Summary

Entrance Year：2017
United Graduate School of Agricultural Sciences
Course：Resource and Environmental Science of Agriculture, Forestry and Fisheries
Name：Zhang Yukun

Title	Nutritional studies on the utilization of probiotics and fermented soybean meal for cultured fish species

Key word（*Bacillus subtilis natto*） （Fermented soybean meal） （Replacement of fishmeal）

CHAPTER 1: Introduction and Purpose

The application of antibiotics as feed additives has a history of many years, with the increasing proportion of animal food consumption in people's lives, the safety of feed directly affects food safety through the transmission of the food chain, which makes the issue of feed safety increasingly prominent. The scientific search for effective solutions to the problems of feeding antibiotics has become a hot topic of research in animal nutrition and feeding science. The main alternatives to antibiotics are acid formulations, micro-ecological formulations, oligosaccharides, and plant extracts but the results of current animal tests on these alternatives vary widely and with varying results.

As a green feed additive, BSN is developing rapidly, which can not only improve the environment of breeding water but also improve the micro-ecological environment of the animal intestine, promote the secretion of digestive enzymes, improve the feed conversion rate, prevent animal diseases, and promote growth performance. This experiment was conducted to investigate the effects of BSN on the growth performance, serum biochemical indexes, non-specific immune indexes, antioxidant indexes, and intestinal function of juvenile sea bream by adding different proportions of BSN to the feeds of juvenile sea bream, to provide an experimental basis for the application of BSN as an alternative to antibiotics in aquaculture, and to explore the differences between different proportions of addition.

The FSM produced by different micro-organisms has different characteristics, among which *B. subtilis* meets the GRAS (Generally Recognized as Safe) principles (Safety principles, US FDA., 1989) and is an aerobic bacterium that produces budding spores under certain conditions. The special structure of the budding spores makes *Bacillus* resistant to acid and alkali, high temperature and extrusion, highly stable in the acidic environment of the intestine, and able to secrete strong active proteases and amylases to promote the digestion of feed nutrients. The ANFs in soya meal are fermented by the micro-organisms to improve their digestibility in the animal body and can therefore partially replace fish meal. The use of FSM prepared with *Bacillus subtilis* can reduce costs and reduce the digestive inhibitory effect of the anti-nutritional factors in FSM on animals.

The current research on the replacement of fishmeal by FSM is mostly focused on the growth

characteristics of FSM for animal bodies and the low replacement rate of fishmeal. However, there is less research on how to prepare high-quality FSM, improve the apparent digestibility of FSM for livestock and animal bodies, and thus improve the proportion of FSM replacing fish meal. Therefore, it is important to study FSM to improve the effective use of soybean protein.

CHAPTER 2: Culture of *Bacillus subtilis natto* and preparation of high-density bacterial agent

Microbial agents have been used for a long time in the brewing, fermentation industry, livestock aquaculture, and other fields. Microorganisms and their metabolites are also added to food as antioxidants, freshness agents, colorants, preservatives, sweeteners, and other roles. Microbial additives as natural food sources have been widely developed and utilized because microorganisms can be obtained quickly and inexpensively through biocultural methods. *Bacillus subtilis natto* (BSN) has been widely used in mammals, and it has been shown that BSN has a growth-promoting effect. And it has various functions such as promoting feed utilization, intestinal probiotics, regulating pH value in the intestine, secreting digestive enzymes, supplementing nutrients, and improving immunity.

In this experiment, BSN was expanded and cultured by fermentation technology, and feed was prepared, and the experiments in this chapter were used to provide test materials for subsequent research on the utilization of BSN in aquaculture.

