Introduction

Tilapia is farmed in 100 countries in tropical and sub-tropical regions around the globe. It is the third most important cultured fish species group after cyprinids and salmonids, with the sector growing at an average rate of 11.2 % per year. The total world production of farmed tilapia has increased from 383,654 mt (metric tonnes) in 1990 to 2.5 million tonnes in 2007 (FAO, 2009). Feed represents a major cost factor in production of farmed tilapia. Global commercial tilapia feed production increased from 1.0 to 3.5 million tonnes from 1995-2007 and it is estimated that it will reach 12.0 million tonnes by 2020 (Tacon, 2010).

Tilapia in intensive aquaculture systems may be exposed to stress, infectious viral and bacterial diseases, parasite infestation and suboptimum poor environmental conditions particularly poor water quality, which may lead to high mortality and serious economic losses (FAO, 2004 and Subasinghe, 2005).

In Egypt, the prevention and treatment of infectious diseases in fish depend on the use of a number of Governmental approved antibiotics, chemotherapeutics and vaccines to control diseases and recommendation for better fish husbandry and strategies to minimize the environmental impact of aquaculture (Aly *et al*., 2008a). The use of antimicrobial drugs, antibiotics, pesticides, and disinfectants for disease prevention and the use of certain antibiotics to improve growth of farmed fish have led to the development of resistant strains of pathogens and also the presence of drug and chemical residues in fish products which may have an impact on human food safety (Boyd and Massaaut, 1999 and Esiobu *et al*., 2002). It is also recognized that antibiotic residues may modulate the immune response of the fish (Lunden *et al*., 2002). The research on alternative strategies to reduce the use of antibiotics and better fish health management by proper nutrition and feeding of tilapia including the use of certain functional ingredients, feed additives and probiotics has been expanded.

Probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, are increasingly viewed as an alternative to antibiotic treatment. Using of probiotics in human and animal nutrition is now well documented (Fuller, 1992; Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000; Irianto and Austin, 2002a; Bache`re, 2003 and Rinkinen *et al*., 2003a). Probiotics have been defined as microbial dietary supplements of benefit to the host (Fuller, 1989). The benefits of probiotics supplements include improved feed utilization, contribution of exogenous enzymes in digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, growth promotion, and increased immune response (Verschuere *et al*., 2000).

It appears that probiotics provide benefits by establishing favorable microbial communities (e.g. lactic acid and *Bacillus* sp.) in the gastrointestinal tract which may alter gut morphology and produce certain enzymes and inhibitory compounds causing improved digestion and absorption of nutrients as well as enhanced immune response (Gatesoupe 1999; Verschuere *et al*., 2000; Kesarcodi-Watson *et al.*, 2008 and Ringø *et al*., 2010). Several studies have demonstrated that the use of probiotics improve the health of larval and juvenile fish, growth performance and body composition. However, the mode of action in fish species may vary between farmed fish species cultured in freshwater and marine environments.

Probiotics have been used as feed additives to improve fish growth including African catfish, *Clarias gariepinu* (Al-Dohail *et al.*, 2009), Senegalese sole, *Solea senegalensis* (Sáenz de Rodrigáñez *et al.*, 2009), tilapia, *Oreochromis niloticus* (Lara-Flores, *et al*., 2003 and El-Haroun *et al*., 2006) Japanese flounder, *Paralichthys olivaceus* (Taoka *et al*., 2006a), and gilthead seabream, *Sparus aurata and* Seabass, *Dicentrarchus labrax*

(Carnevali *et al.*, 2006). The effects of probiotics have been linked to modulation of gut microbiota and establishment of the beneficial microorganisms, higher specific and total digestive enzyme activities in the brush-border membrane which increases the nutrient digestibility and feed utilization. In addition, the production of vitamins by these gut microbiota could also increase vitamin synthesis and improve fish health. (Verschuere *et al.*, 2000; Balcázar *et al.*, 2006a and Kesarcodi-Watson *et al.*, 2008). To date, few studies have been carried out to study the effects of probiotics on the biochemical metabolism of fish.

