THE USE OF SELECTED PREBIOTICS AND PROBIOTIC IN FEED DEVELOPMENT FOR STRIPED CATFISH (*Pangasianodon hypophthalmus*, Sauvage, 1878) JUVENILES: EFFECTS ON GROWTH PARAMETERS AND HEALTH STATUS

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by

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ABBREVIATIONS

ACH50	Alternative haemolytic complement activity
ADM	Apparent dry matter digestibility
ANOVA	One-way analysis of variance
AOAC	Association of official analytical chemists
APD	Apparent protein digestibility
CLRs	C-type lectin receptors
CMC	Carboxy methyl cellulose
CJ	Crypta junction
DNA	Deoxyribonucleic acid
ESR	Erythrocyte sedimentation rate
FAO	Food and agriculture organization
FM	Fish meal
FOS	Fructooligosaccharides
FCR	Food conversion ratio
GE	Gross energy
GI	Gastrointestinal
GOS	Galactooligosaccharides
Hb	Haemoglobin
HSI	Hepatosomatic index
Ig	Immunoglobulin
IPF	Intraperitoneal fat
LAB	*Lactobacillus acidophilus*
LC	Leukocyte count

LM	Light microscopy
MAMPs	Microbe-associated molecular patterns
MAS	Motile aeromonad septicaemia
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MOS	Mannanoligosaccharide
NFE	Nitrogen free extract
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PER	Protein efficiency ratio
PEG	Polyethylene glycol
PGRPs	Peptidoglycan recognition proteins
PRRs	Pattern recognition receptors
PVA	Polyvinyl alcohol
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SBM	Soybean meal
SCFAs	Short chain fatty acids
scFOS	Short-chain fructo oligosaccharides
SGR	Specific growth rate
SPSS	Statistical package for social science
TEM	Transmission electron microscopy

VSI	Viscerosomatic index
V	Villus
VL	Villus lenght
VW	Villus width
WG	Weight gain
WBC	White blood cell
WHO	World health organization

PENGGUNAAN PREBIOTIK DAN PROBIOTIK TERPILIH DALAM PEMBANGUNAN MAKANAN IKAN PATIN BERJALUR (*Pangasianodon hypophthalmus*, Sauvage, 1878) JUVENA: KESAN TERHADAP PARAMETER PERTUMBUHAN DAN STATUS KESIHATAN

ABSTRAK

Satu siri eksperimen telah dijalankan untuk memahami bagaimana probiotik dan prebiotik dapat meningkatkan prestasi pertumbuhan dan menggalakkan status kesihatan ikan patin berjalur juvena. Eksperimen pertama telah dibahagikan kepada empat percubaan untuk menilai kesan pemberian tahap yang berbeza bagi β-glukan (0%, 0.1%, 0.2%, 0.5%, 1%), mannanoligosakarida (MOS) (0%, 0.2%, 0.4%, 0.5 %, 1%), galaktooligosakarida (GOS) (0%, 0.5%, 0.75%, 1%, 1.25%), dan yis (0%, 0.5%, 0.75%, 1%, 1.25%) terhadap prestasi pertumbuhan, penghadaman dan parameter hematologi dan imunologi. Semua diet telah diformulasi untuk mengandungi 30% protein mentah, 12% lipid dan diberi makan kepada ikan patin berjalur juvena yang dipelihara dalam akuarium (berat purata 13-15 g) dua kali sehari. Penambahan 0.1% β-glukan, 0.4% mannanoligosakarida (MOS), 1% galaktooligosakarida (GOS), dan 1% yis dapat meningkatkan pertumbuhan dan juga beberapa parameter hematologi dan imunologi ikan patin berjalur. Kepekatan ini seterusnya digunakan dalam eksperimen kedua, dimana prebiotik digunakan secara tunggal dan secara kombinasi dengan yis. Lapan diet isonitrogenous yang mengandungi probiotik (1% yis), prebiotik (1% GOS, 0.4% MOS, 0.1% β-glukan, secara berasingan), sinbiotik (1% yis + 1% GOS (YGOS), 1% yis + 0.4% MOS (YMOS) dan 1% yis + 0.1% β-glukan (YβG)) telah diberikan kepada ikan di dalam

