EFFECT OF *Lactobacillus acidophilus* AND MANNAN OLIGOSACCHARIDE ON GROWTH PERFORMANCE, DIGESTIVE ENZYME ACTIVITIES, INTESTINAL MORPHOLOGY, HAEMATOLOGY AND RESISTANCE OF STRIPED CATFISH (*Pangasianodon hypophthalmus,* Sauvage, 1878) JUVENILES AGAINST *Aeromonas hydrophila*

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by

MST. NAHID AKTER

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KESAN *Lactobacillus acidophilus* DAN MANNAN OLIGOSAKARIDA TERHADAP PRESTASI PERTUMBUHAN, MORFOLOGI USUS, AKTIVITI ENZIM PENGHADAMAN, HEMATOLOGI DAN KETAHANAN IKAN PATIN BERJALUR JUVENIL (*Pangasianodon hypophthalmus,* Sauvage, 1878) TERHADAP *Aeromonas hydrophila*

Oleh

MST. NAHID AKTER

Tesis yang diserahkan untuk

memenuhi keperluan bagi

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LIST OF ABBREVIATIONS

ADM	Apparent dry matter digestibility
AEP	Acini of the exocrine pancreas
AM	*Aeromonas hydrophila*
ANOVA	One-way analysis of variance
AOAC	Association of official analytical chemists
APD	Apparent protein digestibility
BD	Bile duct
BLAST	Basic local alignment search tool
CAC	Centroacinar cells
CFU	Colony forming unit
CMC	Carboxy methyl cellulose
CN	Central nucleoli
DN	Disappearance of nucleoli
DNA	Deoxyribonucleic acid
EP	Epithelium
ESR	Erythrocyte sedimentation rate
EtBr	Ethidium bromide
FAO	Food and agriculture organization
FM	Fish meal
FOS	Fructo oligosaccharides
FCR	Food conversion ratio
GC	Goblet cells
GC-MS	Gas Chromatography-Mass spectrometry
GE	Gross energy

GI	Gastrointestinal
GOS	Galacto oligosaccharides
H	Hepatocyte
Hb	Haemoglobin
HSI	Hepatosomatic index
Ig	Immunoglobulin
IPF	Intraperitoneal fat
L	Lumen
LAB	*Lactobacillus acidophilus*
LABP	*Lactobacillus acidophilus* pellet
LABS	*Lactobacillus acidophilus* supernatant
LC	Leukocyte count
LP	Lamina propia
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MMC	Melanomacrophage centre
MOS	Mannan oligosaccharide
MRS	De Man, Rogosa and Sharp agar
MV	Microvilli
NFE	Nitrogen free extract
N	Necrosis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume

PER	Protein efficiency ratio
PEG	Polyethylene glycol
PK	Pyknotic nuclei
PN	Periphery nucleoli
PV	Portal vein
PVA	Polyvinyl alcohol
RBC	Red blood cell
SBM	Soybean meal
SCFAs	Short chain fatty acids
scFOS	Short-chain fructo oligosaccharides
SGR	Specific growth rate
SPSS	Statistical package for social science
SR	Survival rate
TEM	Transmission electron microscopy
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VSI	Viscerosomatic index
V	Vacuolization
WG	Weight gain
WBC	White blood cell
WHO	World health organization

KESAN *Lactobacillus acidophilus* DAN MANNAN OLIGOSAKARIDA TERHADAP PRESTASI PERTUMBUHAN, MORFOLOGI USUS, AKTIVITI ENZIM PENGHADAMAN, HEMATOLOGI DAN KETAHANAN IKAN PATIN BERJALUR JUVENIL (*Pangasianodon hypophthalmus,* Sauvage, 1878) TERHADAP *Aeromonas hydrophila*

ABSTRAK

Penyakit dianggap sebagai salah satu faktor yang paling penting membimbangkan dalam akuakultur ikan patin berjalur (*Pangasianodon hypophthalmus,* Sauvage, 1878). Oleh itu untuk itu beberapa siri ujikaji telah dijalankan untuk mengenalpasti tahap yang paling sesuai bagi probiotik *Lactobacillus acidophilus* (LAB) dan prebiotik mannan oligosakarida (MOS), yang dapat meningkatkan pertumbuhan dan tahap kesihatan ikan patin berjalur juvenil. kumpulan tiga replikat ikan patin berjalur juvenil (21.69±0.18g) telah diberi makan diet 0 (kawalan), 103, 10^5, 10^7 dan 10^9 CFU/g LAB, dua kali sehari pada kadar 2.5% daripada berat badan ikan, selama 12 minggu. Prestasi pertumbuhan dan tahap kesihatan ikan berdasarkan parameter hematologi dan immunologi dalam kumpulan ikan yang diberi makan diet yang mengandungi LAB pada kadar 10^5 CFU/g dan keatas menunjukkan peningkatan yang signifikan. Dalam kajian yang lain, apabila ikan patin juvenil (20.41±1.64g) diberi makan lima diet berbeza terdiri daripada 0 (kawalan), 0.2%, 0.4%, 0.6% dan 0.8% MOS, prestasi pertumbuhan dan tahap kesihatan meningkat secara signifikan pada diet yang mengandung 0.6% MOS berbanding kumpulan lain. Kesan suplemen LAB atau MOS terhadap diet penghadaman dan kadar pertumbuhan dan kesihatan ikan (20.50±0.28g) telah dikaji dengan menggantikan 45% protin daripada serbuk ikan (FM) dengan serbuk kacang

soya (SBM). Walaupun ikan yang diberi suplemen LAB atau MOS mengandungi SBM tidak meningkatkan prestasi pertumbuhan dan penghadaman ikan, namun status kesihatan ikan patin berjalur juvenil menunjukkan peningkatan yang ketara terhadap parameter hematologi, jumlah kandungan immunoglobulin dan aktiviti lysozyme. Berdasarkan kepada semua kajian yang dijalankan, boleh dirumuskan bahawa diet yang disuplemen dengan 10^5 CFU/g LAB dan 0.6% MOS adalah mencukupi untuk meningkatkan tahap pertumbuhan dan kesihatan ikan patin berjalur juvenil apabila didedahkan kepada jangkitan *A. hydrophill*. Disamping itu, suplemen 10^5 CFU LAB or 0.6 % MOS pada diet yang mengandungi 55 % protin daripada FM dan 45 % protin daripada SBM dapat meningkatkan tahap kesihatan tanpa memberi kesan buruk terhadap pertumbuhan dan penghadaman ikan patin berjalur juvenil.