Historical review

Environnemental conditions for tilapia culture

In tropical regions, tilapia are cultured commercially most often in earthen ponds. In some countries, intensive culture of tilapia is also carried out in either flow through tanks and raceways or 'recirculation water systems". Water quality criteria for tilapia culture have been defined for number of years (Balarin and Haller, 1979). Although tilapia survive under degraded water quality as compared to other warm water fishes, optimum conditions must be maintained for better growth, feed utilization and disease prevention. When water quality is not adequate for tilapia culture, reduced growth and increased mortality may occur, leading to lower production and profit. Water quality, especially parameters such as temperature, pH, ionized and deionized ammonia, nitrate, nitrite and dissolved oxygen are paramount to the many factors that affect fish health, growth and reproduction**.** Temperature is also a critical water quality parameter for all aquatic animals and optimum temperature must be provided. The main cause of mortality in aquarium fish is the inadequate maintenance of the water environment (Camus *et al.*, 1998).

Nutrient requirements of fish

Fish feeds are formulated to provide essential nutrients (protein, amino acids, fatty acids, minerals and vitamins) and energy for growth, reproduction, and health. The development of feeds for any farmed fish requires sound knowledge of the quantitative nutrient requirements for economic feed formulation. All the essential nutrients required for fish including essential amino acids and fatty acids, fat and water soluble vitamins, macro and trace elements, and energy-yielding macronutrients (protein, lipid and carbohydrate) are important in nutrition and feeding of tilapia. A formulated diet must supply all the essential nutrients and energy required to meet the physiological needs of growing fish and successful reproduction of broodstock fish. In the past three decades, the quantitative requirements for all essential nutrients for several warm water fish species including tilapia have been established (NRC, 1993). The minimum nutrient requirements established will promote optimum growth and prevent nutrient deficiency signs and higher intakes of certain vitamins, minerals, amino acids and essential fatty acids increase the build up of their reserves in the tissues. Certain minerals (e.g. copper and selenium) and fat soluble vitamins (e.g. vitamin A) may be toxic when taken in excess amounts. Deficiencies or excesses of each of the major dietary components have profound effects on disease development and the survival of the fish, largely through their effects on host defense mechanisms (Lall, 2010). A brief introduction to the major nutrient requirements of tilapia has been provided in Table I (NRC, 1993 and Shiau, 2002).

Table I Nutrient requirement of Tilapia

Nutrients	Requirement	Reference
Energy (K cal DE/kg diet)	3000 (12.5 MJ/kg)	NRC, 1993[2]
Protein, crude (digestible), %	32 (28)	
Amino acids (%)		
Arginine	1.18	NRC, 1993
Histidine	0.48	NRC, 1993
Isoleucine	0.87	NRC, 1993
Leocine	0.95	NRC, 1993
Lysine	1.43	NRC, 1993
Methionine + Cystine	0.90	NRC, 1993
Phenylalanine + Tyrosine	1.55	NRC, 1993
Threonine	1.05	NRC, 1993
Yryptophan	0.28	NRC, 1993
Valine	0.78	NRC, 1993
Fatty acids (%)		
n-3 fatty acids	-	NRC, 1993
n-6 fatty acids	0.5-1	NRC, 1993
Macrominerals (%)		
Calcium	R[1]	NRC, 1993
Magnesium	0.06	NRC, 1993
Phosphorus	0.5	NRC, 1993
Potassium	0.21-0.33	Shiau. 2002[3]
Sodium	NT[1]	
Microminerals (mg/kg)		
Copper	R[1]	NRC, 1993
Iodine	NT[1]	
Iron	NT[1]	
Manganese	R[1]	NRC, 1993
Zinc	20	NRC, 1993
Selenium	NT[1]	
Fat soluble vitamins		
Vitamin A, IU/kg	NT[1]	
Vitamin D_3, IU/kg	NT[1]	
Vitamin E, IU/kg	50-100	NRC, 1993; Shiau. 2002
Vitamin K, mg/kg	NT[1]	
Water soluble vitamins(mg/kg)		
Riboflavin	6	NRC, 1993
Pantothenic acid	10	NRC, 1993
Niacin	121	Shiau. 2002
Vitamin B_{12}	NR	Shiau. 2002
Choline	1000	Shiau. 2002
Biotin	0.06	Shiau. 2002
Folate	NT	
Thiamin	2.5	Shiau. 2002
Vitamin B_6	3	Shiau. 2002
Myoinsitol	NT[1]	
Vitamin C	50	NRC, 1993

[1]R: required in diet but quantity not determined, NR: no dietary requirement demonstrated under experimental conditions, NT: not tested
[2]Recommended nutrient requirement values by NRC (1993)
[3]Requirement values summarized by Shiau, 2002.