tangki fiber dua kali sehari, sementara diet kawalan tidak mengandungi sebarang suplemen. Kesan daripada suplemen ini terhadap kerintangan jangkitan *Aeromonas hydrophila* (*A. hydrophila*) juga dikaji. Hasil kajian menunjukkan bahawa diet prebiotik dan sinbiotik mempunyai kelebihan dibandingkan dengan diet kawalan dalam semua parameter yang dikaji. Pengambilan yis tidak berkesan secara signifikan terhadap parameter pertumbuhan ikan patin berjalur, tetapi memberi kesan positif terhadap respon keimunan dan kerintangan terhadap jangkitan *A. hydrophila.* Kajian ini juga menunjukkan bahawa prestasi pertumbuhan lebih baik pada ikan yang diberi diet 1% GOS dan YβG dibandingkan dengan diet yang lain. Dalam percubaan terakhir, kesan penambahan 1% GOS dan YβG dalam makanan mengandungi serbuk kacang soya (SBM) pada ikan patin berjalur telah dinilai. Empat diet telah diformulasi dengan kandungan 100% protein dari serbuk ikan (FM), 55% / 45% FM / SBM protein (FM-SBM), FM-SBM + 1% GOS, dan FM-SBM + YβG. Parameter yang sama seperti pada percubaan kedua telah dinilai dan hasil kajian menunjukkan bahawa parameter pertumbuhan, penghadaman, dan morfologi usus meningkat secara signifikan dalam diet SBM yang ditambah dengan GOS dan YβG. Selepas 3 minggu dijangkiti dengan *A. hydrophila*, tahap kemandirian, parameter hematologi dan imunologi adalah lebih tinggi secara signifikan dalam ikan yang diberi suplemen GOS dan YβG dibandingkan dengan ikan yang diberi makan diet SBM yang tidak disuplemen.

THE USE OF SELECTED PREBIOTICS AND PROBIOTIC IN FEED DEVELOPMENT FOR STRIPED CATFISH (*Pangasianodon hypophthalmus,* Sauvage, 1878) JUVENILES: EFFECTS ON GROWTH PARAMETERS AND HEALTH STATUS

ABSTRACT

A series of experiments were conducted to understand the influence of selected probiotics and prebiotics on growth performance and health status of juvenile striped catfish (*Pangasianodon hypophthalmus*). The first feeding trial was subdivided into four separate experiments to evaluate the effect of different levels of β-glucan (0%, 0.1%, 0.2%, 0.5%, 1%), mannanoligosaccharide (MOS) (0%, 0.2%, 0.4%, 0.5%, 1%), galactooligosaccharide (GOS) (0%, 0.5%, 0.75%, 1%, 1.25%), and yeast (0%, 0.5%, 0.75%, 1%, 1.25%) on growth performance, digestibility, haematological and immunological parameters. All diets were formulated to contain 30% crude protein 12% lipid and were fed to striped catfish (average weight 13 - 15 g) twice daily. The supplementation of 0.1% β-glucan, 0.4% MOS, 1% GOS, and 1% yeast could improve growth, digestibility, and some haematological and immunological parameters of striped catfish. These levels were then supplemented in a second feeding trial, in which the prebiotics were used either singly and in combination with yeast. Eight isonitrogenous diets containing probiotic (1% yeast), prebiotics (1% GOS, 0.4% MOS, 0.1% β-Glucan, respectively) and synbiotics (1% yeast + 1% GOS (YGOS), 1% yeast + 0.4% MOS (YMOS) and 1% yeast + 0.1% β-glucan (YβG)) were fed to fish twice daily, while the control did not contain any supplements. The effect of these diets on the resistance to *Aeromonas hydrophila* (*A. hydrophila*) infection were also observed. The results showed that the prebiotics and

synbiotics diets performed better than the control diet in all parameters studied. Intake of yeast alone did not have any significant influence on growth parameters of striped catfish, but positively affected immune response and resistance to *A. hydrophila*. This study also showed that growth performance was better in fish fed 1% GOS and 1% yeast + 0.1% β-glucan (YβG) diets over the other diets. In the final feeding trial, the effect of 1% GOS and YβG supplementation in diets containing soybean meal (SBM) on striped catfish was evaluated. Four diets were formulated to contain 100% protein from fish meal (FM), 55% /45% FM/SBM protein (FM-SBM), FM-SBM + 1% GOS, and FM-SBM + YβG. The same parameters in the second feeding trial were evaluated and the results showed that growth parameters, digestibility, and intestinal morphology significantly improved in SBM diet supplemented with either GOS or YβG. At 3-week post challenged with *A. hydrophila,* the survival, haematological and immunological parameters were significantly higher in fish maintained on GOS and YβG supplemented diet compared to fish fed unsupplemented SBM diet.

CHAPTER 1

INTRODUCTION

1.1 Background

Aquaculture is the rearing of aquatic organism under the human control to increase production. Its contribution to global food production in the world has increase in the last three decades, particularly in developing countries (FAO, 2014). In 2014, production of food fish from aquaculture amounted 73.8 million tonnes which has been surpassed the capture fisheries (FAO, 2016a). The elevation of fish production achieved by intensification of the existing aquaculture system involving an increase in fish stocking density (Hoseinifar *et al*., 2015). However, the increasing of fish stocking density has induced physiological stress in the cultured fish which accelerate the disease outbreaks in fish (Pohlenz and Gatlin III, 2014) and finally responsible for huge fish production losses and hindered the sustainability of the aquaculture industry (Torrecillas *et al*., 2007).

