in fish fed diets control; 1600 and 3200 TMU kg^{-1} of β-mannanase, differing only from fish fed 6400 TMU kg^{-1} of dietary β-mannanase.

4. Discussion

The study confirms that β-mannanase supplementation improved growth performance of Nile tilapia fed diets with 3200 to 4800 TMU kg^{-1} β-mannanase. Notably, body weight gain, feed efficiency ratio, protein retention, and energy retention efficiency. These results are consistent with emerging studies on β-mannanase effects on growth performance in various fish species (Chen et al., 2016; Dawood and Shi, 2022; Sallam et al., 2020). Together, these findings support the theory that dietary β-mannanase improves growth performance by reducing digesta viscosity and thereby increasing the activity of digestive enzymes.

The present study found that β-mannanase regulated plasma levels of alanine aminotransferase (ALT), glucose, and triglycerides in Nile tilapia, which is in line with previous studies reporting reduced liver damage with β-mannanase in the diet (Chen et al., 2016; Dawood and Shi, 2022; Sallam et al., 2020). High ALT activity reflects abnormal liver function and stress in fish (Wu et al., 2017). This may be due to the greater release of energy and improved access to enzymes and substrates provided by β-mannanase, leading to more nutrients being absorbed (Chen et al., 2016; Sallam et al., 2020).

Concerning plasmatic glucose levels, the study found that increasing levels of β-mannanase in diets led to a linear reduction in plasma glucose levels. However, this is not consistent with previous research, which showed increased plasma glucose levels with the inclusion of β-mannanase in the diet. Glucose levels are related to reduced due intestinal viscosity; however, contrary to this, with increasing β-mannanase levels in diets, we observed a linear decrease in viscosity and plasma glucose levels (El-dakar et al., 2022). In light of such findings, our research casts a new light on the availability of nutrients such as glucose once we can consider an overview of a series of factors capable of influencing the absorption and serum levels of glucose in fish fed with increasing levels of β-mannanase. The first approach is evaluating the effects of β-mannanase on the production of SCFAs and their effects on glucose metabolism (He et al., 2020; Koh et al., 2016). The SCFA can activate FFAR3, stimulating the secretion of intestinal hormones related to glucose regulation, such as PYY in endocrine cells, increasing glucose absorption in muscle and adipose tissue, also causing satiety and consumption reduction, corroborating the performance data in this experiment (Ribola et al., 2017). Furthermore, SCFA activates FFAR2, which stimulates glucagon-like peptide-1 secretion, indirectly regulating blood glucose levels, increasing insulin secretion, and reducing pancreatic glucagon secretion (Barrera et al., 2011; Fujikawa et al., 2013). Activation of FFAR2 by SCFA also increases leptin secretion, which regulates insulin and glucagon levels, regulating consumption, weight gain, and energy metabolism (He et al., 2020; Mazibuko et al., 2013; Ribola et al., 2017). Therefore, it can be considered that the decreasing linear levels observed in this experiment are because β-mannanase anticipates glucose absorption peaks. Since blood plasma collections were performed staggered in the present study, so that all samples were collected 4 hours after feeding, it would not be possible to evaluate the time course of glucose absorption with distinct levels of β-mannanase in juvenile Nile tilapia. It suggests that further research is needed to clarify this mechanism.

Our results suggest that dietary β-mannanase increases energy retention in fish. This is supported by improvements in whole-body crude lipids and plasmatic triglycerides contents in fish fed β-mannanase-contained diets. These findings are in accordance with previous studies with several species, including spinefoot rabbitfish (*Siganus rivulatus*), rainbow trout (*Oncorhynchus mykiss*), hybrid tilapia (*Oreochromis* sp.), Nile tilapia, and common carp (*Cyprinus carpio*), which have also shown a positive association between dietary β-mannanase and triglycerides and whole-body crude lipid modulation (Dawood and Shi, 2022; El-Dakar et al., 2022; Sallam et al., 2020; Taj et al., 2020; Yilmaz et al., 2007). β-mannanase reduced digesta viscosity, which allows better access of digestive enzymes to nutrients, thereby improves digestibility of nutrients and growth performance of fish (Amirkolaie et al., 2005; Kiarie et al., 2021; Leenhouwers et al., 2007b, 2006; Tran-Tu et al., 2018). Together, these findings could explain the higher whole-body crude protein, lipid, and ash in Nile tilapia juveniles. Such data are strongly related to β-mannanase action by breaking the NSPs bonds in the ingredients. As a result, such nutrients, including carbohydrates, are more available in the intestine to be fermented by the microbiota, producing SCFAs in the gut.

In the present study, dietary β-mannanase affected the production of SCFAs, increasing acetic, propionic, and butyric acid levels. The most important means of SCFAs synthesis is endogenous fermentation of carbohydrates in the gut by microbiota present in the intestine (Tran et al., 2020). The profile of SCFAs is mainly dictated by the composition of the carbohydrates that will be fermented by bacterial gut and that will also retro-influence the activity of the microbiota (Flint et al., 2014; Ríos-Covián et al., 2016). The main effects observed with SCFAs include, but are not limited to: improvements in growth performance, feed efficiency, immune response, survival rate, microbiota modulation, and improvements in intestinal morphology (Ebrahimi et al., 2017; Estensoro et al., 2016; Rimoldi et al., 2018; Robles et al., 2013; Tian et al., 2017). Our research shows that β-mannanase directly influences

SCFAs production once acetic, propionic, and butyric acid increase in response to increasing dietary β-mannanase. As each SCFA has a different function in the organism, such differences must be observed following the results found in this study. SCFAs are mainly used in the vicinity of the intestine itself, once, butyric acid is mainly (99 %) used for increasing epithelial barrier and permeability of intestinal cells through the modulation of proteins of the junctions between intestinal cells, protecting intestinal mucosa and increasing villus density, being solely responsible for improvements in gut health (Canfora et al., 2015; Piazzon et al., 2017). SCFA production influences intestinal morphology and pH; thus, intestinal pH fall is the main change expected with NSPs digestion (Tran et al., 2020). Lower pH is responsible for modulating the microbiota and reducing pathogenic organisms, as it can dissociate into gram-negative bacteria, which are primarily related to diseases (MacFarlane and Macfarlane, 2012).

The results showed that β-mannanase significantly impacted the intestinal microbiota composition, with a predominance of *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Fusobacteria* phyla in the gut of juvenile Nile tilapia. *Proteobacteria* and *Fusobacteria* are abundantly found in fish guts, and their association with carbohydrases was previously reported (Egerton et al., 2018; Maas et al., 2020a). The phylum *Proteobacteria* is of high abundance in aquatic environments, which helps to explain the high prevalence of this phylum in the gastrointestinal tract of many fish species, besides, is capable of degrading fiber. (Rawls et al., 2006). *Fusobacteria* are also anaerobic, gram-negative bacilli, and include pathogenic strains (Pelczar et al., 1996). The *Firmicutes* phylum has been reported to positively impact the energy availability of fibrous feeds, leading to growth of Actinobacteria which can enhance secretion of NSP-degrading enzymes (Watanabe et al., 2021). Furthermore, *Firmicutes* are associated with increased body weight and feed efficiency in pigs (Huang et al., 2018). *Actinobacteria* is a major taxonomic phylum among the 18 main lineages, known for its production of extracellular enzymes and secondary metabolites (Ventura et al., 2007).

Bacteroidetes in humans have a direct relationship with mannan utilization (Cuskin et al., 2015) and possess PULs, which encode the necessary apparatus for utilizing complex carbohydrates, specifically mannosidosis bonds(Martens et al., 2009). The addition of β-mannanase significantly increased the presence of the *Bifidobacterium* genus, which tends to increase in response to dietary fiber and has several beneficial effects in fish, including production of bacteriocins that reduce pathogenic (Abudabos et al., 2017; de Figueiredo et al., 2020). Our results further show that β-mannanase reduces the presence of pathogenic genera, such as *Streptococcus* and *Escherichia*, known to cause diseases in swine, poultry, and fish (Petry et al., 2021; Wang et al., 2021). However, whether β-mannanase can stimulate the beneficial bacteria and reduce the presence of potentially pathogenic bacteria in the gut of Nile tilapia is mainly due the pH reduction retroinfluenced by the effects of SCFAs in pH (Wang et al., 2021).

Overall, our study demonstrates the positive effects of β-mannanase supplementation on nutrient digestion and growth performance of juvenile Nile tilapia. β-mannanase also modulated the composition of the fish's gut microbiota by reducing pathogenic genera and increasing beneficial bacteria, as well as improved gut morphology by increasing villus height and width. The results further indicate that β-mannanase enhances the nutritive value of plant-based diets in tilapia by reducing NSPs antinutritional effects and modulating the gut microbiome. The study provides novel evidence for the potential of β-mannanase supplementation to improve the sustainability of tilapia farming.

5. Conclusions

Our findings show that the inclusion of β-mannanase at 4800 TMU kg^{-1} diet reduced digesta viscosity, growth performance, increased digestive enzymes activity SCFA production, and improved gut morphometry. Additionally, β-mannanase positively modulated gut microbiome, by reducing deleterious bacteria, as *Escheria* sp., and increasing the levels of

beneficial bacterias. The current study provides novel evidence that using liquid carbohydrases in tilapia diets offer a promising solution to improve the nutritional value of alternative feed ingredients in tilapia aquaculture.