EFFECT OF *Lactobacillus acidophilus* AND MANNAN OLIGOSACCHARIDE ON GROWTH PERFORMANCE, DIGESTIVE ENZYME ACTIVITIES, INTESTINAL MORPHOLOGY, HAEMATOLOGY AND RESISTANCE OF STRIPED CATFISH (*Pangasianodon hypophthalmus,* Sauvage, 1878) ES AGAINST *Aeromonas hydrophila*

ABSTRACT

Disease has been considered one of the most significant concern for the sustainable development of striped catfish, *Pangasianodon hypophthalmus*, therefore, a series of experiments were carried out to determine the most suitable level of probiotic *Lactobacillus acidophilus* (LAB) and prebiotic mannaan oligosaccharide (MOS), respectively, that can promote growth and improve the health status of e striped catfish. Triplicate groups of e striped catfish (initial weight 21.69±0.18 g) were fed twice daily at 2.5% of body weight, with 0 (control), 10^3, 10^5, 10^7 and 10^9 CFU/g LAB diets, for 12 weeks. Results revealed that the growth performance and health status based on haematology and immunological parameters were significantly improved when fish were fed the diet supplemented with 10^5CFU/g and above LAB. In another study under similar experimental conditions, when striped catfish (20.41±1.64g) were fed with five experimental diets containing 0 (Control), 0.2%, 0.4%, 0.6% and 0.8% MOS, respectively, the growth performance and health status were significantly improved for 0.6% MOS diet fed group than the control fed group. The effect of LAB or MOS supplementation on diet digestibility and subsequently the growth and health status, were evaluated by feeding fish (20.50±0.28 g) with test diets in which 45% of fish meal protein was replaced with soybean meal protein. Although feeding fish with the LAB or MOS supplemented in

the diets containing SBM did not improve the growth performance and nutrient digestibility, the health status of striped catfish was significantly improved by increasing the haematological parameters, total immunoglobulin content and lysozyme activity. Based on the results of all the studies, it can be concluded that supplementation with 10^5 CFU/g of LAB and 0.6% MOS is sufficient to improve growth and health status of e striped catfish when they were exposed to *A. hydrophila* CT19. Further, supplementation of a soybean based diet containing 55% protein from FM and 45% protein from soybean meal with 10^5 CFU LAB or 0.6 % MOS, improves the health status of *P. hypopthalmus* es without compromising growth and nutrient digestibility.

CHAPTER 1

INTRODUCTION

1.1 *Pangasianodon hypophthalmus*

Pangasianodon hypophthalmus, is currently considered to be one of the most important species among commonly cultured freshwater fish. In 2010, global aquaculture production of striped catfish *(P. hypophthalmus)* exceeded 1.40 million tonnes. Vietnam is by far the world's largest producer of striped catfish; in 2010, it produced 1.14 million tonnes, with an estimated value of US$1.40 billion, and exported the fish to 136 countries (De Silva & Phuong, 2011). *Pangasianodon hypophthalmus* which is known as the striped catfish (Sauvage, 1878) (formerly as *Pangasius sutchi* or *Pangasius hypophthalmus*) (Slembrouck *et al*., 2009) played a great role in the rapid growth of aquaculture. This species is flourishing as a significant new source of white fish aquaculture products on the global fish market with several desirable characteristics including its adaptability for intensive culture, acceptance of low input sustainable feeds, adaptability to impaired water quality, and widespread consumer acceptance. This species has a great economic significance in the Southeast Asia region, including Vietnam. It lives in the main water basins of the Mekong and Chao Phraya rivers and has been widely introduced into other cities rivers and ponds for aquaculture. This species is native to tropical weather and suited to water with a 6.5-7.5 pH (Riede, 2004) range, and a temperature range of 22-26 °C (72-79 °F) (Riehl & Baensch, 1996). They desire large bodies of water similar to the deep waters of their native Mekong river basin. Freshwater farming in Vietnam, particularly in the Mekong delta, has shifted from being a rural activity, providing local people with animal proteins, to becoming an aquaculture enterprise. Raised in floating cages in rivers or ponds, striped catfish supply the domestic Vietnamese market and its export market, which is expanding rapidly (Orban *et al.*, 2008) and has become an iconic success story of aquaculture production in Vietnam. Although other Asian countries are producing *Pangasianodon* as a food fish, none have reached a competitive position in the global market.

The intensification process of the culture practices increases the risk of prevalence of stress related disease outbreaks (Bondad-Reantaso *et al*., 2005), which are responsible for huge fish losses. Therefore, aquaculture industry considers the disease occurrence as the state of restriction to aquaculture production at which adversely affects economical development (Ibrahem *et al*., 2010). Several pathogens including fungi, endo and ecto parasites as well as bacteria have been shown to be associated with diseases in various fish species. Stress factors that are responsible for disease outbreaks includes confined environmental degradation, waste released from agricultural activities, low quality of seed, high stocking density and inadequate care that make the stock vulnerable to infectious pathogens (Phuong *et al*., 2007).

For a long time, administration of antibiotics was used as the most common method for dealing with the occurrence of bacterial infections in aquaculture (Li & Gatlin, 2005). The indiscriminate application of these antibiotics as a remedial method for controlling bacterial pathogens has been responsible for the development of resistance bacteria, which has greatly reduced the effectiveness of the treatment options and may be responsible for long term adverse effects in the aquaculture environment (Defoirdt *et al*., 2007; Villamil *et al*., 2014) including accumulation in fish body tissue, immuno suppression and destruction of beneficial microbial flora (Smith *et al*., 2003; Sapkota *et al*., 2008). Among them, the development of antibiotic resistant bacteria (Gómez-Gil *et al*., 2000; Kolndadacha *et al*., 2011) has attracted the greatest concern globally. The use of antibiotics in aquaculture has been reduced over time to fulfil the demands of food safety standards. In fact, in Vietnam the list of banned or restricted antibiotics and chemicals used in aquaculture is closely monitored. Vaccination can be used as a method for controlling many fish diseases instead of antibiotic treatments, but for many diseases vaccines are unavailable or are in the early stages of development (Yousefian & Amiri, 2009). Furthermore, the use of antibiotics or vaccines in fish culture is laborious and expensive. Disease prevention is thus preferable as it is more beneficial than treatment.