To mitigate the problem occurred due to intensification process of aquaculture, vaccination can be used as one of method for controlling fish disease, but the commercial vaccine is costly and its availability is still limited or in the early stage of development (Yoesefian and Amiri, 2009). Therefore, antibiotics are still used as traditional strategy for dealing with fish diseases management (Denev *et al*., 2009). Several antibiotics have been regularly used as a prophylactic and medication to control pathogens (Cabello, 2006) and sub therapeutic doses of antibiotics are also often added in aquafeeds for the improvement of growth (Austin and Austin, 2007). However, these agents may cause a deterioration of the aquaculture environment and adversely affect the health status of fish and consumer particularly for long term application. The inappropriate use of antibiotics in some cases has been responsible for long term negative effects in fish such as increase antibiotic accumulation in fish tissue, develop antibiotic resistant bacteria, suppress immune system, destruct beneficial microbiota in fish and many other problems such as environmental hazards and food safety problems (Sapkota *et al.*, 2008; Li *et al*., 2009; Yousefian and Amiri, 2009; Villamil *et al*., 2014). Considering the adverse effects due to therapeutic and prophylactic use of antibiotic in aquaculture, supplementation of prebiotics, probiotics and synbiotics in fish feed have received increasing attention as eco-friendly strategy for promoting fish growth and health status (Hoseinifar *et al*., 2015). The use of probiotics and/or prebiotics in aquaculture has not only resulted in the reduction of using antibiotics but also improve intestinal microbial balance and growth performance of the fish species towards the increasing of fish production (Gatesoupe, 1999, Robertson *et al*. 2000).

Probiotics are live microbial feed supplement that have beneficial effects on the host by improvement of the intestinal microbiota and feed value, production of enzyme that contribute for digestion, inhibition of pathogenic microorganisms, promotion of growth rate and modulation of immune response (Verschuere *et al*., 2000). Currently, the commercial probiotic products used in aquaculture were prepared from bacterial species such as *Bacillus* sp., *Lactobacillus* sp., *Carnobacterium* sp., *Enterococcus* sp., and from the yeast (*Saccharomyces cerevisae*) (Cruz *et al*., 2012).

Although the use of probiotics for growth improvement and disease control has been extensively promoted, the large-scale use of probiotic in commercial aquaculture may be constrained due to several problems related with handling, pelleting and storing (Merrifield *et al*., 2010a). There is also a possibility that exogenous administration of a single probiotic may not result in long-term microbiota colonization in the gut, particularly when the probiotics used do not belong to the normal dominant intestinal flora (Verschuere *et al*., 2000). In addition, viability after ingestion is difficult to guarantee and hardly to prove. Under these circumstances, the stimulation of beneficial indigenous microbiota by supplementing fish diet with indigestible carbohydrates that act as prebiotic, could be a more sustainable method to improve the proportion of stable and healthy gastrointestinal microbiota in fish which in turn stimulate immune response, metabolism, and growth of the fish (Yousefian and Amiri, 2009).

Prebiotics are non-digestible food ingredients comprising mainly carbohydrate that beneficial to the host by selectively stimulating the growth and/or the metabolism activity of one or limited number of indigenous bacteria in the intestinal tract, thus improving the host's health (Gibson and Roberfroid, 1995). Many substances have been included as prebiotics such as ß-glucan, fructooligosaccharides (FOS), galactooligosacharide (GOS), Inulin, mannanoligosaccharides (MOS), and xylooligosaccharides (XOS) (Qiang *et al*., 2009). The incorporation of these natural compounds in fish diet do not require particular precautions and simpler than required for antimicrobial or chemical therapeutic agents.

Combining probiotics and prebiotics in foods may enhance the effect of the foods and creates a synergy between the two elements rather than the application of individual pre- and probiotic (Merrifield *et al*., 2010a). Recently, there has been a growing interest in understanding the combined effect of probiotics and prebiotics in aquaculture, call synbiotic. Synbiotic is defined as nutritional supplements of combined used of probiotics and prebiotics which beneficially affects the host and improves host welfare by improving the survival and colonization of live microbial dietary supplements in the gastrointestinal tract by selective stimulating the growth and/or metabolism activity of one or few number of health-promoting bacteria (Gibson and Roberfroid, 1995). The application of synbiotic in fish farming have been demonstrated to enhance growth rate, feed efficiency, nutrient digestibility and enzymes activities, disease resistance, and stimulation of the immune parameters (Kesarcodi-Watson *et al.,* 2008; Wang *et al*., 2008; Merrifield *et al*., 2010a).

1.2 Problem Statement

Striped catfish (*Pangasianodon hypophthalmus*) is a fast-growing omnivorous fish that has great economic importance in the South-East Asia countries. In Malaysia, the culture of this has been expanding and is considered as one of the favourite freshwater fish in local consumers (Asdari *et al*., 2011). However, disease in striped catfish culture is the major constraints for its sustainable development (Crumlish *et al*., 2010, Magnadottir, 2010). Several pathogens such as fungi, parasite and bacteria have been shown to be associated with disease in striped catfish. Among the infectious disease, bacteria are the main pathogen that affect the striped catfish culture. The rate of fish loss due to bacterial disease (particularly caused by pathogenic *Aeromonas hydrophila* (*A. hydrophila*), *Edwardsiella ictaluri* and *Flexibacter collunaris*), has been estimated as high as 50 % (Phuong *et al*., 2007), thus responsible for the huge economic loss during disease outbreaks and become a main threat to the sustainability of striped catfish farming industry.