Growth and growth promoters

Growth represents a wide range of biochemical reactions which are reflected on fish body size. It results from a combination of cellular division and increase in cell size so it is regulated by a number of different hormones and nutrients. Growth can be represented as somatic and /or reproductive growth. Growth is affected by environmental parameters such as temperature and water quality, by social parameters such as stocking density and hierarchical structures, by genetic factors and by nutrition and feeding. Deficiency in energy, amino acids or total nitrogen, essential lipids, vitamins or minerals all result in decreased growth (Matty, 1986).

In addition to the nutrient requirements for fish represented in protein, fat, carbohydrate, vitamins and minerals which all are very important for growth, there are also some growth promoters such as antibiotics, hormones and biological growth promoters (probiotics). All these play a role in the improvement and stimulation of fish growth.

Antibiotics

Antibiotics are antibacterial compounds or drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of micro-organisms. In addition to their use as anti-infectious agents, antibiotics are used as growth promoters in farmed animals. Their use in animal feeds can increase the intestinal absorption of nutrients resulting in higher bioavailability of food energy and higher using of ingested nutrients for growth and production, thereby improving the efficiency of nutrient use. The mode of action of antibiotics involves elimination of the pathogenic microorganisms and changes in non-pathogenic intestinal flora which induce beneficial effects on digestion and absorption of nutrients from feeds including a significant increase in the digestibility of dietary protein (FAO, 2005).

The use of antibiotics and drugs in fish industry is complicated and several factors such as the food safety of fish products, the environmental impact, fish welfare, and the safety of the persons administering these compounds must be considered. There are no new antibiotics specifically developed for aquaculture, however, the authorized products prescribed for veterinary use in animal production are used to control infectious diseases in aquaculture (FAO, 2005). Several antibiotics such as oxytetracycline, sulfamerazine and ormethoprim, which have been used in human medicine to control diseases, have been also used for the treatment of bacterial infectious diseases in salmon, catfish, trout and other fish species. Some of fish diseases treated with these antibiotics are skin ulcers, diarrhea and septicemias. However, the pathogens responsible for these infections belong to the same group of bacterial infections that causes disease in humans and its residues in fish products are likely to induce antibiotic resistance in human. It has been observed that bacteria can be transmitted from any aquaculture ecosystem directly to humans either by handling of fish without proper precautions (Bisharat and Raz, 1996 and Weinstein *et al*., 1997) or by consumption of contaminated aquaculture products with bacteria (Ministry of Health and Welfare, 1999).

The use of antibiotics in the aquatic environment may result in increased antibiotic resistance of bacteria in this environment (Petersen *et al*., 2002 and Alcaide *et al*., 2005) and an increase of antibiotic resistance in fish pathogens. The emergence of antibiotic resistance among fish pathogens undermines the effectiveness of antibiotics in aquaculture (L'Abee-Lund and Sørum, 2001 and Sørum, 2006). Resistance to antibiotics is determined by the bacterial genome, this genome may change due to a mutation or by acquisition of new genetic material. Disease resistance to many antibiotics does not arise by mutation of the bacterial chromosome, however rather by the acquisition of new genes who's their products affect resistance by a variety of mechanisms (SVARM, 2001). The resistant bacteria may transfer the resistance determinants to other bacteria (even to bacteria of different genera) of the terrestrial environment, including human and animal pathogens that have never been exposed to the antibiotic, and this phenomenon is known as horizontal gene transfer (Rhodes *et al.*, 2000; SVARM, 2001and Sørum, 2006). Horizontal gene transfer mechanisms involved in exchanging resistance determinants between aquatic and terrestrial bacteria include conjugation or conjugative transposition (Agerso and Guardabassi, 2005 and Casas *et al.*, 2005) and transduction may also play an important role in these processes due to the high concentrations of viruses in seawater and the marine sediment (Bushman, 2002). It has been observed that the use of one antibiotic can lead to increased resistance not only to itself, but also to another unrelated antibiotics, this is known as co-selection (SVARM, 2001). The application of genome-based technologies such as expression profiling and proteomics will lead to search of new antibiotics and better disease prevention.

