EFFECTS OF PROBIOTIC, *LACTOBACILLUS ACIDOPHILUS* ON PATHOGENIC BACTERIA, GROWTH, HEMATOLOGICAL PARAMETERS AND HISTOPATHOLOGY OF AFRICAN CATFISH (*CLARIAS GARIEPINUS*)

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UNIVERSITI SAINS MALAYSIA

2010

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by

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Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

July, 2010

ACKNOWLEDGEMENTS

In the name of Allah, most Gracious, most merciful. All praise for Allah, whose blessings make me able to submit this thesis. With great honour, my profound thanks and deepest appreciation to my supervisor, Prof. Dr. Roshada Hashim, for her unlimited support, excellent guidance, continuous encouragement, and also for putting at my disposal every facility that she had and which I needed during the course of my work. Without her assistance, completion of this thesis would not have been possible and I can never be thankful enough her support.

A special thanks goes to Prof. Dr. Sheikh Ibrahim Amor and Prof. Dr. Darah Ibrahim for helping me by letting me to work in their laboratory during the study. I would like to extend my gratitude to Hadhramout Universitry for Science and Technology (HUST) for providing scholarship and allowing me the opportunity to pursue Doctor of Philosophy degree in Universiti Sains Malaysia (USM); I am also grateful to USM for providing research facilities thus allowing me to complete my research work successfully. I am also thankful to National Fish Health Research Center, Penang, for providing the expert panel for bacteria identification during the study.

I extend my heartfelt thanks to my fellow researchers of Aquaculture Research Group Associate Prof. Dr. Alexander Chong, Mr. Mohammed Aliyu, Mr. Ahmed Bakweta, Miss. Khalidah, Miss. Lavi, Mrs. Sharifa, , Mrs. Finie, Miss. Hassna, Dr. Sarita, Kar Loon, Miss. Nana, Mrs. Preeda, Mrs. Auntie Anna, and Mr. Ancik Jamil, for their valuable discussion, timely help and assistance throughout my study.

I wish to thank Prof. Dr. Abdlhakim Alrawi, Dr. Manssor, Dr. Aqil, Mr. Rais ALtamimi, Dr. Saeed Alfadly, Dr. Khalid Bin Makhashin, Dr. Yasser, Mr. Hassen, Mr. Osan, Mr. Mr. Adel, Mohammed, and all my friends and colleagues who helped me, in any way or form; and also extend my thanks to faculty members, lab assistants and administration officers at the School of Biological Science who helped me during the period of my study.

Finally, I express my best sincere gratitude to all my family members, my relatives and my friends in Malaysia and Yemen for their prayers, assistance and encouragement throughout my study. I think words can never express enough how grateful I am to my parents. I can only say a note of thanks to my father and mother for their prayer, patience and untiring support in every way during my long absence from them. My gratitude is also extended to my wife, sons, brothers, sisters, uncle, and cousins for their motivation and encouragement throughout my study period.

This appreciation is to everybody that involved and supported me throughout my cheers and hardest jiffy. Thank you all so much.

Last, but not least, this work is dedicated to the memory of my father, Abdullah Al-Dohail (1938-2007), who passed away on 4/5/2007, when I was away with my research. I pray to Allah to forgive his sins, bless and accept him, Ameen.

Mohammed Al-Dohail

Penang, Malaysia

July 2010

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AOAC	Association of Official Analytic Chemists
BSA	Bovine Serum Albumin
CFU	Colony forming units
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
FAO	Food and Agricultural Organization
FCR	Feed conversion ratio
Hb	Haemoglobin
HMDS	Hexamethyldisilazane
GE	Gross energy
LAB	Lactic acid bacteria
LC	Leukocyte count
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MRS	Man-Rogosa-Sharpe
NFE	Nitrogen free extract
NPc	Non-probiotic control
NPsx	Non-probiotic diet, fish infected with *Staphylococcus xylosus*
Npah	Non-probiotic diet, fish infected with *Aeromonas hydrophila* gr.2
Npsa	Non-probiotic diet, fish infected with *Streptococcus agalactiae*
OD	Opaque density
Pc	Probiotic control
Psx	Probiotic diet, fish infected with *Staphylococcus xylosus*
Pah	Probiotic diet, fish infected with *Aeromonas hydrophila* gr.2
Psa	Probiotic diet, fish infected with *Streptococcus agalactiae*
PER	Protein efficiency ratio
PEG	Polyethylene glycol
PCV	Packed red cells
RBC	Red blood cell count
RGR	Relation growth rate
SEM	Scanning electron microscope
SGR	Specific growth rate
WBC	White blood cell
WHO	World Health Organization

Kesan Probiotik, *Lactobacillus acidophilus* Terhadap Bakteria Patogen, Pertumbuhan, Parameter Hematological dan Histopatologi Keli Afrika *Clarias gariepinus*

ABSTRAK

Empat eksperimen telah dijalankan bagi menilai penggunaan *Lactobacillus acidophilus secara in vitro* dan *in vivo* sebagai agen kawalan bio terhadap bakteria patogen seperti (*Staphylococcus xylosus, Aeromonas hydrophila* gr.2 dan *Streptococcus agalactiae*) serta kesannya terhadap prestasi pertumbuhan, parameter hematological, kandungan immunoglobin dan histopatologi keli Afrika, *Clarias gariepinus*. Dalam eksperimen yang pertama, aktiviti antimikrob *L. acidophilus* terhadap bakteria patogen ikan umum dipelajari secara *in vitro*. Seterusnya, dalam eksperimen yang kedua, dua makanan dirumuskan terdiri daripada kawalan (makanan bukan probiotik) dan *L. acidophilus* ditambah pada kepekatan 3.01 x10^7 jajahan per gram diet (makanan probiotik). Dalam eksperimen yang ketiga, dua kumpulan dengan dua belas bereplikasi daripada 25 *C. gariepinus* fingerlings (5 g) diberi makan makanan probiotik dan makanan bukan probiotik tiga kali sehari selama 12 minggu. Dalam eksperimen yang keempat, ikan (190 g) yang diperolehi daripada Eksperimen 3 yang dipisahkan ke dalam 8 kumpulan dengan tiga kumpulan yang ditapis di makanan bukan probiotik disuntikkan dengan 1 ml of *S. xylosus, A. hydrophila* gr.2, dan *S. agalactiae* masing-masing dan kumpulan keempat 1ml disuntik dengan air garam fisiologis sebagai kawalan sekali (bukan probiotik kawalan). Tiga kumpulan yang tersisa yang dipertahankan pada diet probiotik juga disuntik dengan 3 yang sama patogen bakteria dan kumpulan keempat 1ml disuntik dengan air garam fisiologis sebagai kawalan (kawalan probiotik). Darah dikumpul untuk parameter hematologi selepas 12 minggu. Sampel darah, hati dan buah pingang diambil pada minggu pertama dan ketiga selepas jangkitan bacteria bagi melihat kesan patohistolgi. Prestasi pertumbuhan (kadar pertumbuhan spesifik dan kadar pertumbuhan relatif), penggunaan nutrient (nisbah keefisienan protein dan

