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Effects of Dietary Supplementation of Synbiotic *Bacillus subtilis* and Fructooligosaccharide on Non-specific Immune Responses and Disease Resistance of Juvenile Nile Tilapia (*Oreochromis niloticus*)

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Abstract

This study was aimed to evaluate the effects of synbiotics, which was created by *Bacillus subtilis* and fructooligosaccharide (FOS) combination on growth performances, immunity improvement, and disease resistance of Nile tilapia (*Oreochromis niloticus*). This study divided the trials into four groups and first trial, juvenile fish (24.5 ± 1.6 g) were fed a basal diet (G1), diets supplemented with 1 g/kg FOS + 1×10^9 CFU/g *B. subtilis* (G2), g/kg FOS + 3×10^9 CFU/g *B. subtilis* (G3) and 5 g/kg FOS + 5×10^9 CFU/g *B. subtilis* (G4) for 56 days. After the feeding trial, the complement C3 gene expressions in the liver were analyzed by a quantitative real-time reverse-transcription polymerase (qRT-PCR), lysozyme activity and respiratory burst activity. Then, fish were infected with *Streptococcus agalactiae*, and the survival rate was recorded for 14 days. The results showed that fish-fed diets supplemented with synbiotics had significant effect ($P < 0.05$) on growth performances including average daily gain (ADG) and survival rate compared with a control. Lysozyme activity and respiratory burst activity were significantly greater in the G3 and G4 groups. The gene expressions of complement C3 in the liver were significantly up-regulated in tilapia fed with G4 ($P < 0.05$) synbiotics. And were higher disease resistance with *S. agalactiae* and survival rates were higher than compare with the control group.

Keywords: Synbiotics, *Bacillus subtilis*, fructooligosaccharide, nile tilapia

Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most popular freshwater fish cultured worldwide. The rapid expansion of Nile tilapia farming has been negatively affected by infectious diseases and climate uncertainties, causing economic losses. Streptococcosis by *Streptococcus agalactiae* (86.67%) caused economic failure due to mass mortality at all culturing stages in Nile tilapia (Jantrakajorn *et al*., 2014). The excessive application of chemicals and antibiotics for prevention and treatment of aquatic diseases leads to negative effects including antibiotic resistance in soil and water environments as well as the undesirable residues in consumed products (Esiobu *et al*., 2002).

Currently, have been demonstrated to have positive effects in many fish species. Fructooligosacharides (FOS) is a common prebiotic and originally used to supplement human diets and farm animals (Mahious *et al*., 2006) but there are a few studies in fish Nile tilapia. Application of dietary FOS in aquaculture provides several positive effects (e.g., nutrient utilization, growth, disease resistance, improved gastrointestinal (GI) microbiota) in various animal species. Climbing Perch (*Anabas testudineu*) fed 1% and 2% FOS supplementary diet had better on growth performance, higher lysozyme activity and percent phagocytosis in climbing perch (*Anabas testudineus*) (Chitmanat *et al*., 2017). The expressions of complement C3 in the liver were significantly higher for tilapia fed 5 g/kg of FOS (Panase *et al*., 2023). Thus application of *B. subtilis* and fructooligosaccharide synbiotic supplement maybe enhancement growth performance, immune responses and disease resistance of juvenile Nile tilapia more than a single use prebiotic or probiotic supplement.

Besides prebiotics associated with aquaculture business, probiotics are living microorganisms such as *Bacillus* sp. and *Lactobacillus* sp. which are applied via the feed or to the rearing water. *Bacillus* spp. are widely used in aquafeeds for feed utilization improvement, growth performance promotion, innate immune response regulation, and disease resistance as well as improvement of water quality for sustainable aquaculture (Kuebutornye *et al*., 2019).

The synbiotic additive feed did not provide any significant improvement in growth rate and feed utilization in juvenile barramundi, *Lates calcarifer*. The different results are probably caused by the types of prebiotics and probiotics, administration dosages, duration, and conditions of experiment, as well as fish ages and species. Most of the experiments have not been further conducted on farms. Fish fed with synbiotic are low mortality rates possible synbiotic could be enhancement non-specific immunity which is discussed in the immune parameter section.

Therefore, the main goal of this study was to investigate the application of *B. subtilis* and Fructooligosaccharide synbiotic supplemented feeds on growth performances, immune responses, and disease resistance against *S. agalactiae* infection of Nile tilapia. The understanding of the various mechanisms of *Bacillus* and FOS to improve a non-specific immune responses and combat against pathogens would be beneficial. This study can contribute to an environmentally friendly alternative to antibiotics which is a promising strategy for a sustainable aquaculture.

