University of São Paulo
"Luiz de Queiroz" College of Agriculture

Synbiotic microcapsules of *Bacillus subtilis* and oat β-glucan in Nile tilapia diets

Angélica Priscila do Carmo Alves

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

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Bachelor of Animal Science

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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Chico Xavier

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

Arthur Schopenhauer

CONTENTS

RESUMO

Microcápsulas simbióticas de *Bacillus subtilis* e β-glucano de aveia em dietas para tilápia-do-Nilo

Probióticos são definidos como aditivos alimentares microbianos que afetam beneficamente o hospedeiro melhorando seu equilíbrio microbiano intestinal. Contudo, a viabilidade dos microrganismos com ação probiótica pode ser prejudicada durante a passagem pelo trato gastrointestinal do hospedeiro ou pela forma e tempo de armazenamento dos alimentos. A microencapsulação surge, portanto, como técnica promissora na proteção dos probióticos, promovendo um microambiente favorável para o microrganismo encapsulado. Entretanto, ainda não se tem o mapa completo dos efeitos do uso da microencapsulação para prolongar a viabilidade de *Bacillus subtilis* em presença de prebióticos. Considerando estes fatores, objetivou-se investigar os efeitos de simbiótico composto de β-glucano de aveia e *Bacillus subtilis*, livres e microencapsulados pela técnica de "spray-drying", em dietas para tilápias-do-Nilo, *Oreochromis niloticus*. O primeiro capítulo desta tese explicita os pressupostos teóricos que permitam a avaliação crítica dos capítulos subsequentes. O segundo capítulo, portanto, consiste no desenvolvimento e caracterização de microcápsulas de *Bacillus subtilis* e β-glucano utilizando o alginato de sódio como biopolímero complementar onde foi avaliado a sobrevivência do probiótico ao processo de microencapsulação e resistência aos fluidos gastrointestinais simulados de tilápia. O terceiro capítulo consiste no estudo da aplicação das microcápsulas em um ensaio de desempenho em delineamento inteiramente ao acaso sendo cinco tratamentos e cinco repetições: C - controle, sem adição de simbiótico; 0,1%S – 0,1% de simbiótico não encapsulado; 0,2%S – 0,2% de simbiótico não encapsulado; 0,1%SM – 0,1% de simbiótico microencapsulado; 0,2%SM – 0,2% de simbiótico microencapsulado. Ao término do ensaio de desempenho foram feitas análises hematológicas, histológicas e de sequenciamento para caracterizar os efeitos da adição dos simbióticos às dietas na melhoria da integridade do epitélio intestinal e desempenho dos animais. Após o período de desempenho, os animais foram submetidos a desafio imunológico pela bactéria *Aeromonas hydrophila* para avaliação da taxa de sobrevivência e análise do sistema imunológico inato para detectar ação do simbiótico na forma livre ou microencapsulada. De modo geral, os probióticos encapsulados pela técnica de spray-drying apresentaram maior viabilidade quando comparados aos probióticos livres ao longo do período de armazenamento e durante os estudos in vitro. Além disso, os probióticos encapsulados foram mais eficazes quando adicionados às dietas das tilápias, o que resultou em melhor desempenho dos animais.

Palavras-chave: Atomização, Spray-drying, Simbióticos, *Oreochromis niloticus*

ABSTRACT

Synbiotic microcapsules of *Bacillus subtilis* and oat β-glucan in Nile tilapia diets

Probiotics are defined as microbial food additives which beneficially affect the host by improving its intestinal microbial balance. However, the viability of microorganisms with probiotic action may be impaired during passage through the host's gastrointestinal tract or by the conditions of food storage. Therefore, microencapsulation appears as a promising technique in the protection of probiotics, eliciting a favorable microenvironment for the encapsulated microorganism. However, there is still no complete map on the effects of microencapsulation to extend the viability of probiotics. Considering these factors, this project aims at investigating the effects of a synbiotic combination of β-glucan and *Bacillus subtilis*, free and microencapsulated by the spray-drying technique, in diets for Nile tilapia, *Oreochromis niloticus*. The first chapter of this thesis explains the theoretical framework that allow the critical evaluation of the subsequent chapters. The second chapter, therefore, consists of the development and characterization of *Bacillus subtilis* and β-glucan microcapsules using sodium alginate as a complementary biopolymer to evaluate the survival of the probiotic to the microencapsulation process and resistance to simulated tilapia gastrointestinal fluids. The third chapter is the application of the microcapsules in a growth trial in a completely randomized design with five treatments and five replicates: C – Control, no synbiotic added; 0.1%S - 0.1% of non-encapsulated synbiotic; 0.2%S - 0.2% of non-encapsulated synbiotic; 0.1%SM - 0.1% microencapsulated synbiotic; 0.2%SM - 0.2% microencapsulated synbiotic. At the end of the growth trial, hematological, histological and sequencing analysis were performed to characterize the effects of the addition of microencapsulated synbiotics to the diets in the improvement of the integrity of the intestinal epithelium and performance of the animals. At the end of the growth trial, fish were submitted to immunological challenge by the bacterium *Aeromonas hydrophila* to evaluate the survival rate and analysis of the innate immune system to detect the action of the synbiotic in the free or microencapsulated form. Overall, probiotics encapsulated by the spray-drying technique showed greater viability when compared to free probiotics during storage and in vitro studies. Also, the encapsulated probiotics were more effective when added to tilapia diets, which resulted in better animal performance.

Keywords: Atomization, Spray-drying, Synbiotics, *Oreochromis niloticus*

1. INTRODUCTION

The use of therapeutic, prophylactic and antibiotic agents as tools for preventive control of pathogens and improvement of animal performance stands out among the strategies to ensure increased yield of aquaculture production systems (Reverter et al., 2014). However, the indiscriminate use of antibiotics has contributed to the progressive increase in bacterial resistance and the transmission of resistant bacteria from the aquatic environment to humans (Belém-Costa and Cyrino, 2006). The use of antibiotics can be a hindrance to the beneficial microbiota of fish and result in residual accumulation in the carcass, impairing the productivity of the fish farming, not to mention making the food unsafe for human consumption. Therefore, the use of functional foods comes out as an alternative to mitigate these impacts (Ganguly et al., 2013; Gatesoupe, 1999; Kesarcodi-Watson et al., 2008).

Functional food is defined as a substance that modulates the biochemical and physiological functions of animals, e.g., prebiotics, probiotics, and synbiotics (Balcázar et al., 2006; Collins and Gibson, 1999; Ziemer and Gibson, 1998). Synbiotics are functional foods that combine the synergistic, complementary and multiplicative association of the isolated actions of microorganism(s) and substrate(s) (Swanson et al., 2020). The synbiotic association can therefore result in a competitive advantage for the probiotic microorganism when consumed together.

Studies have proved the action of different synbiotics on growth, disease resistance, antioxidant activity, and modulation of the immune response of fish (Cavalcante et al., 2020; Devi et al., 2019; Mugwanya et al., 2021; Mohammadi et al., 2022). However, the maintenance of the microorganism's viability throughout the processing and storage stages, and the exposure to conditions inherent in the gastrointestinal tract of animals, is a chief requirement to be considered in the preparation of a probiotic or a synbiotic additive (Pirarat et al., 2015). Therefore, providing probiotics with a physical barrier against adverse conditions is an approach receiving considerable interest.

The microencapsulation technique thus emerges as a tool to promote the protection and viability of probiotics in adverse conditions such as low pH and the presence of bile salts in the digestive tract (Etchepare et al., 2016; Menezes et al., 2013). Microencapsulation using the spray-drying technique offers low operating costs, greater stability, and lower storage cost, potentially being an excellent alternative to producing low-moisture microbial powder cultures (Assadpour and Jafari 2019; Corcoran et al., 2004). Therefore, the present study aimed at understanding the effects of the inclusion of encapsulated and non-encapsulated

synbiotic in diets for Nile tilapia (*Oreochromis niloticus*) juveniles, including: (1) the preparation, characterization and resistance of alginate microcapsules containing a synbiotic compound of oat β-glucan and *Bacillus subtilis* through exposure to simulated gastric fluids and its stability throughout storage for 90 days, and (2) an *in vivo* study, evaluating growth performance, intestinal morphology, hematology, microbiota, and disease resistance of Nile tilapia fed free and microencapsulated probiotics, as discussed in the theoretical framework that follows.

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2. THEORETICAL FRAMEWORK

2.1 Use of probiotics in tilapia culture

The occurrence of diseases in the aquaculture systems not only negatively affects the seafood trade chain but also poses unnecessary risks to ecosystems and human health (Dawood et al., 2020). The interest in the farming and husbandry of tilapia has encouraged research on management, prophylaxis, and adequate nutrition of the species. However, there is still a need for more information about its immunological features, as well as the mechanisms that involve the protection of the organisms and how it connects to nutritional aspects. Thus, the development of diets that would promote resistance of fish to infections through reliable methods that are also safe for the environment and human health, such as probiotics and prebiotics, are fundamental for the development of aquaculture.

Probiotics are beneficial microorganisms used to improve the general health of hosts and enhance fishes' natural defense mechanisms (Giraffa et al., 2010; Nayak, 2010; De et al., 2014;). To be considered a probiotic additive, these microorganisms must (1) be safe for the host, (2) be able to survive gastrointestinal transit, (3) be resistant to gastric juices, and (4) be able to colonize the digestive tract (FAO, 2002). Moreover, the ability of probiotics to promote and/or improve fish health is related to their ability to stimulate the immune response and inhibit the growth of pathogenic bacteria (Dawood et al., 2018; Iwashita et al., 2015).

Many studies have reported the use of probiotics in aquaculture and their ability to synthesize substances that cause positive effects on the health of the host, such as organic acids, short-chain fatty acids, vitamins, natural antibiotics, bacteriocins and hydrogen peroxide (Yan et al., 2002; Zor- riehzahra et al., 2016; Hoseinifar et al., 2018; Chauhan and Singh 2019). Additionally, probiotics may increase food digestibility by stimulating an increase in digestive enzymes such as lipase, amylases, and proteases (Hoseinifar et al., 2018). High digestive enzyme activity was recorded in the intestine of tilapia fed a diet supplemented with four groups of microrganisms *Bacillus subtilis* NIOFSD017, *Lactobacillus plantarum* NIOFSD018, a mixture containing both bacterial isolates, and a yeast, *Saccharomyces cerevisiae* NIOFSD019 (Essa et al., 2010).

Although the mode of action and required quantity of each probiotic microorganism may differ, some mechanisms are reported to be similar. For instance, several probiotics have shown to improve weight gain (Tan et al., 2019; Kuebutornye et al., 2020; Xia et al., 2020), feed conversion (Makled et al., 2017; Midhun et al., 2017), immune response (Saputra et al., 2016; Opiyo et al., 2019; Tachibana et al., 2020) and disease resistance (Saputra et al., 2016; Srisapoome and Areechon 2017) of Nile tilapia. Abdel-Tawwab et al. (2020) reported an

increase in the resistance of tilapia (*O. niloticus*) fed diets supplemented with the probiotic yeast *Saccharomyces cerevisiae* against the pathogenic fungus *Aspergillus flavus* with an optimal inclusion level of 6 g kg^{-1} of diet.

To exert the aforementioned benefits, probiotics must be viable and stable during their transit through the gastrointestinal tract. Dodoo et al. (2017), studying the viability of some commercial probiotics reported a loss of 10^6 colony-forming units (CFU) after exposure to simulated gastric fluids. According to Yao et al. (2019), there is some concern that many probiotic products are ineffective because the beneficial bacteria cannot survive food processing, storage, transport, and passage through the gastrointestinal tract. Therefore, the microencapsulation process may be an effective technique for protecting probiotics from degradation.

2.2 *Bacillus* species as a probiotic

Bacillus species are Gram-positive bacteria of the Fimicutes phylum, Bacilli class, Bacillales order, and Bacillaceae family encompassing more than 293 species (Ciccarelli et al., 2006; Nayak, 2020). These species are broadly used in industry and agriculture because of many advantageous features including their non-pathogenic nature, fast growth rate, easiness of preservation and isolation, ability to sporulate and tolerate harsh conditions, as well as their ability to secrete a multitude of bioactive compounds that can boost host's health (Hong et al., 2005; Ghalwash et al., 2021).

The application of *Bacillus* sp. as probiotics in fish farming has been extensively studied (Abarike et al., 2018; Galagarza et al., 2018; Kuebutornye, et al., 2020; Nayak, 2020). *Bacillus* species are known to promote better nutrient utilization as a result of the secretion of exogenous enzymes and modulation of gut microbiota (Hai, 2015). Therefore, changing the composition of intestinal microbiota for beneficial bacteria communities can also help the immunity of fish and boost the host's intestine integrity (Hoseinifar et al., 2019; Tachibana et al., 2020). Accordingly, Selim and Reda (2015) studying Nile tilapia fed diets containing *Bacillus amyloliquefaciens* for 30 days reported increased incidence of goblet cells in fish's intestinal wall and improved immune responses.

Extensive research carried out with Gram-positive bacteria revealed that *Bacillus* species have a high potential for immunomodulation and disease protection (Aly et al., 2008; Fan et al., 2013; Nayak, 2020; Ghalwash et al., 2021; Wang et al., 2019). Tilapia fed 10^{10} CFU g^{-1} *Bacillus licheniformis* showed high survival after *Streptococcus iniae* challenge (Han

et al., 2015). Similarly, dietary *Bacillus* spp. increased resistance to *Streptococcus iniae* infection in pangasius (Meidong et al., 2018), rohu (Ramesh et al., 2017), and Atlantic salmon (Wang et al., 2019). Seemingly, *Bacillus* species enable the occurrence of many overlapping protection mechanisms such as synergistic, antagonistic, and competitive exclusion of pathogenic bacteria, being the most widely used probiotics in aquaculture (Nayak, 2020).

Because of their unique features, several *Bacillus* species are available in the market for applications in aquaculture. Among those, *Bacillus subtilis* is the most widely used (Alves et al., 2022; Fan et al., 2013; Nayak, 2020; Ghalwash et al., 2021); *B. subtilis* is a well-researched microorganism and one of the first organisms to have complete genome sequenced (Nayak, 2020). Over the years, *B. subtilis* has been broadly used as a probiotic in aquaculture with many positive results on tilapia farms. Won et al. (2020) fed tilapia with diets containing 10^8 CFU g^{-1} *B. subtilis* for eight weeks and reported improved weight gain, feed efficiency, intestinal morphometry, and modulation of immune parameters. According to Aly et al. (2008), *B. subtilis* at 0.5×10^7 CFU g^{-1} diet inhibited the development of *Pseudomonas fluorenscens* improving disease resistance and growth performance of Nile tilapia. Given that many similar results were also reported for Nile tilapia by Abarike et al. (2018), Garcia-Marengoni et al. (2015), Tang et al. (2016) and Telli et al. (2014), it is fair to consider *B. subtilis* eligible for microencapsulation studies, as this technique can not only improve bacterial efficiency through time storage but also improve survival and improve activity under the harsh conditions of the gastrointestinal tract.

2.3 Oat β-glucan as a prebiotic

Prebiotics are food substances that cannot be digested by the enzymes present in the gastrointestinal tract but can be hydrolyzed by specific microorganisms of the intestinal microbiota, promoting benefits to the host. A food additive is considered a prebiotic when it is resistant to gastric acidity and gastrointestinal fermentation, not hydrolyzed nor absorbed in the foregut, and ultimately alters the intestinal microbiota in a favorable way for the host (Pineiro et al., 2008; Kumar et al., 2015). The most studied prebiotics in aquaculture are those derived from bacteria and yeast such as lipopolysaccharides (LPS), muramyl-dipeptide (MDP), oligosaccharides, inulin, and glucans (Alves et al., 2022; Song et al., 2014). Despite the potential benefits of prebiotics to animal health and performance, their use in fish nutrition is at an early stage comparatively to other domestic species such as swine and poultry (Cechim, 2015).

β-glucan are glucose macromolecules found in foods such as oats, barley, fungi and yeast. In cereals such as barley and oats, β-glucan is made from β-(1-3) and β-(-1-4) linkage units, while in fungi and yeast it is formed by β-(1-3) and β-(1-6) linkages (di Luzio et al., 1979; Daou et al., 2012). These structural variations prompt differences in some of the physical properties including solubility, solution viscosity and viscoelasticity and gelation properties (Wang and Ellis, 2014), which may qualify the oat β-glucan as potentially prebiotic and responsive encapsulation material.

As a prebiotic, oat β-glucan has been broadly explored as fermentable fiber in human food as it provides a substrate for microbiota within the large intestine, increases the production of short-chain fatty acids by products, and it has been associated with several physiological and metabolic activities such as cholesterol and glucose metabolism (McRorie and McKeown, 2017; Simpson and Campbell, 2015). In fish, only one study was found with oats nano scale β-glucan as a feed for zebra fish larvae (Udayangani, et al., 2017). These authors evaluated the immunomodulatory properties and survival rate of fish after *Edwardsiella tarda* challenge and reported that fish fed oat β-glucan had a higher survival rate and up-regulation of immune functional genes including TNF-α, IL-1β, β-defensin, lysozyme, IL 10, IL 12.

A few studies have tested the protective capacity of β-glucan as an encapsulating polymer. Sobieralska and Kurek (2019) demonstrated the benefits of using a small quantity of β-glucan for improving the encapsulation efficiency and stability of elderberry anthocyanin extracts compared to the usage of maltodextrin with Arabic gum microcapsules. The addition of β-glucan positively contributes to improving the polydispersity index thus producing health-beneficial effects on the human body. Accordingly, Falco et al. (2017) demonstrated a reproducible build-up of chitosan and sulfated β-glucan as a coating agent to be exploited as carriers for probiotics and delicate nutraceuticals. The authors suggest that coated cells seemed to resist acid gastric conditions, as the coatings were only partially degraded after exposure to simulated intestinal fluid.