nisbah penukaran makanan) dan kemandirian didapati lebih baik secara signifikan pada ikan yang menerima diet yang dibekali probiotik berbanding ynag tidak mengandungi probiotik. Parameter hematologi (PCV, Hb, ESR, RBC and WBC, jumlah protein dalam serum, Ca^{2+}, Mg^{2+}, Cl^{-} , glucose, cholesterol) dan jumlah kepekatan immunoglobin didapati berbeza secara signifikan dalam ikan yang makan diet yang mengandungi probiotik. Keputusan eksperimen yang kelima menunjukkan parameter hematologi: PCV, Hb, ESR, RBC and WBC, jumlah protein dalam serum, Ca^{2+}, Mg^{2+}, Cl^{-}, glucose, cholesterol, jumlah kepekatan immunoglobin dan patohistologi bagi ikan yang diuji dengan probiotik adalah lebih baik secara segnifikan berbanding ikan yang tidak diuji dengan probiotik. Berdasarkan keputusan yang diperoleh, kami mencadangkan bahawa *L. acidophilus* boleh digunakan sebagai agen menentang bacteria pathogen (*A. hydrophila* gr.2*, S. agalactiae* dan *S. xylosus)* sama ada secara *in vitro* mahupun *in vivo* dalam ternakan *C. gariepinus* demi meningkatkan kualiti kesihatan dan keefisienan makanan serta pertumbuhan dan sebagai tambahan dapat merawat bakteria patogen.

Effects of Probiotics, *Lactobacillus acidophilus* on Pathogenic Bacteria, Growth, Hematological Parameters and Histopathology of African Catfish *(Clarias gariepinus)*

ABSTRACT

Four experiments were carried out to evaluate the *in vitro* and *in vivo* used of *Lactobacillus acidophilus* as a biocontrol agent against some common fish pathogenic bacteria (*Staphylococcus xylosus, Aeromonas hydrophila* gr.2 and *Streptococcus agalactiae*) and its effects on growth performance, hematological parameters, and histopathology of African catfish, *Clarias gariepinus*. In the first experiment, the antimicrobial activity of *L. acidophilus* against common fish pathogenic bacteria was studied *in vitro*. In the second experiment, 2 experimental diets were formulated comprising of a control (non-probiotic diet) and a *L. acidophilus* supplemented at a concentration of 3.01 $x10^7$ colonies per gram of diet (probiotic diet). In the third experiment, 2 groups with12 replicates of 25 *C. gariepinus* fingerlings (5g) were fed the probiotic diet and non-probiotic diet 3 times daily for 12 weeks. In the fourth experiment, the fish (190g) obtained from Experiment 3 were redistributed into 8 groups with 3 groups that were maintained on non-probiotic diet were injected with 1ml of *S. xylosus, A. hydrophila* gr.2*,* and *S. agalactiae* respectively and the fourth group was injected with 1ml of NaCl 0.9% as control once (non-probiotic control). The remaining 3 groups that were maintained on probiotic diet were also injected with the same 3 pathogenic bacteria and the fourth group injected with 1ml of NaCl 9% as control (probiotic control). Blood was collected for hematology parameters after 12 weeks. In addition, blood, liver and kidney were collected for histopathology after 7 days and 21 days following infection. The results showed significant (P<0.05) differences in the diameters of the inhibition zone of *L. acidophilus* against pathogenic bacteria *in vitro*. Growth performance parameters and survival were significantly higher in fish maintained on the probiotic supplemented diet compared to those on the control

diet. Hematological parameters were also significantly higher in fish fed with the probiotic supplemented diet than in the control diet. Histopathology and hematological parameters were significantly higher in the infected fish groups maintained on the probiotic diet than in the infected fish groups fed with the non-probiotic diet. From the results, we concluded that *L. acidophilus* can be used as an antimicrobial agent against common fish pathogenic bacteria when used *in vitro* and it can be used as a probiotic agent in *C. gariepinus* culture, to enhance fish health, survival, feed efficiency and growth. In addition, it helps in the treatment of pathogenic bacteria when used *in vivo* condition.

Chapter 1

Introduction

1. 1 Introduction

African catfish (*Clarias gariepinus)* is a native species of tropical and subtropical freshwater of Africa. This fish was introduced to many countries of the world. It belongs to the family of Claridae, and represents one of the most widely produced food fishes in the world (Hecht & Appelbaum, 1988; Van Weerd, 1995; Fasakin *et al.* 2003; Al-Dohail, 2005; Sutriana, 2007). African catfish, *C. gariepinus* are important freshwater fishes, which are widely cultivated for commercial purposes. It is one of the most important cultured fish species in Malaysian waters. Many researchers reviewed that interest in culture of African catfish is increasing due to its high fecundity, fast growth, ability to utilize a wide range of food sources, tolerate a wide range of environmental conditions, and also have the ability for high-density culture (Hogendoorn, 1983; Huisman & Richter, 1987; Hetch *et al*. 1997; Fagbenro *et al.* 1998; Maithya, 1998).

The probiotics could be defined as live microbial feed that improved health of man and terrestrial livestock (Gatesoupe, 1999). Also, probiotics are defined as microbial cells with beneficial effect on the health and well-being of the host (Salminen *et al.* 1999).

The use of probiotics to improve humans and animals health has been comprehensively reviewed (Erickson & Hubbard, 2000; Guarner & Malagelada, 2003). Currently, probiotics have been used in food manufacturing because of their character in helping the humans and animals to digest food and to improve their immunity against diseases. Moreover, the effect of probiotics on some disease resistance as diarrhea

has been studied (Saavedra & Abi-Hanna, 1999; Pathmakanthan *et al.* 2000). Some authors claimed that probiotic strains, *Lactobacillus* and *Bifidobacteria* species have a significant benefit and can reduce the duration of diarrhea in children (Mack *et al.* 1999; Marteau *et al.* 2001; Szajewska *et al.* 2001). According to Gilliland & Speck (1977) and Sandine, (1979) *L. acidophilus* inhibited many common food borne pathogens. Some studies reported that *L. acidophilus* produced antibiotic like substances, which have responsibility for inhibition action (Gilliland, 1989). Gilliland & Speck, (1977) and Silva *et al.* (1987) reported that *L. acidophilus* has the ability to inhibit some pathogens such as *Staphylococcus aureus, Salmonella typhi* and *Escherichia coli.*

Helander *et al.* (1997) and Niku-Paavola *et al.* (1999) reported that *L. acidophilus* produced hydrogen peroxide, lactic acid, acetic acid, and other compounds, which widely inhibit many harmful organisms like *Salmonella*, *E. coli*, *Clostridium*, and *Helicobacter*. Moreover, Gilliland (1989) claimed that probiotics interfered with cholesterol absorption. Other studies suggested that cholesterol level decreased in blood serum when fermented milk or milk containing *Lactobacilli* was orally taken (Mann & Spoerry, 1974; Harrisron & Peat, 1975; Grunewald, 1982). Furthermore, Ouwehand & Vesterlund (2003) reported that probiotics have the ability to prevent urinary tract infections. According to De-Ruiz *et al.* (1996) *Lactobacillus* is used for treating *E. coli* causing urinary tract infection in animals when orally administered.