Materials and Methods

Materials

The fructooligosaccharide (FOS) used in this study was generated by Quantum Hi-Tech Biological Co., Ltd, China. FOS was white or light-

yellow powder without visible impurity. The product composition was combination of 1-kestose (1-kestotriose; GF2), nystose (GF3), and 1F-fructofuranosylnystose (GF4). The undesirable components including bacterial, molds, and yeast were not more than 10 CFU/g. The commercially available probiotic product (Greentech Aquaculture co., LTD, Thailand) comprised of 1×10^9 CFU/g *Bacillus subtilis*.

Diet preparation

The basal diet (HiGrade 9951, CPF) was commercially available containing not less than 30% crude protein, 3% lipid, and 2% crude fiber which have been shown to be acceptable to support the superb growth of Nile tilapia. The four treatment diets were tested including a basal feed used as a control (G1), 1 g/kg FOS + 1×10^9 CFU/g *B. subtilis* supplemented diet (G2), 3 g/kg FOS + 3×10^9 CFU/g *B. subtilis* supplemented diet (G3) and 5 g/kg FOS + 5×10^9 CFU/g *B. subtilis* supplemented diet (G4). The FOS plus *B. subtilis* at a particular concentration were sprayed onto 1 kg of a basal diet, and then all feeds were coated with fish oil at 20 mL per kg basal diet. After that, the experiment feeds were air dried at room temperature for 24 h and stored in sealed plastic bags at 4 °C until use.

Fish and experimental design

Apparently healthy Nile tilapia, an average initial body weight of 24.5±1.6 g, were obtained from a local fish farm and had been acclimated for two weeks in 2 m × 2 m cages. Tilapia were fed to satiation with a commercial diet two times per day (08.00 AM and 16.00 PM). After acclimation, fish were randomly divided into 2m × 2m cages and prepared total 12 cages. Each diet was assigned to triplicate cages and each cage had 20 fish. The first group was fed with basal diet (control group, G1), while the second, third, and fourth groups, were fed basal diet containing 3 different levels of synbiotic groups (1 g/kg FOS + 1×10^9 CFU/g *B. subtilis*, G2; 3 g/kg FOS + 3×10^9 CFU/g *B. subtilis*, G3 and 5 g/kg FOS + 5×10^9 CFU/g *B. subtilis*, G4, respectively. Fish were fed two times per day at 5% of the body weight for 56 days. During the feeding trial, the fish were weighed every two weeks and the amount of feeding was adjusted due to the increased weight.

Growth performances and survival

At the end of the feeding trial, fish in each cage were weighed for growth performance and survival rate measurements. The growth parameters were calculated according to the following formula:

Weight gain (WG, %) = [(final weight–initial weight)/ initial weight] × 100

Average daily gain (ADG g day^{-1}) = 100 × (final body weight – initial body weight) / experimental period

Feed conversion ratio (FCR) = quantity of feed offered/weight gain

Survival (%) = (final number of fish/initial number of fish) × 100

Blood collection

Blood samples were taken after termination at the 56-day feeding trial, fish were randomly taken from 3 fish in each replicate (9 fish per group). Before blood sampling, the fish were deprived from feed for one day. Fish were randomly selected and removed from the cages as soon as possible with minimum disturbance. The caudal peduncle were cleaned using alcohol with special care around the anus in order to avoid any contamination. Then blood was taken from the caudal vein with 1 mL plastic syringe. One part of blood sample was placed into heparinized tube

as an anticoagulant for the determination of respiratory burst activity (superoxide anion assay) and the other part was collected into individual tubes without anticoagulant and allowed to clot at room temperature for 4 h., the serum was then separated and moved into new tubes before being stored -80 °C for further lysozyme activity analysis.

Lysozyme activity

The lysozyme activity assay was slightly modified from (Parry *et al*., 1965). Briefly, 175 µl of *Micrococcus lysodeikticus* suspension [0.2 mg/ml in sodium phosphate buffer (pH 6.2)] was added to 25 µl of fish serum in a 96-well plate. The reaction was determined by measuring absorbance at an optical density at 540 nm by spectrophotometer (Multiskan go, Thermo scientific) and recorded every 1 min for 10 min. The activity of lysozyme (1 unit) in fish serum was calculated as the reduction in A540 of 0.001/min. Lysozyme activity was expressed as U/ml.