In recent years, prebiotics and/or probiotics are under extensive investigation for evaluate their potential beneficial effects on fish health, growth and survival (Verschuere *et al*., 2000; Irianto and Austin 2002; Mussatto and Mancilha, 2007;

Grisdale-Helland *et al*. 2008; Yousefian and Amiri, 2009). Although the finding of several scientific research in aquatic animals suggests that the intake of prebiotic and/or probiotics may have wide range beneficial effects on growth and survival of the host (Fuller, 1989; Gatesoupe,1999; Moriarty, 1998; Verschuere *et al*., 2000; Irianto and Austin, 2002; Mussatto and Mancilha, 2007; Zhou *et al*., 2007; Burr *et al*. 2008; Grisdale-Helland *et al*., 2008; Yousefian and Amiri, 2009; Abd El-Rahman *et al*., 2009), the results have been conflicting. While some researchers have shown that inclusion of prebiotics and/or probiotics in the diet of fish or direct inoculation into the aquatic environment changes gut microbiota, improves growth and survival (Aly *et al*., 2008; Merrifield *et al*., 2010b; Wang *et al.*, 2008), others have reported no beneficial effects (Shelby *et al*., 2006; Marzouk *et al*., 2008; Abd El-Rahman *et al*., 2009; Ferguson *et al*., 2010). Variation among research is probably due to differences in prebiotics and probiotics used, their level in the diet, fish species and age, duration of feeding and feeding management, pathogen dosage and virulence during challenge trial, and methods of challenge (Merrifield *et al*., 2010a).

The use of commercial yeast, *Saccharomyces cerevisiae*, has many proven benefits as feed additives in many terrestrial animals for many reasons such as being a good source of protein and having a probiotic activity (Gatesoupe, 1999; Irianto and Austin, 2002; Abdelhamid *et al*., 2009). Yeasts have several attributes for consideration as good probiotic candidates such as not affected by anti-bacterial compounds, some strains have antagonistic activities against undesirable bacteria (Hatoum *et al*., 2012), can stimulate intestine maturation (Tovar *et al*., 2002), and modulate antioxidant enzyme in host fish (Tovar-Ramírez *et al*., 2010). Moreover, yeast contains various immunostimulant compounds (e.g., β-glucans, nucleic acids, and mannan oligosaccharides), which can explain, in part, the protective effect against pathogens (Li and Gatlin, 2006). Despite their multiple attributes, most of the studies of the effect of baker's yeast on the growth and immune response in different fish species have focused on analysis of the effects of whole yeast cells, or of specific components that were isolated from whole yeast cells, such as nucleotides, mannanoligosaccharide or β-glucan (Welker *et al.*, 2007). So far, the information regarding the use of combination of yeast with commercial prebiotic in striped catfish are not available.

The yeast polysaccharides β-glucan and MOS are two prebiotics that commonly used in aquaculture (Selim and Reda, 2014). However, very limited data are available about their application in striped catfish diets with different in experimental results. Supplementation of 0.1% or 0.2% fungal-derived β-glucan giving optimal immunostimulation in striped catfish after 4 weeks feeding trial compared with the basal diet (Sirimanapong *et al.*, 2015), while feeding β-glucan at concentrations of 0.5%, 1% and 2 % for 9 weeks did not affect the growth performance and survival parameters of striped catfish following cold shock treatment (Adloo *et al.*, 2015). In a study conducted by Tamamdusturi *et al.* (2016) found that supplementation of 0.2% mannanoligosaccharide significantly improve growth performances and immune responses of striped catfish, whereas Akter *et al.* (2015) obtained the optimal growth and feed utilization in striped catfish fed on 0.6% MOS. Based on the aforementioned fact, a series of study is needed to ascertain the responses of striped catfish on supplementation of yeast, β-glucan and MOS in both singly and combination. The effect of GOS, another commonly prebiotic used in aquaculture, was also evaluated either singly or in combination with yeast since the use of this prebiotic has never been reported in striped catfish.

1.3 Research Objectives

The main objective of this study was to determine the effect of supplementation of probiotic yeast and prebiotics GOS, MOS and β-glucan used singly and in combination with yeast in promoting growth and health status of striped catfish (*Pangasianodon hypothalamus*) juvenile.

The specific objectives of this study were:

1. To determine the suitable levels of prebiotics (β-glucan, MOS, GOS) and probiotic yeast (*Saccharomyces cerevisae*) for striped catfish juvenile.
2. To determine the effects of selected probiotic yeast and prebiotics GOS, MOS and β-glucan used singly and in combination with yeast on growth performance, nutrient digestibility and digestive enzyme activity, intestinal morphology, gut microbiota, haematological and immunological parameters.
3. To evaluate the effectiveness of selected prebiotic and/or probiotic supplementation in soybean meal based diet on growth, digestibility, intestinal morphology, microbiota community, haematological and immunological response.
4. To study the resistance of striped catfish fed with prebiotics, probiotic and synbiotics againts *A. hydrophila* infection.