The use of antibiotics in aquaculture causes some hazards and risks to human, which are:

- The first risk is considered as the transfer of resistance, which occurs via the transmission of resistant bacteria from aquaculture environments to human and in turn results in increase the resistance in human bacterial pathogens. It can also develop via the transmission of nonpathogenic bacteria containing antimicrobial resistance genes and the subsequent transfer of such genes to human pathogens (FAO, 2005).
- The second risk is the antibiotic residues in finished farmed products, which results in accumulation of antibiotics in flesh after the long term feeding of these drugs to fish. This practice may cause significant bioaccumulation and chronic toxicity in fish. The incidence of antibiotic residue toxicity depends on if this antibiotic is poorly or highly absorbed by

fish. If the antibiotic is poorly absorbed in the intestinal tract, their residues in fish may be less important. On the other hand, if antibiotics are efficiently absorbed, a substantial increase of their accumulation may occur (SOU, 1997). The presence of antibiotics residual in commercialized fish and shellfish products (Cabello, 2004; Angulo *et al.*, 2004 and Sørum, 2006) may results in undetected consumption of antibiotics by the human consumers of fish which in turn causes a potential alteration of their normal flora that increases their susceptibility to bacterial infections and also the occurrence of antibiotic resistant bacteria (McDermott *et. al.,* 2002; Greenlees, 2003 and Cabello, 2004).

- The third risk is the allergic reaction which may result from human contact with the drug during the treatment of fish with antibiotics. The daily exposure to some antibiotics such as macrolides, quinoxalines and bacitracin can lead to sensitization and in some cases incidence of asthma and dermatitis. The human's exposure to antibacterial feed additive substances may be caused during feed mixing processing (FAO, 2005). It has been reported that allergic reactions occurs frequent in farmers and unprotected workers in the aquaculture industry due to their handling to macrolides, spiramycin and tylosin on regular basis at fish farms (Danese *et al.*, 1994). Moreover, the problems of allergy and toxicity can be generated from undetected consumption of antibiotics residuals in fish which are difficult to diagnose due to the lack of previous information on antibiotic ingestion (Alderman and Hastings, 1998 and Cabello, 2004).

- Another risk is related to the aquatic environment. Many antibiotics used in the animal feed are poorly absorbed in the gut of the animal and approximately 30-90% may be excreted (Alcock *et al.*, 1999). Certain antibiotic metabolites can also be bioactive which may be transformed back to their parent compound after excretion (Langhammer, 1989). It appears that a considerable amount of the administered antibiotics may be excreted into the aquatic environment in an active form. If antibiotics are not efficiently degraded, it is possible that these residues may facilitate growth and development of antibiotic resistant microbial populations (Witte, 1998), this can potentially have deleterious effects on environment and cause adverse effects on human health and terrestrial and aquatic ecosystems.

The existence of large amounts of antibiotics in the environment of water and sediments also can affect the presence of the normal flora and plankton in those niches, resulting in shifts in the diversity of the microbiota (Sørum, 2006).

For all the above mentioned reasons, the impact of antibiotics in aquaculture is well recognized and several countries allow the use of antibiotics in aquaculture under veterinary medicine regulations and their use depends on approved list of antibiotics to be used in fish food (FAO, 2005). The use of antibiotics as growth promoters was banned in Europe in 2006 (Kemper, 2008). In some countries like USA, antibiotics are used in ponds and tanks but not in open-water habitats such as oceans and lakes. It is recommended to the farmers not to drain fish ponds after the harvest which may contain high levels of drugs or antibiotics which in turn may expose newly growing fish to the antibiotic residues and resistant bacteria (Committee on Drug Use in Food Animals, 1999).

So, the unrestricted use of antibiotics in aquaculture has the potential to affect fish and human health on a global scale as well as the aquatic environment. It appears that the transfer of antibiotic resistance determinants among bacteria of different aquatic environment (freshwater, seawater, brackish water) would be attained, mainly as a result of the high concentrations of bacteria in water and aquatic sediments. Likely the abundant presence of bacteriophages may also facilitate such a transfer. The antibiotic use in aquaculture needs to be reduced drastically and replaced with alternative disease control measures such as probiotic feed additives as well improved sanitation in fish husbandry to avoid the emergence of antibiotic resistance in fish pathogens and environmental bacteria and the passing of this resistance to human pathogens which may endanger effective therapy to treat human bacterial infections (Cabello, 2006).

Hormones

Synthetic hormones could shorten the growth time needed for farm-raised fish to reach market size. Insulin and glucagon play important role in carbohydrate metabolism. Both of these hormones are secreted by specific cells in the pancreas in response to high and low blood glucose levels. Insulin plays more important role in amino acid metabolism than in carbohydrate metabolism. Amino acids act as better stimulants of insulin secretion than glucose. Insulin also serves to increase the incorporation of amino acids into protein (Plisetskaya, 1989).