The probiotics must have the ability to survive in an intestinal tract in the present of acidic or alkaline condition. Therefore, to avoid losing probiotics by food when it passes through the gut, the probiotics must grow at a rate faster than their removal or attach themselves to the intestinal wall. Probiotics can adhere to an intestinal surface or to a mucin overlying the epithelial layer (Fuller, 1989). The probiotics are also able to adhere to epithelial layer (Tannock, 1999); thus the probiotics prohibit pathogenic bacteria or reduce adherence of pathogenic bacteria to epithelial layer (Conway *et al.*

1987). The probiotics produce nutrients such as short chain fatty acid, vitamins and antibacterial substance (Jigsaw, 2006).

Recently, in aquaculture, the use of probiotic bacteria to enhance the growth performance and also improve the water quality in which fish are cultured (usually named bioremediation or biocontrol when they act only in water) has received great attention (Verschuere *et al.* 2000). In general, the most commonly used probiotics are lactic acid bacteria (LAB) because they can produce bacteriocins and other compounds that may inhibit the growth of pathogenic bacteria (Juven *et al.* 1992; Gildberg *et al.* 1997; Savadogo *et al.* 2004).

In intensive marine larval culture, the use of large quantities of sterilizer and drugs can negatively affect the development of protective gastrointestinal microflora of cultured fish (Austin & Al-Zahrami, 1988; Strøm & Ringø, 1993). For this reason, reared larvae are more at risk to disease and stress because they are exposed to intestinal flora disruptions (Gomez-Gil *et al.* 2000). As a result of this, it is necessary to search for alternative methods in aquaculture, which could possibly maintain a microbiologically healthy environment of fish and at the same time enhance production and economic profits. Some studies suggested that bacterial probiotics possibly will be controlling potential pathogens, protecting fish health, improve growth performance and preventing fish from disease (Geovanny *et al.* 2007; Irianto & Austin, 2002a; Gatesoupe, 1991; Nogami & Maeda, 1992; Strøm & Ringø, 1993). Carnevali *et al.* (2006) pointed out that the use of *L. delbrueckii* for 70 days as probiotic of sea bass juveniles had positive effects on fish health and growth. Picchietti *et al.* (2007) claimed that administration of various microbial products has enhanced the survival and immune system of cultured fish.

The ability of probiotics to challenge pathogen and digestion of foodstuffs is governed by a series of the bio-physico-chemical process of which probiotics play an important role. For a better understanding, the probiotics that take part in the digestion of these feedstuffs and challenge pathogen *in vitro* and *in vivo* have to be identified and characterized. The concept of probiotic is one of the best innovations introduced in order to solve pathogen challenges to obtain optimal growth performance and fish health.

1.2 Issues for Research in *Clarias gariepinus*

About 10 percent of all cultured fish die due to diseases; and in catfish around 75 percent of newly hatched fries die before achieving market size (Lim & Webster, 2001). This is because fish diseases resulting from the unbalance of microbial community in culture water as well as in the fish gut could result in heavy mortalities in fish farms during a short period of disease outbreaks (Munday, 2002). These diseases may cost the aquaculture industry millions of dollars yearly due to fish mortality, and chemical items used for treating the diseases (Lim & Webster, 2001). The development of aquaculture technology has led to improvement towards better semi-intensive and intensive culture techniques, resulting in higher stocking densities (Holmer *et al.* 2008).

A balanced diet is very important and considered as principal aspect in the aquaculture industry. For this reason, there is a need to improve the quality of fish diets on the cheap cost. It is well known that *C. gariepinus* grows very fast and needs to consume a lot of feed during its life cycle. However, some diseases which have influence on the health and production of this fish during its culture have also been reported in the literature (Thune, 1993; Kori-Siakpere, 2008). Treatment or control of some of these diseases under culture conditions have commonly been through the use of chemical control agents (Wellborn,1985; Vijayakumaran and Radhakrishnan, 2003), some of

which have been reported to disrupt the fish intestinal micro-flora (Strøm and Ringø, 1993) and pollute the environment (Ranjit and Singh, 2003), apart from increasing production costs (Thune, 1993). Therefore, to minimize the use of chemical drugs for treatment of some of these fish diseases and reduce their effects on the fish and the environment and also decrease production costs, cheaper and safer alternatives have become a vital necessity.

Moreover, residual antibiotics mixed in fish feeds are sometimes detected in seafood products, leading to concerns of the compromise to health of human consumers (Nawaz *et al.* 2001). An example of such consumers' concern in the use of antibiotics in animal feeds is the cause of ban in animal feed formulations in the European Union in 2006. Increasing concern regarding health of consumers, the environment and antibiotic resistant microorganisms has therefore, necessitated the search for safer, cheaper and environmentally friendly alternatives, including the use of non-pathogenic microorganisms as probiotic.

Consequently, the search for new techniques to achieve the best growth performance and the best treatment for diseases in the best economic and environmental way is certainly an insistent quest. Presently, there is a gap of information in the application techniques and the use of the probiotic *Lactobaccillus acidophilus* to enhance growth, to improve the immune system and limit disease outbreak, especially in *Clarias gariepinus*, even though there are documented evidences of similar studies conducted with other bacteria species evaluating the effect of toxins in this fish. Several researchers have studied the effects of challenging probiotic with pathogenic bacteria in some cultured fish species where there is limited information regarding the effects of probiotic on growth and diseases of this candidate species; its impact on this species thus merits examining. Therefore, lack of information on the use of *L. acidophilus* to improve growth and the use of *L. acidophilus* as a biocontrol agent against pathogenic

bacteria (*Staphylococcus xylosus, Aeromonas hydrophila* gr.2 and *Streptococcus agalactiae)* and its effects on growth, hematological parameters and histopathology of African catfish, *Clarias gariepinus* led to conduct this study, which is regarded the first known work on the probiotic in this species. Further explanation on the nature of the present study is found in the next section.

1.3 Objectives of Study

The research was conducted to investigate the suitability of probiotic for the African catfish, *C. gariepinus* fingerlings reared to marketable size under laboratory condition. The specific objectives of this study were:

1) To determine the parameters for preparation of live probiotic, *L. acidophilus* for inclusion in fish diet.

2) To determine the effects of dietary inclusion of probiotic, *L. acidophilus* on growth performance and hematological parameters of African catfish *C. gariepinus.*

3) To determine the effects of dietary inclusion of probiotic, *L. acidophilus* on immunoglobin, hematological parameters and histopathology of *C. gariepinus* injected with three common pathogenic bacteria, *Staphylococcus xylosus, Aeromonas hydrophila* gr.2, and *Streptococcus agalactiae*.