CHAPTER 2

LITERATURE REVIEW

2.1 Global Aquaculture Production

The world population is expected to increase from nearly 7 billion in 2011 to approximately 8.5 billion by 2030 and to 9.7 billion by 2050 (UN, 2015) and the aquaculture plays an important role in dietary supply of food and specifically protein for this rising population. Aquaculture has expanded at a remarkable rate over the last three decades which assisted to yield more food fish, keep down the overall fish price, and provide fish and seafood more accessible to consumers (FAO, 2014). While capture fishery production relatively static since the late 1980s, aquaculture production has continued to exhibit impressive increase in the supply of fish demanded by the world's growing population (FAO, 2016b). In 1974, aquaculture provided only 7 percent of fish for human consumption, but it increased to 26 percent in 1994 and 39 percent in 2004, and in 2014 the aquaculture sector contribute to the supply of almost half of worlds's food fish consumption, overtaking capture fisheries for the first time (FAO, 2016a).

Within aquaculture, the freshwater based production is prominent and accounted for nearly 62% of the total aquaculture production in 2010 (increasing from approximately 50% in 1980) whereas marine water based production supplies approximately 30% (declining from 40% in the same period) (FAO, 2014). One of the largest and dramatic changes in aquaculture supply has been from Vietnam pangas catfish farming, which is 1.4 million metric tonnes were produce in 2011 (FAO, 2014). Pangas catfish include 10 different species and the striped catfish

(*Pangasiandon hypophthalmus*) more suited to the intensification of farming practice than traditionally farmed *P. bocourti* (Da *et al*., 2012).

2.2 Striped catfish (*Pangasianodon hypophthalmus*)

Pangasianodon hypophthalmus, formerly known as *Pangasius sutchi* or *Pangasius hypophthalmus*, is a freshwater fish belongs to the familiy of catfish that native live in a tropical climate. The species has a variety of common English names such as iridescent shark-catfish, sutchi catfish, and striped catfish. This fish is from the Chao Phraya and Mekong river basins (Rainboth, 1996) and also found in the Ayeyawady basin of Myanmar (Ha *et al*., 2009), Amazon River, in parts of Russia and in other places of the world with different names (Abbas *et al*., 2006). The fish is extensively cultured by commercial fish farms in Vietnam, Thailand, Malaysia, China, Indonesia, India, Bangladesh and Myanmar (Abbas *et al*. 2006; Dung *et al*., 2008; Islam *et al*., 2006; Rahman *et al*., 2006; Singh *et al*., 2011), however Vietnam remains the largest global producer (Globefish, 2012). The last decade has seen the rapid expansion in pangasius production throughout of Asia India, Myanmar, Indonesia and Bangladesh, but it most notably in Vietnam, which produces more than 1 million tonnes yearly for export purposes (Belton *et al*., 2011).

Striped catfish has scale less skin with a long body, flattened, head relatively small with eyes relatively large (Plate 2.1). The mouth is located in low position with small sharp teeth on jaw and palatal bones. They have two pairs of barbels of which the shorter pair is on the upper (maxillary barbel) and the longer pair in the lower (mandibulary barbel). Fins colour is dark grey or black with one hard fin and six branched dorsal-fin rays (Kottelat, 2001). The juvenile fish have black stripe along lateral line and another long black stripe below lateral line, whereas large adults consistently black or dark grey with light grey in lateral side and silvery in the abdominal side (Rainboth, 1996).

Plate 2.1 Striped catfish (*Pangasionodon hypophthalmus*, Sauvage, 1878)

Striped catfish mostly is a bottom feeder fish, omnivorous in nature that consume fish, crustacean, and plant debris (Riede, 2004). They have a preference to live in large water bodies with shallow depth. As inhabitant in a tropical climate, striped catfish prefer water with temperature of 22-26º C and pH of 6.5-7.5 (Riede, 2004; Riehl and Baensch, 1996). They are migrant fish that moving up the river when ready to spawn during the flood season and traveling down the river as juveniles when water levels decrease (Rainboth, 1996).

According to Eschmeyer (1998), taxonomic hierarchy of striped catfish as follows:

Kingdom	Animalia
Subkingdom	Bilateria
Infrakingdom	Deuterostomia
Phylum	Chordata
Subphylum	Vertebrata
Infraphylum	Gnathostomata
Superclass	Osteichthyes
Class	Actinopterygii
Subclass	Neopterygii
Infraclass	Teleostei
Superorder	Ostariophysi
Order	Siluriformes – silures, catfishes
Family	Pangasiidae – shark catfishes
Genus	Pangasianodon
Species	*hypophthalmus*
Scientific name	Pangasianodon *hypophthalmus* (Sauvage, 1878)

2.3 Fish Disease and Prophylactic Strategy

Disease is a major constraint to the growth of the aquaculture industry and severely hinders both economic and socio-economic development in many producer countries (Austin and Austin, 2007). The occurrence of microbial pathogens, particularly bacterial pathogen, is one of the most common factors that affecting fish farming (Zorilla *et al*., 2003). Fish are continually exposed to bacteria and will normally susceptible to an infection when exposed to prolonged periods of sub-acute stress or shorter periods of acute stress (Shayo *et al*., 2012). Despite continuous efforts by farmers to manage overall health and well-being of their fish, some bacterial pathogens continue to disturb the culture of the fish, resulting in decreased survivability and profitability (Addo, 2013). Therefore, the most important objective in aquaculture production is the maintenance of the fish freedom of disease to sustain high growth rates and survival which in turn leads to a greater profitability.