Respiratory burst activity (superoxide anion assay)

Superoxide anion (O_{2-}) was used to determine respiratory burst activity through nitroblue tetrazolium (NBT) reduction reaction according to the previous protocol with minor modification (Secombes, 1990). Approximately 6×10^6 cells of white blood cells (WBC), 1 ml of blood was taken from fish each fish (3 fish per treatment) were collected from the caudal vein using needle was transferred to heparinised containers. One ml blood sampled mixed with 1 ml of RPMI 1640 and 1 ml of phicol this blend was carefully in a tube. Centrifuged at 4000 rpm/min for 40 min. In order to collect as many WBC was made leukocytes middle layer were removed by a micropipette and remaining WBC in the tube were harvested. The following procedures for cell washing, purification of WBC. One ml of phosphate buffer solution (PBS) was added to each tube the cell were washed by centrifuged at 2,500 rpm/min for 40 min and then adjusted approximately 6×10^6 cells of white blood cells (WBC) under microscope. White blood cells were added to 96-well plate, mixed with 25 µl of NBT, and incubated at room temperature for 2 h. The supernatant was then decanted, and the WBC fixed with 100% methanol for 5 min followed by washing with 70% methanol twice. Potassium hydroxide (2 M KOH) and dimethyl sulfoxide (DMSO) were added to the dried WBC on 96-well plate, mixed well and the reaction of the superoxide anion measuring absorbance at 655 nm (A655) by spectrophotometer (Multiskan go, Thermo scientific).

RNA extraction and cDNA synthesis

Liver tissues were collected from three fish per treatment group after 56-day feeding trial with synbiotic for total RNA extractions. An amount of 20 ng μL^{-1} for the liver was used. According to the manufacturer's protocols, total RNA was extracted using a PureLink RNA Mini Kit (Ambion, USA). The quality of the RNA was measured spectrophotometrically (NanoDrop 2000, Thermo scientific) and with gel electrophoresis (1% agarose gel). Total RNA was converted to complementary DNA (cDNA) using a SensiFAST ™ SYBR® No-ROX Kit (Bioline, UK) following the manufacturer's protocols.

Quantitative real-time reverse-transcription polymerase (qRT-PCR)

Gene expression analyses of the complement C3 of Nile tilapia were conducted with SensiFAST™ SYBR® No-ROX Kit (Bioline, UK) using cDNA 20 ng/µl for liver. The qRT-PCR study of gene expression level with PCRmax Eco 48 Real-time qPCR System (PCRmax, UK) was carried out. The specific primers,

β-actin housekeeping gene and target genes used for qRT-PCR are shown in Table 1. The amplification conditions were as follows: 45 cycles, (95 °C for 10 s, and 60 °C for 30 s). Afterwards, the relative expression levels of complement C3 were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001)

Table 1 Primers used for detection of the target genes

Gene	FWD or REV	Sequence (5′–3′)	Product size (bp)	References
Actin	Forward	TGG CAA TGA GAG GTT CCG	95	(Phumyu
	Reverse	TGC TGT TGT AGG TGG TTT CG		*et al*., 2012)
C3	Forward	TGT GAG TCT ACA GTG AGG AGC	196	(Phumyu
	Reverse	CCC AGA TCT AAA GCC ATT CTG C		*et al*., 2012)

Challenge test with *S. agalactiae*

S. agalactiae used in this study was supported by faculty of fisheries technology and aquatic resources, Maejo university, Thailand were freshly prepared by inoculating a single colony of the bacterium into nutrient broth (NB, Himedia) and culturing at 32 °C for 24 h. Bacterial cells was harvested by centrifugation at 5,000 rpm at 4 °C for 10 min, followed by washing and resuspension in 0.85% NaCl. The *S. agalactiae* suspension was adjusted to 10^8 CFU/ml with 0.85% NaCl before injection. At the end of feeding trial, ten fish were randomly collected from each group and intraperitoneally injected with 0.1 ml of *S. agalactiae* (10^8 CFU/ml). Mortality was daily recorded for 14 days after injection.

Animal ethics

The experiments were conducted according to the norms established by the Maejo University Animal Care and Use Committee.