4) To determine the effects of the probiotic *L. acidophilus* on the viability of three common pathogenic bacteria, *S. xylosus, A. hydrophila* gr.2 and *S. agalactiae, in vitro*.

Chapter 2

Literature Review

2.1 Aquaculture

2.1.1 Introduction

Aquaculture is known as an economic important sector in many developing countries; therefore, it has attracted the attention of many private and public sectors. The most productive countries of fish are aimed to increase fish supplies from aquaculture for the local and export markets and this sector contributes to food security in rural areas.

The aquatic resources are an unlimited gift of nature. The rapid growth in aquaculture production has made the sector important to many countries, particularly in developing countries. Accordingly, FAO's report of the year 2008 stated that aquaculture in 2008 has grown more rapidly than 2007 to meet the growing demand of fish (FAO, 2008).

Because of the fact that aquaculture is one of the fastest-growing food production activities in the world, many authors have given attention to the importance of fish culture as a means of obtaining high food production to meet the needs of a growing world population. Recently, many countries are becoming interested in encouraging fish culture as a part of a global policy, for it improves the efficiency of their populations through the improvement of their nutritional content and their livelihoods. Therefore, world fisheries have changed rapidly during the last decades. New technologies, making of Exclusive Economic Zone, UN meeting of the Law of the Sea in 1982 and

other developing activities have enhanced the fisheries management and production (Ahmed *et al.*1999).

2.2 Catfish (*Clarias gariepinus*)

According to Myers *et al.*(2006) systematize the taxonomy of African catfish as follows:

Kingdom Animalia (animals)

Subkingdom Bilateria (bilaterally symetrical animals)

Branch Deuterostomia (deuterostomes)

Phylum Chordata (chordates)

Subphylum Vertebrata (vertebrates)

Superclass Gnathostomata (jawed vertebrates)

Class Actionopterygii (ray-finned fishes)

Subclass Neopterygii

Infraclass Telestoi

Superorder Ostariophysi

Order Siluriformes (catfishes)

Suborder Siluroidae

Family Clariidae (air breathing catfishes)

Genus *Clarias*

Species *Clarias gariepinus* (African catfish)

Recent revisions of the systematics of African *Clarias* have resulted in several widespread species being synonymised under the name *Clarias gariepinus*. *Clarias gariepinus* has been placed in the subgenus *Clarias (Clarias)* together with *C. anguillaris*, *C. senegalensis* and others (Teugels, 1984).

African catfish, *C. gariepinus* are elongated body, a bony large head with small eyes. Dorsal and anal fins are lengthy with no adipose fin. The mouth is terminal and large. Four pairs of barbells (nasal, maxilar, outer mandibular and inner mandibular) are identifiable. The gills are open wide, and have a deep groove with a strong operculum. The colour varies from sandy-yellow through gray to olive with dark greenish-brown markings while the belly is white (Teugels, 1986; De Vos, 1986; Skelton, 1995; Fasakin *et al.*2003; FAO, 2004).

African catfish is known as a bottom feeder but sometimes feeds at the surface of water. This species is an efficient omnivorous, which is well equipped to exploit whatever resources available for both animal and plant protein (Hecht *et al.*1997). In natural conditions, the fish feed on insects, crabs, plankton, snails and fish and also eat rotting flesh, plants and fruits when prey animals become scarce (FAO, 2004). Catfish are characteristically very adaptable, could stay alive out of water for considerable periods of time if they remain humid (Teugels, 1986; Skelton, 1995).

Clarias gariepinus is a freshwater fish species and the second most important group of cultured fish in the world (Fasakin *et al.* 2003). According to FAO report (2002), *C. garipienus* has been translocated to a number of countries from Africa and is newly farmed either in its pure shape or as a hybrid in the Republic of Korea, the People's Republic of China, Taiwan, Philippines, Vietnam, Cambodia, Laos, Thailand, Malaysia, Indonesia, Brazil and West and Eastern Europe (Bruton, 1986; De Moor and Bruton, 1988).

According to Britz (1988) and Britz and Hecht (1989) African catfish are hardy fishes and can tolerate many environmental factors as such as low or high temperature, salinity, pH, un-ionized ammonia and oxygen as well as high tolerance to widely differing turbidity values.

Clarias gariepinus is an effective air-breather using the epibranchial organ, epibranchial epithelium, gill fans and probably the skin on the dorsum to do so and drowns if denied access to air. Strong resistance to desiccation is due to their air-breathing habit. When the gills breakdown or are closed with mud, the catfish secretes mucus to maintain a moist skin, or dig holes or crude burrows.

This species is highly tolerant of a fluctuating culture environment, and is therefore, recommended for high-density culture. The wide tolerance range of the African catfish shows that it is undoubtedly one of the most suitable species for aquaculture in the world (Britz, 1988; Britz and Hecht, 1989).

2.3 Nutritional Requirements

Accurate nutrition is one of the most important factors, which allow cultured fish to attain their genetic potential for growth, health, reproduction and long life. The nutrient necessities differ between species and with species between the life-cycle (De Silva and Anderson, 1995). Fish need energy to live, digest food, movement, and other physiological activities. They obtain it from the energy stored in the chemical bonds of the food which are consumed; the majority of gross energy, in food is obtained in three types of molecules, the protein, lipid and carbohydrate molecules, the energy liberated when these bonds are broken. This nutrient may come from natural food sources in the water and from whole artificially prepared feeds.

Because of the fact that aquaculture technology has advanced, there has been a trend toward faster growth and higher yield through intensive fish farming, requiring for improvement in the replacement of natural food with prepared feeds (Lovell, 1989). The energy requirements of fishes are influenced by physical, biochemical activities, water temperature, salinity, body size, age, density, reproductive, and stress factors.

Generally, protein requirements reduce with increasing age of fish; in the case of C*. gariepinus,* early juveniles require 55% protein after the post hatchery period of 10-15 days, and the gross dietary protein requirement reduces to between 38% - 42% after around 6 weeks (Machiels and Henken, 1985; Hecht, 1996). Janssen (1984) reported *C. gariepinus* requires 40% crude protein, 10% crude fat, 30% nitrogen-free extracts, 9% ash, and 11% moisture. Al-Dohail (2005) reported that when mixed feeding schedules were used, *C. gariepinus* requires proper diets contain 25% to 35% crude protein and 10% crude lipid. Where natural food is obtainable, catfish require about 36% - 40% of protein levels to grow under intensive conditions. Dry or moist food in different particle sizes appropriate for the life cycle phase or fish size is consumed by catfish (Lee, 1981). Generally, carnivorous fish need higher protein requirements than herbivorous, omnivorous and detritivorous fish.