2.4 Spray-drying technique

The microencapsulation technique is based on the coating of bioactive components in microcapsules made of resistant material. Microencapsulation is used in pharmacology, chemistry, medicine, biotechnology, food science and engineering as it offers a wide range of possibilities to improve the functional properties of different substances (Poshadri and Kuna,

2010; Temiz and Öztürk, 2018). The types of encapsulations correspond to the capsule size being nano-, micro-, and macro-encapsulation. Therefore, being large enough to cover probiotic microrganism with minimal influence on the textural properties of the supplemented product, the recommended microparticle size is smaller than 100 μm (Martín et al., 2015).

The advantages of using encapsulation in animal feeding include, but is not limited to, the controlled/targeted release of bioactive ingredients in commercial products to mask flavors and odors or to extend shelf life whereas protecting compounds with nutritional value (Menezes et al., 2013). Alishahi et al. (2011) evaluated the addition of chitosan and vitamin C nanoparticles in diets for rainbow trout (*Oncorhynchus mykiss*) and registered increased lysozyme activity and also a positive, synergic effect of chitosan and vitamin C regarding the performance of animals. Pirarat et al. (2015) fed diets supplemented with *Lactobacillus rhamnosus* in alginate microparticles to tilapia juveniles and reported longer intestinal villi, better growth rate, in addition to protecting against *Streptococcus agalactiae* infection.

The structure of the encapsulating material will determine the functional properties of the microcapsules. The coating material must be able to withstand the acidic conditions of the stomach allowing bioactive compounds to reach the intestine intact (Champagne et al., 2011). Various polymers such as chitosan, carrageenan, gelatin, and alginate can be used as encapsulating agents in different microencapsulation techniques, such as spray-drying (atomization), spray congealing (cold spraying), extrusion, coacervation, and electrostatic method (Li et al., 2008; Burey et al., 2009; Menezes et al., 2013). Because of its high biocompatibility, low cost, and gelation under ambient conditions, alginate is the natural carbohydrate of choice as encapsulating agent by the atomization technique (Messaoud et al., 2016).

In addition, because of its quick drying process, the atomization technique allows the encapsulation of microorganisms such as probiotics and other thermosensitive compounds such as enzymes, vitamins, and oils (Assadpour & Jafari, 2019). The technique consists of removing moisture through the application of heat, transforming the compound into dry powder in four distinct steps: atomization of the bioactive compound; contact with gas (air); moisture evaporation; and particle separation (Figure 1) (Anandharamakrishnan, 2015).

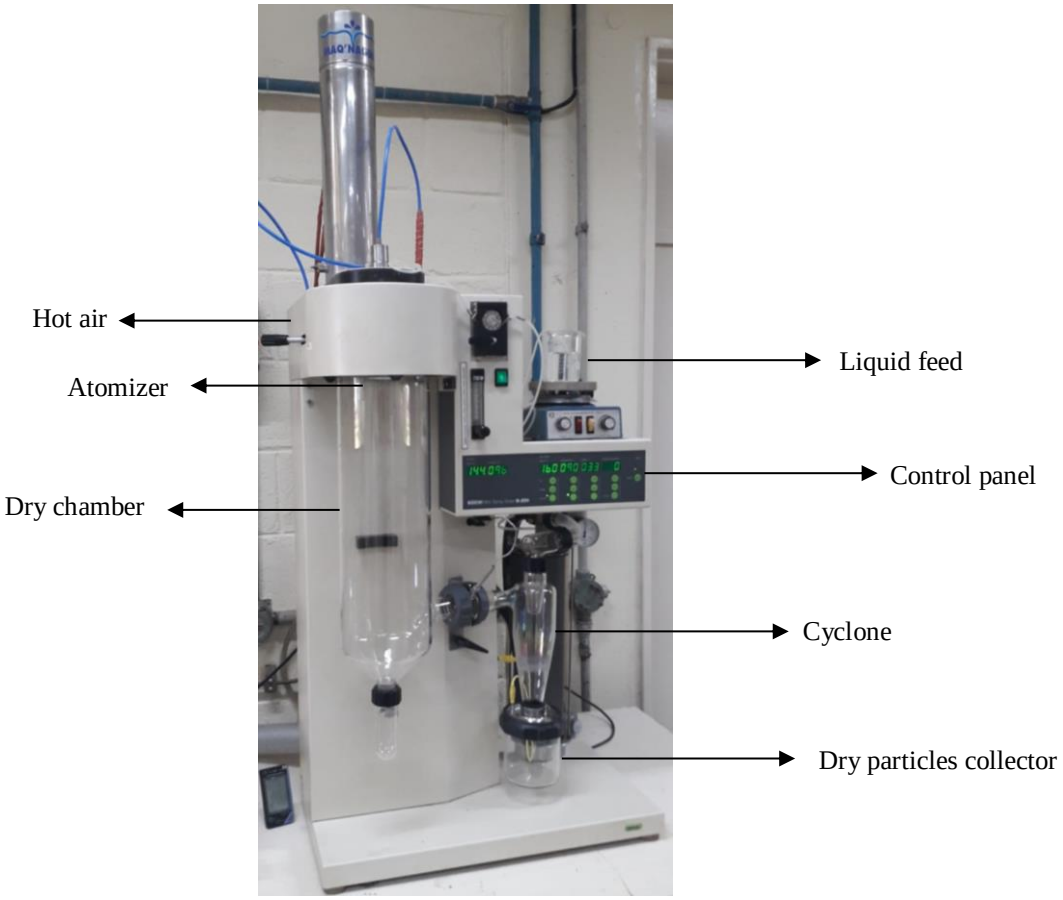

Figure 1 – General spray-drying equipment configuration (Fonte: Autor, 2021).

Although well established, atomization needs further technological development, driven by the increased quest for more sophisticated microparticles by the food industry. Actually, when it comes to animal nutrition, much is yet to be done in the search for ideal conditions for the encapsulation of functional compounds that promote animal health in addition to promoting longer storage time for processed feeds.

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3. CO-ENCAPSULATION OF *Bacillus subtilis* AND B-GLUCAN AND THEIR APPLICATION AS A SYNBIOTIC IN FISH FEED

Abstract

Microencapsulation through a spray-drying technique has been used to enhance the viability of probiotics during processing, storage and during passage through the digestive tract of animals. This work studied the use of oat β-glucan for co-encapsulating *Bacillus subtilis* C-3102 on the alginate matrix using spray-drying technology. The physical characterization included analysis of morphology, zeta potential, size, moisture content, water activity, and hygroscopicity. Survival assays were designed to compare the resistance of free and microencapsulated cells through exposure to simulated gastric fluids (SGF), bile resistance and its stability throughout storage for 90 days with 15 days of intervals at -20, 7, and 25 °C. Cell viability was affected by the spray-drying technique but remained up to 6 log-unit through the 90 days of storage regardless of temperature. Microencapsulation increased survival rate of *B. subtilis* exposed to SGF and bile salts compared to that of free probiotic cells. Microphotographs of the *B. subtilis* microcapsules showed that spherical particles of different sizes were produced with a smooth surface with some concavities. No evident pores or cracks were found in the registered images. Spray-drying combined with the addition of oat β-glucan proved to be an efficient method to protect *B. subtilis* cells in fish feed under storage and simulated gastric conditions.

Keywords: Atomization, Gastrointestinal tolerance, Microencapsulation, Probiotics prebiotics

3.1 Introduction

The intensive farming of tilapia has happened together with ever increased use of antibiotics for the control of outbreaks of numerous diseases (Dong et al., 2015; El-Kady et al., 2022). The use of probiotics, single or combined with prebiotics, has come along as a safe alternative to overcome antibiotics abuse in the management of aquaculture species and systems. Probiotics are endowed with the ability to modulate fish microbiota, and the presence of prebiotics, ingredients that are selectively fermentable, exert complementary beneficial effects on the survival and growth of probiotic cells in the fish gut (Okuro et al., 2013; Fritzen-Freire et al., 2012).

Among probiotic bacteria used in aquaculture, *Bacillus subtilis* has been described as a beneficial agent for biological control of diseases (Alves et al., 2020; Nayak, 2020; Galagarza et al., 2018). These microorganisms can improve fish health by enabling the occurrence of overlapping protection mechanisms such as improvement of the balance of intestinal microbiota through synergistic, antagonistic, and competitive exclusion and

immune-stimulating effects (Nayak, 2020). However, studies indicate that probiotic preparation containing free cells are highly vulnerable to disintegration and may not survive in sufficient numbers when incorporated in animal feed and during their passage through the gastrointestinal tract (And and Kailasapathy, 2005; Sribounoy et al., 2021).

Within this context, microencapsulation of probiotics through a spray-drying technique has been used to enhance their viability during processing, storage and during passage through the digestive tract of animals. Microencapsulation through spray-drying is a commercially viable technique that generates dried powder from a suspension containing microorganism and coating agents, which is nebulized with hot air (Encina et al., 2016; Santos et al., 2019). Among the promising coating agents, prebiotic compounds such as β-glucan from cereals may serve as coating agent as well as a prebiotic ingredient in fish feed.

Oat β-glucan is a linear polysaccharide of monosaccharide D-glucose bonded by β-(1,4) and β-(1,3) glucosidic linkages (Hu et al., 2015). As a prebiotic, oat β-glucan has shown to modulate the gut microbiota in human, animals, and *in vitro* fermentation systems, particularly with bacterial species that can affect host bile acid metabolism and production of fatty acids (Joyce et al., 2019; Ryan et al., 2017). Shah et al. (2016) evaluated barley β-glucan, a prebiotic also bonded by β-(1,4) and β-(1,3) glucosidic linkages, as a coating agent for *Lactobacillus casei*, *L. brevis*, and *L. plantarum* through emulsion technique, and registered an improvement to tolerance when the encapsulated cells were subjected to low pH, heat treatment, simulated intestinal conditions, and storage.

Although several studies have already investigated the use of prebiotic agents as a coating for microencapsulated probiotic cells, to the best our knowledge, there is no report regarding the comparison between the survival of free and microencapsulated *B. subtilis* in fish feed. Therefore, the aim of this study was to develop microcapsules of *B. subtilis* using the combination of sodium alginate and oat β-glucan as coating agents through a spray-drying method. Moreover, the survival of the microencapsulated probiotic under storage and also its viability under simulated gastrointestinal conditions were evaluated and compared to the survival of the free cells.

3.2 Material and Methods

3.2.1 Probiotic culture and growth curve

Lyophilized spores of *Bacillus subtilis* C-3102 (Calsporin®, Biogenic, SP, Brazil) were anaerobically propagated (1g) in 50 mL Luria-Bertani (LB) broth for 24 h in an incubator shaker (Marconi MA830/A) at 150 rpm and 37°C. Resulting culture was

subcultured, checked for purity, and grown overnight. The culture obtained after the second subculture was studied by plotting the cell growth (absorbance at 600 nm) versus the incubation time. The microbial concentration was recorded every 1 h for 12 h and then every 4 hours for 48 h. The growth curve of *B. subtilis* was performed to corroborate its growing stage and determine the appropriate time for the microorganism to be mixed with the wall material (early stationary phase) (Corcoran et al., 2004).

A third subculture was then grown until early stationary phase (10^9 CFU mL^{-1}), harvested by centrifugation at 5000g for 15 min at 4 °C, and then washed twice in 0.85% (w:v) saline solution. The cell pellets were resuspended in PBS (10 mM, pH 7.0) and prepared at a final concentration of 10^{13} CFU mL^{-1}. The cell suspensions were then either subjected to microencapsulation or frozen accordingly until further analysis.

3.2.2 Microencapsulation and coating procedures

Oat β-glucan (B-CAN® Oat Fiber 70%, Garuda International Inc., CA, USA) was used at concentrations of 7g in 100 mL of warm water containing 2g of sodium alginate (FMC BioPolymer, SP, Brazil). The concentration of oat β-glucan was chosen as the maximum level that could practically be atomized in the spray dryer. The wall materials were stirred with a magnetic stirrer for 1 h to hydrate, and then autoclaved at 121°C for 15 min. After cooling the solution, *B. subtilis* strain was added to the wall material solution and stirred with a magnetic stirrer. Final count of *B. subtilis* in the carrier solution was 10^{12} CFU mL^{-1}.

The microcapsules were obtained with a laboratory scale spray dryer (B-290, Büchi, Flawil, Switzerland), operating by co-current, at air inlet temperature of 130 ± 5 °C and outlet temperature of 50 – 60 °C (Nunes et al., 2018; Ma et al., 2015). The feed solutions containing *B. subtilis* were kept under magnetic agitation at room temperature and fed into the main chamber through a peristaltic pump, with feeding flow of 7.5 mL min^{-1}, drying air flow rate of 22.5 m^3 h^{-1}, and compressor air pressure of 0.6 MPa. Unloaded microparticles, used as control, were prepared following the same procedure, except for the addition of *B. subtilis*. The resulting microcapsules were collected from the base of the cyclone and stored for further analysis.

3.2.3 Encapsulation yield

The microencapsulation yield (EY) was calculated according to Mascaraque et al. (2016) using Equation (1):

$$EY\ (\%) = \left(\frac{Mass\ of\ spray\ drying\ products\ recovered\ from\ collector}{Mass\ of\ solids\ in\ the\ processed\ suspension}\right) \times\ 100 \qquad (1)$$

3.2.4 Encapsulation efficiency and viability of probiotics

The encapsulation efficiency of *B. subtilis* was calculated according to a modified method by Martin et al. (2013). To release the entrapped bacteria from the microcapsules, 0.1 g of microcapsules was dissolved in 9.9 mL of PBS (0.1 M, pH 7.5) at 250 rpm for 30 min. with the aid of a magnetic stirrer. The samples were serially diluted with 0.1% peptone (w:v) and dissolved in LB agar. The plate was incubated in anaerobic conditions at 37 °C for 24 h. Plates containing 20 - 300 colonies were counted and recorded as CFU per gram of dried powder or per mL of solution. The encapsulation efficiency (EE) was calculated using Equation (2):

$$EE\ (\%) = \left(\frac{N}{N0}\right) \times\ 100 \qquad (2)$$

where N0 indicates colony forming units (CFU) before the microencapsulation process and N0 indicates colony forming units (CFU) obtained after the process.

3.2.5 Morphology of the microcapsules

The morphology of the microcapsules was examined using a scanning electron microscope (SEM) (JSM-820, Japan Electron Optics Laboratory). Capsules were placed on a specimen aluminum stub with the help of double-sided sticky tape and coated in a sputter coater (SCD 050, Bal-Tec) for 2 min. at an accelerating voltage of 10 and 15 kV.

3.2.6 Water activity and moisture content

Water activities of the microcapsules were measured by using a water activity meter (Aqualab 4TE, Decagon Inc, Pullman, WA, USA) at 25°C. The moisture contents of the microcapsules were determined through oven drying at 105°C until reaching constant weight, according to AOAC (1984) method.

3.2.7 Hygroscopicity

The hygroscopicity of the microcapsules (1 g) was determined gravimetrically as a percentage of weight increase over a week equilibrium period (75% relative humidity) on a desiccator chamber containing saturated NaCl solution at 25 °C. After one week, the samples were weighed and their hygroscopicity was expressed as g of adsorbed moisture per 100 g of dry solids (g 100 g^{-1}) (Fritzen-Freire et al., 2012).

3.2.8 Zeta potential and size distribution

The particle size and the zeta potential of loaded and unloaded microcapsules, free *B. subtilis* cells, glucan, and sodium alginate were measured using a Zetasizer Nano ZS (Malvern Instrument Inc., Worcestershire, UK) at 25℃ (Chang et al., 2020). The measurement principle for particle size was calculated based on the light intensity distribution data of scattered light, while zeta potential was determined using 633-nm laser doppler micro electrophoresis. Microcapsules (0.1 g) were suspended in 5 mL of deionized water, pH 6.8, as a dispersing medium. Early stationary phase cells (10^{10} CFU mL^{-1}) were harvested by centrifugation at 10000 × *g* for 10 min, resuspended twice in 10 mM KNO_3 and analyzed. The dispersion was poured into micro cuvette for particle size and disposable capillary cell (DTS1070), for zeta potential measurement. The particle size measurement results showed the mean of a microcapsule diameter (z-average) and polydispersity index (PdI) value.

3.2.9 Viability test in Nile tilapia pelleted feed under storage at different temperatures

In situ viability test of tilapia feed was performed as previously reported with some modifications (Sribounoy et al., 2021). A basal diet (Table 1) was formulated to meet tilapia's nutritional requirements according to NRC (2011). The diet was processed by extrusion on an experimental, single-screw extruder (Imbramaq Co. 8MX-50), oven-dried (58°C for 24 hr), sterilized by autoclaving (15 min at 121°C), and then divided into two treatments: basal diet added 0.1% encapsulated probiotic and basal diet added 0.1% unloaded microcapsules + free probiotic (FP), top-dressed with soybean oil as binder. A one-gram sample of each supplemented diet was stored in individual airtight plastic containers at -20.0 ± 1 °C (freezing), 7.0 ± 1 °C (refrigeration) and 25.0 ± 1 °C (ambient) for three months (Holkem et al., 2016). Evaluation of *B. subtilis* viability was performed every 15 days. A one-gram sample of each feed was dissolved in 9mL of PBS (0.1 M, pH 7.5) at 250 rpm for 30 minutes with the aid of a magnetic stirrer. The liquefied suspensions were decimally diluted in 0.1% (w:v) peptone water and spread plate on LB agar plates. Viable cells were enumerated as the number of colonies after incubation at 37 °C for 24h and expressed as log colony forming units per gram (log CFU g^{-1}).

Table 1. Composition of the basal diet.

Ingredients	Basal diet (%)
Soybean meal	38.30
Fish meal	16.00
Corn	26.00
Wheat bran	12.90
Starch	3.00
Soy oil[a]	2.00
Cellulose	0.78
Vitamin/mineral mix[b]	0.50
DL-methionine	0.40
BHT	0.02

[a]50% soy oil was included after feed extrusion as microcapsules binder.
[b]Agromix Nutrição Animal, batch number 23099010.