Restrictions or bans on the use of antibiotics as feed additives (Mazlum *et al*., 2011) in aquaculture has sparked the growth of alternative strategies in developing eco-friendly aquaculture for the promision of health and the enhancement of disease control. Therefore, the modern aquaculture industry seeks alternative prophylactics which may help in maintaining a healthy environment, resulting in higher production and better profits (Sáenz de Rodrigáñez *et al*., 2009). In recent years, considerable attention has been given to the modification of intestinal microflora through the use of non-nutrient dietary components, particularly probiotics and prebiotics (termed biotics), to boost the growth, immune system and disease resistance capability of fish (Sakai, 1999; Irianto & Austin, 2002; Gatesoupe, 2005; Kesarcodi-Watson *et al*., 2008; Wang *et al*., 2008; Merrifield *et al*., 2010a). Though the objectives of using these dietary supplements are similar, the manner in which they modify the intestinal microbial community differ.

Probiotic is regarded as “one or more microorganisms which have beneficial effects for the host, and is able to exist in the digestive tract due to its tolerance to acid and bile salts” (Irianto & Austin, 2002). Though using probiotics in aquaculture is relatively new, the interest in them has increased tremendously due to their potential in disease control (Wang *et al*., 2008). Lactic acid bacteria, particularly *Lactobacillus acidophilus*, is considered to be one of the most utilized probiotic strains **(**Faramarzi *et al*., 2011**)**. This group of bacteria produces lactic acid as their main metabolic product during carbohydrate fermentation benefits the host animal by modifying the growth of beneficial microorganisms which ultimately outcompete potentially hazardous bacteria in the intestinal ecosystem and reinforce the natural defense mechanisms (Panigrahi *et al*., 2004).

Another comparatively new but effective approach to disease control in aquaculture is the use of prebiotics. Prebiotics are primarily defined as those non digestible food ingredients that beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, thus improving the host's intestinal balance (Gibson & Roberfroid, 1995). Several well established prebiotic oligosaccharides used in aquaculture include mannan-oligosaccharides (MOS), fructo-oligosaccharides (FOS), short-chain fructo-oligosaccharides (scFOS) and galacto-oligosaccharides (GOS). Among the established prebiotics, MOS is one of the preferable prebiotics used as the dietary supplement for fish and crustacean species (Sang & Fotedar, 2010), which is also used in this current research. Modes of action of MOS differ from other oligosaccharide candidates, such as fructo oligosaccharides and transgalacto oligosaccharides as it functions in an indirect manner within the gastrointestinal tract as opposed to changing the natural intestinal microbiota directly (Flickinger *et al*., 2003). MOS has some important characteristics which make it more attractive as an animal feed additive. Earlier research has stated that heat treatment does not change the ability of MOS to perform normally, which make it possible to include MOS in pelleted diets (Hooge, 2004).

1.2 Problem Statement

Disease in striped catfish is one of the major consequence of the intensification of aquaculture and may eventually become a limiting factor to the economics of a successful and sustainable development of striped catfish farming industry (Crumlish *et al*., 2010). Among the infectious diseases, bacterial agents have been responsible for the major epizootics that affect striped catfish farming. The rate of loss due to bacterial diseases in striped catfish (such as hemorrhagic symptoms caused by pathogenic *Aeromonas hydrophila)* has been estimated to be as high as 50% compared to others (Thinh *et al*., 2004; Phuong *et al*., 2007). Billions of dollars have been lost annually because of disease outbreaks, which has been recognized as a major threat to the sustainability of the aquaculture industry. In the sustainable development of striped catfish farming, outbreaks of disease and the inappropriate use of drugs and chemicals (antibiotics) have been determined as one of the major challenges. As an alternative strategy to antibiotics, the fast growing fish industry urgently needs sustainable approaches for disease control (Gatesoupe, 2005). Among the sustainable approaches available, the use of probiotics and prebiotics have recently attracted extensive attention in aquaculture. Despite numerous studies of *L. acidophilus* and MOS in many cultured fish species, limited information is available on the effects of *L. acidophilus* and MOS on the growth and health status of striped catfish led to conduct this study.

1.3 Purposes of the Study

The principal objective of this research was to determine the feasibility of using the probiotic *Lactobacillus acidophilus* (LAB) and prebiotic mannan oligosaccharide (MOS) to produce healthy striped catfish juveniles and evaluate their influence on the digestibility of a plant based practical diet for striped catfish *P. hypophthalmus* juveniles by comparing with a fish meal based diet.

Specific Objectives

- To determine the influence of dietary probiotic LAB and prebiotic MOS on the growth performance, digestive enzyme activities, intestinal morphology, haematology, immunological response and resistance in striped catfish (*P. hypopthalmus)* juveniles against *A. hydrophila*.

- To determine the effectiveness of selected best level of LAB and MOS respectively, in improving of growth, digestibility, digestive enzyme activities, intestinal morphology, microflora and resistance against *A. hydrophila* fed a practical diet replacing fishmeal with soybean meal.

CHAPTER 2

LITERATURE REVIEW

2.1 Aquaculture

Aquaculture refers to the farming of marine or freshwater organisms, especially food fish, shellfish or aquatic plants, under controlled conditions. Generally, aquaculture is an important economic sector in many developing countries, as it contributes significantly towards the improvement in the livelihoods of the poor either through an improved food supply and/or through employment and increased income.

2.2 World Aquaculture

Currently the world is facing multiple but interlinked challenges due to the impacts of the ongoing economic crisis caused by tremendous climate change. At the same time, another most significant concern is the necessity to meet the food and nutrition needs of the gradually expanding world population. The fisheries and aquaculture sector offers great opportunities to fulfil food and nutrition security, to alleviate poverty, generate economic growth and to ensure proper use of resources.