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CHAPTER III

Article II - Effects of β-mannanase on fecal viscosity, digestibility of nutrients, and digestible energy and protein contents in soybean meal-rich diets fed to juvenile Nile tilapia

ABSTRACT: This study aimed to evaluate graded levels of dietary β-mannanase supplementation on fecal viscosity and pH, and the apparent digestibility coefficient (ADC) of dry matter (DM), gross energy (GE), and nutrients, including amino acids (AAs), as well digestible energy (DE) and digestible protein (DP) contents of plant-based diets fed to juvenile Nile tilapia. Fish (*n* = 504; body weight 7.0 ± 0.43 g) were randomly distributed in 24 aquaria of 70 L each in a recirculation aquaculture system in a completely randomized design with six treatments and four replicates of 21 fish in each aquarium. Fish were fed diets with graded levels of β-mannanase at 0 (control), 1600, 3200, 4800, 6400, and 8000 TMU kg^{-1} diet and hand-fed 12 times a day until apparent satiety for eight weeks. Chromium oxide was used as an indigestible marker. Feces were collected manually by straining the feces through a sieve. Fish fed diet with β-mannanase at 4800 TMU kg^{-1} showed reduced fecal viscosity (−77.1%) and fecal pH (−11.1%), additionally, optimized the ADC of gross energy (+7.2%), crude protein (+3.5%), crude lipid (+1.2%), ash (+19.7%), essential amino acid (+4.0%) and non-essential amino acid (+3.4%). Compared to the control group, fish fed diet with 4800 TMU kg^{-1} diet β-mannanase displayed lower total nitrogen loss (TN_L), organic matter loss (OM_L), inorganic matter loss (IM_L) and nitrogen loss (N_L) of −34.2, −24.6, −9.6 and −2.3 g kg^{-1} of body weight gain (BWG) fish, respectively. Overall, it concluded that β-mannanase at level 4800 TMU kg^{-1} diet improves the digestibility of energy, nutrients, including amino acids, by reducing digesta viscosity. This allows the fish to extract more nutrients from the feed, resulting in increased overall nutrient utilization and improved growth performance. Overall, the use of β-mannanase in tilapia feeds can lead to more efficient and cost-effective aquaculture operations.

Keywords: β-mannans, carbohydrase, *Oreochromis niloticus*, non-starch polysaccharides, sustentability

1. Introduction

Soybean meal (SBM) is widely used as a source of protein in aquafeeds, reducing the competition for food between aquaculture and human consumption (Tacon et al., 2022). However, SBM contains 17 to 27% of non-starch polysaccharides (NSPs) as high molecular weight carbohydrates that serve as the basis for the hardness of cell walls (Choct et al., 2010; Sinha et al., 2011). Therefore, viscous soluble non-starch polysaccharides in cereals and legumes limit their inclusion in fish feed (Kabir et al., 2020). Previous studies have shown that the viscosity of NSPs has adverse effects on nutrient utilization in Nile tilapia, *Oreochromis niloticus* (Haidar et al., 2016; Maas et al., 2018). Noteworthy, in typical non-dehulled SBM, β-mannans account for 1.3 to 2.7% of insoluble NSPs fraction (Hsiao et al., 2006). Recent studies have demonstrated that β-mannans increase digesta viscosity and impair nutrient digestibility in African catfish, *Clarias gariepinus* (Leenhouwers et al., 2007b, 2006) and common carp, *Cyprinus carpio* (Dawood and Shi, 2022). However, the extent to which β-mannans negatively affect viscosity and nutrient digestibility, particularly for amino acids, remains unclear in Nile tilapia.

Exogenous β-mannanase is an enzyme that targets β-mannans bonds, a dietary component in high-fiber feedstuffs such as SBM (Latham et al., 2018). Although β-mannanase can reduce the deleterious effects of β-mannans on growth performance of Nile tilapia (Chen et al., 2016). Despite that, the effects on nutrient digestibility are still conflicting in fish. While previous studies have reported that β-mannanase supplementation in diet of common carp, improved digestible energy content and nutrient digestibility coefficients (Dawood and Shi, 2022). Conflicting literature reported no significant effects of β-mannanase inclusion on

nutrient digestibility in rainbow trout, *Oncorhynchus mykiss* fed SBM-rich diet (Yiğit et al., 2014). Along with fish species specificity, the inconsistent effects of β-mannanase on nutrient digestibility might be influenced by dietary β-mannans content in individual feedstuff composition, which governs digesta viscosity and, subsequently nutrient digestibility (Maas et al., 2020b). Collectively, these investigations indicate that β-mannanase could improve nutrient utilization of fish.

Emerging research indicates that Nile tilapia are highly efficient in converting vegetable feed to food products (Ridha et al., 2020). However, a recent study revealed that Nile tilapia fed NSP-rich diets still produced substantial amounts of undigested nutrients and had negative impacts on the aquatic food (Kabir et al., 2020). Similar findings were reported in striped catfish, *Pangasionodon hypophthalmus* fed soybean meal-based diets, where dietary viscosity promoted by NSPs decreased digestibility and increased fecal waste production (Tu-Tran et al., 2020). Besides, exogenous carbohydrase may be helpful to create environmentally sustainable diets for fish farming in compliance with sustainability principles (FAO, 2020). Furthermore, it may be useful as sustainability indicator of the aquaculture system (Valenti et al., 2018). However, the underlying mechanisms of β-mannanase's impact on digesta viscosity and nutrient digestibility in Nile tilapia are not yet to be fully understood. Thus, the present study aims to investigate the effects of graded levels of dietary β-mannanase supplementation on feces viscosity, digestible energy and protein content, and digestibility of nutrients, including amino acids, in juvenile Nile tilapia fed SBM-rich diets.

2. Material and methods

2.1. Ethics statement

All fish procedures were performed following the Guidelines for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of the State University of Ponta Grossa (Protocol: 22.000024303-4).

2.2. Diets

A basal diet contained 311.2 g kg^{-1} of crude protein and 18.98 MJ kg^{-1} of gross energy, without β-mannanase supplementation (control) was formulated based on soybean meal, broken rice, wheat bran, corn, and poultry by-product meal as primary food ingredients, and formulated to meet the dietary requirements of Nile tilapia (NRC, 2011). From the basal diet, five other diets were elaborated by supplementing 1600, 3200, 4800, 6400 and 8000 TMU kg^{-1} diet of β-mannanase. Exogenous β-mannanase enzyme inclusion replaced an equal silica amount, as shown in Tables 1 and 2.

Table 1. Ingredients composition of the reference diet (g kg^{-1} diet).

Ingredient	g kg^{-1} (as-fed basis)
Broken rice[a]	80
Soybean meal[b]	440
Poultry by-product meal[c]	150
Wheat bran[b]	100
Corn[b]	165
Soybean oil[d]	20
Corn starch[e]	20
DL-methionine 99[f]	2
L-lysine[f]	3
Dicalcium phosphate[g]	10
Mineral and vitamin mix[h]	8
Inert (Silica)[i]	1
Cr_2O_3[j]	1

[a] Armazém São Vito, São Paulo, SP, Brazil.
[b] Bunge, Ponta Grossa, PR, Brazil.
[c] BRF, Toledo, PR, Brazil.
[d] Coamo, PR, Brazil.
[e] Yoki, São Bernardo do Campo, São Paulo, Brazil.
[f] Ajinomoto Animal Nutrition Division, SP, Brazil.
[g] Sarfos, Goiás, Brazil.
[h] Customized premix (Composition per kilogram of feed (IU or mg kg^{-1} of diet): Vitamin A (retinyl acetate), 6,000 IU; vitamin D_3, (cholecalciferol), 1,000 IU; vitamin E (DL-α-tocopheryl acetate), 60 mg; vitamin K_3 (menadione Na-bisulphate), 12 mg; vitamin B_1 (thiamine HCl), 24 mg; vitamin B_2 (riboflavin), 24 mg; vitamin B_6 (pyridoxine HCl), 20 mg; vitamin B_{12} (cyanocobalamin), 0.05 mg; folic acid, 6 mg; D-calcium pantothenate, 60 mg; ascorbic acid (ascorbyl polyphosphate), 350 mg; D-biotin, 0.24 mg; choline chloride, 800 mg; niacin, 120 mg; ferrous sulfate ($FeSO_4.H_2O.7H_2O$), 50 mg; copper sulphate ($CuSO_4.7H_2O$), 3 mg; manganese sulphate ($MnSO_4.H_2O$), 20 mg; zinc sulphate ($ZnSO_4.7H_2O$), 30 mg; potassium iodide (KI), 0.4 mg, cobalt sulphate ($CoSO_4.4H_2O$), 0.25 mg; sodium selenite (Na_2SeO_3), = 0.1 mg, BHT, 200 mg; calcium propionate, 1000mg.
[i] Merck Company, Germany.
[j] Sygma-Aldrich Brazil Ltda, 99.5%, São Paulo, SP, Brazil.

All diets were ground through a 0.8-mm screen in a centrifugal mill (Viera MC 680B, Tatuí, SP, Brazil). The extrusion process was performed through a 1.5-mm die diameter in a single screen extruder with die temperature set at 92°C (Exteec EX30, Ribeirão Preto, SP, Brazil), obtaining pellets with 2.5-mm of diameter and floatability rate higher than 99%. After that, the pellets were dried in a drying drum with rotary drier at 55ºC (pellet temperature) for 10 min (Model E-62, Ferraz Máquinas e Engenharia LTDA, Ribeirão Preto, SP, Brazil).

547 **Table 2.** Analyzed composition of the basal diet (g kg^{-1} dry matter basis).