Growth can be regulated by some hormones and factors that involved in growth regulation in fish. These involve a series of releasing and inhibiting factors regulating the levels of the molecule that has the growth promoting effect. Thyroid hormones and their regulatory hormones play an important role in the regulation of growth. Thyroid releasing hormone (TRH) hormone is released from the hypothalamus and has a direct effect on the pituitary gland. This effect on the pituitary gland is induced to release thyroid stimulating hormone (TSH) or the thyrotropin. An increase in the blood levels of TSH, the thyroid gland is stimulated to release thyroxine (T4). In the peripheral tissues such as the liver, T4 is modified by the action of monodeiodinase enzyme to produce triiodothyronine (T3). T3 has the somatotrophic or growth-promoting effects. The circulating T3 and T4 in the blood have a negative feed –back effect on the release of TSH by the pituitary gland (MacLatchy and Eales, 1990).

Another growth regulatory hormone mechanism involves growth hormone (GH) and its regulatory hormones. GH is involved in the regulation of somatic growth and the maintenance of protein, lipid, and carbohydrate metabolism (Ganong 1983). It has also been reported that GH may be the most promising agent for growth promotion in aquaculture (Zohar, 1989). The secretion of GH is mediated by a number of factors such as gonadotropin releasing hormone (GnRH) and neuropeptide Y which stimulate the pituitary gland for the secretion of GH and also the somatoststin which inhibit the release of growth hormone. All these compounds, (GnRH), neuropeptide Y and somatoststin are secreted by the hypothalamus. GH acts upon a variety of tissues, inducing the secretion of insulin-like growth factors (ILGFs) which is called somatomedins, which have the somatotropic effects. Some studies have been shown that GH can promote the growth by increasing protein synthesis and via the mobilizing lipid with a subsequent sparing of amino acids that will be used for the protein deposition (De Silva and Anderson, 1995).

Several natural and synthetic hormones including growth hormone, thyroid hormones, gonadotropin hormone (GnH), prolactin and various steroids have been evaluated in fish growth experiments (Higgs *et al*., 1982 and Matty, 1986). However, due to the limited availability of natural GH because of its preparation from fish pituitary glands is not considered economical. The complementary DNA (cDNA) encoding fish GH has been sequenced and the recombinant GH (rGH) has also been shown to be potent in enhancing the growth rate of fish by intraperitoneal injection (Tsai *et al*. 1994; 1995) or by immersion (Agellon *et al*., 1988). But it appears that the administration of these rGH via injection or immersion is not practical for fish farming. In another study rGH were added to the basic meal pellet of Juvenile black seabream *Acantlmpagrus schlegeli* at different concentrations and it was found that the meal containing 0.05% rGH showed a significant increase in percentage weight gain (60%) and feed efficiency (41%) as compared to the control group (Tsai *et al*.,1997). Administering rGH together with its expression host as a feed additive appears to be the most practical method. In a trial designed to enhance fish growth and to prevent the purification steps, it was found that specific growth rate of turbot fed the feed supplemented with 1.0% transgenic *Synechocystis* sp. PCC6803 (blue-green alga) containing the *Paralichthys olivaceus* growth hormone (GH) gene was 21.67% higher than that of control fish (Liu *et al*. 2007).

Steroid hormones have growth promoting properties; these hormones are derived from cholesterol. Several fish species have shown anabolic responses to steroid (Matty, 1986). The inclusion of steroids in the diet of salmonids increased the growth rate of this fish (De Silva and Anderson, 1995). The most effective among these steroids is the 17α-methyltestosterone (Gannam and Lovell, 1991). The action of this hormone may be due to the increase in the rate of protein synthesis, appetite and the increase in the rate of digestion and absorption of nutrients from diet. However, prolonged steroid treatment for growth acceleration may cause detrimental side effects including skeletal deformity, early development of gonads, and increased susceptibility to infectious and pathological changes in the liver, kidney and digestive tract (Zohar, 1989, and Gannam and Lovell, 1991). Estrogen and corticoids are another two groups of steroids but they have a negative effect on the growth due to the increase of protein degradation and lipolysis (De Silva and Anderson, 1995).

It is obvious that the use of hormones as growth promoters in aquaculture may not be either practical or economical. Additional research is necessary in the area of biotechnology such as genetic engineering to fully explore the potential of hormones for aquaculture use.

Probiotics in aquaculture

Significant amount of researches have been conducted on the use of "probiotics" as functional ingredients for the health of humans, animals and fish (Fuller, 1989; Miettinen *et al*., 1996 and Kesarcodi-Watson *et al*., 2008).