In general, global fish production has grown faster than world population growth. Therefore, aquaculture has remained one of the fastest food production sectors. According to FAO global food fish aquaculture production had increased by 5.8 percent to 70.5 million tonnes in 2013 with the production of farmed aquatic plants which were mostly seaweeds estimated at 26.1 million tonnes. Besides, China alone had contributed 43.5 million tonnes of food fish and 13.5 million tonnes of aquatic algae in 2013 (FAO, 2014). “Food fish” refers to those types of food which are used as food for human consumption, including finfishes, crustaceans, molluscs, amphibians, freshwater turtles and other aquatic animals such as sea cucumbers, sea urchins, sea squirts and edible jellyfish.

Aquaculture production over the last fifteen years has expanded tremendously to augment supplies from natural fisheries. According to FAO reports, the contribution of aquaculture to the world fisheries and aquaculture production in 2012 outpaced the 50% of the total capture fisheries which is now the world's leading source of aquatic products (FAO, 2014). Freshwater species, striped catfish plays a significant role to this rapid growth of aquaculture which is flourishing as valuable new sources of white fish aquaculture products in the global fish market with positive attributes including the adaptability to intensive culture, acceptability of low input sustainable feeds, adaptability to impaired water quality and the widespread of consumer acceptance. Since the dramatic augment of the *Pangasianodon* in the world aquaculture markets, Vietnam has become an iconic success story and remains the world's largest producer of striped catfish; in 2010, it produced 1.14 million tonnes, with an estimated value of US$1.40 billion, and exported the fish to 136 countries (De Silva & Phuong, 2011). Although other tropical Asian countries also produce *Pangasianodon* spp. as food fish, yet none has reached a competitive position in the world market.

2.3 Taxonomy, Distribution and Morphology of *Pangasianodon hypophthalmus*

Sutchi catfish, *Pangasianodon hypophthalmus*, was formerly known as the iridescent shark, *Pangasius hypophthalmus*. It is also known as the ikan patin in Malaysia, Thai pangus or striped catfish in Bangladesh, the Siamese shark or swai in Thailand and ca tra in Vietnam (Plate 2.1).

Plate 2.1 *Pangasianodon hypophthalmus* juvenile used in this study.

The scientific classification of *Pangasianodon hypophthalmus* is given below (Sauvage, 1878)

Kingdom: Animalia

Phylum: Chordata

Super-class: Osteichthyes

Class: Actinopterigii (ray-finned fishes)

Sub-class: Neopterigii

Infra-class: Teleostei

Super-order: Ostariophysi

Order: Siluriformes (catfish)

Family: Pangasiidae (shark catfishes)

Genus: *Pangasianodon*

Species: *Hypophthalmus*

Scientific name: *Pangasianodon hypophthalmus (*Sauvage, 1878)

Synonym: *Helicophagus hypophthalmus* (Sauvage, 1878),

Pangasius hypophthalmus (Sauvage, 1878), *Pangasius sutchi*, (Fowler, 1937)

The striped catfish (*P. hypophthalmus*), the native freshwater fish of the main South-East Asia water basin, such as the Mekong delta river region of Vietnam, Laos, Cambodia, Chao Phraya rivers in Thailand (Kabir, 2012) and Ayeyawady basin of Myanmar (FAO, 2009). Basically, this species is a migratory fish that moves upstream for breeding purpose during the rainy season when the water levels are high and return to downstream for finding the rearing habitat when the water levels recede (Rainboth, 1996). However, this fish has also been introduced for farming in many countries in Southeast Asia including Malaysia (Phumee *et al*., 2011), India, China, Indonesia (FAO, 2009) and Bangladesh (Ahmed *et al*., 2010). Most of the fish available in the market have been mass-bred for the purpose, and also cultivated for food in most of its native countries. It is omnivorous in nature, feeding principally on plants, algae, zooplankton, insects, fruits, crustaceans, and fish, and it prefers water with a 6.5-7.5 pH (Riede, 2004) and a temperature range of 22-26 °C (72-79 °F) (Riehl & Baensch, 1996). They have a preference to live large water bodies similar to the deep waters of their native Mekong river basin.

The striped catfish is the member of the freshwater Siluriformes in the *Pangasidae* family (Slembrouck *et al*., 2009). This fish has scale less skin with a comparatively long and laterally flattened body. Its head is comparatively small, whereas the mouth is quite broad with small sharp teeth on jaws, vomerine and palatal bones. The eyes of this species are relatively large. Two pairs of barbells are present, the upper pairs (maxillary barbel) are smaller compared to the lower pairs (mandibulary barbel) (Robert & Vidthayanon, 1991; Rainboth, 1996). The fins are generally dark grey or black in color. The dorsal fin consists of one hard fin ray (spine) and six soft fin rays. An adipose fin is present in between the dorsal and caudal fin and the caudal fin is isocoetoral. Generally, the body colour of this fish is black to dark gray on the dorsal side, whereas it is light gray in the lateral side and silvery in colour in the abdominal side (Kabir, 2012).

2.4 Diseases of *Pangasianodon hypophthalmus* Farming

For the last few years, fish health has become a major global problem to aquaculturists. In an intensive fish culture, fish diseases are a common phenomena. Any imbalance among the three important factors such as the existence of the pathogen in fish body and the quality of environment and the general health status of the fish, are the possible reasons of the outbreak of disease (Plate 2.2). Good fish health can be maintained without the use of chemotherapeutic agent when the above three conditions can be assured of its balance. The required water quality, the appropriate stocking densities and a balanced diet are the key factors for maintaining the good health status of fish.