Composition	g kg^{-1}
Dry matter	932.1
Gross energy (MJ kg^{-1})	18.98
Crude protein	311.2
Crude fiber	38.24
Crude lipid	31.40
Ash	64.3
Amino acid	
Essential amino acid	
Arginine	1.910
Histidine	0.811
Isoleucine	1.149
Leucine	2.536
Lysine	1.796
Methionine	0.582
Phenylalanine	1.620
Threonine	1.409
Tryptophan	0.366
Valine	1.687
Non-essential amino acid	
Alanine	1.722
Aspartic acid	2.829
Cysteine	0.511
Glutamic acid	4.756
Glycine	1.892
Proline	0.000
Serine	1.807
Tyrosine	0.944

548

549 Liquid β-mannanase (Natupulse® TS, BASF, Ludwigshafen am Rhein, Germany;
550 8000 TMU g^{-1})was top-sprayed onto each kilogram of diet to supply 1600; 3200; 4800; 6400,

551 and 8000 TMU kg^{-1} diet of endo-1,4-β-mannanase, being applied 0.2; 0.4; 0.6; 0.8 and 1.0 g
552 kg^{-1} of Natupulse . The same procedure was applied to unsupplemented diet to receive the same
553 treatment, but without the commercial β-mannanase inclusion in soybean oil.

554 *2.3. Fish and Experimental Design*

555 The experiment was conducted at the Aquaculture Laboratory of the State University
556 of Ponta Grossa, Ponta Grossa, PR, Brazil. All-male masculinized Nile tilapia juveniles (*n* =
557 1500; 3.0 ± 0.5 g; Premium strain) were obtained from Aquabel Fish Farm (Rolândia, PR,
558 Brazil). Fish were acclimated for a 4-week period in a circular tank (500 L), with temperature
559 and dissolved oxygen set at 28°C and 6 mg L^{-1}, respectively. Fish were hand-fed a commercial
560 extruded diet (Supra, 1.0 mm Ø; Alisul Alimentos, Maringá, PR, Brazil), with 460 g kg^{-1} of
561 crude protein, six times daily for 21 days. Afterward, fish (*n* = 504; 7.0 ± 0.43 g; mean ± SD)
562 were grouped-weighed and randomly distributed into 24 plastic aquaria (70 L each) equipped
563 with a recirculating system composed of a decanter to remove solids, a mechanical filter with
564 bio-balls, heater (3000W) and a central UV-light disinfection system (55W). The aeration
565 system was comprised of a centrifugal 0.5-HP blower (Sulpesca, Toledo, PR, Brazil) fitted with
566 silicone airline tubing, with a porous stone in each experimental aquarium. Each aquarium was
567 siphoned daily to maintain 10% of the water volume and remove fish metabolites. Temperature
568 was set at 28 ± 0.5°C, dissolved oxygen was kept at 6.2 ± 0.2 mg L^{-1}, and water flow was kept
569 at 1.2 L min^{-1} per aquarium throughout the trial. Data of individual aquarium temperature and
570 dissolved oxygen were monitored daily using YSI Multi-Parameter Water Quality Meter (YSI
571 Incorporated, Ohio, USA). Water quality parameters were monitored weekly with a pH-meter
572 (TEC-2, Piracicaba, SP, Brazil) and kept at 7.0 using calcium carbonate and phosphoric acid;
573 ammonia, nitrite, and nitrate analysis were performed using commercial kits (Alfakit,

574 Florianópolis, SC, Brazil), and were kept at 0.01; 0.02 and 0.01 mg L^{-1}, respectively. Fish were
575 hand-fed from 8:00 to 18:00 h, 12 times daily, until apparent satiety for 60 days.

576 *2.4. Chemical composition*

577 The proximate composition of diets and feces samples was performed according to
578 standard methods of the Association of Official Analytical Chemists (AOAC, 2002). Moisture
579 analysis was determined by oven-drying at 105°C until constant weight and crude lipid by the
580 ether-extraction method (Folch, 1957). Crude protein (N × 6.25) analysis was performed using
581 the macro Kjeldahl method (Tecnal, MA-036, Piracicaba, SP, Brazil) after acid hydrolysis. The
582 analysis of ash was achieved by overnight combustion in a muffle furnace at 550°C (Tecnal,
583 2000B, Belo Horizonte, MG, Brazil). The crude fiber analysis was performed according to loss
584 on ignition of dried lipid-free residues following digestion with 1.25% H_2SO_4 and 1.25%
585 NaOH. Chromium analysis was performed by inductively coupled plasma optical emissions
586 spectrometry using an internally validated method of analysis (AOAC, 1990). Gross energy of
587 diets and feces was carried out by adiabatic bomb calorimeter (Parr 6400; Parr Instruments Co.,
588 Moline, IL, USA), using benzoic acid as a calibration standard The profile of dietary amino
589 acids and amino acids in feces were determined by High Performance Liquid Chromatography
590 (HPCL) (Hitachi, Tokyo, Japan), at the Laboratory of Ajinomoto do Brasil Indústria e Comércio
591 de Alimentos Ltda, Division of Animal Nutrition (São Paulo, SP, Brazil) (Rayner, 1985).
592 Tryptophan was determined after alkaline hydroxylation of the sample with lithium hydroxide.

593 *2.5. Digestibility measurements*

594 The apparent digestibility coefficients (ADC) of gross energy and nutrients were
595 measured using chromic oxide (Cr_2O_3) as an external inert marker (Guimarães et al., 2008).
596 After one-month of the feeding trial, feces were collected from each aquarium twice daily in

the morning (08:30 h) and in the afternoon (18:30 h) until the last day before the end of the experimental trial. All aquaria were cleaned daily before the feces collection. The collection was done manually by siphoning the fecal matter and straining through a 1-mm meshed net. For this, the laboratory lighting system was turned off to prevent excessive fish movement, and the fecal collection followed a single handheld flashlight light. After the centrifugation cycle, the supernatant was discarded, and the solid sediment was dried in a ventilated oven at 55°C for 24 h. Thus, the feces sample was fine-grinded (0.5-mm diameter) in a laboratory willye mill (Tecnal R-TE 648, Piracicaba, SP, Brazil) and stored at −20°C until analysis. The ADC was calculated following previously established expression (Forster, 1999; NRC, 2011) as ADC = 1 − [(Cd/Cf) × (Nf/Nd), where ADC is the apparent digestibility coefficients; Cd is the concentration of chromium oxide in the diet; Cf is the concentration of chromium oxide in the feces (g kg^{-1} DM); Nf is the concentration of nutrient or energy in the feces (g kg^{-1} or MJ kg^{-1} DM); Nd is the concentration of nutrient or energy in the diet (g kg^{-1} or MJ kg^{-1} DM). The digestible energy (DE) and digestible protein (DP) contents were calculated as the product of gross energy and crude protein ADC of the diets.

2.6. Fecal pH and viscosity

Fecal pH was measured using a pH-meter (Kasvi – ATC-K39-0014PA, São José dos Pinhais, PR, Brazil), placed directly in the feces. The samples of feces were centrifuged at 3000 rpm x g for 10 min (Kasvi – SKU K14-1215, São José dos Pinhais, PR, Brazil) to obtain the liquid phase. The supernatant obtained was placed in the viscometer (Brookfield Digital Viscometer, Model DV-II Version 2.0, Brookfield Engineering Laboratories Inc., Stoughton, MA), set at 28°C. The viscosity measurement was the average 50.0/s shear rate, and the viscosity values were recorded as apparent viscosity in centipoise (cP).

621 *2.7. Fecal loss*

622 At the start and end of the feeding trial, all fish were fasted for 24 h, anesthetized with
623 tricaine methanesulphonate (MS-222; Sigma-Aldrich; 200 mg L^{-1}), counted, and bulked weighed.
624 The feed intake were daily recorded from each aquarium. The total nutrient loss (TN_L), organic
625 matter loss (OM_L), inorganic matter loss (IM_L) and nitrogen loss (N_L) were determined as
626 follows:

627 TN_L (g kg^{-1} of BWG fish) = FCR x DM_D – [(FCR x DM_D) x ADC_{DM}]

628 OM_L (g kg^{-1} of BWG fish) = TN_L – IM_L

629 IM_L (g kg^{-1} of BWG fish) = FCR x MM_D – [(FCR x MM_D) x ADC_{MM}]

630 N_L (g kg^{-1} of BWG fish) = FCR x N_D – [(FCR x N_D) x ADC_N]

631 TN_L, OM_L, IM_L, N_L is total nutrient loss, organic matter loss, inorganic matter loss and nitrogen
632 loss, respectively (g kg^{-1} of BWG of fish), FCR is feed conversion ratio, DM_D, MM_D, N_D is dry
633 matter, mineral matter and nitrogen content of diets (%), and ADC_{DM}, ADC_{MM}, ADC_N are
634 apparent digestibility coefficient of dry matter, mineral matter and nitrogen, respectively (%).

635 *2.8. Statistical analysis*

636 All results were described as least square means and pooled standard error of means
637 (SEM). All data were tested for normality using Kolmogorov–Smirnov test, and homogeneity
638 was tested using Levene's test. Data were analyzed as a two-way ANOVA using the General
639 Linear Model (GLM) procedure. The dose-response effect of supplemental β-mannanase was
640 determined using an orthogonal polynomial contrast for linear and quadratic effects (SAS,
641 version 9.2). In addition, Dunnett's test procedure was used to compare data from each β-
642 mannanase supplementation level with the non-supplemented diet (control). The Welch test (P
643 < 0.05) was applied for microbiome analysis, followed by the Bonferroni correction test. The
644 analyses were performed using the statistical metagenomics program STAMP for statistical

645 analysis of metagenomic profiles (Parks et al., 2014). The averages for biodiversity between
646 treatments were compared using the number of observed OTUs and the Chao1 index by the
647 Kruskal Wallis test ($P < 0.05$) once a non-parametric distribution was detected by the Shapiro-
648 Wilk test. Multivariate analysis was employed to conduct principal component (PC) analysis,
649 and the score and loading plot were utilized to ascertain the correlation among individual
650 variables of the first two eigenvalues (PC 1 and 2). All data were analyzed according to the
651 Proc GLM of the Statistical Analysis System (Version 9.0), and values were presented as mean
652 ± standard error.