Statistical analysis

Results expressed as the mean values ± standard deviation (SD). Differences among treatments were determined using a one-way analysis of variance (ANOVA) with the statistical software SPSS Version 15.0. A post hoc, Duncan test was further applied to examine significant differences between treatments. Significant differences were accepted at $P < 0.05$.

Results and Discussion

Growth performances and survival rate

In principle, probiotics and prebiotics have been combined to provide the survival of lived microbial nutritional supplements at the digestive tract of the fish (Gibson and Roberfroid, 1995) leading to the growth enhancement.

Fructooligosacharides (FOS) is a common prebiotic and originally used to supplement human diets and farm animals, but there are a few studies in fish species (Mahious *et al*., 2006). Besides prebiotics associated with aquaculture, probiotics are living microorganisms such as *Bacillus* sp. which are applied via the feed or to the rearing water. However, our study had no significant improvement in growth and feed conversion ratio compared to fish fed a control diet. The growth performances of Nile tilapia after 56-day feeding trial with synbiotic between FOS and *B. subtilis* were presented in Figure 1. The average weight (Figure 1), weight gain, WG (Figure. 1) and feed conversion ratio, FCR (Figure 1) were

not significantly different compared with the control group ($P>0.05$). However, the average daily gain, ADG of fish in G2 and G3 groups was significantly higher compared with the control group and other groups (Figure 1). The survival rate of tilapia in G4 all synbiotic groups g was significantly different compared with the control group ($P<0.05$) (Figure 2). Our results agree with Addo (2013), who combined the probiotic strains and prebiotics used as feed additives and showed the reduced mortality in Nile tilapia and channel catfish under the conditions of laboratory (Addo, 2013).

The results of our study indicated that dietary synbiotic could significantly boost the average daily gain, ADG possible due to probiotics and prebiotics have been combined to provide the survival of lived microbial nutritional supplements at the digestive tract of the fish (Gibson and Roberfroid, 1995) and survival rate of Nile tilapia. These results may be correlated with the high digestive enzymatic activities provided by the synergistic action of prebiotic and probiotic and subsequently promote the growth. Prebiotics are hydrolyzed to their respective sugars in the intestinal tract of the host and are then utilized as a source of carbon to increase the biomass of bacteria and prebiotics are utilized by intestinal bacteria under diverse mechanisms dependent on sugar linkages and the bacterial strains (Goh and Klaenhammer, 2015). Among the prebiotics and probiotics widely adopted, both oligosaccharides and *Bacillus* have been successfully used in aquaculture. It was noted that the supplementation of synbiotics yielded significantly better results than individual applications.

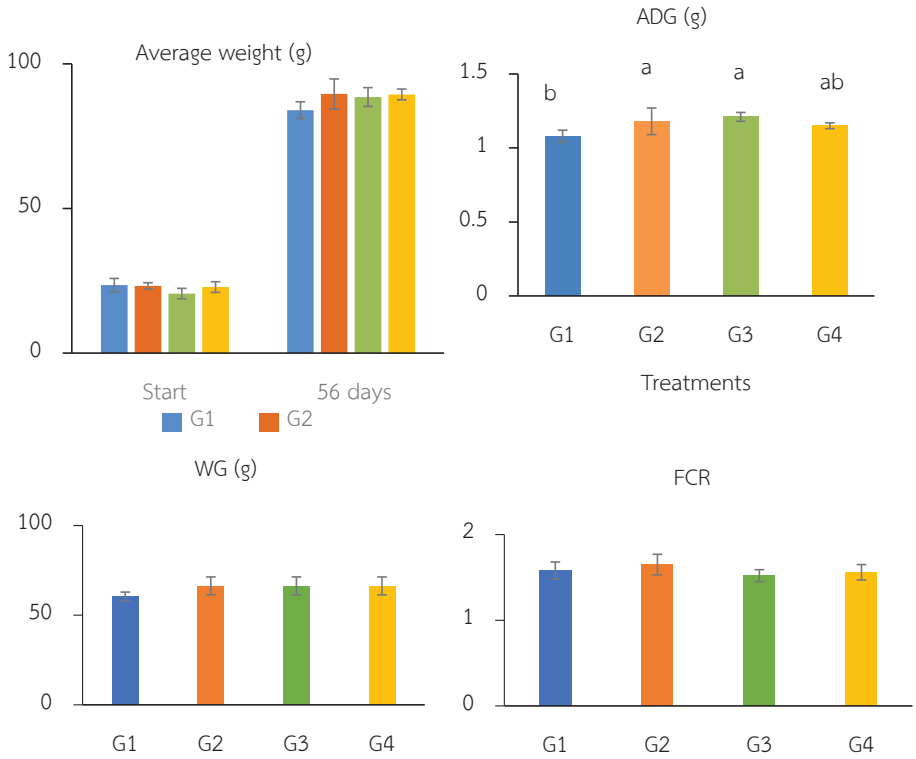

Figure 1 Growth performances of Nile tilapia fed a control feed and diets supplemented with different concentrations of synbiotic for 56 days. Error bars = standard deviation *P<0.05*