Understanding the food habits, that is the composition of preferred foods of fish, is an important factor for the evaluation of their nutritional requirements, which provide some indications of how a prepared diet should be formulated to the optimal protein, lipid, carbohydrate and energy levels (Stickney, 1993). According to Haylor (1989) nutritional requirement of African catfish was summarized in Table 2.1.

Table 2.1 Nutritional data of African catfish (fry, fingerling and adult)

Nutritional requirement (%)	African catfish		
	Fry	Fingerling	Adult
Crude protein	50	40	40
Crude lipid	9.5	10	10
Carbohydrate	20	30	30
Fiber	1.5	<20	<20

2.4 Feeding Mechanism and Digestive System

Fish are usually classified based on the nature of the food ingested as follows: herbivorous, detritivores, omnivorous, and carnivorous and it is important to note that feeding behavior can change throughout a fish's life cycle. For instance, zooplankton may be eaten by a species when it is a juvenile, but it may change to feed largely on plants when it grew up. Moreover, fish may also be classified into ecological groups either according to the prevailing feeding conditions i.e. pelagic plankton feeders, benthos feeders or according to, functional adaptations, which are associated with the nature of food and feeding behavior. The digestive system of teleosts fish shows in Plate 2.1. The system could either be a straight tube from the mouth to the anus, or it may form loops that are divided into different parts having different functions (De Silva and Anderson, 1995). Dabrowski (1984) and Person-Le Ruyet (1989) summarized the important changes in the digestive tract during ontogenesis of fish larvae. At hatching, the digestive tract is a straight tube which is closed at the mouth and also stays quite unchanged until the completion of yolk absorption. This is followed by the development of a buccopharynx, foregut, midgut and hindgut. The larval phase is completed when a stomach with gastric glands and pyloric caeca developed. The liver and the pancreas are created at hatching and functions at first feeding.

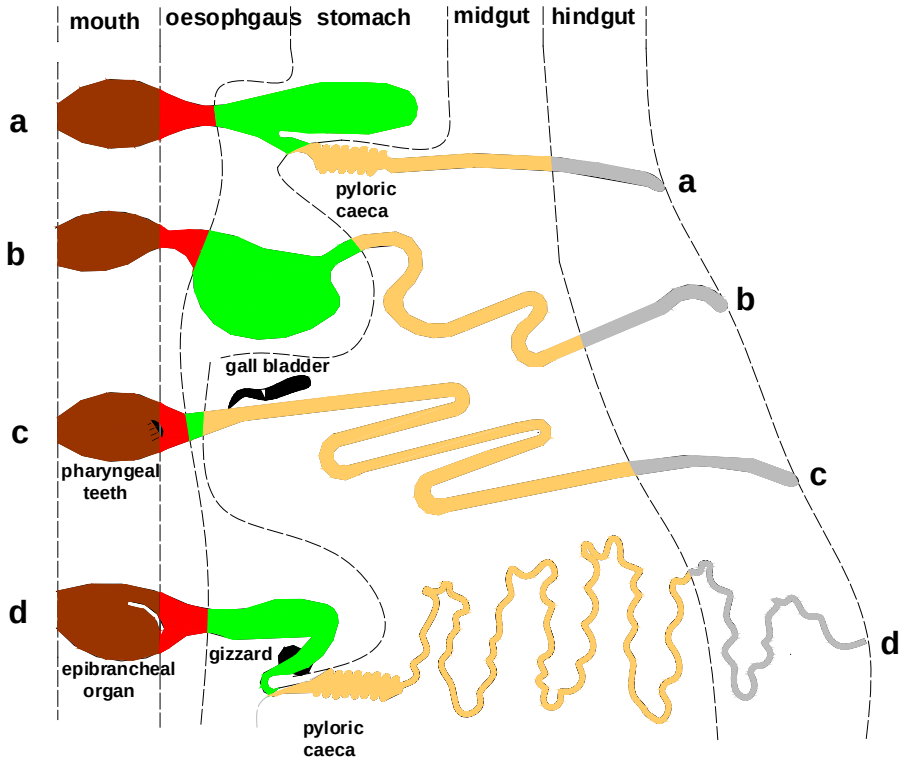

Plate 2.1 The digestive systems of four fish, according to feeding habits: (a) Rainbow trout (carnivore). (b) Catfish (omnivore emphasizing animal sources of food). (c) Carp (ominivore, emphasizing plant sources of food). (d) Milkfish (microphagous plaktivore). Source: De Silva and Anderson (1995); Al-Dohail (2005).

Consecutive studies by optical and electron microscopy on the structure and function of the cod, *Gadus morhua,* stomach has been carried out (Mattison and Holstein, 1980; Holstein and Cederberg, 1980). In fish, intestine is a simple absorbing epithelium lined with a brush border of microvillus, which is typical of absorptive tissues. In most fish, the intestine may be distinguished and categorized according to their physiological functions in that the anterior part, which received the bile and pancreatic juices, is distinguished by the presence of great numbers of chylomicrons in the epithelial cells and the distal part is distinguished by pinocytotic activity and cells containing granules consisting of absorbed nutrients (De Silva and Anderson, 1995). Pyloric caecae is a supplementary appendage in teleosts fish, having a cellular structure like that of the intestinal caecae. Buddington and Diamond (1986), De La Noüe *et al.* (1989) and Bauermeister *et al.*(1979) reported that the pyloric caece enhances the absorption of

amino acids, carbohydrates and lipids, whereas De La Noue *et al.* (1989) failed to observe such an effect on protein.

2.5 Probiotics

2.5.1 Definition

The word probiotic which means “for life” is derived from the Greek language (Reque *et al.* 2000). Many definitions for probiotics have been improved with time in the course of 13 years as follows: Fuller (1989) defined the probiotic as living microorganisms that promote the health of their host by improving the balance of the intestinal microbial flora. Havenaar and Huis (1992) also defined the probiotics as mono- or mixed living microbial cells that progress towards the health of the host by improving the natural microflora. Salminen (1996) defined the probiotic as living microbial cells that improve health and nutrition of the host. Schrezenmeir and De Vrese (2001) defined probiotics as living microbial cells that develope the health of the host when it is taken orally in adequate numbers. Gatesoupe (1999) redefined probiotics for aquaculture as living microbial cells added as dietary supplements, which improve health of the host. Additionally, the World Health Organization (FAO/WHO, 2001) defined probiotics as living microorganisms that promote the health of the host when it is applied in sufficient adequate numbers.

The probiotics must have the ability to survive in an intestinal tract in the present of acidic or alkaline condition. In addition, to avoid losing probiotics by food, the probiotics either have to grow at a rate faster than their removal or attach themselves to the wall of the intestine. Probiotics can adhere to surface of the intestine or overlying the epithelial layer (Fuller, 1989). The probiotics have to be able to adhere to epithelial layer (Tannock, 1999), thus, they prohibit pathogenic bacteria or reduce adherent of

pathogens (Conway *et al.* 1987). The probiotics also produce nutrients and such as short chain fatty acid, vitamins and antibacterial substance (Jigsaw, 2006).