653 **3. Results**

654 *3.1. Fecal pH and viscosity*

655 The effects of dietary β-mannanase supplementation on fecal pH and viscosity of
656 juvenile Nile tilapia are displayed in Figure 1. The pH tended to decrease in a quadratic pattern
657 ($P = 0.001$; $R^2 = 0.856$; $Y_{min.} = 5840$ TMU kg^{-1} diet β-mannanase), while the viscosity
658 decreased linearly ($P < 0.001$).

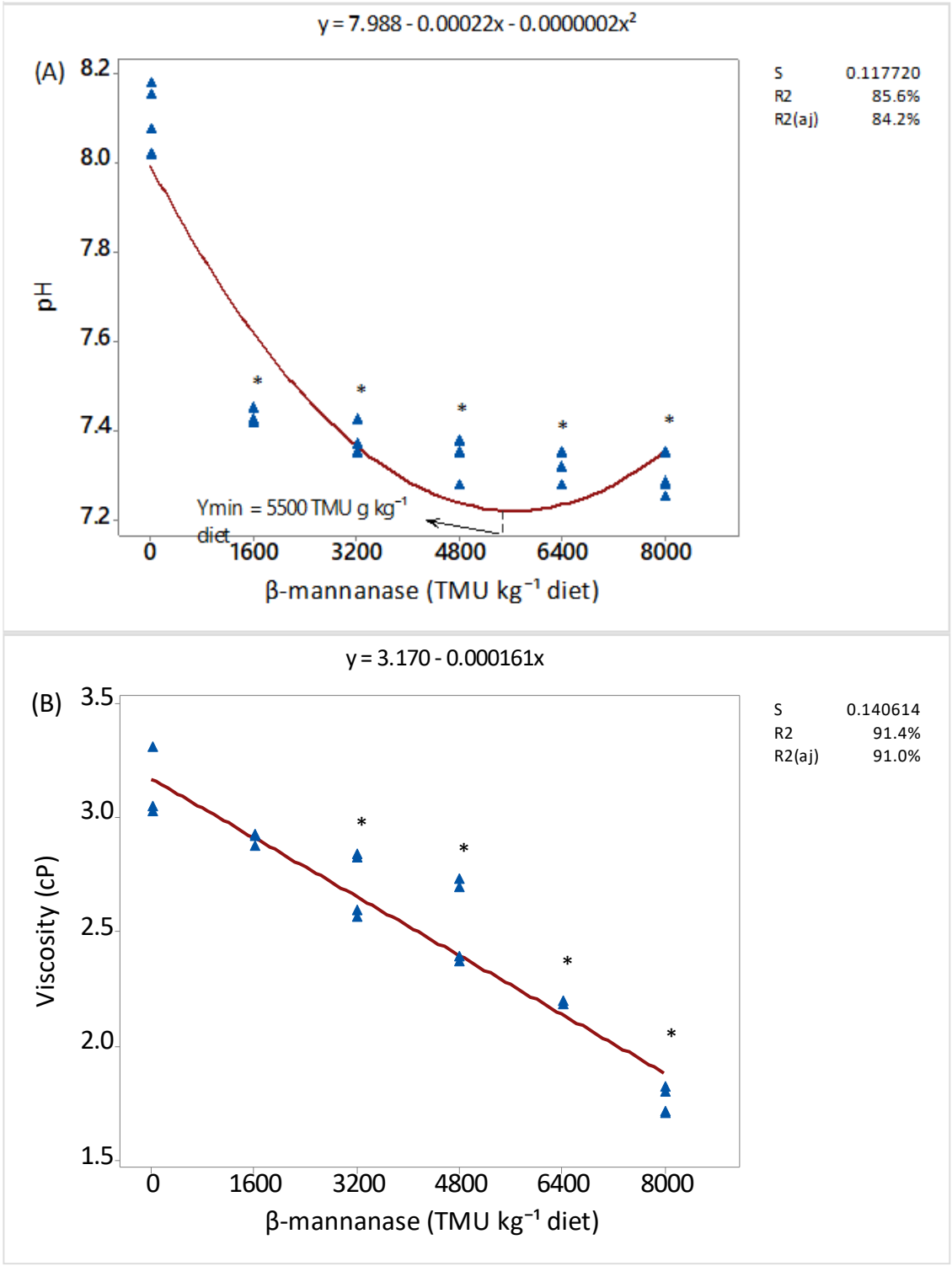

Figure 1. Fitted orthogonal polynomial contrast for quadratic and linear plots of digesta pH (A; quadratic) and viscosity (B; linear) content as a function of the level of supplemented β-mannanase on top of diets fed to juvenile Nile tilapia. Each dot point represents mean of each replicate of 21 fish.

665 Based on Dunnet's test, fish fed diet with 1600 to 8000 TMU kg^{-1} β-mannanase
666 showed lower fecal pH ($P < 0.001$) than fish fed diet control. Besides, β-mannanase promoted
667 lower fecal viscosity ($P < 0.001$) in fish fed diets with 3200 to 8000 TMU kg^{-1} β-mannanase
668 than those fish fed diets control and diet with 1600 TMU kg^{-1} β-mannanase.

669 *3.2. Digestibility of energy and nutrients*

670 The effects of graded levels of β-mannanase supplementation on ADC of dry matter,
671 gross energy, and nutrients are shown in Table 3. The ADC of dry matter ($P = 0.002$; $R^2 =$
672 0.377; $Y_{max.} = 3760$ TMU kg^{-1} β-mannanase), gross energy ($P < 0.001$; $R^2 = 0.676$; $Y_{max.} =$
673 4160 TMU kg^{-1} β-mannanase), crude protein ($P < 0.001$; $R^2 = 0.848$; $Y_{max.} = 4080$ TMU kg^{-1}
674 β-mannanase), crude lipid ($P < 0.001$; $R^2 = 0.767$; $Y_{max.} = 4000$ TMU kg^{-1} β-mannanase), and
675 ash ($P < 0.001$; $R^2 = 0.752$; $Y_{max.} = 5360$ TMU kg^{-1} β-mannanase diet) increased in a quadratic
676 manner in fish fed graded β-mannanase levels.

677

Table 3. Apparent digestibility coefficients (%) of juvenile Nile tilapia fed the experimental diets[1].

	β-mannanase [2] (TMU kg^{-1} diet)							*P-value*		
Parameter	0	1600	3200	4800	6400	8000	SEM[3]	L[4]	Q[4]	Dunnet[5]
Dry matter	69.5	69.9	69.9	70.2*	70.1	69.6	0.082	0.626	0.002	0.041
Gross energy	66.4	68.0	72.5*	71.2*	70.6*	66.7	0.554	0.484	<0.001	<0.001
Crude protein	82.7	83.7*	86.2*	85.7*	85.0*	82.7	0.297	0.620	<0.001	<0.001
Crude lipid	94.2	95.0*	95.4*	95.3*	95.0*	94.2	0.113	0.841	<0.001	<0.001
Ash	57.2	62.9*	69.4*	68.5*	67.3*	63.6*	0.930	0.016	<0.001	<0.001

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1} diet) by Dunnet´s test ($P < 0.05$).

Based on Dunnet's test, only fish fed diet with 4800 TMU kg^{-1} of β-mannanase showed higher ADC of dry matter ($P < 0.041$), while fish fed diets with 4800 and 6400 TMU kg^{-1} dietary β-mannanase revealed higher ($P < 0.05$) ADC of dry matter and gross energy, respectively, than fish fed diet control. Consistently, fish fed diets with 1600 to 6400 TMU kg^{-1} β-mannanase demonstrated higher ADC of crude protein, crude lipids, and ash than fish fed diet control ($P < 0.05$).

3.3. Fecal loss

The effects of graded levels of β-mannanase supplementation on total nutrient loss, organic matter loss, inorganic matter loss and nitrogen loss are shown in Table 4. The total nutrient loss ($P < 0.001$; $R^2 = 0.377$; 75.2; Y_{min} = 4896 TMU kg^{-1} diet β-mannanase), organic matter loss ($P = 0.001$; R2 = 61.1; Y_{min} = 4755 TMU kg^{-1} diet β-mannanase), inorganic matter loss ($P < 0.001$; R2 = 92.3; Y_{min} = 4842 TMU kg^{-1} diet β-mannanase), nitrogen loss ($P < 0.001$; $R^2 = 0.767$; R2 = 90,91; Y_{min} = 4528 TMU kg^{-1} diet β-mannanase) decresead in a quadratic manner in fish fed graded β-mannanase levels.

Table 4. Effect of graded levels of β-mannanase on the apparent digestibility coefficients of essential amino acids in juvenile Nile tilapia[1].