Probiotics have been defined as ''organisms and substances which contribute to intestinal microbial balance'' (Parker, 1974). Fuller (1989) proposed probiotic as ''a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance''. Another definition for probiotics was suggested by Havennar *et al*. (1992) which was "mono or mixed cultures of live microorganisms which when applied to animal or man, beneficially affect the host by improving the properties of the indigenous micro flora.

Tannock (1997) proposed that ''living microbial cells administered as dietary supplements with the aim of improving health''. Gatesoupe (1999) suggested an alternative definition of probiotics as: microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be keeping alive, with the aim of improving health. Verschuere *et al*. (2000) proposed the definition as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment".

Apart from the requirement of the probiotic to be a live culture, this definition is a lengthy way of describing a probiotic as defined by Irianto and Austin (2002a) thus "a probiotic is an entire or component(s) of a microorganism that is beneficial to the health of the host". The latter definition is in accordance with that given by Salminen *et al.* (1999).

The use of microbial probiotics in aquaculture is now widely accepted (Sharma and Bhukhar, 2000; Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000; Irianto and Austin, 2002b; Wang and Xu, 2006; Vine *et al*., 2006; Wang, 2007 and Kesarcodi-Watson *et al*., 2008). Several commercial probiotics are available and they are widely used in fish and shellfish farming either as feed additives or introduced in pond water (Wang *et al*., 2005).

Probiotic Mode of action

In order to use probiotics as feed additives, it is important to know their mode of action and explain if the isolate(s) to be used may compete with other probiotics and/ or pathogenic microbes. The screening methods used with human probiotics have been focused on procedures used to process the product, production system, biosafety and taking into account the location in the gut where the microorganism will be bioactive (Huis in't Veld *et al*., 1994). On the other hand, when probiotic will be produced for aquaculture purpose, it is necessary to consider the ability of microorganisms to compete with other pathogenic strains (Vine *et al*., 2004a), assessment of pathogenicity and evaluation of *in vivo* activity in addition to the economics of their use and total cost of the product (Gomez-Gil *et al*., 2000).

The mode of probiotic action requires an early attention, because, if the probiotic prevent the pathogenic microbes by extracellular metabolites, the pathogen could eventually produce a resistance to such metabolite. Therefore, it is important for probiotic to have more than one mode of action to compete the pathogen. These mode of actions may include the enhancement of the humoral and /or cellular immune response, modification of the pathogenesis by changes at metabolic level particularly enzyme activities (Fuller, 1987), the ability to produce an antimicrobial metabolite (i.e., antagonistic compound), the ability to grow rapidly, the ability to colonize (attachment) at the intestinal mucus and also the production of other metabolites that are important for the host (Verschuere *et al*., 2000).

Probiotics could also enhance the non-specific immunity (Gill, 2003), they have the ability to stimulate cytokine production, which influences the T-cell production (Maassen *et al*., 2000 and Perdigon *et al*., 2002). All of these criteria could be used for the *in vitro* screening of potential probiotics (Verschuere *et al*., 2000). In summary, the mode of action of probiotics include the production of inhibitory compounds to pathogenic microorganisms, competition for chemicals or available energy, competition for adhesion site, enhancement of immune response, improvement of water quality, interaction with phytoplankton, source of macro-and micronutrients, improved utilization of feed, enzymatic contribution to digestion and absorption, antimutagenic and anticarcinogenic activity, growth-promoting factors, and increased immune response (Verschuere *et al*., 2000; Balcázar *et al*., 2006b and Gómez and Balcázar, 2008).

Production of Antagonistic compounds

Antagonistic compounds or substances are either primary or secondary extracellualr metabolites or compounds that are produced by some microorganisms (actionmycetes, bacteria and /or fungi), which have an inhibitory effect towards other microorganisms resulting in a wide spectrum mode of action. The most common among such compounds are antibiotics, which are chemicals usually produced as secondary metabolites of microorganisms (Brock and Madigan, 1997). However, the productions of antimicrobial substances by the selected isolates *in vitro* may not guarantee the inhibitory effect *in vivo* (Gibson *et al*., 1998; Ringø and Gatesoupe, 1998 and Gram *et al*., 2001). This limitation in their mechanism of action may be due to either the selective ingestion by the host (Riquelme *et al*., 2000) or the failure of probiotic to grow in the gastrointestinal tract.