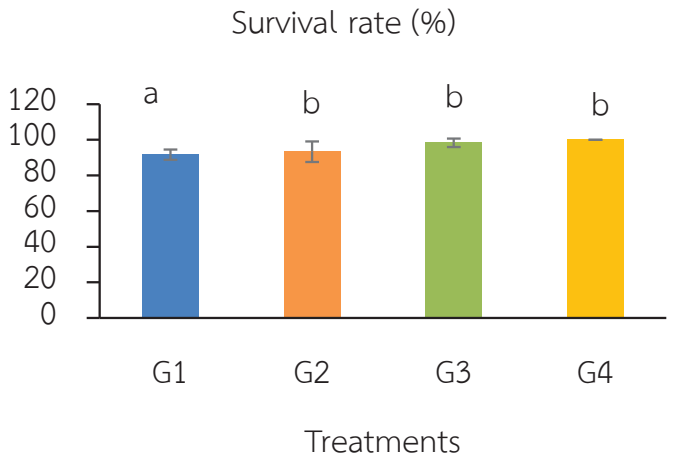

Figure 2 Survival rate of Nile tilapia after fed a control feed and diet supplemented with different concentrations of synbiotic for 56 days. Error bars = standard deviation *P<0.05*

Effect of synbiotics on immune parameters

Lysozyme activity

Lysozyme is an enzyme that degrades the peptidoglycan in bacterial walls and plays an important role in controlling infectious fish pathogens. In the present study, lysozyme activity showed the significantly higher in fish fed Diet G3 (3 g/kg FOS + 3×10^9 CFU/g *B. subtilis*) and G4 (5 g/kg FOS + 5×10^9 CFU/g *B. subtilis*) compared with control group (Figure 3) ($P<0.05$). Like our results, zebrafish (*Danio rerio*) fed with polysaccharide gel extracted from the rind of durian fruit which encapsulated with *Bacillus subtilis* and co-inoculation with Artemia nauplii showed a positive effect in lysozyme activity (Priya *et al*., 2021). Moreover, several studies reported Japanese eel, *Anguilla japonica* fed with *B. subtilis* at 0.5×10^7 CFU/g and mannanoligosaccharide at 5 g/kg had a significantly higher lysozyme activity than those fed with other diets (Lee *et al*., 2018). Thus, prebiotic and probiotic supplementation at an appropriate concentration possibly enhanced lysozyme activity in fish.

Respiratory burst activity

A respiratory burst is an indication of the oxidative potential of reactive oxygen species including hydrogen peroxide, superoxide anions, and hydroxyl radicals. These reactive oxygen species are produced by activated phagocytic cells and they are responsible for killing engulfed pathogens. Reactive oxygen species have been widely used to evaluate the ability of the host to defend against pathogens (Abbas *et al*., 2014). Significant differences ($P<0.05$) in respiratory burst activity were observed in G3 and G4 after 56 days of the feeding trial (Figure 4)

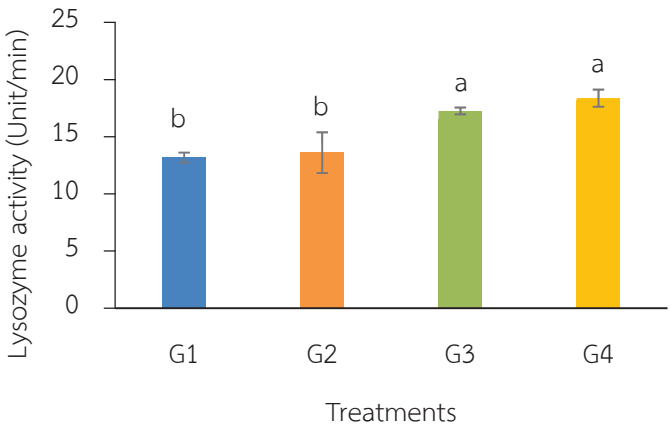

Figure 3 Lysozyme activity of Nile tilapia fed with synbiotic for 56 day (*n* = 3). Error bars = standard deviation *P<0.05*