Disease by pathogen generally occurs when several factors coincide such as the presence of virulent pathogen (bacteria, viruses, fungi and parasites) that cause disease in the host (Grimholt *et al*., 2003). The other factor is the susceptibility of the fish to the pathogen and endogenous factor such as age or the genetic status of a fish may have an effect on susceptibility of the fish (Houghton *et al*., 1991; Wiegertjes *et al.*, 1995; Arkush *et al*., 2002; Grimholt *et al*., 2003). The conditions in the fish environment which can impair normal physiological processes may also cause disease. Stressors include adverse water conditions, high stock density, handling (harvesting, grading, transport, and therapeutic treatments), frequent disturbances, inadequate nutrition (nutrient deficiencies, feeding regime, toxins in the diet) and a general lack of sanitation (Schaperclaus, 1991; Gatlin III, 2002).

The intensification of pangasius production nowadays is also accompanied with the emergence of some disease agents such as bacteria and parasites (Phan *et al*., 2009). One of the common bacteria cause diseases reported in pangasius farming is *Aeromonas hydrophila* (Subagja *et al*., 1999).

Aeromonas hydrophila (*A. hydrophila*) is a ubiquitous gram-negative rod-shaped bacterium in aquatic habitats which commonly attacks freshwater fish both farmed and wild fish such as catfish, and many species of tropical or ornamental fish (Zhang *et al*., 2014). This bacterium causes disease in fish known as motile aeromonad septicaemia and haemorrhagic septicaemia (da Silva *et al*., 2012) and epizootic ulcerative syndrome (Austin and Adam, 1996) depending on the lesions caused by this bacterium which include septicaemia within numerous organs of the fish and ulcers of the fish skin.

Although Aeromonas are well known as pathogens of fish, it is important to note that these bacteria also constitute part of the normal intestinal microflora of healthy fish (Cahill, 1990). Therefore, the presence of these bacteria by itself is not indication of disease and stress factors due to culture conditions, such as poor water quality, overcrowding and rough handling, enhance the susceptibility of fish to these bacteria (Shayo *et al*., 2012) and lead to outbreaks of disease (Cipriano, 2001). The pathological lesions attributed to *A. hydrophila* infections may be only seen in the skin or internal organs but sometimes the lesions spread to other body sites causing systemic infection (Majumdar *et al*., 2006). When disease is acute, as with disease challenge studies, fatal septicaemia may occur rapidly that fish die before the development of anything but a few symptoms of the disease may be occurred such as haemorrhages at the base of fins, operculum or on the skin, distended abdomens, swollen eyes, accumulation of fluid in the abdomen and intestines, swollen liver and spleen (Plumb, 1999). It is commonly known that several interrelated factors influence severity of the disease, including stress factors, virulence level of Aeromonas strains, the physiologic condition of the host, and the degree of genetic resistance inherent within specific populations of fishes (Kozinska and Pekala, 2012).

Depending on the type of Aeromonas bacteria the fish has, usually antibiotics were use as medication to eliminate the infection either be injected into the fish, added to the fish water, or added in the feed (Jeeva *et al*., 2011). The antibiotic agents and other chemical additive such as anabolic steroid and growth promotors were also commonly administered in feed to control the outbreak of other bacterial diseases in commercial aquaculture (Defoirdt *et al*., 2011; Faggio *et al*., 2015; Gaunt *et al*., 2010). However, new regulations and consumer preferences prohibit the aquaculture industry from the use of antibiotics and other synthetic additives (Addo, 2013). Furthermore, the use of antibiotics in aquaculture has received considerable attention because their abuse has led to the emergence antibiotic resistance, reducing drug efficacy, can pose potential risk to consumers and the environment (Carrias *et al*., 2012) and depress the fish immune system (Hoseinifar *et al*., 2016a). To meet the increasing consumer demands for fish products that have not been treated with antibiotics and at the same time maintaining good health and growth, it is needed to seek the alternative control strategies such as addition of supplements to substitute the use of antibiotic (Merifield *et al*., 2010a). In recent years, prebiotics, probiotics and their combination are under extensive investigation for their potential as antibiotic substitution due to their beneficial effects on fish health and growth (Denev *et al*., 2009).