3.2.10 Viability test in simulated gastric conditions and 10% tilapia bile

Simulated gastric fluids (SGF) of Nile tilapia were prepared using saline solution (0.6% NaCl) with adjusted pH to 1.5, using HCl (5M) solution. A 0.5 g sample of *B. subtilis* microcapsules and 0.5mL of free cells were placed into separated test tubes containing 4.5 mL of SGF. One mL of each sample was taken after incubation at 25 °C in a water bath (Dubnoff TE053) for 0, 1, 2, and 3 h and enumerated for the cell counts on LB agar using a pour- plate method (Pirarat et al., 2015).

For determination of bile tolerance, 0.5 g of *B. subtilis* microcapsules and 0.5 mL of *B. subtilis* free cells were added to 4.5 mL of SGF at pH 1.5 and incubated at 25 °C for 1 h. After the incubation, samples were centrifuged at 10,000 *g* for 10 min. Then, SGF was removed and replaced with 4.5 mL of tilapia bile salt (10%). One mL of each sample was taken after further incubations of 1, 2, and 3 h at 25 °C and enumerated on LB agar using a pour-plate method to determine the number of viable cells (Nikoskelainen et al., 2001; Pirarat et al., 2015).

3.2.11 Statistical analysis

Trials were carried out in triplicates and the data reported as mean ± SD. The obtained results were exposed to the analysis of variance (ANOVA) and Tukey's tests ($p \leq 0.05$) using SAS version 9.4 (SAS Institute, Inc.; Cary, NC, USA).

3.3 Results

Growth kinetics of *B. subtilis* was used to determine the early stationary phase - time when the cultures were most suitable for the spray-drying process (Figure 1). Under these conditions, the early stationary phase was reached at 24 h with 2.9×10^{9} CFU mL^{-1}.

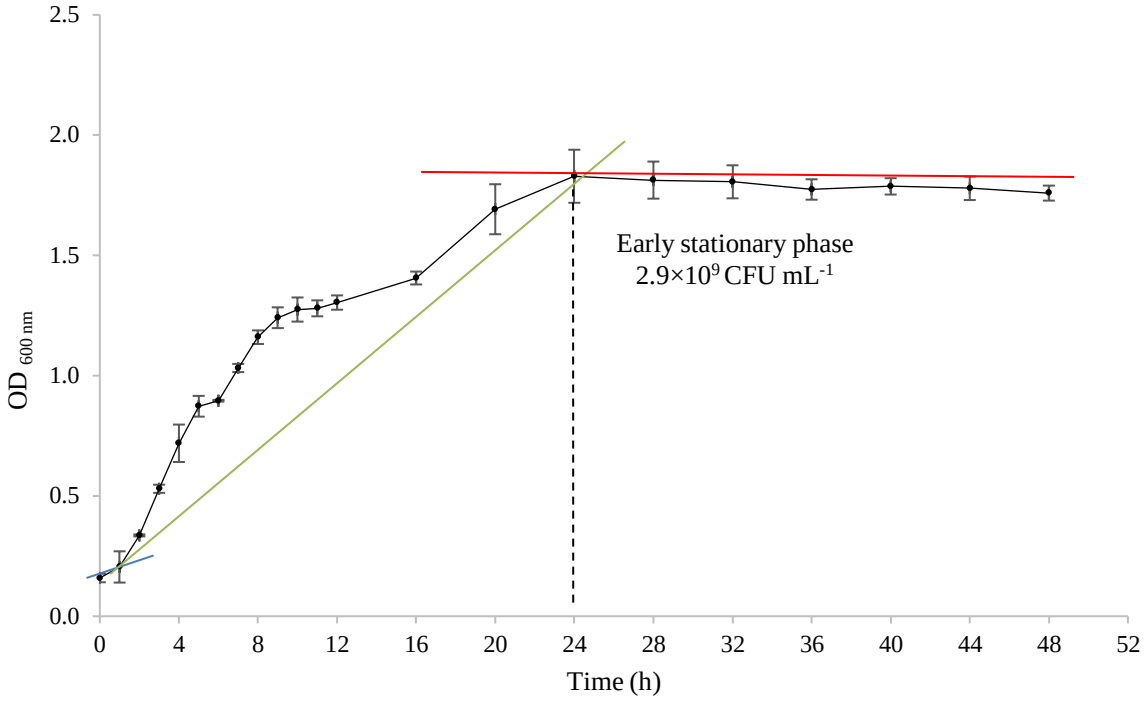


Figure 1. The growth curve (OD 600) of *B. subtilis* (C-3102) in Luria-Bertani (LB) medium to determine the early stationary phase.

For characterization of microcapsules and cell viability (Table 2), the number of free cells was counted before and after the inoculums underwent the spray-drying process. The spray-drying method resulted in the loss of 3 log-unit of viable cells, approximately 22% of loss in viability.

Table 2. Characterization and viability of *Bacillus subtilis* after encapsulation process.

Cell viability (log CFU/mL)						
Before drying	After drying	Encapsulation yield (%)	Encapsulation efficiency (%)	Moisture content (g 100 g^{-1})	Water activity a_w	Hygroscopicity (g 100 g^{-1})
12.39±0.08	9.65±0.12	62.03±10.75	77.9±3.06	3.98±0.87	0.289±0.002	10.01±0.28

Values are presented as the mean value ± standard deviation (SD) (n = 3).

The size or PDI of microcapsules made with and without *B. subtilis* did not differ (Table 3). The formed microcapsules had a suitable size for addition to animal feed products (1.55 ± 0.3 μm). Zeta potential analysis (Table 3; Figure 2) showed that the surface electrical

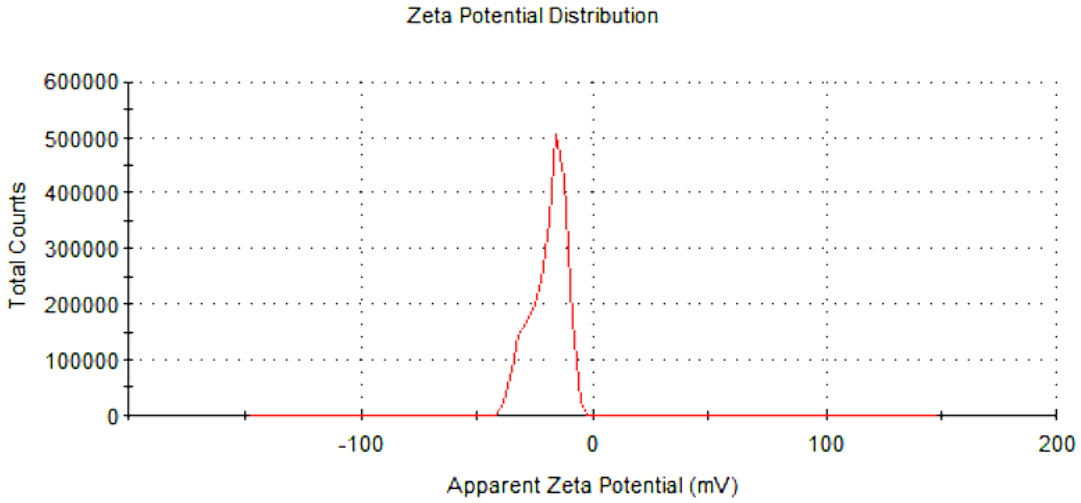
charge of both microparticles (with and without *B. subtilis*) was negative and did not differ, which is consistent with the negative charge of sodium alginate ions combined with the negative charge of oat β-glucan. The morphological analysis of SEM microphotographs of the *B. subtilis* microcapsules showed spherical particles of heterogenous sizes with variation from 1.12 to 14.56 μm (Figure 3). The surface of some microcapsules had a smooth surface while others had concavities on the surface. No evident pores or cracks were found in the registered images.

Table 3. Particle size, polydispersity index (PDI) and zeta potential of loaded microcapsules (with β-glucan and *Bacillus subtilis*) and unloaded microcapsules (with β-glucan and without *Bacillus subtilis*).

Treatment	Particle size	PDI	Zeta potential
	nn		
Loaded microcapsules	1548±342,4	0.67±0.17	-21.6±1.9
Unloaded microcapsules	1412±238,3	0.61±0.16	-19.0±2.1
Free cells	nd.	nd.	-19.3±2.6
β-glucan 7%	nd.	nd.	-2.33±0.2
Sodium alginate 2%	nd.	nd.	-51.16±0.8

Values are presented as the mean value ± standard deviation (SD) (n = 3).

(a)



(b)

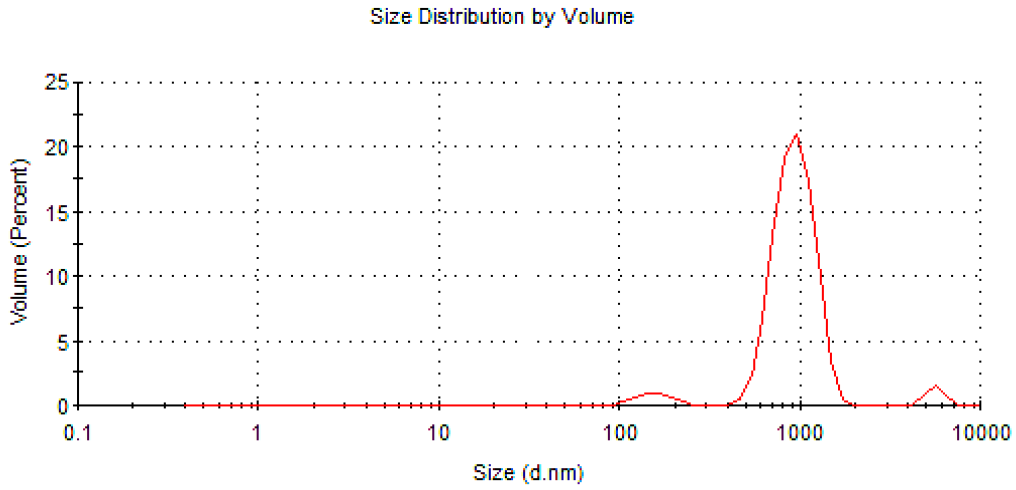


Figure 2. Zeta-potential (a) and particle size distribution (b) for a representative sample of alginate microcapsules loaded with β-glucan and *Bacillus subtilis*.

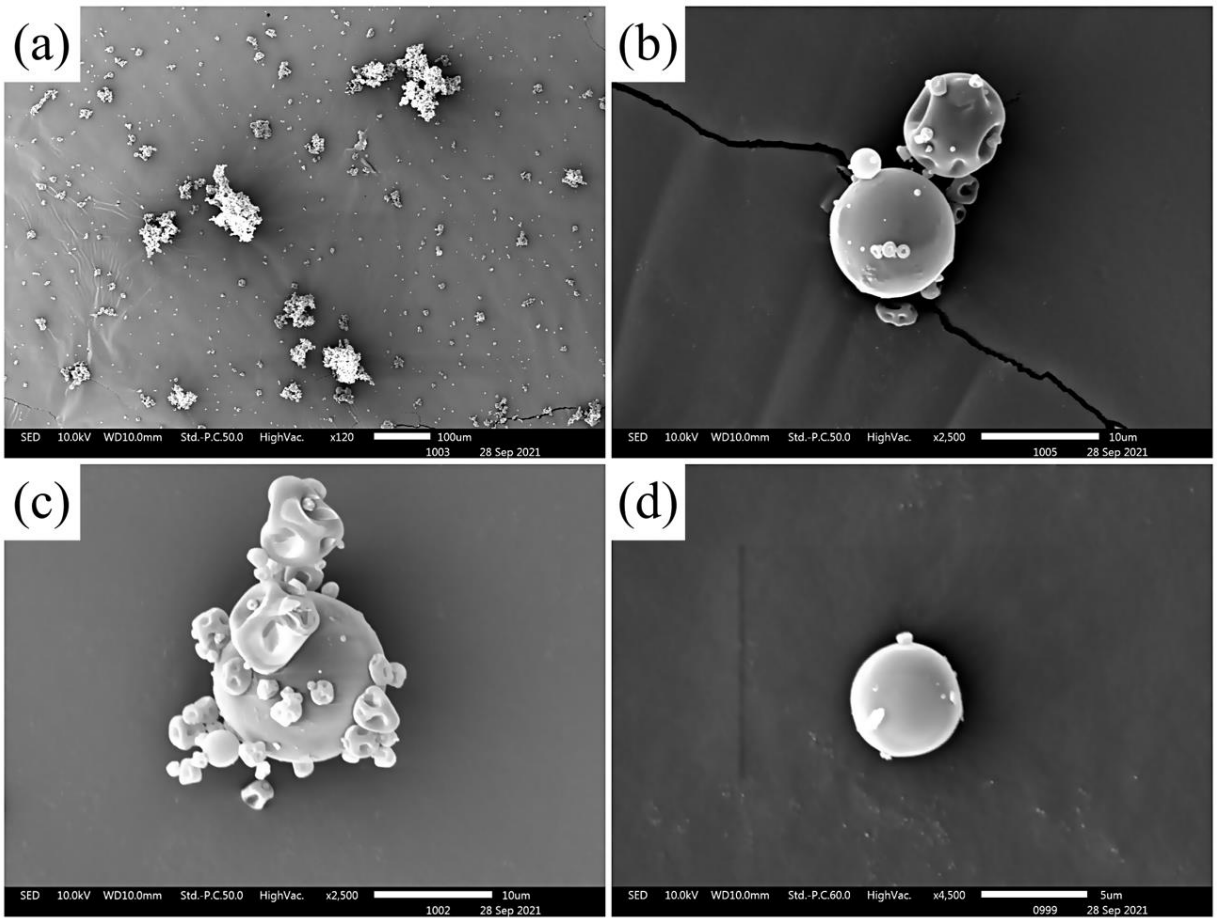


Figure 3. SEM micrographics of the microcapsules containing *Bacillus subtilis* co-encapsulated with oat β-glucan and alginate at 120× (a), 2500× (b; c) and 4500×(d).

Microencapsulation exerted a protective effect on *B. subtilis* cells as compared to free cells added to the Nile tilapia pelleted feed under storage for three months at -20, 7, and 25 °C (Figure 4). After 90 days of storage, both free and microencapsulated cells were viable, but only microencapsulated cells remained above 6 log-unit of viability. Comparing the storage temperature, microcapsules stored at freezing (-20 °C) and ambient (25 °C) for 90 days showed the highest counts of 6.9±0.09 log CFU mL^{-1} and 6.8±0.02 log CFU mL^{-1}, respectively. For 45 days, microencapsulated bacteria stored at 25 °C were stable, followed by 30 days of stability of microencapsulated bacteria at -20 and 7 °C, while free bacteria showed marked logarithmic reduction regardless of storage time. Overall, the lowest reduction in viability of both encapsulated and free microorganisms were obtained at 7°C, with 6.02±0.06 log CFU mL^{-1} of microencapsulated bacteria compared to 3.17±0.12 log CFU mL^{-1} of free cells.

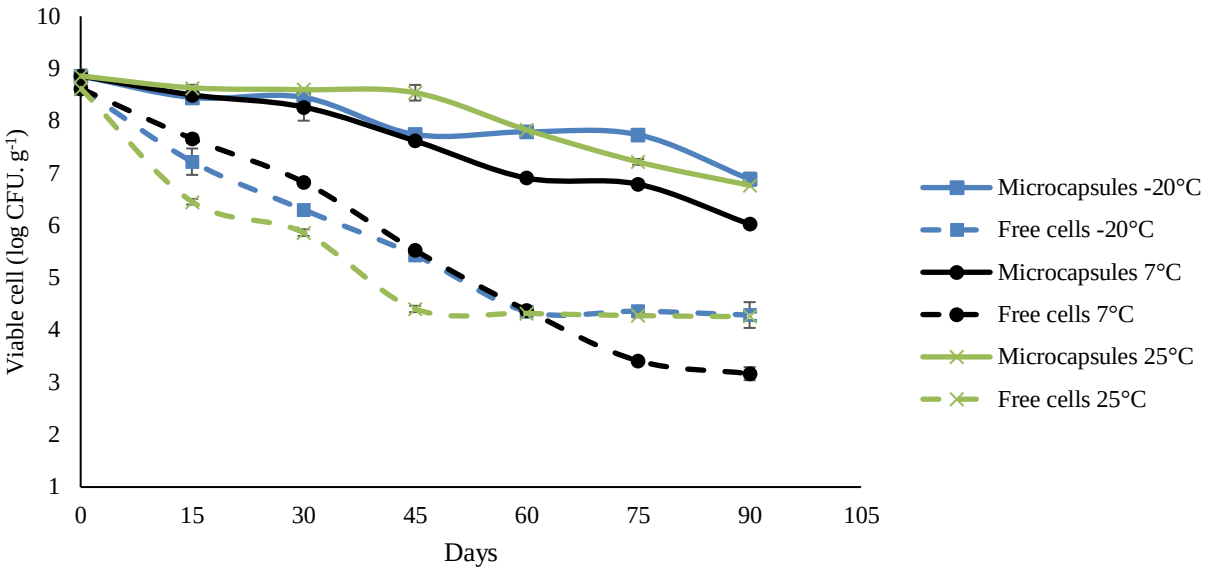


Figure 4. Viability test of free cells and microencapsulated *Bacillus subtilis* in Nile tilapia pelleted feed under storage for three months in freezing (-20 °C), refrigeration (7 °C) and ambient (25 °C) temperatures. Results are shown as μ ± SD (n = 3).

To evaluate the possible benefits of the microencapsulation process, free cells of *B. subtilis* were used as a reference in the same conditions of the simulated gastric fluids and bile tolerance (Figure 5a). After three hours of incubation with simulated gastric fluids (SGF), there was a significant decrease in the survival of free bacteria from 9.4±0.14 to 7.6±0.06 log CFU mL^{-1}, while microencapsulated cells presented better tolerance, 9.5±0.12 to 8.4±0.07 log CFU mL^{-1}. The viability of microencapsulated *B. subtilis* cells was significantly higher than the free probiotics after 1, 2, and 3 h post-treatment with 10% tilapia bile (Figure 5b). The survival rate of free probiotics reduced 4 log-unit while microencapsulated probiotic reduced only 3 log-unit after being exposed to simulated gastric and tilapia bile conditions, confirming the loss of viability during transit to the action site in the tilapia gut.