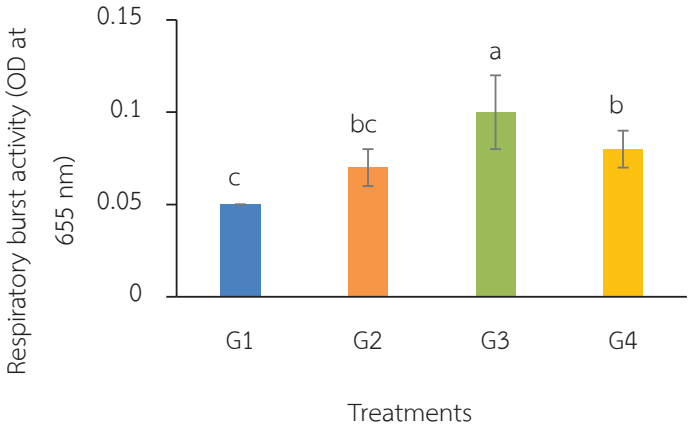

Figure 4 Respiratory burst activity of Nile tilapia fed with synbiotic for 56 day (*n* = 5). Error bars = standard deviation *P<0.05*

Gene expression in the liver of Nile tilapia

The immune status of tilapia fed synbiotic diets was investigated by determining the expression of complement C3 gene expression in the liver, the results are shown in Figure 5. Complement C3 gene expression was significantly up-regulated in the liver of tilapia fed with 5 g / kg FOS and 5×10^9 CFU/g of *B. subtilis* (G4) and fish fed with 3 g/kg FOS + 3×10^9 CFU/g *B. subtilis* (G3) additive diets ($P<0.05$)

The complement system is a vital innate immune barrier in pathogen prevention and regulates humoral immune responses (Beutler, 2004). The complement component C3 gene is responsible in an inflammatory response and monocyte/macrophage phagocytosis. After activation or pathogen infection, the C3 molecule is decomposed into C3a and C3b, and modulates the inflammatory response to defend against pathogen infection. The present study indicates that increased the serum complement C3 may be significantly beneficial in fish fed with 3 g/kg FOS + 3×10^9 CFU/g *B. subtilis* (G3) and 5 g FOS/kg feed and 5×10^9 CFU/g of *B. subtilis* (G4).

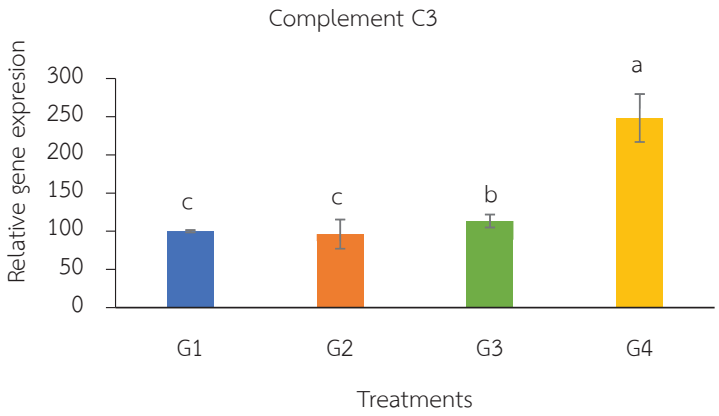

Figure 5 Gene expression of complement C3 in the liver of Nile tilapia. Error bars = standard deviation *P<0.05*

Challenge test with *S. agalactiae*

In this part, mortality of fish after challenge test with *s. agalactiae* is pathogenic bacteria causing high mortality and economic losses in tilapia. The challenge test is used as an eventual assay to assess the fish disease resistance. At the end Nile tilapia the feeding trial with synbiotics for 56 days, fish were challenged with *S. agalactiae*. The survival rate of Nile tilapia were recorded for 14 days (Figure 6). The highest survival rates were found in the G4 group was significantly ($P<0.05$) while the lowest survival rates were observed in a control group. Clinical signs of infected fish included abnormal swimming, darkened color, less appetite, hemorrhage on the surfaces of the body andhepatomegaly. The combination of mannan oligosaccharides (MOS) and commercial probiotic DBA® (*Bifidobacterium* sp, *Lactobacillus acidophilus* and *Enterococcus faecium*) reduced the mortality of Nile tilapia infected with *A. hydrophila* (Cavalcante *et al*., 2020). The synergetic effect of *Bacillus subtilis* and the prebiotic Previda®, a commercial hemicellulose extract was reported in Nile tilapia (8 weeks of feeding) against *A. hydrophila* infection (Addo *et al*., 2017). The higher survival of these fish probably higher innate immune responses which was noticed from enhanced immune parameter.