	β-mannanase [2] (TMU kg^{-1} diet)							*P-value*		
Parameter	0	1600	3200	4800	6400	8000	SEM[3]	L[4]	Q[4]	Dunnet[5]
Total nutrient loss	283.4	258.0*	253.5*	249.2*	244.7*	257.2*	2.891	0.001	<0.001	<0.001
Organic matter loss	255.5	235.9*	235.6*	230.9*	226.1*	235.8*	2.304	0.003	0.001	<0.001
Inorganic matter loss	27.9	22.1*	17.9*	18.3*	18.6*	21.4*	0.744	0.003	<0.001	<0.001
Nitrogen loss	8.7	7.5*	6.2*	6.4*	6.6*	7.8*	0.191	0.082	<0.001	<0.001

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1} diet) by Dunnet´s test ($P < 0.05$).

Based on Dunnet's test, fish fed diet with 1600 to 8000 TMU kg^{-1} of β-mannanase showed lower total nutrient loss ($P < 0.01$), organic matter loss ($P < 0.01$), inorganic matter loss ($P < 0.01$) and nitrogen loss dry matter ($P < 0.01$), respectively, than fish fed control diet.

3.4. Digestibility of amino acids

The effects of dietary β-mannanase on ADC of essential and non-essential amino acids are presented in Table 5, respectively. The ADC of essential amino acids ($P < 0.001$; $R^2 = 0.682$; $Y_{max.} = 5120$ TMU kg^{-1} β-mannanase), arginine ($P < 0.001$; $R^2 = 0.689$; $Y_{max.} = 4720$ TMU kg^{-1} β-mannanase), histidine ($P < 0.001$; $R^2 = 0.657$; $Y_{max.} = 5520$ TMU kg^{-1} β-mannanase), isoleucine ($P = 0.001$; $R^2 = 0.634$; $Y_{max.} = 5280$ TMU kg^{-1} β-mannanase), leucine ($P < 0.001$; $R^2 = 0.625$; $Y_{max.} = 5360$ TMU kg^{-1} β-mannanase), lysine ($P = 0.001$; $R^2 = 0.638$; $Y_{max.} = 5360$ TMU kg^{-1} β-mannanase), methionine ($P < 0.001$; $R^2 = 0.753$; $Y_{max.} = 5200$ TMU kg^{-1} β-mannanase), threonine ($P = 0.003$; $R^2 = 0.465$; $Y_{max.} = 4880$ TMU kg^{-1} β-mannanase), tryptophan ($P < 0.001$; $R^2 = 0.525$; $Y_{max.} = 4080$ TMU kg^{-1} β-mannanase) and valine ($P = 0.004$; $R^2 = 0.458$; $Y_{max.} = 4960$ TMU kg^{-1} diet β-mannanase) showed a quadratic behavior. Conversely, the ADC of e phenylalanine increased linearly ($P = 0.016$) in fish fed graded levels of dietary β-mannanase.

Table 5. Effect of graded levels of β-mannanase on the apparent digestibility coefficients of essential amino acids in juvenile Nile tilapia[1].

	β-mannanase [2] (TMU kg^{-1} diet)							*P-value*		
Amino acid	0	1600	3200	4800	6400	8000	SEM[3]	L[4]	Q[4]	Dunnet[5]
Essential amino acid	85.2	88.6*	88.9*	88.6*	89.1*	88.5*	0.308	0.003	<0.001	<0.001
Arginine	90.6	92.5*	92.8*	92.6*	92.9*	92.1*	0.185	0.022	<0.001	<0.001
Histidine	87.5	90.9*	91.3*	90.8*	91.8*	91.3*	0.332	0.001	<0.001	<0.001
Isoleucine	83.7	86.9*	87.8*	87.5*	87.9*	87.3*	0.364	0.003	0.001	<0.001
Leucine	86.6	88.8*	89.2*	89.0*	89.6*	89.0*	0.244	0.001	<0.001	<0.001
Lysine	89.1	91.4*	91.7*	91.7*	92.2*	91.6*	0.743	0.002	0.001	<0.001
Methionine	81.8	90.7*	91.2*	90.7*	91.5*	90.6*	0.448	0.001	<0.001	<0.001
Phenylalanine	84.2	87.5	88.2*	87.9*	88.7*	87.8*	0.311	0.016	0.017	0.028
Threonine	81.4	85.2*	84.9*	84.4*	84.7*	84.4*	0.240	0.043	0.003	<0.001
Tryptophan	85.9	87.8*	87.6*	87.5*	87.1*	86.5	0.168	0.816	<0.001	0.001
Valine	81.2	84.4*	84.3*	84.2*	84.7*	83.8*	0.329	0.033	0.004	0.006
Non-essential amino acid	81.9	85.1*	85.3*	84.8*	85.4*	85.4*	0.312	0.003	0.011	<0.001
Alanine	82.1	85.0	83.8	82.7	82.6	83.9	0.321	0.905	0.676	0.073
Aspartic acid	87.9	89.8*	90.0*	89.8*	90.0*	89.5*	0.194	0.030	0.001	0.001
Cysteine	75.6	81.7*	81.7*	80.9*	82.2*	82.8*	0.628	0.002	0.048	0.001
Glutamic acid	76.7	80.2*	81.0*	80.7*	81.6*	81.1*	0.416	0.001	0.005	0.001
Glycine	93.1	94.1*	94.1*	93.9*	94.2*	94.1*	0.108	0.016	0.031	0.009
Serine	75.6	79.1*	79.3*	78.9*	79.2*	78.9*	0.341	0.013	0.004	0.001

Tyrosine	82.6	85.8	87.1	86.9	88.1*	87.6	0.568	0.005	0.077	0.050

[1] Values are means and standard error of the mean of four replicate cages of 21 fish each.
[2] Endo-1,4-β-mannanase (Natupulse TM®, 8000 TMU kg^{-1}, BASF Corporation, Ludwigshafen, Germany).
[3] Pooled standard error of the means.
[4] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of β-mannanase.
[5] Means within a row with different superscripts differ significantly from Control diet (β-mannanase = 0 TMU kg^{-1} diet) by Dunnet´s test ($P < 0.05$

Based on Dunnet´s test, the ADC of arginine ($P < 0.001$), histidine ($P < 0.001$), isoleucine ($P < 0.001$), leucine ($P < 0.001$), lysine ($P < 0.001$), methionine ($P < 0.001$), threonine ($P < 0.001$), tryptophan ($P = 0.028$), valine ($P = 0.006$) and mean of total essential amino acids ($P < 0.001$) were higher in fish fed diets with 1600 to 8000 TMU kg^{-1} β-mannanase than fish fed control diet. Differently, the ADC of phenylalanine was higher ($P = 0.028$) in fish fed diet with 3200 to 8000 TMU kg^{-1} diet β-mannanase than fish fed diet control. The ADC, cysteine ($P = 0.002$), glutamic acid ($P = 0.001$), glycine ($P = 0.016$), and tyrosine ($P = 0.005$) as well the mean of non-essential amino acids ($P = 0.003$) were higher than fish in diet control. Considering the ADC of non-essential amino acids, aspartic acid ($P = 0.001$; $R^2 = 0.531$; $Y_{max.} = 4880$ TMU kg^{-1} diet β-mannanase), and serine ($P = 0.004$; $R^2 = 0.499$; $Y_{max.} = 5120$ TMU kg^{-1} diet β-mannanase) presented a quadratic distribution. According to Dunnett's test, the ADC aspartic acid ($P = 0.001$), cysteine ($P = 0.001$), glutamic acid ($P = 0.001$), glycine ($P = 0.009$), serine ($P = 0.001$) and mean of non-essential amino acids ($P < 0.001$) were higher in fish fed diet with 1600 to 8000 TMU kg^{-1} dietary β-mannanase than fish fed diet control. Besides, the ADC of tyrosine was significantly higher ($P = 0.050$) in fish fed diet with 6400 TMU kg^{-1} β-mannanase than fish fed diet control. However, the ADC was unaffected by dietary treatments, neither by orthogonal polynomials ($P = 0.905$) nor Dunnett's test ($P = 0.073$) analysis.

3.5. Digestible energy and protein

Figure 2 presents the effects of graded levels of β-mannanase on digestible energy and protein contents of diets. A quadratic response was observed for digestible energy ($P < 0.001$; $R^2 = 0.676$; $Y_{max.} = 4286$ TMU kg^{-1} β-mannanase) and digestible protein ($P < 0.001$; $R^2 = 0.848$; $Y_{max.} = 4143$ kg^{-1} diet β-mannanase) contents.