In addition to the antibiotics, there are also several bacterial metabolites such as organic acids, hydrogen peroxide (Ringø and Gatesoupe, 1998), carbon dioxide (Gill, 2003) and siderophores (Yoshida *et al*., 1995 and

Braun and Braun, 2002) which may have specific inhibitory actions. In particular, siderophores play an important role in the nutrition and metabolism of fish. These iron chelating or binding compounds are produced by some bacterial and fungal species (Braun and Braun, 2002). The major role of siderophores is to scavenge iron from the environment and deliver this mineral which is essential to the microbial cell (Winkelmann, 1991). These isolates which have the ability to produce siderophores can utilize non-soluble Fe^{3+} from the surrounding medium (Braun and Killmann, 1999). Thus, siderophores bacteria could be benefit by controlling the pathogenic microorganisms those have a critical requirement of iron but their iron uptake is low (Gatesoupe *et al*., 1997).

Adherence of probiotic bacteria to intestinal mucus

The adherence to the intestinal epithelium and mucus and the production of antimicrobial agents by lactic acid bacteria are the crucial factors that prevent pathogens in humans (Reid, 1999 and Gill, 2003). A similar mechanism also occurs in fish, when lactic acid bacteria are attached to the mucus layer, this blocking the pathogen infection (Ringø *et al*., 1998), where the attachment is considered the main prerequisite for colonization. There is a relation between the pathogen virulence and its attachment ability to the mucus. Hence, attachment is considered to be the first step in pathogenesis of infection (Olsson *et al*., 1996; Joborn *et al*., 1997 and Ringø and Gatesoupe, 1998). Several researchers have investigated the capability of probiotics to attach to the intestinal mucus of the fish (Olsson *et al*., 1992; Vazquez-Juàrez *et al*., 1997; Andlid *et al*., 1998; Rinkinen *et al*., 2003b and Vine *et al*., 2004b). It is now become clear that the attachment acts as one of the competitive mechanisms by which probiotics can inhibit the pathogen to colonize or to bind the tissue. This mechanism appears to have an advantage over the antibiotic production which may result in mutational resistant pathogenic isolates (Reid *et al*., 2001).

Other beneficial effects (Production of other beneficial compounds)

Several probiotic bacterial isolates have been involved in the production of several digestive enzymes and secondary metabolites such as vitamins, pigments and short chain fatty acids

- Most probiotics may provide their host with one or more of digestive enzymes. The microbial flora isolated from the fish digestive tract showed chitinase (MacDonald *et al*., 1986) amylase (MacDonald *et al*., 1986 and Gatesoupe *et al*., 1997), protease (Gatesoupe *et al*., 1997), and cellulase activities (Erasmus *et al*., 1997 and Bairagi *et al*., 2002). It has been observed that probiotic induced the production of larval digestive enzymes during the ontogeny of their digestive system (Gawlicka *et al*., 2000 and Zambonino-Infante and Cahu, 2001).
- Vitamin synthesis has been observed in several bacterial species isolated from aquatic animals. It has been reported that channel catfish, *Ictalurus punctatus* (Limsuwan and Lovell, 1981), carp, *Cyprinus carpio* (Sugita *et al*., 1991a), various tilapia species (Lovell and Limsuwan, 1982 and Sugita *et al*., 1991a) and rainbow trout (Sugita *et al*., 1991b) have the ability to produce vitamin B_{12} in their digestive tract.
- Carotenoid pigments act as antioxidants by scavenging lipid free radicals that oxidize lipid (Holmstrom *et al*., 2002).
- The intestinal bacteria in fish can be involved in nutrition and metabolism (Clements, 1997), where these intestinal bacteria serve as lipids producers (Ringø *et al*., 1992 and Shirasaka *et al*., 1995) and play a vital role in the diet for tilapia, *Oreochromis niloticus* by changing the digestive system morphology (Kihara and Sakata, 1997).

Beneficial effects of probiotics on fish immune system

Among the several beneficial effects of probiotics, modulation and enhancement of immune system have been observed in carp (Stosik and Szenfeld, 1996), shrimp, *Penaeus monodon* (Rengpipat *et al*., 2000) and rainbow trout (Irianto and Austin, 2002b and Panigrahi *et al*., 2004). Typical microroganisms of the 'normal' intestinal flora may possess a range of beneficial features such as the ability to degrade certain food components, produce certain B vitamins, stimulate protective enzymes and immune system and produce digestive enzymes. The normal flora may be also involved in the metabolism of some potential carcinogenic substances and they may play a role in efficacy of drugs. Furthermore, the colon mucosa is dependent on short chain fatty acids (SCFA) produced by the colonic microflora. (Kvietys and Granger, 1981; Roediger, 1982 and Hoverstad, 1989).