2.5.2 Characters of Probiotics

It has been comprehensively reviewed on the use of probiotics to improve the health of humans and animals (Erickson and Hubbard, 2000; Guarner and Malagelada, 2003). Currently, probiotics have been used in food manufacturing because of their character in helping the humans and animals to digest food and to avoid many diseases.

The researches related to probiotics for aquaculture are summaried in Table 2.2. The effect of probiotics on some disease as fish bacteria disease has been studied (Gram *et al.*1999; Olafsen, 2001; Vanbelle *et al.*1990). Gram *et al.*1999 reported that probiotic bacteria reduced the mortality rate in rainbow trout infected with *Vibrio anguillarum* significantly; Olafsen (2001) also reported that improved disease resistance in cod fry; Vanbelle *et al.* (1990) reported that inhibited colonisation and proliferation of opportunistic and obligate pathogenic bacteria. Moreover, probiotic strains, *Lactobacillus* and *Bifidobacteria* species have the significant benefit and can reduce the duration of diarrhea in children (Mack *et al.*1999; Marteau *et al.* 2001; Szajewska *et al.* 2001).

According to Gilliland and Speck (1977) and Sandine (1979) *Lactobacillus acidophilus* inhibited many commonly food borne pathogens, *Lactobacillus* sp. inhibited bacterial pathogens and *Lactobacillus fermentum* (Aly Savadogo *et al.* 2004) inhibited growth of pathogenic bacteria. Although the inhibition of pathogen occurs clearly, the mechanism of inhibition against intestinal pathogens is not completely understood. Some studies reported that *L. acidophilus* produced antibiotic like substances, which have responsibility for inhibition action (Gilliland, 1989). Silva *et al.*

(1987) and Gilliland and Speck (1977) reported that *L. acidophilus* has the ability to inhibit some pathogenic bacteria such as *Staphylococcus aureus, Salmonella typhi* and *Escherichia coli*. Also, Helander *et al.* 1997 and Niku-Paavola *et al.* (1999) reported that *L. acidophilus* produces hydrogen peroxide, lactic acid, acetic acid, and other compounds, which widely inhibit many harmful organisms like *Salmonella*, *E. coli*, *Clostridium*, and *Helicobacter*. Furthermore, Aly Savadogo *et al.* (2004) reported that *Lactobacillus fermentum* has inhibitied *S. aureus* ATCC 25293. Klaenhammer (1993) showed that *Lactobacillus lactis 99 and Lactobacillus brevis* have inhibited *Streptococcus xylosus.*

Gilliland, (1989) claimed that microflora interfere with cholesterol absorption. Other studies suggested that cholesterol level decrease in the serum of blood when fermented milk or milk containing *Lactobacilli* was orally taken (Mann and Spoerry 1974; Harrison and Peat, 1975; Grunewald, 1982).

Ouwehand and Vesterlund (2003) reported that probiotics have the ability to prevent urinary tract infections by killing *E. coli* causing urinary tract infection. According to De-Ruiz *et al.* (1996) *Lactobacillus* has removed E*. coli* causing urinary tract infection in animals when *Lactobacillus* was orally administered.

Table 2.2 Summary of research related to probiotics for aquaculture

Fishes tested	Probiotic tested	Pathogen tested or study conducted	Method used	Reference
Atlantic cod	*Carnobacterium divergens*	*V. anguillarum*	*In vitro* and *in vivo*	Gildberg *et al.* (1997)
Atlantic cod	*Carnobacterium divergens*	*V. anguillarum*	*In vitro* and *in vivo*	Gildberg and Mikkelsen (1998)
Atlantic salmon	*Lactobacillus plantarum*	*A. salmonicida*	*vitro* and *in vivo*	Gildberg *et al.* (1995)
Atlantic salmon	*Carnobacterium* sp. *(K1)*	*V. anguillarum, A. salmonicida*	*In vitro* and *in vivo*	Jöborn *et al.* (1997)
Atlantic salmon	*Ps. fluorescens*	*A. salmonicida*	*In vitro* and *in vivo*	Gram *et al.* (2001)
Atlantic salmon, rainbow trout	*Carnobacterium* sp.	*V. anguillarum, V. ordalii, Y. ruckeri, A. salmonicida*	*In vitro* and *in vivo*	Robertson *et al.* (2000)
Eel	Commercial product: Cernivet® LBC (Ent. Faecium SF68), Toyocerin® (B. toyoi)	*Ed. tarda*	*In vivo*	Chang and Liu (2002)
Eel	*A. media*	*Saprolegnia* sp.	*In vitro* and *in vivo*	Lategan and Gibson (2003)
Eel	*A. media*	*Saprolegnia parasitica*	*In vivo*	Lategan *et al.* (2004b)
Gilthead sea bream	*Cytophaga* sp., *Roseobacter* sp., *Ruergeria* sp., *Paracoccus* sp., *A.* sp., *Shewanella* sp.	*Natural larval survival study*	*In vivo*	Makridis *et al.* (2005)
Gilthead sea bream	*V.* sp., *Micrococcus* sp.	*L. anguillarum*	*In vitro* and *in vivo*	Chabrillón *et al.* (2006)
Goldfish	*Dead cells of A. hydrophila*	*A. salmonicida*	*In vivo*	Irianto *et al.* (2003)
Indian major carp	*B. subtilis*	*A. hydrophila*	*In vivo*	Kumar *et al.* (2006)
Nile tilapia	*Str. faecium, Lactobacillus acidophilus, Sacc. cerevisiae*	*Growth study*	*In vivo*	Lara-Flores *et al.* (2003)
Pollack	Commercial product: Bactocell (*Pediococcus acidilactici*), Levucell (*Sacc. cerevisiae*)	Pollack growth study using enriched Artemia	*In vivo*	Gatesoupe (2002)
Rainbow trout	*Ps. fluorescens*	*V. anguillarum*	*In vitro* and *in vivo*	Gram *et al.* (1999)