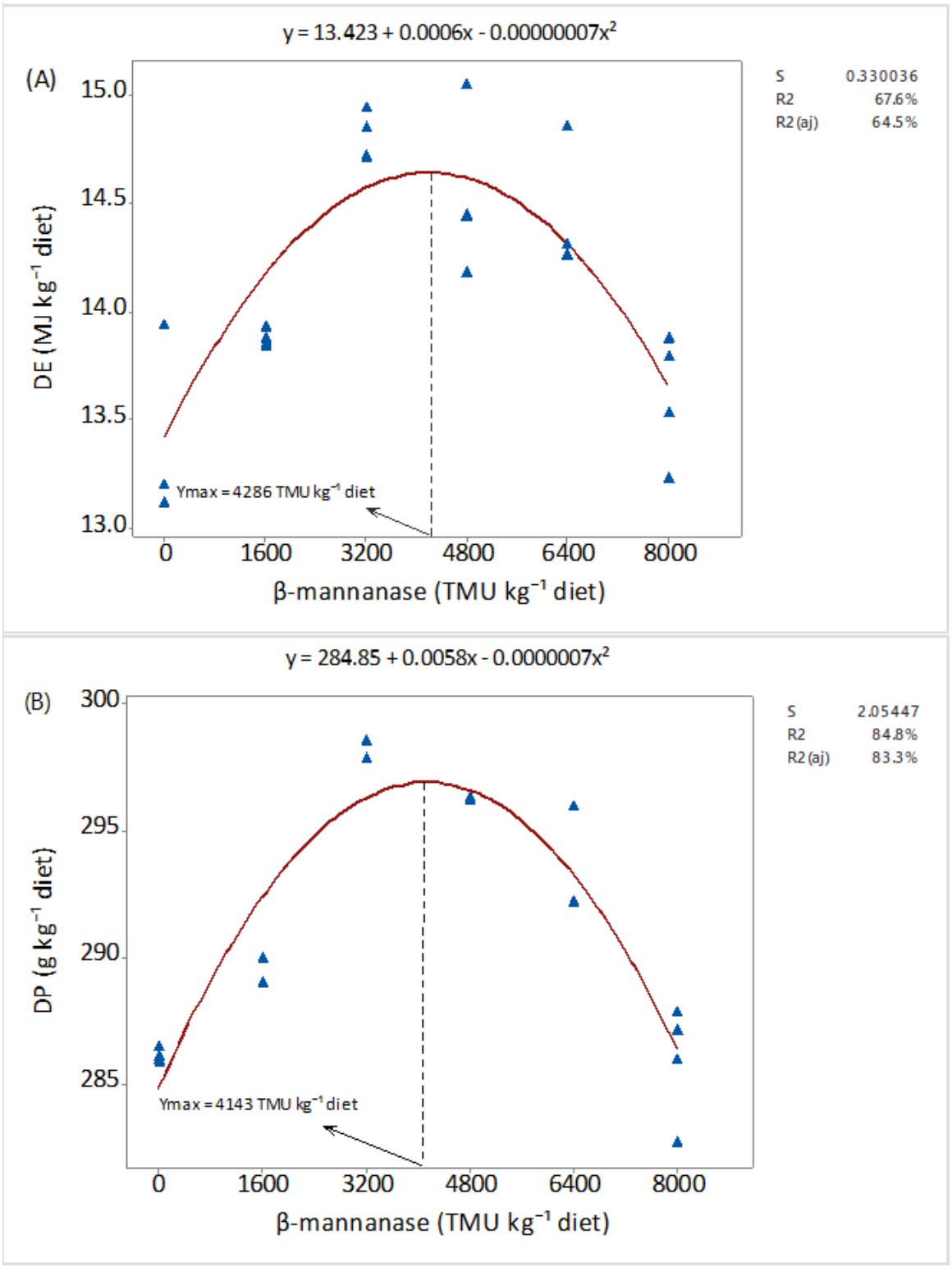

Figure 2. Effect of graded levels of β-mannanase on the digestible energy (DE) and digestible protein (DP) content of diets fed to juvenile Nile tilapia. Each dot point represents mean value of 21 fish as replicate. Orthogonal polynomials were used to evaluate quadratic responses to the levels of β-mannanase. Means within a row with asterisks superscripts differ significantly from control diet (β-mannanase = 0 TMU kg^{-1} diet) by Dunnet´s test ($P < 0.05$).

Dunnett's test showed that the dietary digestible energy contents in fish fed 3200 to 6400 TMU kg^{-1} dietary β-mannanase was significantly higher ($P < 0.001$) than fish fed the control diet. Fish fed a diet with 1600 to 6400 TMU kg^{-1} dietary β-mannanase also significantly increased ($P < 0.001$) the dietary digestible protein content relative to that fish fed the control diet.

3.6. Principal component analysis

Figure 3 shows the principal component analysis of fish fed the experimental diets over eight weeks. Principal component analysis shows the main effects observed in the present study and accounts for 61% of the total effects, and the second component 25.7%, respectively. The main effects observed in ADC responses were primarily represented by dietary β-mannanase levels of 0, 1600, 3200 and 4800 TMU kg^{-1}. The control diet was negatively correlated with pH and viscosity of feces, and the diets 3200, 4800 and 6400 TMU kg^{-1} were more responsible for the effects on ash content, dry matter, gross energy, crude protein and crude lipids of feces (Figure 3A). The gross visualization of the PC analysis score loading plots is presented in Figure 3B.

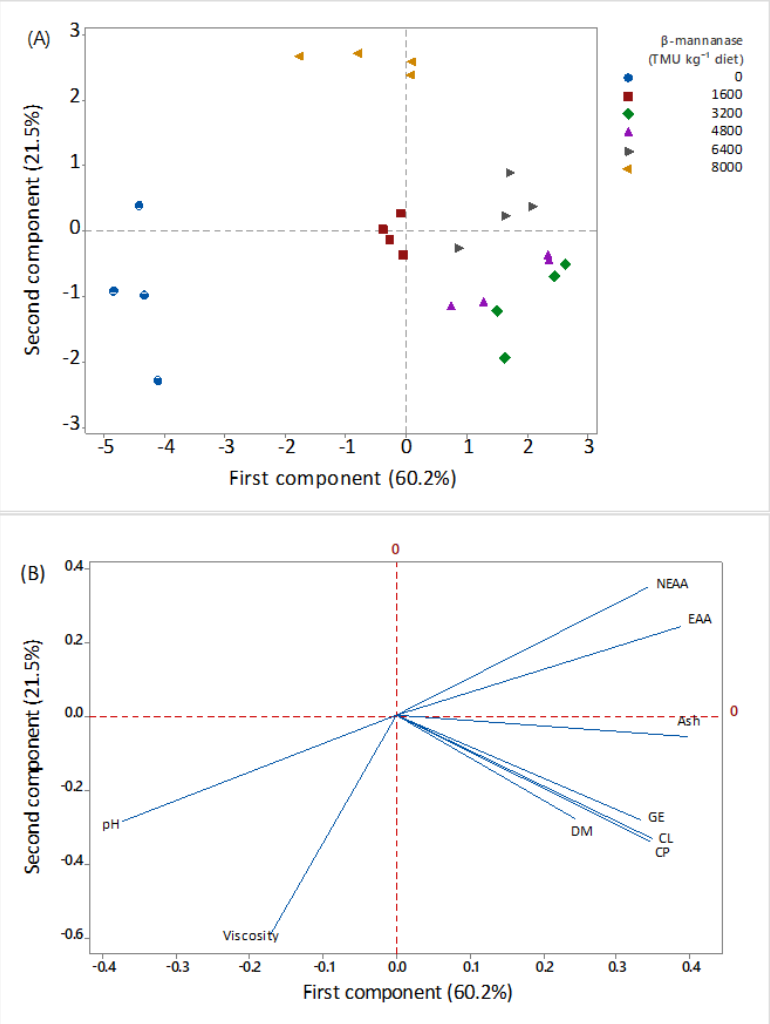

Figure 3. Principal component analysis (PCA) of score plots of dietary treatments with graded levels of β-mannanase (A) and loading plots (B) of fecal pH and viscosity and apparent digestibility coefficients of dry matter (DM), gross energy (GE), crude protein (CP), crude lipids (CL), ash, essential amino acids (EAA) and non-essential amino acids (NEAA) of Nile tilapia fed diets with graded levels of β-mannanase. The figure was constructed using the Bray-Curtis distance method and represents the distance between samples, a summary of the main effects composition. Each point represents the entire treatment in four replicate aquaria. Distant points indicate more different influences.

A negative correlation between digesta pH and viscosity was observed. It has been demonstrated herein by the large angles between the variables dry matter, gross energy, crude lipid, crude protein, ash, and essential and non-essential amino acids.

4. Discussion

The results of this study support the hypothesis that supplementation of β-mannanase can mitigate the antinutritional effects of NSPs in aquafeeds by reducing fecal viscosity, as evidenced by the ADCs of energy and nutrients. These results align with previous findings demonstrating the positive effects of β-mannanase on digestibility in various fish species, primarily due to the reduction of digesta viscosity (Kiarie et al., 2021; Kim et al., 2017; Mok et al., 2015). The mechanism by which β-mannanase improves digestibility is primarily attributed to reduced fecal viscosity (Castillo and Gatlin, 2015). The digesta viscosity, which affects nutrient digestibility, is influenced by the chemical structure and association of NSPs with cell wall components and their physical effects on digestion and absorption (Sternemalm et al., 2008). Furthermore, high viscosity may obstruct the access of digestive enzymes to their substrates and create a barrier to nutrient availability by increasing the rate of passage of digesta through the digestive tract (Leenhouwers et al., 2006). Another possibility is that increased endogenous nutrient losses and the thickness of the layer of unstirred water adjacent to the mucosa may be promoted by high digesta viscosity leading to decreased digestion and absorption of nutrients (Balasubramanian et al., 2018; Lange, 2000; Leenhouwers et al., 2007a).

The present study found a reduction in fecal pH in fish fed a diet containing 4800 TMU kg^{-1} β-mannanase. This reduction is attributed to the improved digestibility of NSPs, as increased nutrient availability, which drives microbiota fermentation and the production of SCFAs, decreasing intestinal pH (Bown et al., 1974; Kihara and Sakata, 1997). Previous research has shown that changes in fecal pH within an optimal range result in significant changes in gut microbiota composition, as a lower pH reduces the abundance of harmful bacteria (Hossain et al., 2019).