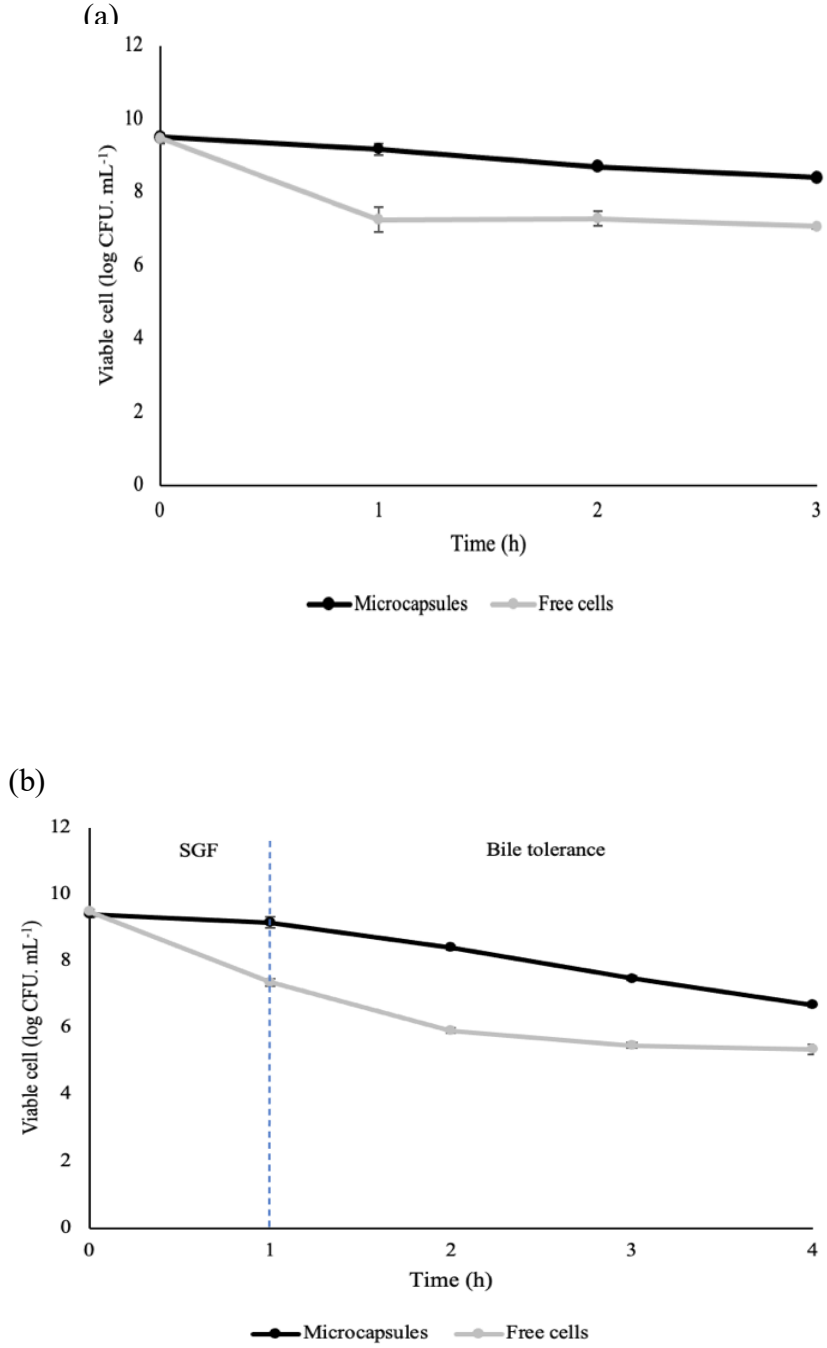


Figure 5. Viability loss of free and microencapsulated *B. subtilis* under (a) simulated gastric fluids (SGF) for 1, 2, and 3 hours and (b) bile tolerance for 1, 2, and 3 hours after 1 hour of exposure to SGF. Results are shown as μ ± SD (n = 3).

3.4 Discussion

Co-encapsulation of *Bacillus subtilis* and oat β-glucan in alginate microcapsules was studied as a method to improve stability of probiotic cells by protecting them from adverse environments such as storage time and, therefore, during transit to the action site in the diverse conditions in fish gut. The air inlet temperature of microencapsulation of 130 °C was

chosen based on studies reporting that probiotic cells such as *Bacillus subtilis* B99-2, *Lactobacillus acidophilus* La-5, *Bifidobacterium* BB-12 presented higher survival rates and storage viability at 130 ℃ or between 125 ℃ and 145 ℃ (Ma et al., 2015; Nunes et al., 2018).

The air inlet temperature is directly proportional to microcapsules drying rate and final moisture contents (Gharsallaoui et al., 2007). Lower water quantities (<6%) are required to prolong the shelf life of probiotic powders (Liao et al., 2017), which partially agrees with the storage results. The water activity (a_w) value indicates the water allowed for biochemical reactions, and is related to moisture contents. According to Barajas-Álvarez et al. (2022), a_w value lower than 0.60 is considered a safe value to reduce the microbial growth rates. To avoid agglomeration problems during storage of probiotics, the recommended moisture content and a_w for a long-term storage should be lower than 5% and 0.3, respectively (Ananta et al., 2005). The values herein registered were below the suggested values and in line with the hygroscopicity values registered. The hygroscopic property of the microcapsules can be attributed mainly to the polymeric structure of D-glucose units of β-glucan, which has hygroscopic properties, although the hygroscopic values were comparatively lower than other probiotics encapsulated in the presence of prebiotics (Fritzen-Freire et al., 2012; Yonekura et al., 2014).

Doherty (2011) suggests that during the drying process, water may be removed from bacterial cells, leading to membrane transition and leakage, resulting in cell death. Although the drying process resulted in loss of 22% of viable cells, the final result of 9.65 log CFU mL^{-1} remained within the recommended dose for the majority of probiotic strains added to animal feed (Simon et al., 2005).

In fact, the viability of probiotic after spray-drying depends upon several factors, such as the outlet temperature, strain, storage temperature, and the carrier agent into the drying medium (Lian et al., 2002). For the encapsulation matrix purposes, the combination of biopolymers and prebiotics have been explored as carrier agents for microencapsulation (Bhagwat et al., 2020; Pinto et al., 2015). As a matter of fact, polysaccharides with prebiotic properties such as inulin, pectin, maltodextrin, and glucan have been used to protect probiotic bacteria during encapsulation process and under storage conditions (Bhagwat et al., 2020; Oliveira et al., 2007; Roselen et al., 2019; Shah et al., 2016).

The oat β-glucan (1,3 and 1,4 glycosidic bonds) is a polysaccharide considered as a well-known prebiotic component in cereals which stimulates the growth of probiotic bacteria. The application of cereal β-glucan as wall material is valuable because of its physicochemical

and nutritional properties. β-glucans have elicited improvement in growth and health of different animal groups, including fish and other land animals (Ferreira et al., 2018; Sealey et al., 2008). In aqueous solutions, cereal β-glucan may have medium to high viscosity, facilitating the entrapment of materials or living cells by the viscous matrix and, therefore, forming a film as registered by Kurek et al. (2018) using barley β-glucan as a carrier agent for fish oil.

Accordingly, besides the formation of concavities registered on the surface of the microcapsules which is typical of spray-dried samples as a result of the rapid water evaporation (Rosenberg et al., 1985), actually no pores or cracks were found in the registered SEM micrographics. However, the process yield was adversely affected by the film-forming capacity of β-glucan with expressive loss of material, approximately 32%, resulting from their adhesion to the atomization chamber. It suggests that the D-glucose units of β-glucan and free water in the microcapsules may have caused a plasticizing effect. According to Ozmen and Langrish (2003) under some conditions, spray-dried powder adheres to the surface of the drying chamber leading to possible blockage and loss of material during the process.

Average size of the microparticles was 1.5 μm, varying between 0.4 and 14.56 μm. At higher magnifications, no bacteria were observed on the surface of the microcapsules and a smooth external surface was noticed, which may prevent leakage and provide protection for encapsulated bacteria. According to Martín et al. (2015), being large enough to cover probiotic cells with minimal influence on the textural properties of the supplemented product, the recommended microparticle size is smaller than 100 μm. Zeta potential analysis showed that the electrical charge of both microparticles (load and unloaded capsules) surfaces was negative as expected, and as a result of the negatively charged sodium alginate ions present on the surface of the particles (Ji et al., 2019; Vega-Carranza et al., 2021). Interestingly, the presence or absence of probiotic cells did not affect the zeta potential of the microcapsules, evidencing the coating capacity of alginate and β-glucan combined. Furthermore, the high zeta potential registered in the microcapsules was a desirable trait since it increases the stability of the microcapsules while decreasing the chances of agglomeration, which may impair product packaging and manufacturing (Ahmad et al., 2019).

Viability of free and encapsulated *B. subtilis* decreased during the 90-day storage period. Microcapsules average survival rate varied from 6.0 to 6.9 log CFU mL^{-1}, and ranged on 22% to 32% viability loss in all storage temperatures while survival rate of free cells varied from 3.1 to 4.3 log CFU mL^{-1} and ranged on 50% to 63% of viability loss. Microcapsules remained stable for 45 days at ambient temperature and 30 days at 7 and -

20°C, suggesting that temperature influenced the stability of bacteria during storage. After 45 days, only microencapsulated probiotic count was higher than 6 log-unit, the minimum recommended value of probiotic in functional foods (FAO/WHO 2002). The remaining number of *B. subtilis* above 6 log CFU mL^{-1} is sufficient to still be considered as a product of probiotic value, with high potential to function as a protective agent in fish farming (Adorian et al., 2019; Telli et al., 2014).

Results of studies evaluating cell viability of spray-dried probiotic microcapsules under storage have differed markedly (Roselen et al., 2019; Fritzen-Freire et al., 2012). Metabolic activity, enzymatic reaction, and molecular mobility (driven by water activity) are the primary reasons that impact the stability of probiotics (Bhagwat et al., 2020; Santivarangkna et al., 2008). When evaluating the effect of storage temperature (-20, 4, 25 and 37 °C) on dried *L. cas*ei LK-1, Liao et al. (2017) reported high viability at -20°C when compared to other temperatures. Differently, Oliveira et al. (2007) reported no differences in microencapsulated *L. acidophilus* LAC 4 stored for six months at 7 and 37 °C. In the present study, free and microencapsulated *B. subtilis* showed similar behavior with high viability at ambient and freezing temperatures. Although low storage temperatures (4 - 7 °C) are described as prompting better bacteria viability (Dianawati et al., 2016), the inclusion of prebiotics seems to minimize mechanical, oxidative and osmotic stress of probiotic submitted to spray-drying process and, thus also, during storage at room temperature. Furthermore, *B. subtilis* has been described to be a robust bacterium that can successfully adapt to various changes in the environment as it is able to initiate many survival mechanisms such as uptake of exogenous DNA, biofilm formation, and sporulation (Tan and Ramamurthi, 2014).

To evaluate the possible benefits of the microencapsulation process, free cells were used as a reference for the simulated gastric conditions and bile tolerance in Nile tilapia. Microencapsulated cells were stable during the first hour of incubation showing the lowest reductions with a decrease in viability of 11% compared to the free cells (19%) after three hours. Similar results were reported by Sribounoy et al. (2021) analyzing pelleted fish feed containing microcapsules of *L. rhamnosus* GG and the prebiotic Jerusalem artichoke exposed to simulated gastrointestinal conditions. The encapsulation process was effective in protecting the probiotic bacteria and, under *in vivo* condition, would allow a high number of cells to reach the target site in the tilapia gut and provide the probiotic positive health effects.

Subsequently, when in contact with bile salts, it was registered a lower logarithmic reduction in viability of microencapsulated cells (28.7%) as compared to free cells (44.2%). The *in vitro* results reveal that the combination of alginate and β-glucan effectively protected

Bacillus subtilis during the gastric simulation, resulting from the slow degradation of β-glucan and its stability at low pH range which lowered the decrease in probiotic survival. These results are in accordance with those obtained for *L. casei*, *L. brevis*, and *L. plantarum* encapsulated in β-glucan matrix using emulsion technique (Shah et al., 2016), and also using other prebiotic matrix such as fructooligosaccharide (FOS) as a wall material for *Lactobacillus plantarum* MTCC 5422 encapsulated by spray-drying (Rajam and Anandharamakrishnan, 2015).

3.5 Conclusion

Compared to free probiotic cells, the microencapsulation process in sodium alginate matrix of probiotic bacteria *Bacillus subtilis* combined with oat β-glucan via spray-drying improved the survival of probiotic bacteria in fish feed under simulated gastrointestinal conditions of tilapia after a storage period of 90 days. The microbial count of microcapsules was above the minimum level of 10^6 log CFU mL^{-1} during the storage period and at the point of delivery, after 3h of bile tolerance. Additionally, the conditions used in this study created microcapsules with acceptable physicochemical properties for further incorporation into animal feed products. For future studies, a comparison of microcapsules with and without oat β-glucan in alginate matrix as well as tests with different inlet/outlet temperatures during the drying process are recommended.

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4. CO-ENCAPSULATION OF SYNBIOTIC *Bacillus subtilis* AND β-GLUCAN ON GROWTH PERFORMANCE, MICROBIOTA, AND IMMUNITY OF NILE TILAPIA

Abstract

Probiotic survival in processed feed can be affected by several factors including length of feed storage period and the gastrointestinal transit of ingested feed. Therefore, providing probiotics with a physical barrier against adverse environmental conditions may improve the stability of the microorganisms used as probiotic additives. This study investigates the effect of free and spray-dried *Bacillus subtilis* C-3102 (BS) in oat β-glucan microcapsules on growth performance, hematology, intestinal microbiota, immunology, antioxidant enzymes activity, and disease resistance of Nile tilapia, *Oreochromis niloticus*. Four hundred Nile tilapia (39.96±2.8 g) were randomly stocked into 25 aquaria (n=16 fish) in a totally randomized experimental design (n=5) and fed one of the following diets: (C) Control diet (without synbiotics); (0.1%S) 1g kg^{-1} diet of unloaded microcapsules and 2.7×10^9 of free BS CFU kg^{-1} diet; (0.2%S) 2 g kg^{-1} diet of unloaded microcapsules and 5.4×10^9 of free BS CFU kg^{-1} diet; (0.1%SM) 1g of microcapsules loaded with 2.7×10^9 BS CFU kg^{-1} diet; (0.2%SM) 2g of microcapsules loaded with 5.4×10^9 BS CFU kg^{-1} diet. After 60 days, fish fed 0.2%SM showed the highest growth performance and best feed utilization ($p<0.05$) compared to fish fed free probiotics and control diet. Intestinal villi were longer and submucosa layer was thicker in fish fed free and microencapsulated probiotics ($p<0.05$) than the control group. Fusobacteriota, Firmicutes, and Bacteroidota were the dominant phyla across all samples accounting for more than 90% of the gut microbiota. Bacteroidota showed higher relative abundance ($p<0.05$) in fish fed 0.2%S and 0.2%SM as compared to the control group, mainly represented by Bacteroides genus. No differences were registered in hematological parameters ($p>0.05$) before challenge and only leukocyte count was altered in fish fed the control diet and 0.1%S diet after bacterial challenge with *Aeromonas hydrophila* ($p<0.05$). There were no differences in serum immunological parameters and antioxidant enzymes activity after challenge ($p>0.05$) and phagocytic activity was enhanced in fish fed both 0.2%S and 0.2%SM diets. Survival rates did not differ among groups. Results highlight that microencapsulation has the potential to protect *B. subtilis* and may constitute a valuable approach for the viability of probiotics as additives for fish feeds.

Keywords: Spray-drying, Microencapsulation, Probiotics, Prebiotics, Aquaculture

4.1 Introduction

Tilapia is one of the main farmed fish species in the fastest growing agribusiness in the world aquaculture (FAO, 2022). Along with the increasing demand for aquatic food, disease outbreaks have been considered a major constraint to the expansion of fish farms

(Dawood et al., 2020). Historically, aquaculture has mostly relied on the use of chemotherapeutic treatments to control infectious diseases. However, the indiscriminate overuse of conventional drugs for disease control has been widely criticized given their negative impacts, especially the rise of antibiotic-resistant pathogens strains, damage to the natural environment, and accumulation of residues in aquatic products (Dawood et al., 2020; Mohapatra et al., 2013). To overcome such adverse impacts, feed additives such as probiotics, prebiotics, and synbiotics have been used as alternative to improve growth, health, and disease resistance of fish (Nayak, 2010; Hoseinifar et al., 2018).

Synbiotics are defined as "a combination of live microorganisms and substrate(s) selectively used by host microorganisms that confers health benefits to the host" (Swanson, 2020). When combined, probiotics and prebiotics can positively affect the hosts' growth by enhancing the production of useful by-products required by the host and core microbiota such as fatty acids, vitamins, and antimicrobial agents (Mugwanya, et al., 2021). *Bacillus subtilis* stands out amongst the probiotics used in aquaculture as one of the most researched bacteria because of its benefits such as promoting better nutrient utilization, improving the balance of the intestinal microbiota, and strengthening mucosal defenses against pathogens (Nayak 2020; Tachibana et al., 2021).