2.4 Application of Probiotics, Prebiotics, and Synbiotics in Aquaculture

A numerous feed additives, including probiotics and prebiotics and their combination (synbiotics) pose beneficial effects to the host and was used in aquaculture to fight disease, improve growth, stimulate immunity response of the host, as well as act as an alternative antimicrobial compound (Irianto and Austin, 2002; Yousefian and Amiri, 2009).

Even the use of prebiotics, probiotics and their combination in aquaculture is now widely accepted; however, some results on their efficiency from a few studies have been conflicting (Gatesoupe, 2005; Grimoud *et al*., 2010). Variation among research is probably due to differences in prebiotics and probiotics used, their level in the diet, fish species and age, duration of feeding and feeding management, pathogen dosage and virulence during challenge trial, and methods of challenge. In addition, fish stocking densities, fish handling, and environmental conditions may also affect the results. All these factors can influence the success or failure of the use prebiotics, probiotics and their combination in the enhancement of growth, immunity and/or disease resistance in fish (Merrifield *et al*., 2010a).

2. 4. 1 Probiotics

The definition of probiotic in aquaculture is a live, dead or component of a microbial cell that when administered via the feed or the rearing water exert beneficial effect to the host by improving either growth performance, feed efficiency, stress response, disease resistance, health status, or general potency which is achieved through improving the hosts microbial balance or the microbiota balance of the ambient environment (Faramarzi *et al*., 2011). The aim of administering probiotics is manipulation of the gut microbiota to improve the beneficial impacts of the commensal microbes through exclusion of opportunistic pathogens and stimulation of the immune system (Balcazar *et al*., 2006; Ringø *et al*., 2010a; Verschuere *et al*., 2000). The ideal candidates for probiotics are dominant members of the normal microbiota in the fish species (Verschuere *et al*., 2000; Olivia-Teles, 2012).

The other criteria for the selection of potential probiotics include:

- Have ability to resist and survive of high pH effect in the stomach, biliary secretions and pancreatic secretions in order to reach the small and large intestines
- Non-pathogenic and non-toxic to the host
- Well-accepted by the host through ingestion, may colonize and proliferate in the gut
- Exert beneficial effects on the host such as provide essential nutrient to the host
- Stabilize the intestinal microflora
- Adhere to the intestinal epithelial cell lining and produce antimicrobial substances towards pathogen (Verschuere *et al*., 2000; Spanggaard *et al.*, 2001; Balcázar *et al*., 2006; Vine *et al*., 2006; Gómez and Balcázar, 2008).

There are several proposed modes of action by which probiotics may protect the host from the intestinal disorder (Lee *et al*., 1999; Rolfe *et al*., 2000) and exert beneficial effect to the host. Firstly, probiotic produce several inhibitory substances and antibacterial molecules such as organic acid, hydrogen peroxide and bacteriocins that directly inhibit other bacteria or viruses in the gut and actively contributing in the fight against pathogen (Fuller, 1989). Secondly, probiotic may competitively inhibit the bacteria adhesion on the intestinal epithelial surface which will allow the probiotic to rapidly colonize the intestinal tract (Conway *et al*., 1987), thus may prevent the utilization of nutrients by pathogenic bacteria. Thirdly, probiotic can protect against disease by stimulating the production of immune molecules or modulating the inflammatory agents (Fekushima *et al*., 1998).

In aquaculture, a wide range of microorganisms as probiotic have been investigated in numerous study in which both Gram-positive and Gram-negative bacteria have been administered effectively (Hai, 2015), but the best documented so far are lactic acid bacteria, *Bacillus* spp. and yeasts (Gatesoupe, 2008; Nayak, 2010; Dimitroglou *et al*., 2011). Nowadays, several probiotics either as monospecies or multispecies supplements are also commercially available for aquaculture practices (Nayak, 2010). One of the first evaluations of commercial products in fresh water fish focused on a bacterial preparation called Biostart that is derived from Bacillus isolates which was used during the production of catfish (Queiroz *et al*., 1998). Further, Taoka *et al*. (2006) observed that the use of Alchem Poseidon and Alchem Korea CO and Wonju Korea CO, which have mixed cultures of bacteria (*Bacillus subtilis*, *Lactobacillus acidophilus*, and *Clostridium butyricum*) and yeast (*Saccharomyces cerevisiae*), enhanced nonspecific immune parameters of tilapia (*Oreochromis niloticus*) such as lysozyme activity, migration of neutrophils, and plasma bactericidal activity, resulting in improvement of resistance to *Edwardsiella tarda* infection. Meanwhile, Biogen (a *Bacillus subtilis* strain combined with hydrolytic enzymes) was used to supplement the feed of *Oreochromis niloticus* to increases their productivity (Haroun *et al*., 2006).

2.4.1(a) The role of probiotics

Effect on growth and feed utilization

Applications of probiotics may enhance the growth rate of the fish by direct increase in nutrient uptake (Irianto and Austin, 2002), and by providing the nutrients (Sakata, 1990; Kolndadacha *et al*., 2011). The probiotic can stimulate the appetite of the fish (Abdelhamid *et al*., 2009) by improve quality of diet (Al-Dohail *et al*., 2009), or by improved the digestibility (Bidhan *et al*., 2014) and as consequence will enhance growth rate. Probiotics also have a beneficial effect on production of essential nutrients such as biotin and vitamins B 12 (Sugita *et al*., 1991), fatty acids

(Vine *et al*., 2006), and amino acids (Balcazar *et al*., 2006) all leading to improved growth performance (Welker and Lim, 2011).