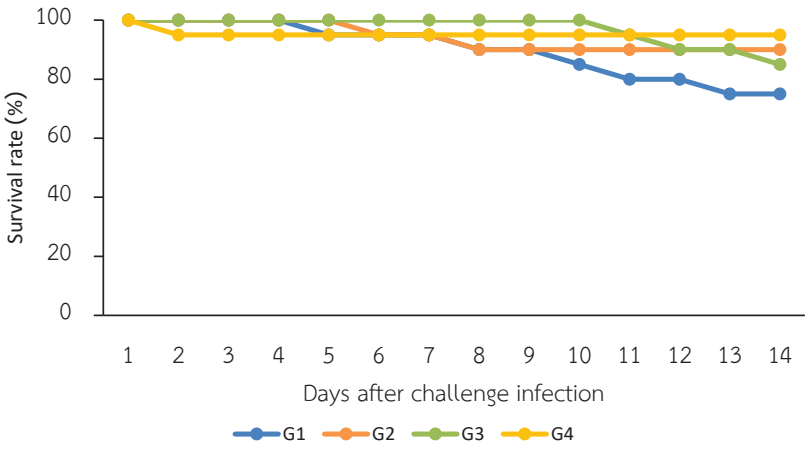

Figure 6 Survival rates (%) of Nile tilapia fed with synbiotic after challenge with 1×10^8 CFU/ml of *S. agalactiae* (n = 20) for 14 days

Conclusion

In conclusion, the growth performances of Nile tilapia after 56-day feeding trial with synbiotic between FOS and *B. subtilis*. The average daily gain, ADG G2 and G3 group, survival rate was significantly compared with the control group. And significant effects were observed on the immune responses. It may also increase their resistance to *S. agalactiae* infection. At present, the feed additives lead to additional expenditures, fish farmers have to be concerned before application and also the use of new microbial strains has to safely conduct to avoid potential negative side effects.

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References

Abbas, A.K., A.H. Lichtman and S. Pillai. 2014. Cellular and molecular immunology Ebook. Elsevier Health Sciences. Elsevier Health Sciences, Nederland.

Addo, S., A.A. Carrias, M.A. Williams, M.R. Liles, J.S. Terhune and D.A. Davis, 2017. Effects of *Bacillus subtilis* strains on growth, immune parameters, and Streptococcus iniae susceptibility in Nile tilapia, *Oreochromis niloticus*. Journal of the World Aquaculture Society 48(2): 257-267. Available: https://doi.org/10.1111/jwas.12380.

Beutler, B. 2004. Innate immunity: an overview. Molecular immunology 40(12): 845-859. Available: https://doi.org/10.1016/j.molimm.2003.10.005.

Cavalcante, R.B., G.S. Telli, L. Tachibana, D. de Carla Dias, E. Oshiro, M.M. Natori, W.F. da Silva and M.J. Ranzani-Paiva. 2020. Probiotics, prebiotics and synbiotics for Nile tilapia: growth performance and protection against *Aeromonas hydrophila* infection. Aquaculture Reports 17: 100343. Available: https://doi.org/10.1016/j.aqrep.2020.100343.

Chitmanat, C., S. Thongsri and T. Phimpimon. 2017. The Influences of Dietary Supplementation with Fructooligosaccharide on Growth and Immune Responses of Climbing Perch (*Anabas testudineus*) Int. International Journal of Agriculture and Biology19(4): 787-791. Available: DOI: 10.17957/IJAB/15.0350.

Das, S., K. Mondal and S. Haque. 2017. A review on application of probiotic, prebiotic and synbiotic for sustainable development of aquaculture. Journal of Entomology and Zoology Studies 5(2): 422-429.

Doan, H.V., S.H. Hoseinifar, W. Tapingkae, S. Tongsiri and P. Khamtavee. 2016. Combined administration of low molecular weight sodium alginate boosted immunomodulatory, disease resistance and growth enhancing effects of *Lactobacillus plantarum* in Nile tilapia (*Oreochromis niloticus*). Fish & Shellfish Immunology 58: 678-685. Available: https://doi.org/10.1016/j.fsi.2016.10.013.