Several factors have been claimed to affect the viability of probiotics, including the harsh conditions through the gastrointestinal transit of ingested feed, time and temperature of storage, and oxygen content (Fritzen-Freire et al., 2012; Yao et al., 2019). Studying the viability of commercial probiotics during the gastrointestinal transit Dodoo et al. (2017) reported that several strains suffered a reduction of over a 10^6 CFU within five minutes of incubation in gastric fluids. As a result of the concern that many probiotics are ineffective because the bacterial cells do not survive most food processing techniques, time of storage, and passage through the gastrointestinal tract, the microencapsulation process has been receiving more and more attention to improve the stability of microorganisms used as a probiotic additive. The microencapsulation technique by spray-drying is a cost-effective process that consists of the atomization of a solution or emulsion containing the desired product and coating agents into droplets by rapidly drying with hot gas into solid powder at a certain temperature and pressure (Encina et al., 2016; Santos et al., 2019).

Protection of probiotics by substances considered as prebiotics can be an alternative to increase their viability during the microencapsulation process. Oat β-glucan, a natural polymer built up of individual glucose molecules that are linked together by 1,3 and 1,4 linkages, is one within a class of nondigestible polysaccharides credited with prebiotic

properties (Daou, et al., 2012). Although extensive information has been reported related to the health benefits of yeast-derived β-1,3/1,4-glucan, limited information is available on the use of oat β-glucans in animal feeds. The objective of this study was thus to evaluate growth, intestinal microbiota, innate immune responses, antioxidant defense, and histology of the intestinal tract of Nile tilapia fed free and microencapsulated *Bacillus subtilis* in oat β-glucan capsules.

4.2 Material and Methods

This study comprised two phases – before (I) and after (II) challenge evaluation. In Phase I, fish were fed experimental diets containing two levels of free and microencapsulated *B. subtilis* and β-glucan in the aim to nutritionally prepare them to withstand bacterial challenge. In Phase II, fish were subjected to bacterial challenge with *Aeromonas hydrophila* in the aim to better understand the immunological and nutritional effects of either free or microencapsulated *B. subtilis* on Nile tilapia (*Oreochromis niloticus*) health.

4.2.1 Preparation of microcapsules of *Bacillus subtilis* and β-glucan

The synbiotic microcapsules were prepared as described by Nunes et al. (2018) with minimal modifications. In brief, 7g of oat β-glucan (B-CAN® Oat Fiber 70%, Garuda International, Inc., CA, USA) were added to warm water containing 2 g of sodium alginate (FMC BioPolymer, SP, Brazil). The concentration of oat β-glucan was chosen as the maximum level that could practically be atomized in the spray dryer. The wall materials were hydrated for 1 h under continuous stirring, and then autoclaved at 121 °C for 15 min. After cooling the solution, *Bacillus subtilis* C-3102 cells in the early stationary phase were added to the wall material solution and homogenized with the aid of a magnetic stirrer. Final count of *B. subtilis* in the carrier solution was 10^{12} CFU mL^{-1}.

The microcapsules were obtained with a laboratory scale spray dryer (B-290, Büchi, Flawil, Switzerland), at air inlet temperature of 130 ± 5 °C and outlet temperature of 50-60 °C. Unloaded microcapsules were also prepared following the same procedure, except for the addition of *B. subtilis*. The synbiotic microcapsules were analyzed to contain 9.65±0.12 log CFU g^{-1} with mean microparticle size of 1.55 μm.

4.2.2 Animals and experimental design (Phase I)

Animal handling and feeding procedures were performed under the guidelines of the Ethics Committee of Animal Welfare of the Luiz de Queiroz College of Agriculture (CEUA-ESALQ), protocol number 8600200521 (ID 000241). Trial was carried out in the Fish Nutrition Laboratory of the Animal Science Department at ESALQ, Piracicaba, SP. Fish were fed a basal diet (32% crude protein) for three weeks before the start of the trial. Water quality was monitored three times a week throughout the experiment and maintained within optimal conditions for Nile tilapia as follows: water temperature at 28 ± 2°C, dissolved oxygen at 6.4 ± 0.5 mg L^{-1}, total ammonia 0.25 ± 0.02 mg L^{-1}, pH: 7.8 ± 0.2. A 12h photoperiod was used during the trial.

Four hundred male, juvenile Nile tilapia, *Oreochromis niloticus* (39.96 ± 2.8 g) were stocked in 25 experimental units (300-L tanks, open system), 16 fish per tank, in a completely randomized experimental design with five treatments (n=5). Diets were formulated to meet tilapia's nutritional requirements based on standards of the National Research Council (NRC, 2011). The treatments were:

- **(C)** control group – diet devoid of synbiotics;
- **(0.1%S)** synbiotic – 1g kg^{-1} diet of unloaded microcapsules and 2.7×10^9 *Bacillus subtilis* CFU kg^{-1} diet;
- **(0.2%S)** synbiotic – 2 g kg^{-1} diet of unloaded microcapsules and 5.4×10^9 *B. subtilis* CFU kg^{-1} diet;
- **(0.1%SM)** synbiotic microcapsules – 1g of microcapsules loaded with 2.7×10^9 *B. subtilis* CFU kg^{-1} diet;
- **(0.2%SM)** synbiotic microcapsules – 2g of microcapsules loaded with 5.4×10^9 *B. subtilis* CFU kg^{-1} diet.

Free and microencapsulated *B. subtilis* were mixed in soybean oil (50%) and sprayed over the diets. The basal diet was sprayed with 50% of synbiotic-free oil (Table 1). The actual counts of *Bacillus subtilis* measured in the experimental diets were 0, 2.3×10^9, 5.2×10^9, 2.4×10^9, and 5.3×10^9 CFU kg^{-1} diet (air dried weight), respectively. Throughout the 60 days of feeding trial, fish were fed twice a day (0800 and 1600 h) until apparent satiation, and the feed intake was recorded. The chemical composition of the experimental diets was analyzed according to AOAC protocols (2000).

Table 1. Ingredients and proximate composition of experimental diets.

Ingredients	Treatments [¤]				
	Control	0.1%S	0.2%S	0.1%SM	0.2%SM
	---------- % dry weight ----------				
Soybean meal	38.30	38.30	38.30	38.30	38.30
Fish meal	16.00	16.00	16.00	16.00	16.00
Corn	26.00	26.00	26.00	26.00	26.00
Wheat bran	13.00	12.90	12.80	12.90	12.80
Starch	3.00	3.00	3.00	3.00	3.00
Soy oil [Ω]	2.00	2.00	2.00	2.00	2.00
Cellulose	0.78	0.78	0.78	0.78	0.78
Vitamin/mineral mix [¶]	0.50	0.50	0.50	0.50	0.50
DL-methionine	0.40	0.40	0.40	0.40	0.40
BHT	0.02	0.02	0.02	0.02	0.02
Synbiotic	-	0.1	0.2	0.1	0.2
Proximate composition					
Dry matter	94.1	92.1	92.7	92.9	93.4
Protein	31.8	31.9	31.9	31.8	32.1
Lipid	5.44	5.36	5.49	5.51	5.38
Ash	9.01	9.12	8.75	8.83	9.02
Energy (MJ kg^{-1})	14.6	13.3	14.8	14.8	13.7

[¤] (C) control group – diet devoid of synbiotics; (0.1%S) synbiotic – 1g kg^{-1} diet of unloaded microcapsules and 2.7 × 109 *Bacillus subtilis* CFU kg^{-1} diet; (0.2%S) synbiotic – 2 g kg^{-1} diet of unloaded microcapsules and 5.4 × 109 *B. subtilis* CFU kg^{-1} diet; (0.1%SM) synbiotic microcapsules – 1 g of microcapsules loaded with 2.7 × 109 *B. subtilis* CFU kg^{-1} diet; (0.2%SM) synbiotic microcapsules – 2 g of microcapsules loaded with 5.4 × 109 *B. subtilis* CFU kg^{-1} diet.

[Ω] 50% soy oil was included after feed extrusion as microcapsules binder.

[¶] Agromix Nutrição Animal (Jaboticabal, SP, Brazil), contents per kg of product: folic acid 1200.00 mg; Nicotinic Acid 20.00 g; Pantothenic Acid 10,000.00 mg; Biotin 200.00 mg; Co 80.00 mg; Cu 3,500.00 mg; Choline 100.00 g; Fe 20.00 g; I 160.00 mg; Inositol 25.00 g; Mn 10,000.00 mg; Se 100.00 mg; Vitamin A 2,400,000.00 IU; Vitamin B_1 4,000.00 mg; Vitamin B_2 4,000.00; Vitamin B_{12} 8,000.00 mcg; Vitamin C 60.00 g; Vitamin B_2 4,000.00 mg; Vitamin B_6 3,500.00 mg; Vitamin D_3 600,000.00 IU; Vitamin E 30,000.00 IU; Vitamin K_3 3,000.00 mg; Zn 24.00 mg.

4.2.3 Sampling procedures and performance indices (Phase I)

At the end of the feeding trial, three fish were randomly sampled from each tank and anesthetized with benzocaine solution (50mg L^{-1}) after 12 h of fasting for blood withdrawal from the caudal vein. The collected blood sample was divided into two sets: one blood set was added to a tube with EDTA as an anticoagulant for hematological procedures and another blood set was added to an EDTA-free tube, left to clot for one hour at room temperature, and centrifuged at 4.800 g for 5 min for the harvesting of blood serum. Fish were then

immediately euthanized with benzocaine overdose (250 mg L^{-1}), weighed and laparotomized for the collection of intestinal tract tissue samples.

Performance parameters and survival rate were calculated as follows: WG: Weight gain (g) = final weight – initial weight; SGR: Specific growth rate (% day^{-1}) = [(final mean weight - initial mean weight)/ length of feeding trial (days); FI: Feed intake, (g) = [dry feed intake (g $fish^{-1}$)]; FCR, Feed conversion ratio = [feed fed/weight gain]; Survival rate (%) = [(final number of fish/ initial number of fish) x 100].

4.2.4 Histological analysis (Phase I)

Tissue samples from the proximal intestine were fixed in formaldehyde solution (4 %). After complete fixation, tissues were preserved in 70% ethanol, dehydrated, cleared in xylene and embedded in paraffin (56-58°C). Histological serial sections (4 μm) were then stained with Alcian blue (AB, pH 2.5) and periodic acid Schiff (PAS), counterstaining with Carazzi's haematoxylin (Luna, 1968; Suvarna et al., 2012).

Pictures of each slide were captured using a microscope (Olympus - BX5, Tokyo, Japan) and camera (Olympus - DP72, Tokyo, Japan) combination for the intestinal measurements. Villus height (VH), villus width (VW), crypt depth (CD), submucosa (SM), intestinal muscle layers (ML), and goblet cells count (GC) were determined using Image J 1.8 software (National Institute of Health, USA). The VH of 30 of the tallest, well-oriented villi were measured per slide from the top of the villus to the crypt mouth. The VW was measured across the base of the villus at the luminal surface and CD were measured from the crypt mouth to the base (Hampson, 1986; Kuebutornye et al., 2020). The MT was measured from the inner edge of the muscularis mucosae to the outer edge of the serosa. Goblet cells were counted across a standardized distance of 100 μm and then calculated by averaging the cell numbers from all samples within each treatment (Standen et al., 2015).

4.2.5 Characterization of the intestinal microbiota (Phase I)

The transient microbial population from the gut of tilapia were examined by sequence analysis. Digesta contents were aseptically collected from the whole intestine of fifteen fish from each treatment (n=3 per replicate) by stripping with sterilized tweezers, after a 12-h post feeding period. The intestinal contents were stored in sterilized microtubes, flash

frozen in liquid nitrogen and stored (-80 °C). The DNA was extracted from 200 mg of the intestinal content of fish using a commercial kit (QIAmp DNA from stool Mini Kit, Qiagen Inc., Toronto, Ontario) following the manufacturer's recommendations. The extracted DNA was quantified using Qubit®2.0 fluorometer (Life Technologies, Carlsbad, CA, USA), and DNA integrity was confirmed by agarose gel electrophoresis. The V3 and V4 regions of the bacterial 16S rRNA gene were amplified by PCR using tagged primers 515F (5′-barcode-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′), where each barcode with an eight-base sequence was specific to each sample using a miseq sequencer (Illumina, Inc., California, USA; illumina miseq) (Zhai et al., 2016; Yu et al., 2019). All reactions were adjusted to a final volume of 25μL and amplified under the following conditions: 95°C for 3 min, followed by 25 cycles of 95 °C; 55°C and 72 °C for 30 sec each with a final elongation step at 72 °C for 5 min. All purified amplicon products from different samples were mixed and sequenced on the Illumina Miseq sequencing platform 250 bp (2×).

4.2.6 Bioinformatics and statistical analysis (Phase I)

The raw data were quality-filtered and aligned using DADA2 package implemented in QIIME2 (v.2019.10). The DADA2 algorithm corrects Illumina sequencing errors, remove chimeric sequences and resolves exact amplicons sequence variants (ASVs) with single nucleotide resolution from the full length 16rRNA gene. The ASV is a higher-resolution product, analogue of the traditional OTU, which records the number of times each exact amplicon sequence variant was observed in each sample. After the initial processing of the sequencing data, taxonomies were assigned to each ASV using a DADA2 program implementation of the Naive Bayesian classifier method (Wang et al., 2007). The taxonomies generated by DADA2, and their quantifications, were imported into the phyloseq program also implemented in RStudio (v.3.6.1). The reverse strand matching option was enabled and all the reads that did not hit against SILVA database were excluded from downstream analyses (Glöckner et al., 2017). To compute the alpha diversity of fish gut microbiota, two metrics were calculated: Shannon and Simpson indices, calculated with QIIME2. Before diversity analysis, sequences were normalized to the minimum number of reads. The alpha diversity was calculated at sequence depth of reads per sample with 10,000 iterations and then were averaged. Beta diversity was estimated by computing the relationships between samples according to the weighted UniFrac distance metric and visualized using analysis of Detrended

correspondence analysis (DCA) and multidimensional scaling (MDS). To identify the bacterial taxa that were differentially abundant across microbial population of the intestine of tilapia the DESeq2 package was used, performing pairwise comparisons between the five treatments (Lozupone and Knight, 2005; Anderson, 2001; Love et al., 2014). Venn diagrams were created using ConceptDraw PRO 7 (http://www.conceptdraw.com) by transforming to relative abundance and filtering taxa to those that occur greater than 0.1%.

4.2.7 Blood sampling and biochemical analysis (Phase I and II)

Blood samples were drawn from the fish caudal vein by sterile syringe before and after *A. hydrophila* challenge. The total erythrocytes and leukocytes counts were performed in an improved Neubauer hemocytometer and Natt-Herrik solution (Natt and Herrik, 1952). Hemoglobin (Hb) was determined by the cyanomethemoglobin colorimetric method using a commercial kit (Labtest Kit - Centerkit, SP, Brazil) according to the kit instructions. The hematocrit (Htc, %) was determined by the standard microhematocrit method (Goldenfarb et al., 1971). Total plasma protein (TPP) was measured using refractometer with a plasma drop from the microhematocrit capillary by breaking it just above the leukocyte layer after the hematocrit reading (Hrubec, and Smith, 2010). The mean corpuscular volume [MCV = (Htc × 10)/erythrocytes] and the mean corpuscular hemoglobin concentration [MCHC = (Hb × 100)/Htc] were calculated according to Wintrobe (1934). Blood serum glucose was determined by the glucose oxidase method using a commercial kit (Labtest Kit - Centerkit, SP, Brazil) according to kit instructions.

4.2.8 Bacterial challenge (Phase II)

The *Aeromonas hydrophila* strain used in this assay was kindly provided by the Laboratory of Aquatic Organisms Pathology (LAPOA) strain collection, “Centro de Aquicultura da Universidade Estadual Paulista” (Aquaculture Center of Sao Paulo State University - CAUNESP, Jaboticabal, SP, Brazil). *A. hydrophila* was cultured in TSA (28 °C for 24 h), inoculated in brain–heart infusion (BHI) at 28 °C for 18 h. The bacteria were subsequently centrifuged (3000 × *g*, 4 °C for 10 min.), washed three times in PBS solution, and the concentration was adjusted to 4.6×10^7 CFU mL^{-1} (Assane et al., 2019). At 60 days of feeding trial, 12 fish of each tank were anesthetized in aqueous solution of benzocaine

(1:10,000), and inoculated intraperitoneally with 1mL kg^{-1} of the bacterial suspension. Fish were kept under observation for two weeks to record the clinical signs and mortality rate (Schaperclaus et al., 1992; Abdel-Tawwab et al., 2008). After the *A. hydrophila* challenge, four fish from each tank were anesthetized in aqueous solution of benzocaine and blood samples were drawn from each fish using EDTA-coated vials, while non-coated vials were used for serum collection as described. Hepatic tissue samples were also collected from the same fish for enzyme activity assays, and stored at -80 °C.

4.2.9 Phagocytic activity (Phase II)

Phagocytic activity was measured using the method described by Martins et al. (2009). In summary, 0.5 mL of fish blood was dropped into centrifuge tubes, to which was added 0.25 ml of *Enterococcus* (1.0×10^6 CFU mL^{-1}). The tubes were homogenized and kept at 28 °C in a water bath for 30 min, and shaken every 10 min. After this time, duplicate blood smears were prepared and stained by Giemsa/May-Grunwald (Rosenfeld, 1947). The number of leukocytes that engulfed bacteria was counted as percentages in relation to total leukocyte number in the smears.

4.2.10 Lysozyme activity (Phase II)

Lysozyme activity was determined in fish serum using a turbidimetric assay (Jørgensen et al., 1993) with an adjustment of the pH of the *Micrococcus lysodeikticus* (M0508, ATCC No 4698, Sigma Aldrich) to 6.2 (Ellis, 1990). Briefly, 10 µL of the sample was mixed with 200 µL *Micrococcus lysodeikticus* suspension in PBS. Lysozyme activity (units mL^{-1}) was calculated using the following formula: $[(\Delta absorbance_{(5\text{-}1\ min)}/3)/0.001] \times 100$. One unit of lysozyme activity was defined as the quantity of enzyme that caused a 0.001 decrease in absorbance per minute measured at 450 nm.