Our research demonstrated the beneficial effects of β-mannanase on energy and nutrient digestibility. Our results align with previous studies evaluating the effects of β-

mannanase in different fish species, which observed improvements in the digestibility of energy and nutrients (Caldas et al., 2018; Dawood and Shi, 2022; Leenhouwers et al., 2007a; Magalhães et al., 2016). These results might be attributed to the reduction in digesta viscosity, which enables the action of digestive enzymes with their respective substrates (Magalhães et al., 2016). These results may support a protein-sparing effect, leading to improved growth performance (Kim et al., 2017). Concerning crude lipid digestibility, β-mannans modify intestinal functions, impairing endogenous secretion of water and lipids (Angkanaporn et al., 1994). β-mannans can increase bile acid secretion and result in significant loss of bile acids in the feces (Ikegami et al., 1990) and may justify increased hepatic synthesis of bile acids from cholesterol to restore homeostasis, influencing the absorption of lipids and cholesterol in the intestine and causing a drop in blood cholesterol levels (Hossain et al., 2003). Additionally, β-mannans can trap bile salts, thus reducing their efficiency in fat solubilization and, consequently, impairing lipid absorption (Ebihara and Schneeman, 1989). Furthermore, the presence of β-mannans in fish diets is known to reduce the digestibility of protein and amino acids (Leenhouwers et al., 2006).

In the present study, the β-mannanase increased the ADC of ten essential AAs, such as arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Additionally, it improved the ADC of six non-essential AAs, such as aspartic acid, cysteine, glutamic acid, glycine, serine, and tyrosine. There is no previous research evaluating the individual amino acid digestibility with dietary β-mannanase in Nile tilapia. Despite that, our results corroborate other studies with broiler, swine, and tilapia, showing that mixtures of carbohydrases or enzymatic complexes containing carbohydrases like xylanase and β-glucanases increased the ADC of amino acids (de Brito et al., 2021; Ferreira et al., 2016; Romero et al., 2013). The mechanism whereby β-mannanase improves the ADC of AAs has been attributed to the capacity to reduce fecal viscosity (Castillo and Gatlin, 2015;

Maas et al., 2018; Sinha et al., 2011). Additionally, NSPs can increase the endogenous excretion of AA due to the high viscosity of feces, which stimulates the endogenous secretion of AA and increases mucin production (Angkanaporn et al., 1994). To date, mucin is produced in intestinal cells, is composed mainly of threonine, and compromises the digestibility of AA (Pirgozliev et al., 2010). Thus, the higher ADC of threonine in the present study suggests that the improved ADC of amino acids may be explained by a decrease in the fecal viscosity and reduction in mucin production, which allows a more significant contact between enzyme and substrate, facilitating protein and amino acids digestibility (de Brito et al., 2021; Ferreira et al., 2016). Indeed, dietary amino acids are one of aquafeeds' most costly ingredients. The exogenous dietary β-mannanase has been shown to increase the digestibility of crude protein and amino acids, so this research supports future diet formulations based on the ADC values of amino acids with the addition of β-mannanase at the levels recommended in the present study. Finally, increasing the digestibility of all essential amino acids is of great value since more amino acids are available to be absorbed and metabolized, reducing the impairment effects of amino acid deficiencies and reducing industrial amino acid additions. Additionally, reducing nitrogen excretion in the environment corroborates the activity's economic and environmental sustainability (Furuya et al., 2005; Schaafsma, 2005).

The present study supports previous findings of improved energy and protein digestibility with β-mannanase supplementation in various fish species (Romero et al., 2013; Ferreira et al., 2016; Jeon et al., 2019). Accurate estimation of digestible energy is critical to formulating less cost-effective diets, considering the effect of β-mannanase on the energy and protein content of the diet, especially when diets are deficient in energy relative to protein, which reduces growth rate. Further, considering the crucial correlation between digestible energy and protein in diets with β-mannanase is essential for the optimization of feed utilization

The results of the PC analysis in the present study indicated the impact of graded levels of β-mannanase in the diet on fecal viscosity and pH, ADCs of energy and nutrients, and digestible energy and protein in juvenile Nile tilapia. The findings showed a strong negative correlation between pH and viscosity with the ADC of dry matter, gross energy, crude protein, crude lipids, ash, essential and non-essential amino acids, and digestible energy and protein. This correlation supports the previous studies that evaluated the effects of β-mannanase in several species (Dawood et al., 2022). These findings underscore the importance of including β-mannanase, a carbohydrase, in the diet of juvenile Nile tilapia to reduce digesta and feces viscosity and improve the digestive process. However, it is crucial to determine the optimal level of β-mannanase addition since varying levels had differing effects on the fish in the present study.

Overall, this study evaluated the potential of supplementing tilapia aquafeeds with β-mannanase in NSPs-rich diets. The results indicate that β-mannanase may mitigate the adverse effects of NSPs on nutrient digestibility in juvenile Nile tilapia. Additionally, this approach has the potential to reduce feeding costs and optimize tilapia farm operations through the use of sustainable alternative feedstuffs in industrial-scale production. The study provides novel evidence that exogenous supplementation of β-mannanase may be a practical strategy to improve the nutritive value of economic and environmental sustainability with plant sources in juvenile Nile tilapia feeds.

5. Conclusions

The inclusion of liquid β-mannanase at a concentration of 4800 TMU kg^{-1} in the diet of juvenile Nile tilapia resulted in a reduction of fecal pH and viscosity. This reduction led to an optimization of energy and nutrient digestibility, including amino acids. Furthermore, the addition of 4800 TMU kg^{-1} of β-mannanase to the diet improved the dietary contents of DE

and DP. This study demonstrates that the inclusion of liquid β-mannanase at a concentration of 4800 TMU kg^{-1} is a useful nutritional tool, effectively improving the nutritive values of plant-based diets for precision feeding of Nile tilapia.

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CHAPTER IV

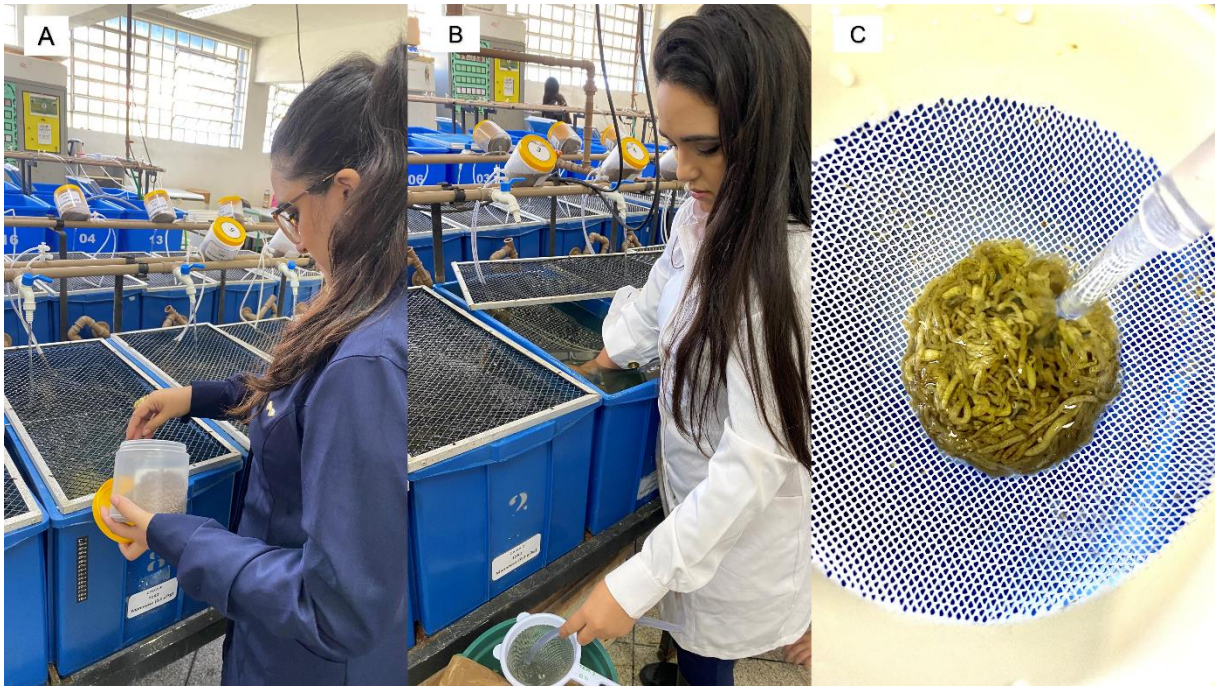
CONCLUSIONS AND IMPLICATIONS

Recent research in aquaculture nutrition has focused on replacing fishmeal, a limited and expensive protein source, with plant-based alternatives to sustain the growing aquaculture sector and align with sustainability goals. To achieve this, using vegetable-based feed ingredients such as soybean meal is crucial in creating practical diets for Nile tilapia. However, mannans, a non-starch polysaccharide in soybean meal, can negatively impact fish growth and feed efficiency. It is therefore important to investigate methods to minimize the effects of mannans, such as adding exogenous enzymes, to create precise and sustainable diets that meet fish nutritional requirements. β-mannanase is an important exogenous enzyme in aquaculture and fish nutrition, breaking down β-mannan bonds to reduce digesta viscosity, increasing the accessibility of digestive enzymes to substrates, and elevating nutrient availability for absorption and metabolism. The present study demonstrates that β-mannanase at 4800 TMU kg^{-1} diet enhanced performance parameters such as body weight gain, feed efficiency, protein, and energy retention efficiency in juvenile Nile tilapia, supported by increased apparent digestibility coefficients of energy and nutrients, including amino acids. The increase in nutrient availability also leads to improved intestinal morphology, resulting from changes in short-chain fatty acid production by beneficial bacteria in the gut microbiome. Microbiome analysis presents a novel approach to examining nutritional interventions' effects on Nile tilapia bacteria populations. It has the potential to enhance gut health, nutrient utilization, and growth