Esiobu, N., L. Armenta and J. Ike. 2002. Antibiotic resistance in soil and water environments. International Journal of Environmental Health Research 12: 133-144. Available: https://doi.org/10.1080/09603120220129292.

Gibson, G.R. and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. The Journal of nutrition 125(6): 1401-1412. Available: https://doi.org/10.1093/jn/125.6.1401.

Goh, Y.J. and T.R. Klaenhammer. 2015. Genetic Mechanisms of Prebiotic Oligosaccharide Metabolism in Probiotic Microbes. Annual review of food science and technology 6(1): 137-156. Available: https://doi.org/10.1146/annurev-food-022814-015706.

Jantrakajorn S., H. Maisak and J. Wongtavatchai. 2014. Comprehensive investigation of streptococcosis outbreaks in cultured Nile tilapia, *Oreochromis niloticus*, and red tilapia, *Oreochromis* sp., of Thailand. Journal of the world aquaculture society 45(4): 392-492. Available: https://doi.org/10.1111/jwas.12131.

Kuebutornye, F.K., E.D. Abarike and Y. Lu. 2019. A review on the application of Bacillus as probiotics in aquaculture. Fish & shellfish immunology 87: 820-828. Available: https://doi.org/10.1016/j.fsi.2019.02.010.

Lee, S., K. Katya, A. Hamidoghli, J, Hong, D-J. Kim, and S.C. Bai. 2018. Synergistic effects of dietary supplementation of *Bacillus subtilis* WB60 and mannanoligosaccharide (MOS) on growth performance, immunity and disease resistance in Japanese eel, *Anguilla japonica*. Fish & shellfish immunology 83: 283-291. Available: https://doi.org/10.1016/j.fsi.2018.09.031.

Livak K.J. And T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25(4): 402-408.

Mahious A.S., FJ. Gatesoupe, M. Hervi, R. Metailler and F. Ollevier. 2006. Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, C. 1758). Aquaculture International 14: 219-229. Available: https://doi.org/10.1007/s10499-005-9003-4.

Panase, A., M. Thirabunyanon, J. Promya and C. Chitmanat. 2023. Influences of *Bacillus subtilis* and fructooligosaccharide on growth performances, immune responses, and disease resistance of Nile tilapia, *Oreochromis niloticus*. Frontiers in Veterinary Science 9: 1094681. Available: https://doi.org/10.3389/fvets.2022.1094681.

Parry Jr, R.M., R.C. Chandan and K.M. Shahani. 1965. A rapid and sensitive assay of muramidase. Proceedings of the Society for Experimental Biology and Medicine 119(2): 384-386. Available: https://doi.org/10.3181/00379727-119-30188.

Phumyu, N., S. Boonanuntanasarn, A. Jangprai, G. Yoshizaki and U. Na-Nakorn. 2012. Pubertal effects of 17α-methyltestosterone on GH–IGF-related genes of the hypothalamic–pituitary–liver–gonadal axis and other biological parameters in male, female and sex-reversed Nile tilapia. General and Comparative Endocrinology 177(2): 278- 29. Available: https://doi.org/10.1016/j.ygcen.2012.03.008.

Priya, P.S., A. Ashwitha, K. Thamizharasan, M. Harishkumar, S. Dinesh, N.T.G. and M. Kamaraj. 2021. Synergistic effect of durian fruit rind polysaccharide gel encapsulated prebiotic and probiotic dietary supplements on growth performance, immune-related gene expression, and disease resistance in zebrafish (*Danio rerio*). Heliyon. 7(4): 1-6. Available: DOI: 10.1016/j.heliyon.2021.e06669

Secombes, C. and T. Fletcher. 1992. The role of phagocytes in the protective mechanisms of fish Annual Review of Fish Diseases 2: 53-71. Available: https://doi.org/10.1016/0959-8030(92)90056-4.

Sookchaiyaporn, N., P. Srisapoome, S. Unajak and N. Areechon. 2020. Efficacy of *Bacillus* spp. isolated from Nile tilapia *Oreochromis niloticus* Linn. on its growth and immunity, and control of pathogenic bacteria. Fisheries science 86: 353-365. Available: https://doi.org/10.1007/s12562-019-01394-0.