4.2.11 Haemolytic activity of the alternative complement system (Phase II)

Haemolytic activity of the alternative complement system (HACS) was measured using sheep red blood cells as targets (Sutili et al., 2016). In brief, tilapia serum (10 µL) was incubated at room temperature for 1 hour with 2%-sheep blood (25 µL). After the incubation

time, 100 μL of cold-PBS was added and centrifuged at 5,000 × *g* for 10 min at 4°C. The supernatant (100 μL) was transferred to 96-well microplates and the absorbance of the samples was read at 405 nm. The percentage of haemolysis of each sample was calculated using the following calculation: % hemolysis = $[(A_{405\ sample} - A_{405\ no\text{-}haemolysis}) / (A_{405\ total\ haemolysis} - A_{405\ no\text{-}haemolysis})] \times 100$.

4.2.12 Enzyme Activity Assays (Phase II)

Liver samples were homogenized (1:9 w:v) using a turrax homogenizer (Marconi MA-102) in ice-cold PBS (pH 7.2). The resulting homogenate was centrifuged at 12,000 ×*g* for 10 min., at 4°C. The supernatant was aliquoted and used to determine total protein, catalase (CAT), superoxide dismutase (SOD), and total glutathione (tGSH).

Protein concentration was measured at an absorbance of 595 nm according to the Bradford method (1976) with bovine serum albumin as standard. CAT activity was measured by ultraviolet spectrophotometry (Nelson and Kiesow, 1972). The assay mixture consisted of 2.0 mL PBS (50 mM, pH 7.2), 0.05 mL H_2O_2 (0.3 M), and 0.05 mL homogenate. Changes in H_2O_2 absorbance after 60 seconds were measured at 240 nm. For SOD assay, homogenates were supplemented with 0.03 mol L^{-1} KCl and centrifuged for 10 minutes at 12,000 ×*g* at 4°C. In brief, 1.0 mL of 0.05 mol L^{-1} PBS buffer (pH 7.2) containing 1 mmol L^{-1} DTPA was added to 40 μL of tissue sample (2 mg of total protein mL^{-1}). The reaction was initiated by the addition of 0.2 mmol L^{-1} pyrogallol, and the change in optical density at 570 nm was recorded after 5 min. SOD activity was calculated with 1 U mg^{-1} protein of SOD defined as the amount that inhibited the rate of pyrogallol autoxidation by 50 % (Del Maestro and McDonald, 1985; Dieterich et al., 2000). Total glutathione concentration (tGSH) was measured using DetectX® Glutathione Fluorescent Detection Kit (Arbors Assay, MI, USA) following the manufacturer's instructions. The kit utilizes a colorimetric substrate that reacts with the free thiol group on GSH.

All measurements were done in triplicates using 96-well microplates and samples were analyzed using a microplate reader (Multiskan FC Microplate Photometer). Control samples were used in each plate in order to check the inter-assay precision.

4.2.13 Data analysis

Data for each parameter was tested for normality and homoscedasticity. Analysis of variance (ANOVA) was used to detect differences in intestinal measurements, growth performance, hematological, and immunological parameters followed by Tukey test. Post hoc analysis was performed using the independent samples Student's t-test to determine the differences between before and after challenge with significance set at $p \leq 0.05$. Analyses that did not meet the assumptions of residual normality or homogeneity of variance were subjected to nonparametric Kruskal-Wallis' analysis, followed by Dunn's test ($p \leq 0.05$). The statistical analyses were performed using SAS Studio statistical software (SAS Institute, Cary, NC, USA) and values are expressed as $\mu \pm$ SEM.

Richness (alpha diversity) and community composition (beta diversity) measurements were performed using a one-way ANOVA followed by Tukey test if significant differences were found ($p \leq 0.05$). Analysis of differential abundance between treatments were performed using a DESeq2 package, performing pairwise comparisons between all treatments. The microbiome diversity was calculated based on the number of observed ASVs. A PERMANOVA test was performed to test whether the centroids and dispersions of the Beta diversity of each treatment are equivalent or different. A Venn 5-set diagram was generated to display microbial assemblages common to the five groups. The statistical analyses were performed using RStudio (RStudio, Inc., PBC, Boston, MA, USA), values are expressed as $\mu \pm$ SD.

4.3 Results

Growth performance

The overall growth performance of Nile tilapia was affected by synbiotic inclusion (Table 2). The fish fed 0.2%SM showed similar weight gain to the 0.1%SM treatment and higher weight gain when compared to fish fed 0.1%S, 0.2%S, and control group. Tilapia fed diets with microencapsulated synbiotic also displayed better feed utilization in terms of SGR and FCR when compared to fish fed control diet. No differences were registered on FI among the treatments and the survival rate was not influenced by free or microencapsulated *Bacillus subtilis* in Nile tilapia diets during the 8 weeks of the trial.

Table 2. Growth performance and feed utilization of Nile tilapia fed diets with free and microencapsulated *Bacillus subtilis* for 8 weeks.

	Experimental diets					
Variable	Control	0.1%S	0.2%S	0.1%SM	0.2%SM	p-value
IBW (g)	40.3±0.3	39.96±0.3	40.13±0.3	39.85±0.3	40.26±0.4	0.7233
FBW (g)	106.1±0.4^{c}	110.74±0.9^{bc}	109.52±43^{bc}	117.53±2.2^{ab}	124.39±5.1^{a}	0.0031
WG(g)	65.8±1.25^{c}	70.78±1.89^{bc}	69.3±1.85^{bc}	77.7±2.39^{ab}	84.1±1.28^{a}	0.0027
SGR (%)	1.09±0.02^{c}	1.17±0.01^{bc}	1.15±0.03^{bc}	1.29±0.04^{ab}	1.41±0.09^{a}	0.0027
FI (g)	73.03±1.2	70.34±1.5	71.33±1.5	72.65±1.3	74.93±1.3	0.0826
FCR	1.14±0.01^{c}	1.03±0.02^{ab}	1.07±0.04^{bc}	0.99±0.04^{ab}	0.89±0.07^{a}	0.0080
Survival (%)	100	100	100	100	100	

Values expressed as $\mu \pm SEM$ of five replicates. Means in the same row followed by different superscripts are significantly different ($p < 0.05$). IBW, initial body weight; FBW, final body weight; WG, weight gain; SGR, specific growth rate; FI, feed intake; FCR, feed conversion ratio.

Histological analysis

Light microscopy micrographs revealed normal and healthy morphology of the proximal intestine of Nile tilapia fed the experimental diets. The intestines of the fish showed intact epithelial barrier with well-organized mucosal folds and several abundant goblet cells showing a strong positive stain for Alcian blue and PAS (Figure 1). Dietary synbiotic diets resulted in taller villus on 0.2%SM, 0.1%SM, and 0.2%SM compared to the 0.1%S followed by the shorter villi results registered on the control group. The dietary treatments had no significant effect on the VW, CD, GC, and ML. Submucosa (SM) measurements were higher in fish fed either free or microencapsulated probiotics diets compared to the control group (Table 3).

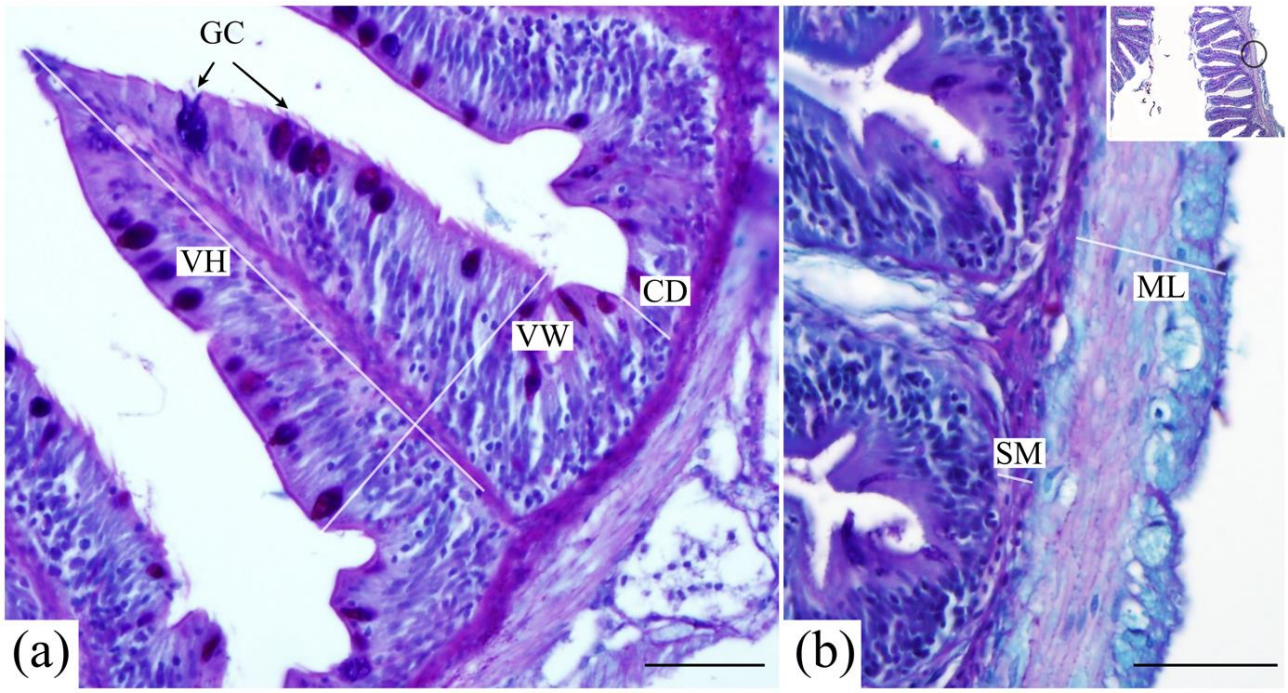


Figure 1. Photomicrographs of the proximal intestine of Nile tilapia *O. niloticus* in cross sections. (a) Magnification of the villi showing the goblet cells (GC) and the morphometric measurements of the villi height (VH), width (VW), and crypt depth (CD) (bar = 50 µm). (b) Higher magnification showing the thickness of the submucosa (SM) and muscle layers (ML; inner circular and out longitudinal layer of smooth muscle) (PAS/AB; bar = 30 µm).

Table 3. Measurement of intestinal parameters of Nile tilapia fed the experimental diets with free and microencapsulated *Bacillus subtilis*.

	Experimental diets					
Variable	Control	0.1%S	0.2%S	0.1%SM	0.2%SM	p-value
VL(µm)	222.45±2.88^{c}	242.05±2.53^{b}	265.85±3.12^{a}	265.66±3.05^{a}	261.43±3.0^{a}	<.0001
VW (µm)	140.32±3.70	140.77±3.68	133.70±3.64	135.12±3.74	133.01±3.80	0.4116
CD(µm)	21.76±1.39	21.68±1.42	25.54±1.36	24.62±1.40	25.21±1.39	0.1379
GC (cells/100µm)	4.97±0.56	5.73±0.54	5.97±0.59	5.80±0.56	5.53±0.58	0.8881
SM (µm)	7.90±0.87^{b}	10.74±0.81^{a}	10.86±0.83^{a}	11.73±0.83^{a}	10.329±0.81^{a}	0.0295
ML(µm)	38.23±2.73	37.43±2.73	39.35±2.81	42.39±2.68	38.14±2.75	0.7259

Values expressed as means of five replicates ± SEM. VL: villus length; VW: villus width; CD: crypt depth; GC: goblet cells; SM: submucosa; ML: muscular layers. Means in the same row with different lowercase superscripts are significantly different ($P < 0.05$).

Characterization of intestinal microbiota

The microbial communities collected from intestines contents of Nile tilapia fed the experimental diets were successfully extracted. From these, a total of 851,824 sequences were obtained with an average of 34,073 sequences per sample using Illumina Miseq 250bp (2×) of the V3-V4 region of the 16S rRNA gene. In total, 23 phyla, 30 classes, 65 orders, 112 families and 199 genera were detected of which 31 genera had a relative abundance above 0.1% of community. Sequence coverage was above 99% in average for all the sequenced samples suggesting acceptable resolution of bacterial communities.

The most abundant taxonomic groups for tilapia gut samples across all samples were: Fusobacteriota (63% – 78%) and Firmicutes (12% – 24%) at phylum level, Fusobacteriia (63% - 78%), Clostridia (12% – 24%), and Bacteroidia (1.2% – 4.9%) at class level, Fusobacteriaceae (57% – 76%) and Peptostreptococcaceae (11% – 23%) at family level and Cetobacterium (63% - 78%), Romboutsia (11% – 23%), Akkermansia (0.9% - 2.2%), and Bacteroides (0.5% - 2.1%) at genus level (Figure 2). At phylum level, Bacteroidota showed higher relative abundance in 0.2%SM (4.9%) and 0.2%S (4.8%) compared to the Control group (1.2%), mainly represented by Bacteroides genus. A large percentage of unassigned bacteria was also found ranging from 1.4% (Control group) to 3,4% (0.2%SM) followed by 4.1% (0.2%S).

Mean ASVs and indices of Shannon, Simpson, Chao 1, and Pielou showed no significantly larger biodiversity in fish fed the experimental diets compared to the control group (Table 4). Nonparametric analysis (PERMANOVA) revealed no significant differences in the structure (PERMANOVA, $F = 0.42$, $p = 0.83$) of gut microbiota between the five dietary groups. However, the results of hierarchical clustering of beta diversity based on weighted Unifrac distance showed that the 5 samples of control were clustered together, while samples of the probiotic treated groups were very diverse (Figure 3).

Analysis of the Venn diagram of intestinal microbiota showed that 10 (32.3%) of the 31 ASVs that were greater than 0.1% were shared among all tilapia gut samples (Figure 4). The samples had 22 (Control), 19 (0.1% S), 20 (0.2% S), 17 (0.1% SM), and 11 (0.2% SM) ASVs registered. Moreover, a few ASVs were unique to some groups only, e.g., 5 ASVs in the control group [Aureimonas (Proteobacteria phylum), Rhodopirellula (Planctomycetota), Nesterenkonia (Actinobacteriota phylum), Phreatobacter (Proteobacteria phylum), and not identified genus (Proteobacteria phylum)]; 1 ASV in the 0.1%S group [Pedomicrobium (Proteobacteria phylum)]; 2 ASVs in the 0.2%S group [IMCC26207 (Actinobacteriota

phylum; Nocardioide (Actinobacteriota phylum)], 1 ASV in the 0.1%SM group [Lamia (Actinobacteriota phylum)], and 1 ASV in the 0.2%SM group [not identified genus (Firmicutes phylum)].

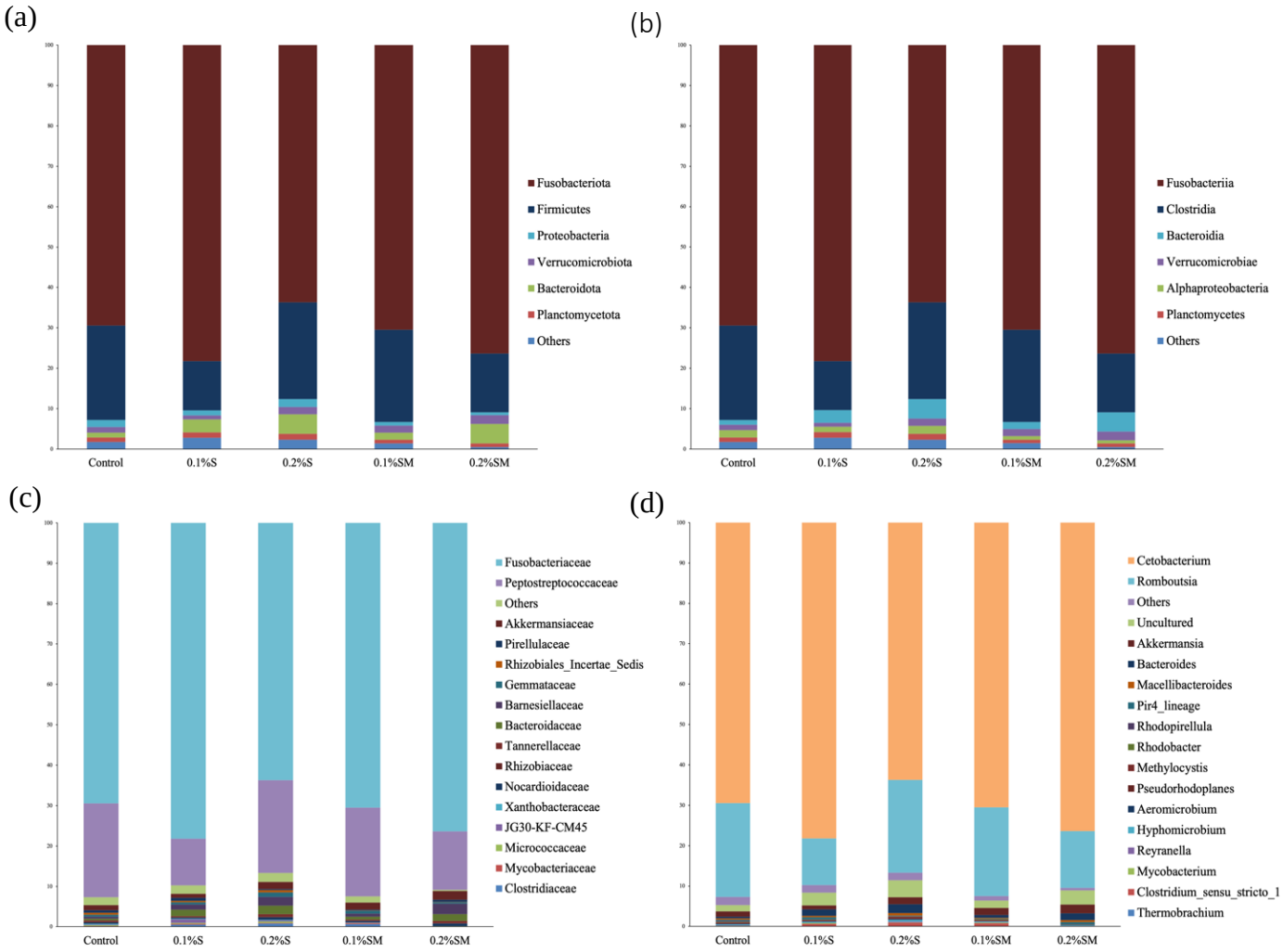


Figure 2. Relative abundance of ASVs with abundance >0.1% of all the sequenced samples grouped by treatment at: a) phylum, b) class, c) family and d) genus levels. The bars represent the average for each treatment.