Table 2-2 Summary of research related to probiotics for aquaculture

Fishes tested	Probiotic tested	Pathogen tested or study conducted	Method used	Reference
Rainbow trout	*Lactobacillus rhamnosus.*	*A. salmonicida* sp. *salmonicida (furunculosis)*	*in vivo*	Nikoskelainen *et al.* (2001a)
Rainbow trout	*Ps.* sp.	*V. anguillarum*	*In vitro* and *in vivo*	Spanggaard *et al.* (2001)
Rainbow trout	*A. hydrophila, V. fluvialis, Carnobacterium* sp.	*A. salmonicida*	*In vitro* and *in vivo*	Irianto and Austin (2002b)
Rainbow trout	*Dead cells of A. hydrophila, V. fluvialis, Carnobacterium* sp.	*A. salmonicida*	*In vivo*	Irianto and Austin (2003)
Rainbow trout	*Lactobacillus rhamnosus*	Immune enhancement	*In vivo*	Nikoskelainen *et al.* (2003)
Rainbow trout	*Commercial product: BioPlus2B (B. subtilis, B. licheniformis)*	*Y. ruckeri*	*In vivo*	Raida *et al.* (2003)
Rainbow trout	*Lactobacillus rhamnosus*	Natural immunostimulation measured	*In vivo*	Panigrahi *et al.* (2004)
Rainbow trout	*Pediococcus acidilactici, Sacc. boulardii*	*Prevention of vertebral column compression syndrome*	*In vivo*	Aubin *et al.* (2005)
Rainbow trout	*A. sobria*	*L. garvieae, Str. iniae*	*In vivo*	Brunt and Austin (2005)
Rainbow trout	*Lactobacillus rhamnosus*	Natural immunostimulation measured	*In vivo*	Panigrahi *et al.* (2005)
Rohu	*B. circulans, B. subtilis*	Digestive enzyme study	*In vivo*	Bairagi *et al.* (2004)
Sea bass	*Debaryomyces hansenii, Sacc. cerevisiae*	Digestive enzyme study	*In vivo*	Tovar *et al.* (2002)
Senegalese sole	*V.* sp., *Ps.* sp., *Micrococcus* sp.	*V. harveyi*	*In vitro* and *in vivo*	Chabrillón *et al.* (2005)
Silver perch	*A. media*	*Saprolegnia* sp.	*In vivo*	Lategan *et al.* (2004a)
Tilapia	Commercial product: Alchem Poseidon, Korea	*Ed. tarda*	*In vivo*	Taoka *et al.* (2006)
Turbot	2 unidentified marine bacteria	GIT colonization study	*In vivo*	Makridis *et al.* (2000)
Turbot	Marine bacteria	Natural survival study	*In vivo*	Huys *et al.* (2001)
Turbot	*Roseobacter* sp., *V.* sp.	*V. anguillarum, V. splendidus, Psalt.* sp.	*In vitro* and *in vivo*	Hjelm *et al.* (2004)

Ps. = *Pseudomonas, A. = Aeromonas, V. = Vibrio, Alt. = Alteromonas, Pa. = Pasteurella, Ed. = Edwardsiella, Y. = Yersinia, Psalt. = Pseudoalteromonas, S.= Staphylococcus, Pr. = Proteus, Ca. = Candida, Ent. = Enterococcus, E. = Escherichia, L. = Lactococcus, P. = Photobacterium, Bif. = Bifidobacterium, Fl. = Flavobacterium, Str. = Streptococcus, Sacc. = Saccharomyces, B. = Bacillus, IHNV = infectious hematopoietic necrosis virus, OMV =Onchorhynchus masou virus.*

2.6 Probiotic Bacteria in Aquatic Feed

Probiotic bacteria have been already added to diet of several fishes (Table 2.3) and it was more attractive due to its advantages. Different types of lactic acid bacteria have been added to animal feed (Jiraphocakul *et al.* 1990), fish feed (Gildberg *et al.* 1997) and added to drinking water (Watkins and Kratzer, 1984).

Probiotic bacteria have been added to the several fish feeds i.e. *Lactobacillus rhamnosus* used in the diet of Rainbow trout (Nikoskelainen *et al.* 2001; Panigrahi *et al.* 2004); *Enterococcus faecium* added to diet of *Anguilla anguilla* (Chang and Liu, 2002); *Bacillus circulans* added to diet of Labeo rohita (Ghosh *et al.* 2004). Also, *Carnobacterium* has been used as a probiotic in fish dry feed (Gildberg *et al.* 1997; Gildberg and Mikkelsen, 1998, Robertson *et al.* 2000). On the other hand, probiotic bacteria have been added to fishes cultured water i.e. *Bacillus megaterium, B. subtilis, B. polymyxa* and *B. licheniformis* used in cultured water of Channel catfish (Queiroz and Boyd, 1998); *Vibrio pelagius* added to cultured water of Turbot (Ringø and Vadstein, 1998); *Roseobacter* sp. strain 27-4 inserted in cultured water of Turbot larvae (Hjelm *et al.* 2004).

Furthermore, Anadón *et al.* (2006) reported that probiotics i.e. *Bacillus cereus var. toyoi, B. licheniformis*, *B. subtilis*, *Enterococcus faecium, L. acidophilus*, *L. casei*, *L. farciminis*, *L. plantarum*, *L. rhamnosus, Pediococcus acidilactici* and *Streptococcus infantarius* have been used in animal feed in the European Union. In general, proiotic bacteria have been improving a body weight gain and food conversion ratio of fishes and animals.

Table 2.3 Probiotics used in fishes diets

Fishes tested	Probiotic tested	Probiotic source	Method used	Reference
Turbot larvae (Scophthalmus maximus)	*Streptococcus lactis and Lactobacillus bulgaricus*	Unknown	Enrichment of live food	Banda *et al.* (1992)
Turbot larvae	*Lactobacillus* sp. and *Carnobacterium* sp.	Rotifers (Brachionus Plicatilis)	Enrichment of rotifers	Gatesoupe (1994)
Atlantic salmon (Salmo salar L.)	*Vibrio alginolyticus*	Commercial shrimp hatchery	Bathing in bacterial suspension	Austin *et al.* (1995)
Atlantic cod fry	*Carnobacterium divergens*	Intestines of Atlantic salmon	Addition to diet	Gildberg and Mikkelsen (1998)
Oreochromis niloticus	*G-probiotic*	Commercial product	Addition to diet	Naik *et al.* (1999)
Atlantic salmon	*Carnobacterium* sp.	Intestines of Atlantic salmon	Addition to diet	Robertson *et al.* (2000)
Rainbow trout	*Lactobacillus rhamnosus ATCC 53103*	Culture collection	Addition to diet	Nikoskelainen *et al.* (2001)
Rainbow trout	*Aeromonas hydrophila, Vibrio fluvialis, Carnobacterium* sp., *Micrococcus luteus*	Digestive tract of rainbow trout	Addition to diet	Irianto and Austin (2002b)
Anguilla anguilla	*Enterococcus faecium SF68*	Commercial product (Cernivet)	Addition to diet	Chang and Liu (2002)
Rainbow trout	*L. rhamnosus JCM 1136*	Culture collection	Addition to diet	Panigrahi *et al.* (2004)
L. rohita	*Bacillus circulans*	Intestines of Labeo rohita	Addition to diet	Ghosh *et al.* (2004)

2.7 Types of Probiotics

There are many types of micro-organisms that have been used as probiotics for animals and humans. The important lists of the known probiotics available were presented in Table 2.4 (Mombelli and Gismondo, 2000; Holzapfel and Schillinger, 2002). The known *Lactobacilli* and *Bifidobacteria* are the two major types of probiotic,

which was designed for human and animal use. Also, *Saccharomyces*, (yeast) have been used for human and animal (Holzapfel *et al.* 1998). Although *Lactobacilli* and *Bifidobacteria* are reported as safe probiotic organisms due to their long safety usage and non-pathogenic behaviors, the safety is still important and have been continuously investigated and revised (Blanquet *et al.* 2005; Ross *et al.* 2005, Leverrier *et al.* 2005).