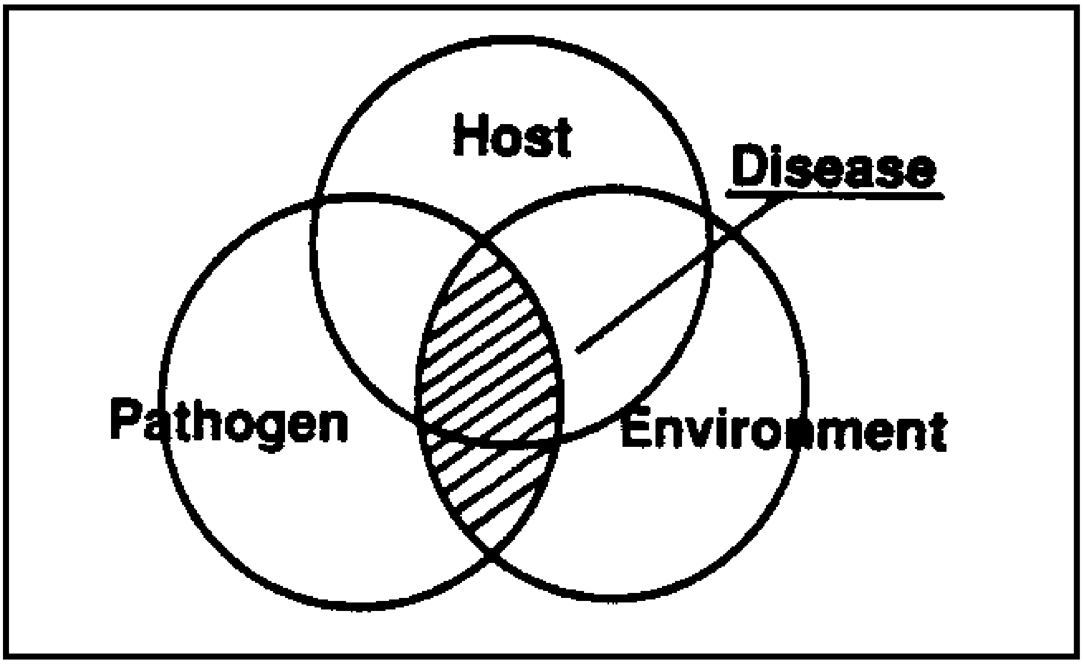

Plate 2.2 Disease is the end result of the host due to interaction and interrelationship of above 3 factors (Source: Francis-Floyd, 1997).

The continuous decline of fish in the wild stocks tend to increase the necessity to develop aquaculture in order to assure the supply of high quality protein to fulfil the world's growing population demand. Tolerance to high stocking densities, rapid growth, high production as well as good palatability and high market value are some important characteristics, which should be taken into consideration for pond aquaculture (Phuong & Oanh, 2009).

As the disease outbreak of aquaculture species increases with the increasing farming intensity (Bondad-Reantaso *et al*., 2005), aquaculturists have set two most important goals, the rapid growth and high disease resistance of aquaculture species. Fish diseases are commonly caused by pathogenic microorganisms such as protozoa, fungus, bacteria and virus which are common in water during the culture period. Among the various pathogenic microorganisms, bacterial agents, particularly *Aeromonas hydrophila*, has been observed as being responsible for the major epizootics affecting the striped catfish farming (Subagja *et al.*, 1999). Therefore, the conventional techniques of disease management which are commonly applied in aquaculture are the prophylactic and therapeutic control of bacterial diseases based on the use of chemical disinfectants and the oral administration of antimicrobial drugs (Verschuere *et al.*, 2000). However, due to apply the conventional disease management techniques, microbial populations are facing several problems and the most significant problem is the development of antibiotic resistant bacteria (Aoki *et al*, 1985; Gómez-Gil *et al.*, 2000). Besides, this can cause a potential hazard to public health as well as to the environment because of the imbalance of intestinal microflora due to the accumulation of excess antibiotic in the fish body (Esiobu *et al.*, 2002). Therefore, many researchers have proposed several alternative approaches to develop environmental friendly aquaculture, such as the use of probiotics and prebiotics as biological control agents in place of the use of chemotherapeiutents in aquaculture (Robertson *et al.*, 2000; Gatesoupe, 2005; Merrifield *et al*., 2010a).

2.5 Probiotic

"Probiotic" term originate from the Greek words '*pro;* and '*bios'* which means "for life" (Schrezenmeir & de Vrese, 2001) and this term carries different meanings over the years. Dr. Elie Metchnikoff was the first person who clarified the useful role played by some bacteria as observed among farmers who consumed milk containing pathogen and that "reliance on intestinal microbes for food makes it possible to take steps to change the flora of our bodies and to replace harmful microbes by beneficial microbes" (Metchnikof, 1907). However, the term probiotic was first introduced by Lilly and Stillwell (1965) after modification of the original word "probiotika." This term was used to illustrate the substances secreted by a microorganism which stimulates the growth of another. It was also described as an agent which functions as a contrary to antibiotics. In 1974, Parker (Parker, 1974) defined it as "organisms and substances which have a beneficial effect on the host of animal by contributing to its intestinal microbial balance." Later in 1989, Fuller attempted to improve Parker's definition by clarifying that it is "a live microbial feed supplement which beneficially affects the host animals by improving its intestinal microbial balance" and mentioned that it would be useful in a range of extreme temperatures and salinity changes (Fuller, 1989). Subsequently, it was recommended that probiotics were "monocultures or mixed cultures of microorganisms which can be applied to humans or animals, that do good to the host by improving the properties of indigenous microflora" (Havenaar & Huis, 1992). Because of high interest of probiotics used in aquaculture, Moriarty (1998) suggested to extend the definition of probiotics to "living microbial additives that benefit the health of hydrobionts and therefore increase productivity." The following year, Gatesoupe (1999) defined probiotics as "in a certain way of microbial cells administered, so that they can reach the gastrointestinal tract and be alive with the aim of improving health". The inhibition of pathogens by using probiotics was carried out in the same year and this led the definition to be expanded to"… live microbial supplementation can give benefits to the host by improving its microbial balance" (Gram *et al*., 1999). An expert with the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) stated that probiotics are live microorganisms which when consumed in adequate amounts confer a health benefits for the host (FAO, 2001).

Recently, it has been established that probiotic organisms have shown antimicrobial activity by altering the intestinal microflora thereby secreting a wide range of antibacterial substances such as bacteriocins and organic acids and competing with pathogens for adhesion to the intestine, competing for nutrients which are necessary for pathogen to survive. They are also capable of modulating the immune system. Because of these criteria, when viable probiotics are administered at certain concentrations, they will favorably affect the health of the host (Myers, 2007). In addition, nowadays probiotics are defined commonly as "friendly or healthy bacteria," (Wang *et al*., 2008).