CHAPTER 3: Effects of dietary *Bacillus subtilis natto* supplementation on growth, digestive enzyme activity, immune response, and intestinal microorganisms of red sea bream, *Pagrus major*

In this study, *Bacillus subtilis natto* (BSN) was evaluated on the growth, digestive enzyme activity, blood chemistry, immune response, and intestinal microorganisms of red sea bream (*Pagrus major*). Fish fed five different levels of BSN at 0 (BN0), 1×10^7 (BN1), 1×10^8 (BN2), 1×10^9 (BN3) and 1×10^{10} (BN4) CFU kg^{-1} diet for 56 days. The fish of BN3 and BN4 groups displayed better growth performance than the BN0 groups ($p < 0.05$). BN3 and BN4 groups significantly improved the specific activities of amylase and protease enzymes when compared to the control group ($p < 0.05$), while the lipase enzyme was increased significantly in fish fed BN3 diet when compared with the control diet. Furthermore, the plasma total protein was increased significantly in the fish fed BN3 diet when compared with the control diet ($p < 0.05$). Hematocrit values were significantly improved in BN2, BN3, and BN4 diets group ($p < 0.05$). When compared to the control, the number of *B. subtilis* and *Lactobacillus* sp. in intestinal content were significantly increased, and *Escherichia coli* was decreased in the BN4 group compared to all other groups ($p < 0.05$). The specific growth rate analysis and expressional regulation of the growth-related genes stimulated by BSN suggest the potential application of BSN in improving the growth performance on the red sea bream. Additionally, the supplementation of BSN in the diet of red sea bream at 1×10^9 and 1×10^{10} CFU/kg diet could improve the growth, health condition, and immune response.

CHAPTER 4: Effects of *Bacillus subtilis natto* and *Lactobacillus plantarum* on growth, intestinal microflora, immune and digestive enzyme gene expression in Japanese flounder, *Paralichthys olivaceus*

In this study, a 60-day feeding trial was conducted to evaluate the effects of a basal control diet (CON), a 10^{10} CFU/kg diet (BSN) of *Bacillus subtilis natto*, and a 10^{10} CFU/kg diet (LP) of *Lactobacillus plantarum*, and a mixed diet (BSN+LP) on Japanese flounder. The initial body weight of the fish was 5.81 ± 0.03 g (mean ± SD) and was fed twice daily. Weight gain, specific growth rate, feed efficiency, protein efficiency ratio, and lysozyme activity were significantly higher in fish fed BSN, LP, and BSN+LP diets than in fish fed CON diets. Superoxide dismutase was significantly higher in fish fed the BSN and BSN+LP diets than in fish fed the CON diet. Also, gene expression of interleukin (IL-1β) and tumor necrosis factor (TNF-α) were significantly higher in all probiotics provide groups than in fish fed the CON diet. The expression level of mRNA for trypsin (*try*) was significantly higher in probiotics provide groups compared to the CON group. Based on these results, 10^{10} CFU/kg of *B. subtilis natto* (BSN) and *Lactobacillus plantarum* (LP) exerted beneficial effects on growth, immunity, and disease resistance in Japanese flounder.

CHAPTER 5: Optimization of soybean meal fermentation for aqua-feed with *Bacillus subtilis natto* using the response surface methodology

This study aimed at improving the nutritional value of soybean meal (SBM) by solid-state fermentation (SSF) using *Bacillus subtilis natto* (BSN) to overcome the limitations of SBM usage in aquafeed. The response surface methodology (RSM) was employed to explore the relationships of fermentation conditions such as temperature, time, water-substrate ratio, and bacterial inoculation on the degree of protein hydrolysis (DH) and the crude protein content (CP). The optimum conditions for achieving the higher DH (15.96%) and CP (55.76%) were 43.82 °C, 62.32 h, 1.08 of water-substrate ratio, and a layer thickness of 2.02 cm. CP and DH in the fermented meal (FSM) increased by 9.8% and 177.1% respectively, and crude fiber decreased by 14.1% compared to SBM. The protein dispersibility index (PDI) decreased by 29.8%, while KOH protein solubility (KPS) was significantly increased by 17.4%. Flavonoids and total phenolic acid content in FSM were increased by 231.0% and 309.4% respectively. Neutral protease activity (NPA) also reached the highest level (1723.6 U g^{-1}). Total essential amino acids (EAA) in FSM increased by 12.2%, higher than the 10.8% increase of total non-essential amino acids (NEAA), while the total free amino acid (FAA) content was 12.76 times higher than that of SBM. Major antinutritional factors in SBM were significantly reduced during the process and almost all SBM protein macromolecules were decomposed. Together with the cost-effectiveness of SSF, BSN-fermented soybean meal products have great potential to improve the plant composition and replace high-cost ingredients in aquafeed, contributing to food security and environmental sustainability.