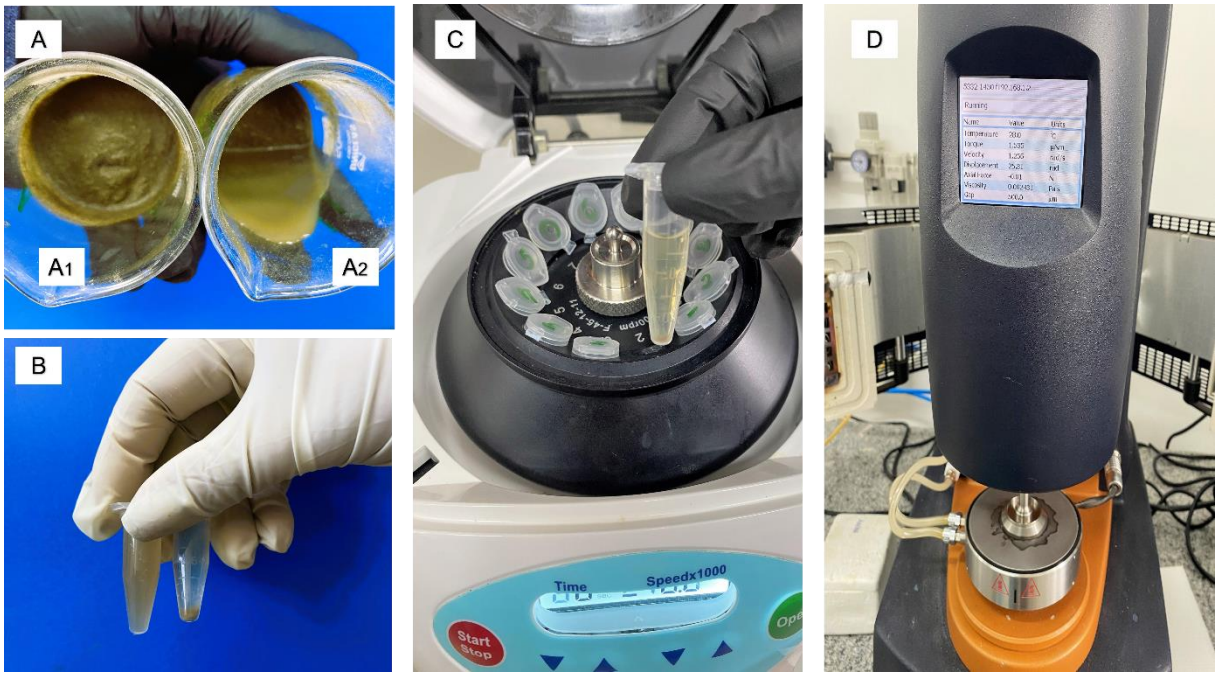
performance in aquaculture. These results highlight the significance of considering the gut microbiome in creating sustainable and precise nutrition for tilapia farming.

APPENDICES

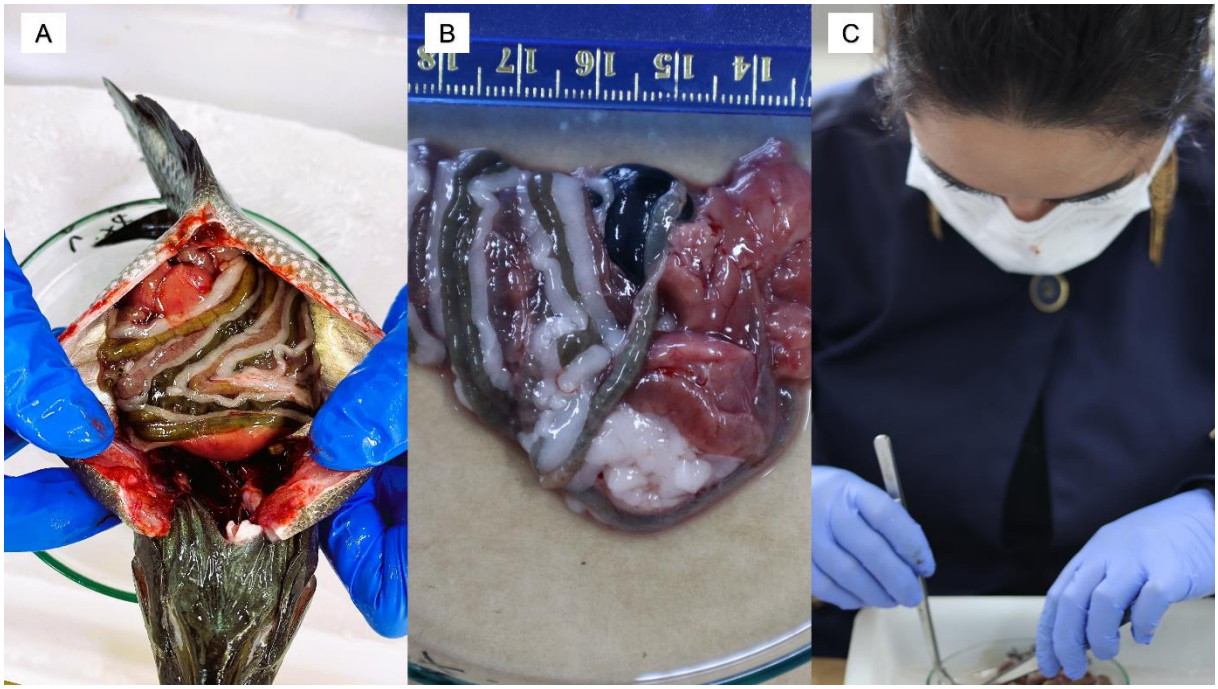
Appendix A. Illustration n of the experimental recirculation aquaculture system – RAS (A), fish utilized (B) and extruded diet (C) emplloyed in the growth and digestibility assay.



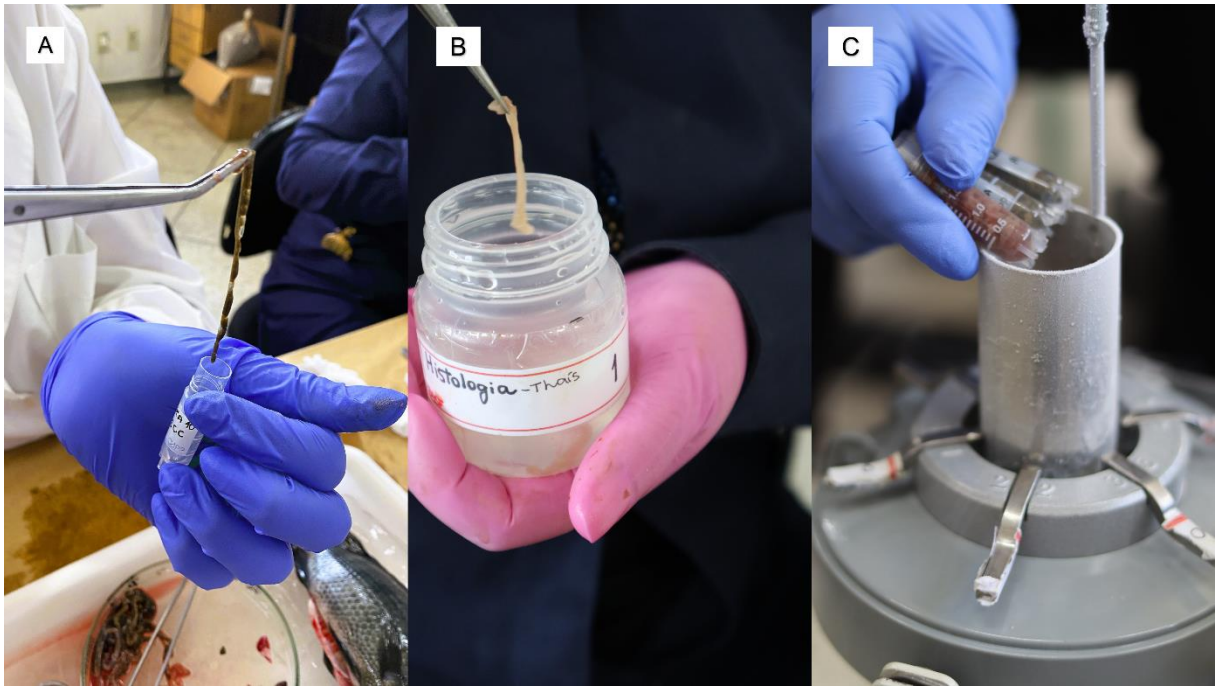
Appendix B. Illustration of the feeding allowance (A) and feces collection management (B; C) management employed in the growth and digestibility assay.



Appendix C. Illustration of the digesta viscosity in fish fed the control diet without (A1) or with 4800 TMU kg^{-1} β-mannanase (A2), centrifugation of feces to obtain the supernatant for determining the digesta viscosity (B, C), and the viscosity analysis performed using a Brookfield Digital Viscometer (D), in the growth and digestibility assay.



Appendix D. Illustrations showing the fish dissection (A), the collection of visceral fat and liver contents (B), and an overview of the sample collection process (C) of visceral fat and liver.



Appendix E. Illustrations showing the collection of digesta for short-chain fatty acids and microbiome analysis (A), the collection of a middle intestine portion for morphological analysis (B), and the preservation of samples for short-chain fatty acids and microbiome analysis by freezing in liquid nitrogen (C).