Effect on digestive enzyme

The digestive organs and enzyme activity to be influenced by ingested feed ingredients, which are simultaneously related with the fish growth and health (Shan *et al*., 2008). Fish may adapt their metabolic functions to the dietary substrates through regulation of enzyme secretion in order to improve the utilization of feed ingredients (Bidhan *et al.*, 2014). There is evidence that probiotics effectively participate in the digestive process by producing extracellular enzymes such as proteases, amylase and lipases (Balcazar *et al*., 2006), thus improve the nutrient digestibility of food (ten Doeschate *et al*., 2008) by increasing the digestion of protein, starch, and fat (Essa *et al*., 2010). Therefore, nutrients are absorbed more efficiently when the feed is supplemented with probiotics (El-Haroun *et al*., 2006), which was reflected by the better growth of fish. Increased digestive enzyme activities including amylase, lipase and protease were reported in rohu (*Labeo rohita*) fed a combination of *Bacillus subtilis*, *Lactococcus lactis* and *Saccharomyces cerevisiae* (Mohapatra *et al*., 2012). In other trials, fish growth and enzyme were enhanced in European sea bass larvae (*Dicentrarchus labrax*) after fed diet supplemented with probiotic yeast (*Saccharomyces cerevisiae*) (Tovar-Ramirez *et al*., 2010). Moreover, probiotics also positively affect the assimilation of food components (Irianto and Austin, 2002).

Effect on intestinal morphology

Probiotics could colonize and implement their beneficial effect in the intestinal region of animals which is being considered as a complex harbour of commensal, non-pathogenic and pathogenic microorganisms (Nayak, 2010). Probiotics are capable of retaining the healthy intestinal condition by reducing the amount of harmful pathogenic microorganisms that persist within the GI tract of fish and which can develop the structure of the intestinal epithelial layer by lowering the mucosal damage and increasing of absorption of nutrients (Merrifield *et al*., 2010a). Abu-Elala *et al*. (2013) observed that fish treated with yeast (*Saccharomyces cerevisiae*) showed yeast colonization in the intestine, accompanied by an increase in the length and density of the intestinal villus. Also, supplementation of yeast added diet to *Senegalese sole* significantly increased villus length compared to unsupplemented group (Batista *et al*., 2014). Yeast cells have been reported to be a source of nucleotides, which contribute for the intestinal maintenance in aquatic animals by improving mucosal flora and mucosal surfaces with relative elongation of the intestinal tract (Li *et al*., 2006). An increase in villus length and villus width are directly related to enlarge of surface area, which may indicate improvement of intestinal absorptive capacity of nutrients (Caspary, 1992).

Effect on intestinal microbiota

The microbiota present in fish intestine plays a major role on the growth performance and health of fish (Ringø & Gatesoupe, 1998, Merrifield *et al*., 2010b; Nayak, 2010; Pérez *et al*., 2010; Vadstein *et al*., 2013; Llewellyn *et al*., 2014) by assisting in breaking down ingested food or inhibiting the colonization of the intestine by pathogens (Manzano *et al*., 2012). Fish microbiota community comprises viruses, archaea, protozoa, yeasts, and bacteria. In terms of abundance, bacteria are typically the dominant micro-organisms present in the gut and species of the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Tenericutes* are reported among the most dominant members present in fish gut (Merrifield and Carnavelli, 2014). Gut microbial community can be divided in two groups: allochthonous microbiota, which is the group that passes through the gut with the feed or digesta, and autochthonous microbiota, which is closely associated with the host tissues, is resident (Llewellyn *et al*., 2014). Autochthonous microorganisms have significant effects in the improvement of GI tract of fish, including development and maturation of the intestine and immune system (Bates *et al*., 2006; Nayak, 2010) and resistance to infectious pathogenic microbiota (Ringø *et al*., 2007). The ability of some probiotics to adhere to the intestinal mucus may block colonization of some pathogenic bacteria (Ringø *et al*. 2010; Gatesoupe 1999) using several mechanisms such as competition for space and food, secreting antimicrobial compounds as well as being receptors at mucosal surfaces (Nayak, 2010). In that way, probiotic species provide the most favorable positive impacts on the host body by modulating its intestinal microbial balance (Julio and Marie-Jose, 2011).

Effect on haematological parameters

Haematological parameters, particularly package cell volume, total and differential leukocyte count in the blood, provide an indication of the fish health status (Sampath *et al*., 1998). Dahiya *et al*. (2012) reported the positive effect of 3 probiotics namely *Lactobacilus sporogeneses*, *Saccharomyces boulardii* and mixture many bacteria on the haematological parameters of *Clarias batracus* L., in which