2.5.1 Characteristics of Probiotic

According to Fuller (1989) a good probiotic should have the following characteristics:

- Should be a strain which is capable of exerting a beneficial effect on the host animal, e.g. increased growth or resistance to disease.

- Should not have any side effect; should neither be pathogenic nor toxic, not only with regard to the host species but also with regard to aquatic animals in general and human consumers.
- Should be viable under normal storage conditions and able to survive during industrial process.
- Should be capable of surviving and metabolizing in the gut environment, e.g. resistant to bile and low pH due to organic acids enrichment.
- Possess high ability to multiply in the intestine.
- Should have strong adhesive capability with the digestive tract of the host.
- Should have antagonistic characteristics against one or more pathogenic microorganisms.

2.5.2 Types of Probiotics

There are various types of microorganisms that have been shown probiotic characteristics and commonly used for both human and animal consumption is presented in Table 2.1. Among them two most important types of probiotics are *Lactobacilli* and *Bifidobacteri* (Robertson *et al*., 2000). Besides, various types of yeast species, particularly, under the genus of *Saccharomyces* have been considered as significant probiotic candidate for human and animal uses (Holzapfel *et al*., 1998).

Table 2.1 Common probiotics used for human and animal consumption

***Lactobacillus* species**	***Bifidobacterium* species**	**Other species**
Lactobacillus acidophilus	*Bifidobacterium bifidum*	*Bacillus cereus*
L. rhamnosus	*B. breve*	*Enterococcus faecium*
L. casei	*B. infantis*	*Lactococcus lactis*
L. bulgaricus	*B. longum*	*L. cremoris*
L. gasseri	*B. adolescentis*	*Streptococcus thermophilus*
L. crispatus	*B. lacis*	*Saccharomyces cerevisiae*
L. plantarum	*B. animalis*	*S. boulardii*
L. salivarius		
L. buchneri		
L. johnsonii		
L. reuteri		
L. fermentum		

2.5.3 Lactic Acid Bacteria

Considering the above probiotic characteristics, lactic acid bacteria are claimed to be the most utilized probiotic (Robertson *et al*., 2000). These probiotic bacteria are characterized as gram positive, non-sporulating cocci or rod shaped and produced lactic acid as their main metabolic product during carbohydrate fermentation. Previous study claimed, lactic acid bacteria as the normal microflora in the gastrointestinal (GI) tract of healthy animals such as mammals and aquaculture species (Ringø, 2008; Aly *et al*., 2008; Wang, 2011) and have been considered as being safe for feeding fish (El-Ezabi *et al*., 2011). These probiotic bacteria are provided benefit to the host animal by modifying the growth of beneficial microorganism that can contribute inhibitory compounds such as lactic acid, hydrogen peroxide, diacetyl, acetaldehyde and bacteriocin, which would ultimately outcompete the potentially hazardous bacteria from the intestinal ecosystem and would reinforce the natural defence mechanisms of organisms (Ringø & Gatesoupe, 1998; Gatesoupe, 1999; Balcázar *et al*., 2007a). Among the lactic acid bacteria, *Lactobacillus acidophilus* has been considered one of the important probiotic because of its inhibitory effect against numerous pathogenic microorganism (Al-Dohail, 2010; Talpur *et al*., 2014).

2.5.4 Mechanisms of Action

Probiotics can influence the decline of the prevalence and reduce the duration of diseases by stimulating the resistance to colonization and inhibitory effects against pathogens. Both *in-vitro* and *in-vivo* tests after using several probiotic strains, exhibited the inhibition of pathogenic bacteria through various mechanisms. For a few years, many research were conducted on the beneficial effects of probiotics. It is difficult to understand the real mechanism of actions of probiotics, and only some explanations are available.

The possible modes of action of using probiotic include:

i) Competition for adhesion site/competitive exclusion of pathogenic bacteria (Gómez-Gil *et al*., 2000; Vine *et al*., 2004; Bagheri *et al*., 2008).

ii) Colonization (Bagheri *et al*., 2008).

iii) Production of antimicrobial substances/antibacterial activity (De Keersmaecker *et al*., 2006; Aly *et al*., 2008; Vila *et al*., 2010; Enany *et al*., 2012).

iv) Source and competition for nutrients (Prieur *et al*., 1990; Verschuere *et al*., 2000; Bagheri *et al*., 2008).

2.5.4.1 Competition for Adhesion Sites / Competitive Exclusion

Competition for adhesion sites and colonization on the intestine and other tissue surfaces are the most common and significant mechanism of probiotics action to combat against harmful pathogens (Ringø *et al*., 2007). This is referred to as competitive exclusion of pathogenic bacteria (Ohashi & Ushida, 2009). The appropriate adhesion of pathogenic bacteria to the enteric mucus and intestinal wall surface is very important for any pathogen to cause damage to the host animal (Olsson *et al*., 1992; Vine *et al*., 2004). The physical blocking of pathogenic bacteria colonization by probiotic bacteria from their favorite adhesion site, such as intestinal villus, goblet cells and colonic crypts is referred to as competitive exclusion (Chichlowski *et al*., 2007). The adhesion of the probiotics on the epithelial cells is normally performed in two ways. One is non-specific depending on the physico-chemical factors and another is specific which is based on the adhesion of the probiotics on the surface of the adherent bacteria and receptor molecules (Salminen *et al*., 1996). One of the most important mechanisms of the intestinal microflora is to resist the adhesion of pathogenic bacteria to the host intestine, thereby creating of a physiologically negative environment, with regard to pH, redox potential, and hydrogen sulfide enrichment (Fons *et al*., 2000). However, by maintaining good husbandry practices and environmental conditions, the composition of microbial communities can be altered, which ultimately will be able to excite the proliferation of selected bacterial species. It is well established that the presence/existence of microflora in the intestinal tract of aquatic animals can be altered by supplying beneficial microorganisms through feeding, which ultimately will reduce the availability of opportunist pathogens (Deven *et al*., 2009). In fact, there are some evidence that lactic acid bacteria, particularly *L. lactis* and *L. plantarum* can successfully reduce the adhesion of pathogenic bacteria *A. hydrophila*, *A. salmonicida* and *V. anguillarum* in intestinal mucus of fish (Balcázar *et al*., 2008).