CHAPTER 6: Utilization of *Bacillus subtilis natto* fermented soybean meal for fishmeal substitution in diets of juvenile red sea bream, *Pagrus major*

This study aims to estimate the efficiency of BSN fermented soybean meal (FSM) for red sea bream. Firstly, optimized FSM solid-state fermentation process by response surface analysis (Chapter 4). Then, evaluated the effects of replacing fishmeal with FSM on growth performances, feed utilization, immune responses, intestinal bacteria, and intestinal morphology of red sea bream.

Triplicate groups of fish received five isonitrogenous diets where fishmeal was replaced with FSM at 0% (FM), 18% (FSM1), 36% (FSM2), 54% (FSM3), and 70% (FSM4), respectively. After 56 days, FSM2 and FSM3 treatments did not affect weight gain, feed conversion efficiency, and survival compared to control. The FSM4 treatment showed reductions in final body weight, specific growth rate and feed intake ($p < 0.05$). Furthermore, feed conversion ratio and survival were not affected by the FSM4 diet. Phagocytosis activity in FSM1 and FSM2 groups were significantly higher than in FM or FSM4 groups. Additionally, the number of goblet cells in the proximal intestine exhibited significantly higher levels in FSM1 and FSM2 compared to FM.

These findings suggested that protein from FSM can substitute up to 54% of fishmeal without negative effects on growth, nutrient utilization, immune response, or intestinal morphology of red sea bream. And the substitution of fishmeal by the FSM promoted immune responses at a moderate level (18% FSM, 36% FSM).

CHAPTER 7: General summary and conclusion

The present study examined the effect of *B. subtilis natto* on the growth performance and health level of red sea bream, and the beneficial effects of *B. subtilis. natto* and *Lactobacillus plantarum* in Japanese flounder feeds. Then a study on the fermentation of SBM by BSN was conducted. After evaluating the nutritional value of FSM, examined the potential applications of FSM as practical ingredients in diets for red sea bream (*Pagrus major*). Various growth, nutrient utilization, blood health, immune response, and stress resistance parameters were employed to evaluate the influence of microbial fermentation on the utilization of SBM by the *B. subtilis natto*.

To summarize the present study results from different experiments, it was found that:

1. The dietary administration of *B. s. natto* at the level of 1×10^9 and 1×10^{10} CFU/kg of feed has a positive effect on the growth performance, digestive enzymes activity, hematology parameters, and intestinal flora homeostasis of red sea bream.

2. Dietary separately or mixed administration of *B. s. natto* and *L. plantarum* at 1×10^{10} (CFU/g) could enhance growth performance, immune responses, and digestive enzyme related gene expression in Japanese flounder.

3. Fermentation temperature 43.82°C, fermentation time 62.32 h, water-material ratio 1.08 and SBM layer thickness 2.02 cm were the most suitable solid state fermentation condition for obtaining high protein content and the degree of protein hydrolysis of SBM with *B. subtilis natto*.

4. The *B. subtilis natto* FSM had low anti-nutritional factor levels, higher protein level, higher free amino acid level, relatively high antioxidant activity and neutral protease activity.
5. *B. subtilis natto* FSM can substitute up to 54% of fishmeal without negative effects on growth, nutrient utilization, immune response, or intestinal morphology of red sea bream juvenile.
6. At a moderate level (18% FSM, 36% FSM) the substitution of fishmeal by the *B. s. natto* FSM promoted immune responses.

There is increasing evidence that natural feed additives can have beneficial effects on aquaculture animals by supporting well-balanced gut microflora and improving fish health. Microbial fermentation enhances the nutritional quality of SBM, offering an effective approach to improving the quality of plant proteins sources for aqua-feed. The results of this study suggest that it is possible for FSM containing probiotic bacteria to have a high inclusion rate in marine fish feeds, providing an opportunity to develop practical feeds with high levels of plant protein sources. However, with the focus on growth, resistance, and immune response, further research is needed on how microbially FSM regulates gene expression in fish and the feed-attracting of fermented products.