Table 4. Intestinal microbial diversity indices of Nile tilapia fed diets with free and microencapsulated *B. subtilis*. Valid reads, total mean number of amplicons sequence variants (ASVs), estimated ASV richness (Chao1), evenness index (Pielou), and diversity index (Shannon and Simpson).

Index	Treatments					p-value
	Control	0.1%S	0.2% S	0.1%SM	0.2%SM	
Valid reads	172,204±4211	333,966±4211	629,763±4211	932,068±4211	402,637±4211	0.5133
ASVs	39.6±14.8	35.0±27.5	35.6±22.8	30.4±24.5	23.4±20.9	0.8690
Diversity estimators						
Chao 1	40.84±11.4	40.39±12.8	37.39±11.4	30.11±11.4	35.78±14.7	0.9700
Pielou	0.25±0.04	0.24±0.04	0.28±0.04	0.20±0.04	0.24±0.04	0.8657
Shannon	0.92±0.22	0.82±0.22	0.98±0.22	0.73±0.22	0.79±0.25	0.9416
Simpson	0.50±0.10	0.38±0.10	0.39±0.09	0.32±0.09	0.33±0.04	0.8140

Values expressed as means of five replicates ± SD.

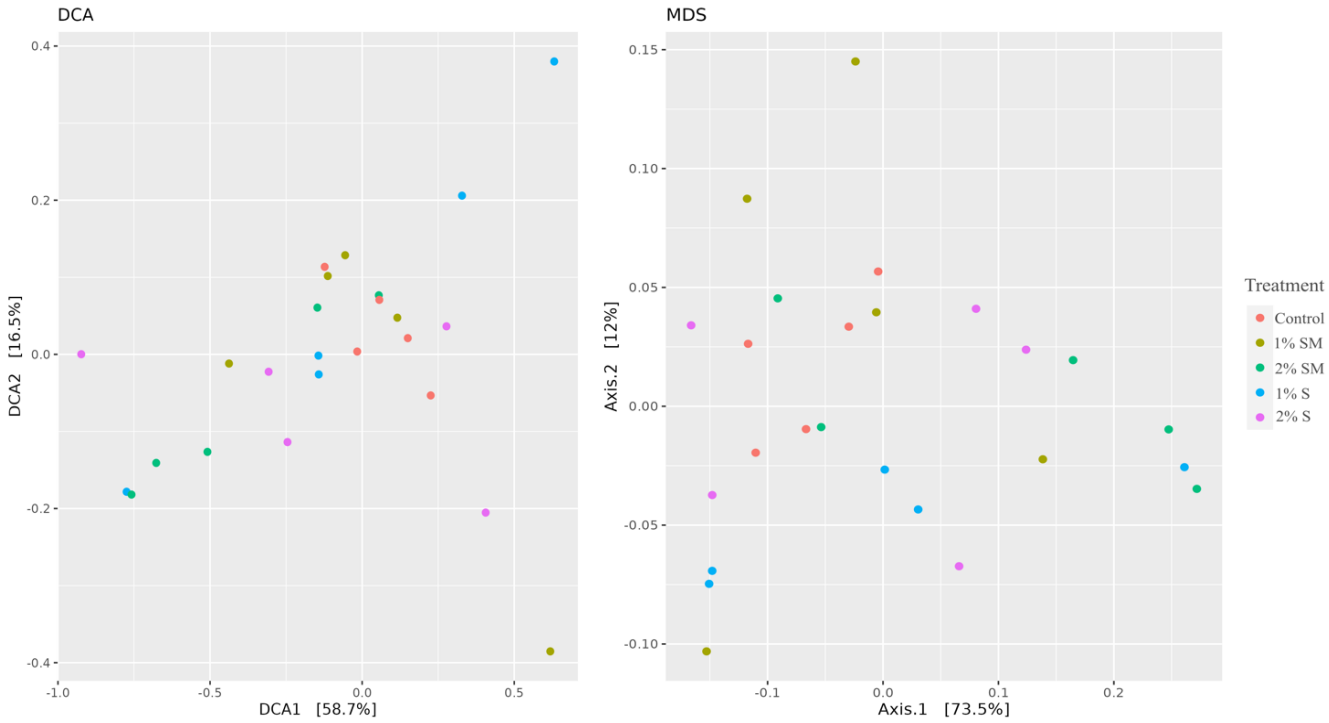


Figure 3. Detrended correspondence analysis (DCA) and multidimensional scaling (MDS) graphs showing the relationships between intestinal samples based on weighted UniFrac metric. The percentage of variation explained by DCA and MDS are indicated in the axis

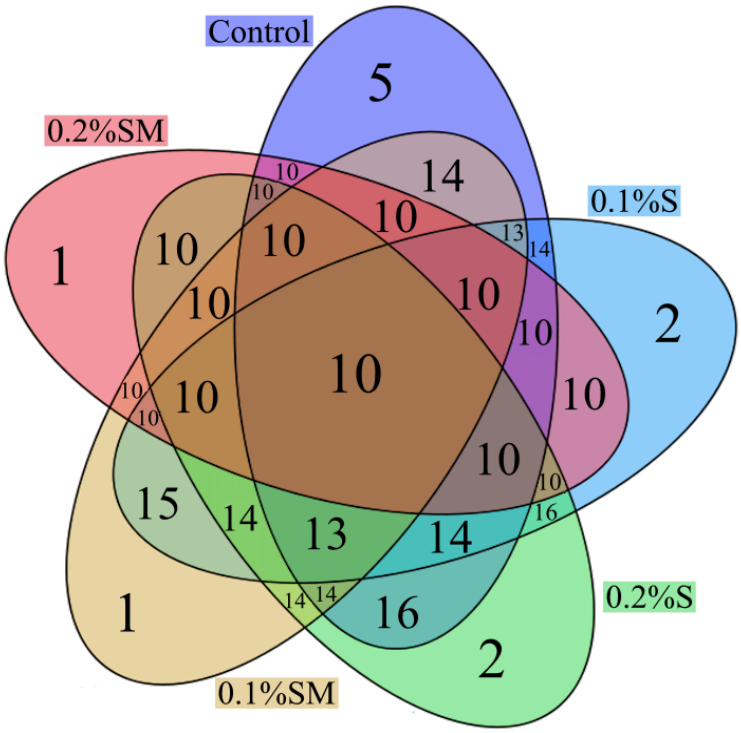


Figure 4. Five-set Venn diagram distribution of all ASVs shared by Nile tilapia fed diets with free and microencapsulated *Bacillus subtilis*. Each ellipse represents one group. The overlapping regions between the ellipses represent the ASVs that is shared between the treatments.

Hematological parameters

No significant differences in RBC, WBC, Htc, TPP, Hb, MCV, MCHC, and Glu were displayed among the groups before the challenge (Table 5). After challenge WBC showed a significant increase in all treatments compared to the results before challenge. Comparing treatments, WBC count remained similar in 0.2%SM, 0.1%SM, and 0.2%S before and after challenge while the control group followed by the 0.1%S were increased.

Table 5. Hematological parameters of Nile tilapia fed diets with free and microencapsulated *Bacillus subtilis* before and after *Aeromonas hydrophila* challenge.

		Experimental diets					
Variable		Control	0.1%S	0.2%S	0.1%SM	0.2%SM	p-value
RBC	Before	1.15±0.08	1.13±0.07	1.10±0.03	1.15±0.04	1.14±0.03	0.9298
(10^6 μL^{-1})	After	1.23±0.09	1.13±0.05	1.17±0.03	1.18±0.04	1.16±0.06	0.7978
	p-value	0.2748	0.5806	0.1420	0.5295	0.4812	
WBC	Before	2.47±0.21^{A}	2.33±0.18^{A}	2.08±0.17^{A}	2.30±0.14^{A}	2.04±0.12^{A}	0.2273
(10^3μL^{-1})	After	3.38±0.16^{Bb}	3.20±0.28^{Bb}	2.19±0.10^{Aa}	2.55±0.19^{Aa}	2.09±0.08^{Aa}	<.0001
	p-value	0.0084	0.0298	0.6404	0.4320	0.8812	
Htc (%)	Before	33.7±1.6	32.6±0.7	31.5±0.9	32.8±0.7	32.0±1.1	0.4495
	After	32.0±1.1	33.9±0.8	32.2±1.1	34.6±0.8	33.1±0.8	0.2133
	p-value	0.3653	0.1879	0.6252	0.2008	0.3896	
TPP	Before	5.38±0.4	5.85±0.2	5.75±0.3	5.28±0.3	5.52±0.4	0.2678
(gL^{-1})	After	6.43±0.3	5.76±0.3	5.58±0.3	6.42±0.4	5.81±0.5	0.1136
	p-value	0.2317	0.4811	0.6741	0.2122	0.4980	
Hb	Before	8.97±0.4	8.42±0.4	8.69±0.2	8.5±0.3	8.7±0.2	0.8675
(g dL^{-1})	After	9.6±0.9	9.1±0.4	9.2±0.5	9.5±0.6	9.4±0.6	0.8371
	p-value	0.2191	0.3466	0.7828	0.1323	0.2118	
MCV (fL)	Before	298.7±21.2	291.9±19.4	287.7±9.7	287.2±12.9	280.4±15.3	0.9507
	After	264.4±17.1	304.1±15.7	275.3±12.5	293.3±16.7	286.9±12.2	0.4015
	p-value	0.2438	0.6378	0.5118	0.6891	0.3895	
MCHC	Before	26.48±0.8	25.87±0.6	27.75±1.1	25.92±0.8	27.49±0.8	0.4234
(%)	After	30.18±2.5	26.84±1.6	29.21±1.9	27.45±2.6	28.41±1.9	0.6230
	p-value	0.1528	0.5426	0.2109	0.1205	0.3254	
Glu	Before	31.63±0.7	33.72±1.6	34.03±3.3	34.81±2.7	33.48±2.9	0.8442
(g dL^{-1})	After	32.38±1.8	34.36±1.8	34.21±1.2	33.49±2.1	34.25±1.4	0.9289
	p-value	0.8751	0.6788	0.8431	0.8218	0.3875	

Values expressed as μ ± SEM, five replicates. RBC: red blood cell count; WBC: white blood cell count; Htc: hematocrit; TPP: total plasma protein; Hb: hemoglobin; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; Glu: glucose. Means in the same row with different lowercase superscripts are significantly different ($P < 0.05$). Uppercase superscripts compare the hematological response of fish in the same treatment before and after challenge by the independent samples t-test ($p \leq 0.05$).

Bacterial challenge

The cumulative mortality rate, after two weeks of *A. hydrophila* infection, is shown in Figure 5. No difference among the treatments were registered, and the mortalities lasted for 6 days. The relative percentage of survival was Control group 85.0%, 0.1%S group 91.6%, 0.2%S group 86.7%, 0.1%SM group 90%, and 0.2%SM group 90%. Although low mortality rates were registered, the typical clinical signs of aeromonose, such as depigmentation, abdominal swelling and cutaneous hemorrhage, were registered in a few fish during the 15-day challenge (Figure 6).

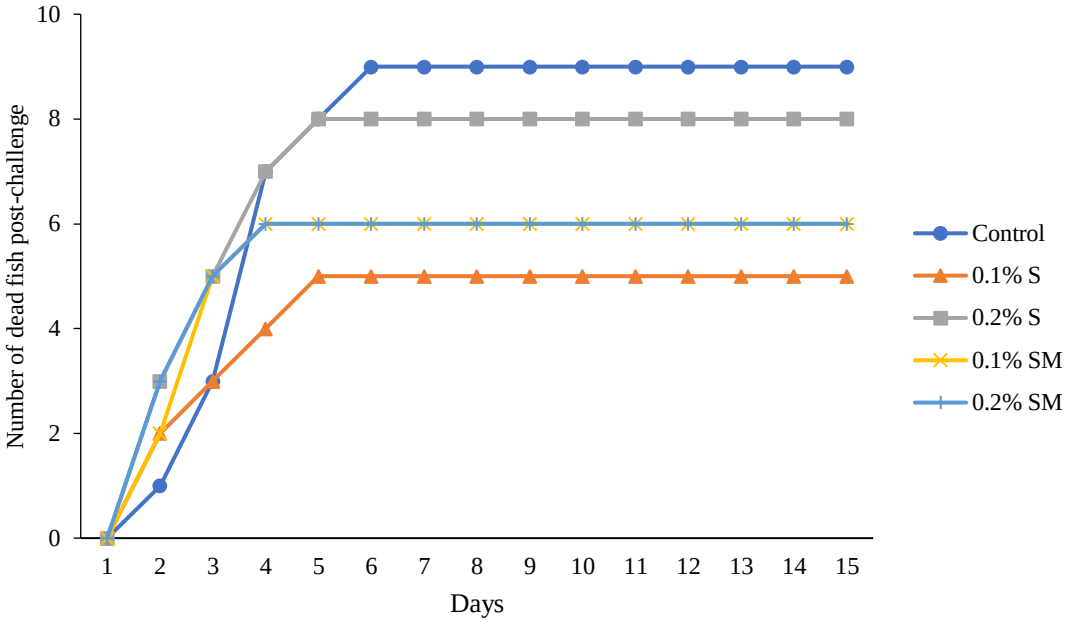


Figure 5. Number of dead fish after 15 days of *Aeromonas hydrophila* challenge.

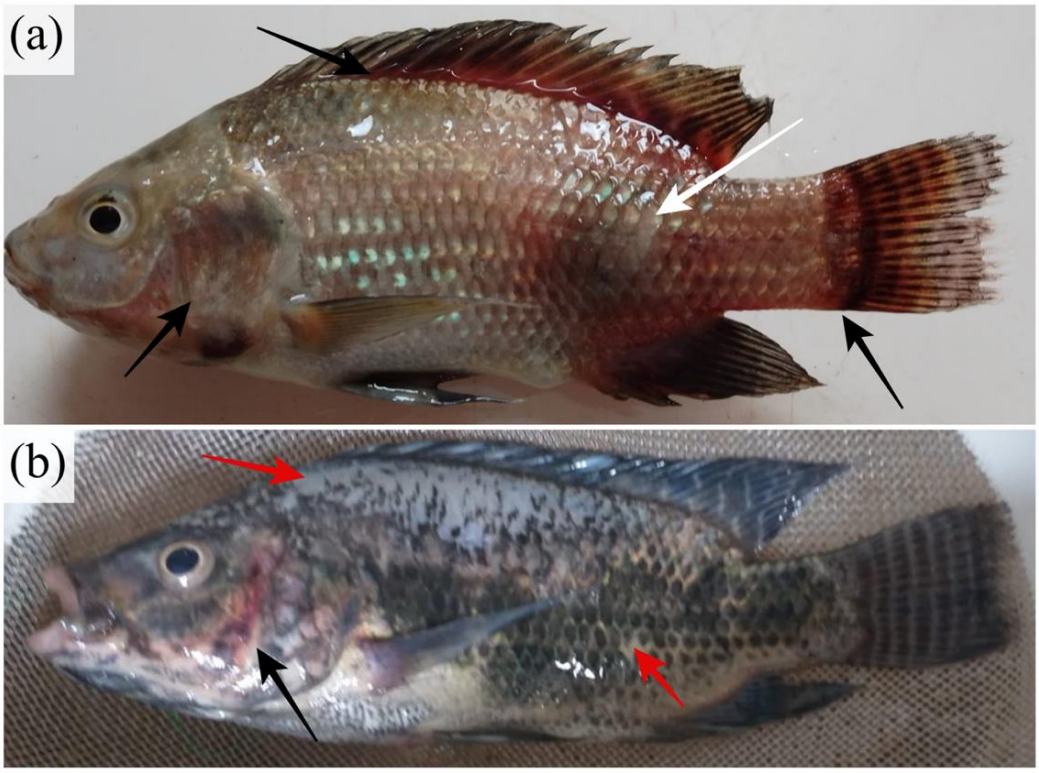
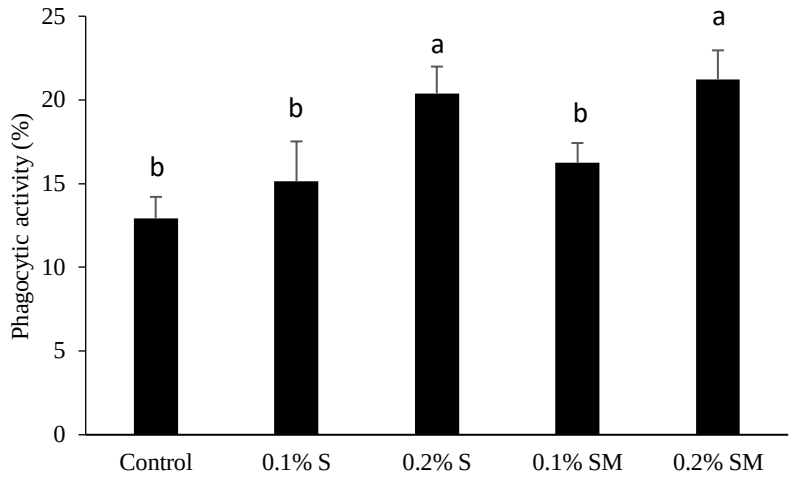


Figure 6. Nile tilapia after intraperitoneal inoculation with *Aeromonas hydrophila*. (a) Inflammatory foci (white arrow) and hemorrhagic signs in gills, dorsal fin, and tail fin (black arrows). (b) Body depigmentation (red arrows) and hemorrhagic signs in gills (black arrows).