2.5.4.2 Colonization

Probiotics execute their activity through colonization and the secretion of many growth-promoting substrate in the host intestine (Bagheri *et al*., 2008). Probiotics colonization of the gastrointestinal tract of host animals is probable only after birth, and prior to a very competitive indigenous microflora that has been installed. After being installed, only the supplement of high levels of probiotic can activate its temporary and artificial dominance. After the intake had stopped, the population of probiotics in the gastrointestinal tract of mature animals show a rapid decrease within days (Fuller, 1992). A microorganism is capable to colonize in the gastrointestinal tract whenever it can stay there for a long time by possessing a higher multiplication rate than its expulsion rate (Conway, 1997). The attraction of bacteria to the mucosal surface association within the mucous gel or attachment to epithelial cells is the main reason of this colonization. The adhesion and colonization of the mucosal surfaces by probiotics are possible protective mechanisms against pathogenic organisms through a competition for nutrients and the binding sites (Westerdahl *et al*., 1991), or modulation of immune (Salminen *et al*., 1998). Several factors responsible for the colonization of microorganisms are as follows**:**

(i) Host related factors: Thses include body temperature, redox potential levels, enzymes, and genetic resistance. For instance, bacteria can enter the host body through the mouth, either being mixed with water or food particles, and while passing along the alimentary tract, some of them are able to adhere as part of a resident microflora. Among the rest of the microflora some are destroyed throughout the digestive process while passing through the intestine, and the rest is eliminated via the faeces. Another factor which may be able to suppress bacterial growth is the production of any antimicrobial substances by the host.

ii) Microbe-related factors: These are the effects of antagonistic microorganims, bacteriocins, lysozymes, proteases, formation of hydrogen peroxide, ammonia, and sudden changes of pH values due to production of organic acids (Gram *et al*., 1999; De Vrese & Marteau, 2007; Vila *et al*., 2010). It is well recognized that lactic acid bacteria produce substances, for example bacteriocins, that are capable to inhibit the growth of some pathogenic microorganisms.

2.5.4.3 Production of Antimicrobial Substances / Antibacterial Activity

Bacterial antagonism is a familiar event in nature; therefore, the interactions of microbes play a significant role to maintain the equilibrium between useful and potentially pathogenic micro-organisms (Balcázar *et al*., 2004). Probiotics secret certain inhibitory substances such as bacteriocins, organic acids (lactic, acetic and butyric acid) and H_2O_2 (De Keersmaecker *et al*., 2006; Vila *et al*., 2010) which are able to show antagonistic activity against the pathogenic microorganisms, and consequently will prevent their propagation in the host bodies. It was previously reported that bacteriocins acidophilin, lactocidin and acidolin are secreted by one of the most important probiotic strain *L. acidophilus,* whereas *L. plantarum* produces lactolin (Vila *et al*., 2010). They are also able to show antimicrobial activity by lowering the pH of the intestine (De Keersmeacker *et al*., 2006), the agglutination of pathogenic microorganisms, binds with toxic metabolites (Fonden *et al*., 2000; Oatley *et al*., 2000; Haskard *et al*., 2001), and the production of mucus (Mattar *et al*., 2002; De Vrese & Marteau, 2007). Several researches have reported that probiotics especially *Lactobacillus* sp., *Bifidobacterium* sp. and *Bacillus* sp. have been able to secrete a wide range of chemical substances which have shown an inhibitory effect on pathogenic bacteria (Oscáris *et al*., 1999; Gotteland *et al*., 2006; Vila *et al*., 2010). There is evidence that the probiotic strain of *L. salivarius* sub sp. *salivarius* UCC118 secrete a peptide, which has shown the inhibition capability against a broad range of pathogens, including *Staphylococcus, Enterococcus, Listeria, Bacillus,* and *Salmonella* species (Flynn *et al*., 2002).