Table 2.4 Types of common probiotics for human and animal uses

Lactobacillus species	*Bifidobacterium* species	Another species
Lactobacillus acidophilus	*Bifidobacterium bifidum*	*Bacillus cereus (toyoi)*
L. casei	*B. lactis*	*Saccharomyces cerevisiae*
L. crispatus	*B. longum*	*Saccharomyces boulardii*
L. plantarum	*B. breve*	*Lactococcus lactis*
L. rhamnosus	*B. infantis*	*Stryptomyces* (yeast)
L. salivarius	*B. adolescentis*	*E. coli Nissle*
L. gasseri	*B. animalis*	
L. buchneri		
L. johnsonii		
L. reuteri		
L. fermentum		

Source: (Mombelli and Gismondo, 2000; Holzapfel and Schillinger, 2002)

2.8 *Lactobacilli*

Lactic acid bacteria are classified under family of *Lactobacilli*. About 65 species of *Lactobacillus* have been documented (Hammes and Vogel, 1995). Axelsson (1993) and Klein *et al.* (1998) reported that *Lactobacilli* is a gram positive, non-motile, non-spore-forming anaerobic or aerobic rods and usually catalase negative. *Lactobacillus* mainly occurs in the oral cavity, intestinal tract, and vargina in humans and animals (Bello and Hertel, 2006).

2.8.1 *Lactobacillus acidophilus*

The *L. acidophilus* is single cells or chains (Holt *et al.* 1994), non-motile, non-sporing, bile tolerant, and catalase negative (Femandes and Shahani, 1988; Klein, 1998; Holt *et al.* 1994). Furthermore, *L. acidophilus* grows at pH values between 5-7 and the optimum growth between 5.5 and 6.0 (Mead and Adams, 1975), at low temperature 15°C, at higher temperature 45°C and the optimal growth between 35–38°C (Hammes and Vogel, 1995).

Holt *et al.* (1994) reported that *L. acidophilus* produces about 85% of DL-lactic acid isomers and less than 10% of other products. Furthermore, *L. acidophilus* produces enzymes such as proteases, which digest proteins and lipases, which digest lipid (Fernandes *et al.*1987) and also generated some vitamins (Jigsaw, 2006). Also, *L. acidophilus* has the ability to produce sufficient amounts of hydrogen peroxide and bacteriocins, which have inhibitory actions against various pathogenic microorganisms (Dahiya and Speck, 1968; Nettles and Barefoot, 1993).

2.9 Antimicrobial Compounds of *Lactobacillus acidophilus*

There are many antimicrobial compounds that are produced by lactic acid bacteria. The antimicrobial compounds produced by lactic acid bacteria can inhibit the growth of pathogenic bacteria in its fermented products (Raccah *et al*. 1979, Smith and Palumbo 1983, Cintas *et al.* 1998). According to Smith and Palumbo (1981), Juven *et al.* (1992) and Soomro *et al.* (2002) the mechanisms of *Lactobacilli* to inhibit the other pathogenic bacteria are not completely understood. Smith and Palumbo (1981), Juven *et al.* (1992) and Soomro *et al.* (2002) connected the mechanisms of *Lactobacilli* to the production of lactic acid and other antimicrobial compounds. The antimicrobial compounds such as hydrogen peroxide, carbon dioxide, diacetyl uncharacterized

compounds, and compounds like bacteriocins, which produced by *L. acidophilus,* as following (Piard and Desmazeaud, 1991) :

2.9.1 Acidic Medium

In carbohydrate fermentation, the final main production of *L. acidophilus* is acetic and lactic acids. The pH value is decreased as a result of acetic and lactic acids, which have the ability to inhibit several types of pathogenic microorganisms (Jin *et al.* 1996; Altaf *et al.* 2006). Bhatia *et al.* (1989) and Midolo *et al.* (1995) claimed that *Lactobacillus* strains inhibited the growth of *Helicobacter pylori*, *Campylobacter jejuni*, *E. coli* and *Clostridium difficile* because of the acid productions. It also has the ability to inhibit the growth of *Salmonella enteritidis*, *S. typhimurium*, and *S. enteritidis* (Jin *et al.*1996). Moreover, Niku-Paavola *et al.* (1999) reported that *Lactobacillus plantarum* produces antibacterial compounds such as benzoic acid, which is active in the presence of lactic acid with the ability to inhibit the gram-negative organisms, and fungus such as *Fusarium.*

2.9.2 Bacteriocin

It is known that lactic acid bacterial species produces a bacteriocin composition of proteinaceous and bactericidal. The bacteriocin is classified into different classes: antibiotics which are small peptides (e.g. nisin), small heat-stable peptides, large heat-labile proteins, and complex bacteriocins, which are not well defined (Klaenhammer, 1993). Wei and Hansen (1993); Cleveland *et al.* (2001) reported that bactericidal have a wide rang of activity against pathogenic bacteria including *Streptococcus*, *Mycobacterium*, *Listeria monocytogenes*, *Clostridium botulinum*, *Staphylococcus aureus* and *Bacillus cereus*.

2.9. 3. Hydrogen Peroxide

It is known that lactic acid bacteria produce hydrogen peroxide (H_2O_2) in the presence of oxygen. Cords and Dychdala (1993) reported that the *Lactobacillus* and *Lactococcus* strains that product H_2O_2 could inhibit *Staphylococcus aureus, Pseudomona* sp. and various psychotrophic microorganisms in food. Moreover, Kong and Davison (1980) claimed that the antimicrobial effect of H_2O_2 might be due to the oxidation of sulfhydryl groups which caused denaturing of a number of enzymes and from the peroxidation of membrane lipids hence the increased membrane permeability.

2.9.4 Carbon Dioxide

It is known that lactic acid bacterial species produce carbon dioxide (CO_2) during fermentation processes. At the moment, the mechanism of CO_2 antimicrobial activity is not known accurately. According to Hotchkiss *et al.* (1999) CO_2 has the ability to inhibit the growth of gram negative bacteria, psychrotrophic. Eklund (1984) and Røssland (2005) claimed that CO_2 plays an important function in creating an anaerobic environment; therefore, the anaerobic environment inhibits enzymatic decarboxylations and the accrual of CO_2 in the cell may cause a dysfunction in permeability. Carbon dioxide can inhibit the growth of many microorganisms that food spoilage, particularly gram-negative psychrotrophic bacteria (Farber, 1991; Hotchkiss, 1999).

2.9.5 Aromatic Compounds

Diacetyl compound produces by lactic acid bacteria during fermentation processes. According to Jay (2000) diacetyl compound inhibits the growth of gram-negative bacteria due to its reaction with the arginine-binding protein of pathogenic bacteria. He