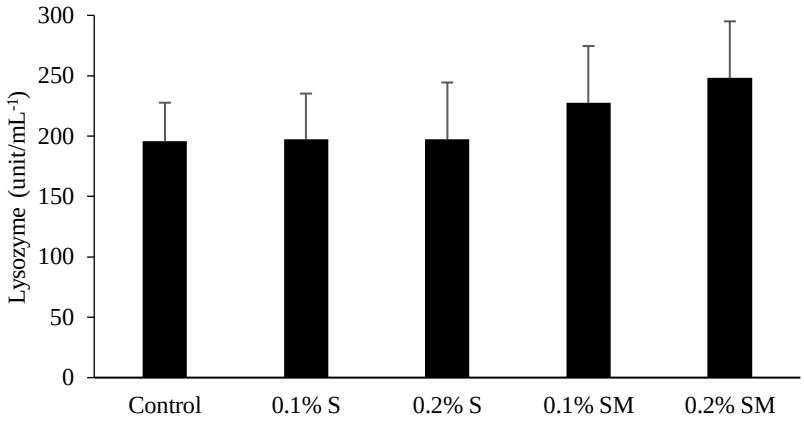
Immunological parameters

After the *A. hydrophila* challenge, a significant difference was registered for the phagocytic activity in the blood of fish fed 0.2%SM and 0.2%S compared to the control group. No differences were registered in lysozyme activity and hemolytic activity of the alternative complement system (HACS) after *A. hydrophila* challenge (Figure 7).

(a)



(b)



(c)

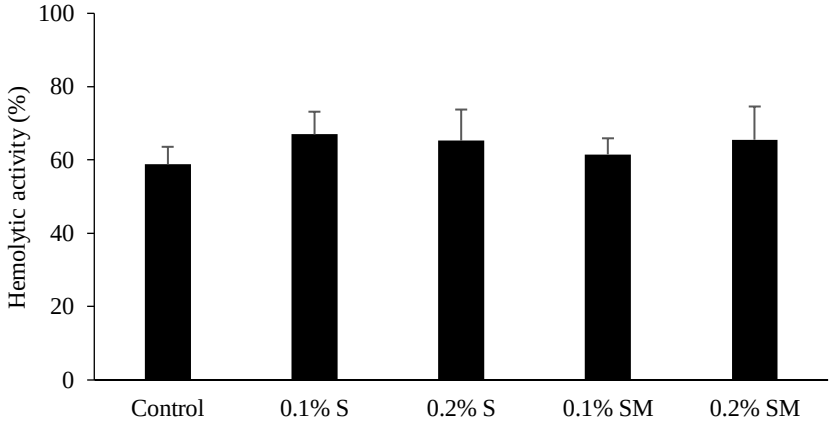


Figure 7. Immunological blood parameters: (a) phagocytic activity, (b) lysozyme activity, and (c) hemolytic activity of the alternative complement system of Nile tilapia fed diets with free and microencapsulated *Bacillus subtilis* and challenged with *A. hydrophila*. Values are

expressed as μ ± SD from five replicates (four fish per replicate). Bars with lowercase letters are significantly different from those of control group ($p = 0.05$).

Antioxidant enzymes activity

No differences were registered in protein estimation and the antioxidative enzymes CAT, SOD, and tGSH activity in the liver of Nile tilapia from the five treatments after *A. hydrophila* challenge (Figure 8).

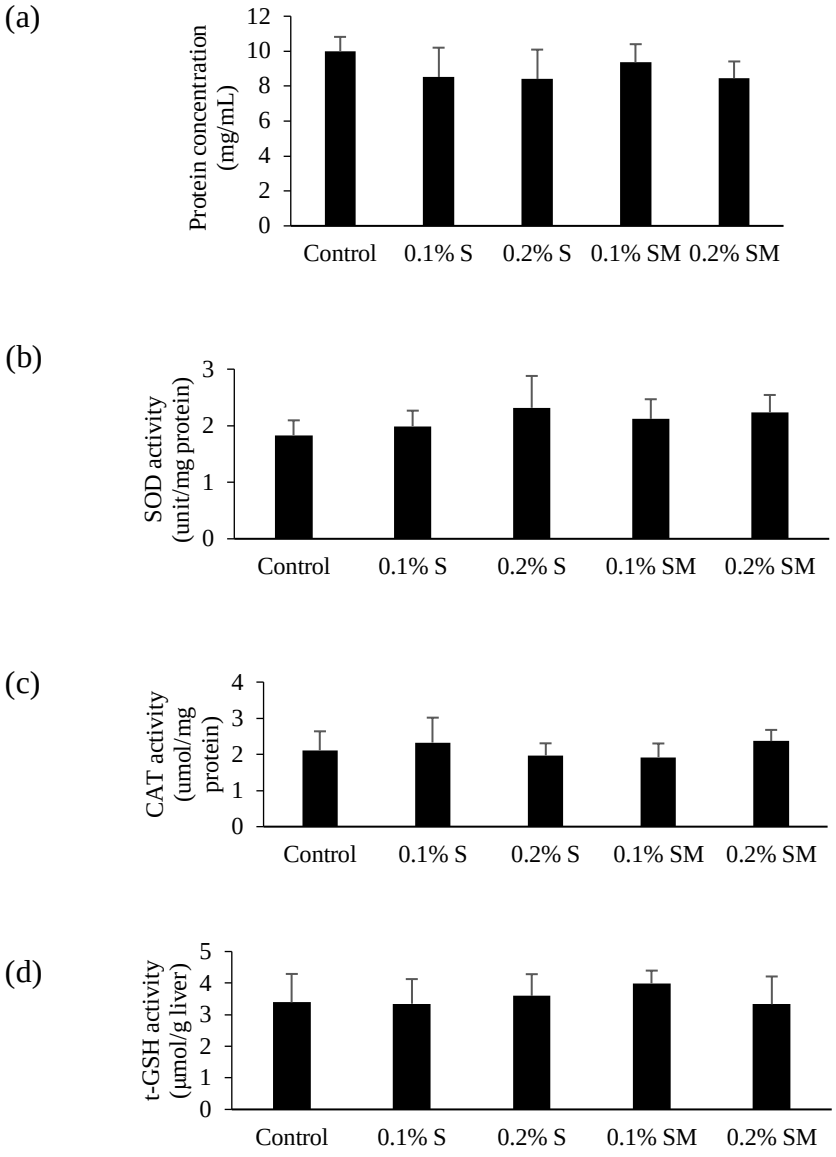


Figure 8. Protein concentration (a) and activity of antioxidative enzymes SOD (b), CAT (c), and tGSH (d) of Nile tilapia fed diets with free and microencapsulated *Bacillus subtilis* and challenged with *A. hydrophila*. Values are expressed as µ ± SD from five replicate groups (four fish per group). Bars with lowercase letters are significantly different from those of control group (p = 0.05).

4.4 Discussion

The combination of prebiotics and probiotics has become a promise for a variety of health purposes in aquaculture. In this study, *Bacillus subtilis* and oat β-glucan were

combined as a synbiotic additive in Nile tilapia diets to enhance their isolated beneficial effects and to assess the protective aspects of microencapsulation on the viability of probiotic cells. *B. subtilis* is a well-researched probiotic with some advantages such as ease dissemination, safety, cost-effectiveness of production, ability to tolerate a broad range of physiological conditions, and the ability to produce several bioactive compounds (Kuebutornye et al., 2020; Nayak, 2020). Cereal β-glucans, such as oat-derived β-1,3/1,4-glucan, are water-soluble polysaccharide with prebiotic properties that so far has not been much exploited in microtechnology and polymer science (Falco et al., 2017).

Addition of co-encapsulated *B. subtilis* and alginate-β-glucan to diets improved growth performance of Nile tilapia when compared to non-encapsulated probiotic cells and the control group. Several studies have already proved the ability of *Bacillus* probiotic strains on the improvement of growth performance of tilapia (Abarike et al., 2018; Kuebutornye et al., 2020; Tachibana et al., 2021), but in this case, the microencapsulation process helped fish to utilize the tested diets more efficiently, which enhanced the growth performance and feed utilization. Similarly, Tamamdusturi and Yuhana (2016) reported improved growth rate with low values of feed conversion rate in pangasius *Pangasianodon hypophthalmus* fed diets containing microcapsules of probiotic *Bacillus* sp. NP5 and prebiotic mannan oligosaccharide (MOS).

Bacteria belonging to the *Bacillus* genus are known to utilize several nutrients in the prebiotics for proliferation, growth, and release of bioactive compounds which facilitate nutrient assimilation, resulting in nutrient retention that led to a high growth rate (Djauhari et al., 2017). However, the potential benefits of probiotics may be way underrated because of the substantial reduction in their viability during storage and gastrointestinal transit (Yao et al., 2019). Therefore, the improvement in the growth performance of tilapia fed 2%SM diets suggest the capability exerted by the microencapsulation process of protecting probiotics from degradation.

The inclusion of probiotic in fish diets may change gut health by altering the surface area of the intestinal villi and the thickness of the intestinal muscle layers (Madreseh et al., 2019). In this research, villous height and the vascularization of the submucosa have increased in all treatments except for the control group. Similarly, Elsbagh et al. (2018) studying Nile tilapia fed commercial blend of *Bacillus* strain reported greater villi heights and higher goblet cells count on the proximal intestine. Higher intestinal villi and high vascularization may suggest change of digestive activity, nutrient absorption and ultimately reflected as improved growth performance of fish (Pirarat et al., 2011; Najafabad et al., 2016). As groups 0.2%S,

0.1%SM, and 0.2%SM showed the highest villi increase, it can be infered that the inclusion of microencapsulated cells stimulated cell hypertrophy regardless β-glucan concentration and it partially corroborates the results of growth performance.

The intestinal microbiome of fish are complex communities which markedly influences homeostasis and digestion, modulating not only the host's physiology and immune responses, but also the morphology and function of the intestinal epithelium (Tarnecki et al., 2017; Butt and Volkoff, 2019). These communities are sensitive to farming environmental conditions and diet changes, including probiotic and prebiotic inclusion (Merrifield et al., 2010; Standen et al., 2015). The present study used a high-throughput sequencing approach to assess the microbial diversity and abundance in the gastrointestinal tract of Nile tilapia fed synbiotics. From the 199 ASVs identified, only 10 were common to all groups with relative abundance higher than 0.1%. It suggests the occurrence of a small yet dominant core microbiota, which persist regardless of synbiotic treatment. Fusobacteriota, Firmicutes, and Bacteroidota were the dominant phyla across all samples accounted for more than 90% of the gut microbiota. These phyla have been shown to be dominant in several fish species including tilapia (Standen et al., 2015; Adeoye et al., 2016; Butt and Volkoff, 2019; Yu et al., 2019).

Bacteroidota showed higher relative abundance in fish fed 0.2%S and 0.2%SM compared to the control group, mainly represented by Bacteroides genus. Bacteroides are particularly interesting for their protective mechanisms of competitive exclusion of potential pathogens and also for their role in processing highly complex carbohydrates (Wexler, 2007). In humans, some bacteria from this genus have been shown to be able to degrade and ferment different β-glucan such as those derived from oat, barley, and fungus (Carlson et al., 2017; Martens et al., 2011; Temple et al., 2017). Although the sequencing analysis could not classify bacteria up to species level, the abundance of the Bacteroidota phyla, mainly the Bacteroides genus, in fish fed both 0.2% synbiotic treatments may suggest that tilapia intestinal microbiota could ferment dietary oat derived β-1,3/1,4-glucans. Similarly, Petit et al. (2021) found high abundance of Bacteroides in common carp fed curdlan (β-1,3-glucan) and *S. cerevisiae* (60% of β-1,3/1,6-glucan), suggesting the β-glucan-fermenting capacity.

At the genus level, *Cetobacterium* (Fusobacteriota phylum) and *Romboutsia* (Firmicutes phylum) were dominant across all samples, accounting for, at least, 86% of total genera. The dominance of these bacteria reflected in lower microbial diversity as registered in alpha diversity indices in all treatments. Despite the reduced microbial diversity in the gut, *Cetobacteria* and *Romboutsia* are broadly related to the enhancement of gut health and immune response in Nile tilapia (Sakyi et al., 2020; Pereira et al., 2021). According to

previous studies (Maas et al., 2021; Yu et al., 2019), the administration of a single probiotic strain may not cause a relevant impact on the richness of the whole gut microbiota of Nile tilapia, but it may induce alterations in the abundance of specific genera as previously discussed.

No significant differences were registered regarding hematological variables among individuals receiving control and experimental diets. The values of hematological parameters (RBC; WBC; Htc; TPP; Hb; MCV; MCHC; Glu) were similar to those reported in recent studies on tilapia fed prebiotics and probiotics (Dawood et al., 2020; Cavalcante et al., 2020; Kord et al., 2021) and are, therefore, within the normal range for the species. Cavalcante et al. (2020) also did not register significant changes in the hematological and immunological parameters of tilapia fed diets supplemented with commercial blend probiotics (*Bifidobacterium* sp, *Lactobacillus acidophilus*, and *Enterococcus faecium*), prebiotics (mannan oligosaccharides and chitosan), and those combined as synbiotics.

Mortality started being registered one day after the *A. hydrophila* challenge. Similarly, Azzam-Sayuti et al. (2021) reported mortalities early as 24 h post-challenge with *Aeromonas spp.* in red hybrid tilapia highlighting the ability of Aeromonas to cause acute infection in fish. Despite the low mortality rates registered through the challenge period, the pathogenic Aeromonas was able to cause disease, which was confirmed by the observation of typical clinical signs of aeromonose, such as abdominal swelling, depigmentation, and cutaneous hemorrhage. Low mortality rates were reported previously by Dong et al. (2015), who infected tilapia with *S. agalactiae* isolated from outbreaks in tilapia farms and, also, by Brum et al. (2017), who infected Nile tilapia with pathogenic bacteria isolated from fish that were fed diet supplemented with essential oils of clove basil *Ocimum gratissimum* and ginger *Zingiber officinale*. In fish farms, mortality outbreaks caused by pathogenic bacteria are registered especially in situations of thermal stress when fish become more susceptible to disease (Brum et al., 2017). Therefore, it is fair to infer that, besides a possible low virulence of the bacteria, low mortality rates occurred because (1) fish were in the zone of thermal comfort during the challenge period (28± 2°C), (2) the core microbiome were mostly composed by *Cetobacteria* and *Romboutsia* that are related to the enhancement of gut health and immune response in Nile tilapia, and (3) fish were healthy and well-fed and no mortalities were registered during the trial before the challenge.

Free and microencapsulated probiotics also did not improve the hematological and serum immunological parameters evaluated after challenge, except for the higher leukocyte counts in fish fed 0.1%SM and control diet. Although, some clinical signs of aeromonose

where registered on the initial days of infection, mortalities lasted only for six days and, possibly, on the 15th day fish were completely recovered. For instance, the high leukocyte count in fish from 0.1%SM and control diet may suggest a long time spent by those groups to recover compared to fish fed 0.2%S, 0.1%SM, and 0.2%SM diets asserting that there was stimulation of the innate immune response. On post-challenge period, fish strive to defend from infection and, therefore, leukocytes play an essential role of defense against infiltrating pathogens (Uribe et al., 2011).

Regarding the phagocytic capacity, fish fed 0.2%SM and 0.2%S registered higher percentage of phagocytosis comparatively to the control group. These treatments showed higher numbers of granulocytes and macrophages that were able to engulf the foreign cells. Considering the highly immune-stimulant properties of β-glucan (Rodrigues et al., 2017), it is safe to suppose that 0.2% of oat β-glucan combined to *B. subtilis* were able to engage its receptors and improve phagocytosis against *Enterococcus* cells. The mechanisms of β-glucan effect on fish immunity are not completely understood, but they might involve improved phagocytosis and increased expression of cytokines in macrophage and neutrophils (Domenico et al., 2017). Similar to the findings herein registered, Dawood et al. (2020) concluded that Nile tilapia fed probiotic combined to β-glucan had high phagocytic activities and greater tolerance against infectious pathogens.

The oxidative stress induced by pathogenic bacteria in fish is a result of overproduction of reactive oxygen species (ROS) and/or weakening of the defense antioxidant system (Baldissera et al., 2018; Souza et al., 2019). Once the production of ROS exceeds the cellular antioxidant capacity, it results in severe cellular injury and, eventually, in cell death as a result of cellular osmotic pressure (Baldissera et al., 2019; Dawood et al., 2020). Antioxidants enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) are the main molecules to inhibit ROS production. In this study, no significant differences were registered in SOD, CAT, and GSH activity among fish receiving control and experimental diets. It is a difficult result to explain as *Bacillus* spp. has been reported to upregulate antioxidant enzymes in Nile tilapia (Dawood et al., 2020; Midhun et al., 2019; Wang et al., 2017) and oat β-glucan is known to protect organs from effects of oxidative stress (Błaszczyk et al., 2015). However, similarly to our findings, Ghalwash et al. (2021) registered no differences in the activity of antioxidant enzymes in fish fed *Bacillus*-based probiotics compared to a control group under normal conditions. When the physiological homeostasis of fish is altered, the oxidative stress can be triggered through the overproduction of ROS, suppressing the antioxidant system. Therefore, it suggests that the recovered fish

were under homeostasis and the synbiotic treatments may have had low or no effects on the antioxidant enzymes.

4.5 Conclusion

Dietary co-encapsulated *Bacillus subtilis* and oat β-glucan at 2g kg^{-1} (5.4×10^9 CFU kg^{-1} diet) improved growth performance and feed utilization of Nile tilapia whereas free and microencapsulated probiotic had positive impact on intestinal morphology. These results highlight that microencapsulation has the potential to protect *B. subtilis* and may constitute a valuable approach for the viability of probiotic strains through the adverse conditions of the gastrointestinal tract of fish and feed storage. Additional studies are necessary to understand the effective protective abilities of microencapsulated *B. subtilis* challenged with stressful factors, investigating the triggered antioxidant and immune systems in tilapia. Future studies should also investigate the microbiota modulation after challenge and the activity of digestive enzymes to better understand the process of nutrient metabolism.

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