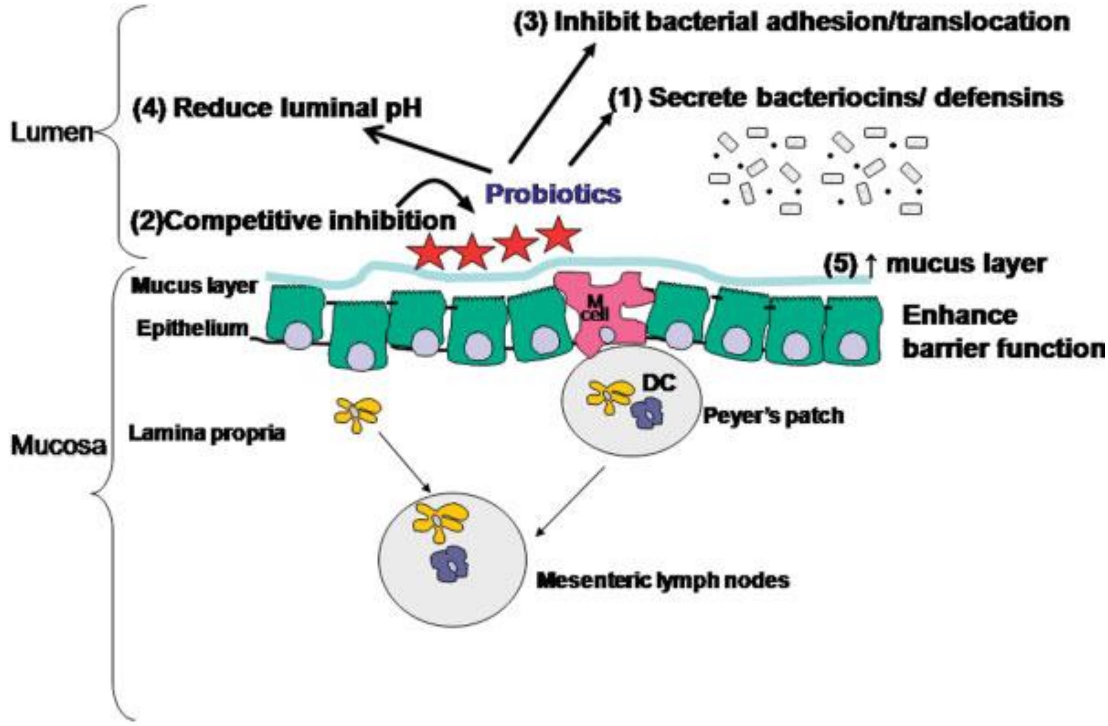

Plate 2.3 Inhibition of enteric bacteria and enhancement of barrier function by probiotic bacteria. Schematic representation of the crosstalk between probiotic bacteria and the intestinal mucosa. Antimicrobial activities of probiotics include the (1) production of bacteriocins/defensins, (2) competitive inhibition with pathogenic bacteria, (3) inhibition of bacterial adherence or translocation, and (4) reduction of luminal pH. Probiotic bacteria can also enhance the intestinal barrier function by (5) increasing mucus production. [The picture taken from www.interscience.wiley.com.]

2.5.4.4 Sources and Competition for Nutrients

Intestinal microorganisms play a crucial role in nutrition as well as the well-being of several animals (Floach *et al*., 1970) including fish (Sugita *et al*., 1989). The ability of intestinal microorganism to synthesize vitamins, essential growth factors (fatty acid, amino acid) and digestive enzymes are well documented (Teshima & Kashiwada, 1967; Clements, 1997). Microorganisms particularly anaerobic bacteria play a significant role in carp (*Cyprinus carpio*) nutrition by contributing fatty acids (Clements, 1997) which are the end products of anaerobic fermentation (Smith *et al*., 1996). There is also evidence that the useful microflora (aerobic, anaerobic) can serve as a source of food by providing vitamins, such as B12 and essential amino acids, which play a significant role in the host's nutrition (Sugita *et al*., 1991a, 1991b).

In fact, probiotics improve the health or well-being of the host by adhering to the mucus membrane, epithelial cells, gastrointestinal tract as well as other tissues (Gatesoupe, 1999; Farzanfar, 2006). Several researches have been conducted to evaluate the *in-vitro* and *in-vivo* attachment ability of many beneficial microorganisms and their results recommended that the some potential probiotic have ability to displace the pathogens by competing for necessary nutrients, space, etc. (Verschuere *et al*., 2000). They can cause the unavailability of nutrients and energy sources for essential growth of some pathogenic bacteria, by effectively utilizing those nutrients which would otherwise can be consumed by pathogenic microbes.

2.5.5 Beneficial Effects of Probiotics

2.5.5.1 Effect of Probiotics on Intestinal Ecosystem

The gastrointestinal (GI) tract of fish considered are as harbours of a complex microbial community, especially two distinct groups, i.e. allochthonous (exogenous) and autochthonous (indigenous) (Nayak, 2010). Autochthonous microorganisms are capable of playing significant roles within the GI tract such as to contributing to the development/maturation of the intestine and immune system (Bates *et al*., 2006; Picchietti *et al*., 2007; Nayak, 2010), and managing resistance to infectious pathogens (Birkbeck & Ringø, 2005; Ringo *et al*., 2007). They are also able to hinder the colonization of bacteria through several modes of action including competition for food and space as well as being receptors at mucosal surfaces, and secreting antimicrobial compounds (Nayak, 2010). Thereby, probiotics provide the beneficial effects on the host by improving its intestinal ecosystem (Julio & Marie-José, 2011). However, very few studies have been conducted to evaluate the effects of probiotics supplementation on the intestinal autochthonous microflora of fish (Nayak, 2010; Merrifield *et al*., 2010a). Previous researches only focused on cultivation-based techniques to evaluate intestinal microbial population which noticeably only permit the investigation of culturable bacteria, while the non-culturable bacteria, which may account a great effect in the intestine of fish, remained unclear (Nayak, 2010; Merrifield *et al*., 2010a; Zhou *et al*., 2009).

2.5.5.2 Effect of Probiotics on Digestive Enzymes

It is well documented that digestive organs are greatly influenced by the composition of food and are responsible for instantaneous changes in the activities of the digestive enzymes (Bolasina *et al*., 2006; Shan *et al*., 2008), which is finally linked with fish health and growth. In fact, the efficiency of feed utilization in the fish is greatly influenced by the presence of digestive enzymes. Protease, amylase, and lipase are three different types of enzyme which are responsible for the breakdown of protein, carbohydrate and fat respectively. There is evidence that dietary probiotics are able to increase the digestion of protein, starch and fat by increasing digestive enzyme activities in Nile tilapia, which ultimately increase the growth and feed utilization (Essa *et al*., 2010). Elevated enzyme activities (protease, amylase and lipase) were also noticed in *Labeo rohita* after being fed with a combination of probiotics (*Lactococcus lactis*, *Bacillus subtilis* and *Saccharomyces cerevisiae)* (Mohapatra *et al*., 2012). Microorganisms and their secreted enzymes play a significant role on digestion process (Munilla-Moran & Stark, 1990) by raising the total enzyme activity of the intestine (Ziaei-Nejad *et al*., 2006; Wang, 2007). The enzymes secreted by probiotic cells are known as exogenous enzymes which can help to synthesize endogenous enzymes (Mohapatra *et al*., 2012). These exogenous enzymes are able to tolerate a wide range of pH compared to endogenous enzymes, which can delay the digestion period (Essa *et al*., 2010) thereby assuring the maximum utilization of nutrients. However, it is complicated to separate between exogenous enzymes secreted by the probiotics and endogenous enzymes synthesized by fish (Essa *et al*., 2010). The exoenzymes produced by probionts are able to increase the digestive utilization of feed (Mohapatra *et al*. 2012). Probiotics not only have a beneficial impact on the digestive systems, but also in the assimilation of food components (Irianto & Austin, 2002).

2.5.5.3 Effect of Probiotics on Intestinal Morphology

It was reported that probiotics could establish and execute their activity in the intestine of animals considered as a harbour of the diverse population of pathogenic,