The most important functions of probiotics include immune modulation and strengthening the gut mucosal barrier by 1) gut microflora modification, 2) adherence to the intestinal mucosa with capacity to prevent pathogen adherence or pathogen activation, 3) modification of dietary proteins by the intestinal microflora, (4) modification of bacterial enzyme capacity especially of those involved in tumor induction, and 5) influence on gut mucosal permeability (Salminen *et al*., 1996). The probiotic may prevent potential pathogens from colonizing the gut by production of antimicrobial compounds, or by effectively competing them for nutrients or mucosa. The benefit to the host may arise as a nutritional effect, whereby the bacteria are able to break down toxic or otherwise non-nutritious components of the diet, which the host can then digest (Robertson *et al*., 2000).

Effect of Probiotics on growth and digestive enzymes activities

Bacteria are commonly distributed in the aquatic environment of aquaculture systems which is enriched by the nutrients either by their leaching in water or excretion in feces. Bacteria are introduced in the digestive tract of fish through food and water. Being rich in nutrients, the digestive tract of fish confers a favorable culture environment for the growth of microorganisms. The importance of intestinal bacteria in the nutrition and well-being of their hosts has been established early for homeothermic species such as birds and mammals (Floch *et al.*, 1970). However, there was limited information available on fish, poikilothermic vertebrates. The distribution of endogenous digestive enzymes in fish has been studied by several workers (Kawai and Ikeda, 1972; Das and Tripathi, 1991; Hidalgo *et al*., 1999; Bezerra *et al*., 2005; Jun-sheng *et al*., 2006 and Chan *et al*., 2008). However, information regarding the enzyme producing intestinal bacteria particularly their origin and significance in digestion and absorption of nutrients in fish is scarce.

In aquaculture, antibiotics have been widely used to prevent and treat infectious diseases, however, they are also considered as causative factors on development of antibiotic resistant bacteria in fish as well as accumulation of their residues in the body. As an alternative measure to overcome these problems, probiotics have been recently introduced in aquaculture to inhibit growth of pathogens as well to improve growth and feed utilization of aquatic organisms (Byun, 1997 and Rengpipat *et al*., 1998). De Schrijver and Ollevier (2000) reported that the protein digestion in juvenile turbot, *Scophthalmus maximus* was improved by supplementation of probiotic bacteria, *Vibrio proteolyticus* in feeds.

Probiotics have been shown to produce a positive effect on the growth and feed utilization of farmed fish. When Nile tilapia fry were fed experimental diets containing two levels of protein (27, and 40 %) and two types of probiotic 0.1% of a commercial bacterial mixture containing *Streptococcus faecium* and *Lactobacillus acidophilus* and 0.1% yeast, *Saccharomyces cerevisiae*, diet containing 40 % protein was considered optimum for tilapia fry whereas low protein (27 %) was used to reduce growth and induce stress in these fish (Lara-Flores *et al*., 2003). The same authors noticed that the addition of 0.1% probiotics in tilapia fry diets improved growth, and mitigated the effects of stress factors. In this study, the diet containing yeast produced the best results, being the most viable option for optimizing growth and feed utilization in intensive tilapia culture. Feed utilization was highest in tilapia fry fed with the yeast supplemented diets. An another study by El-Haroun *et al.* (2006), when the commercial probiotic (Biogen) was incorporated in the experimental diets for Nile tilapia at 0%, 0.5%, 1.5%, 2.0% and 2.5% of diets, the growth performance and nutrient utilization of fish including weight gain, specific growth rate, protein efficiency ratio, protein productive value and energy retention were significantly higher in the groups receiving probiotic as compared to the control diet.

It has been observed that the administration of probiotics containing *Bacillus* sp. enhanced the enzyme activity in the digestive tract of Indian white shrimp, *Fenneropenaeus indicus* (Zieai-Nejad *et al*., 2006). These authors reported that endogenous enzymes synthesized in the digestive tract of shrimp were stimulated rather than exogenous enzymes synthesized by probiotic bacteria mainly because high enzyme activity was observed under the low population of *Bacillus* sp.

The effect of probiotics on the growth performance and digestive enzyme activities of common carp was studied by Yanbo and Zirong (2006). They found that the addition of probiotics in common carp diets resulted in an improvement in growth, feed utilization and activities of digestive enzymes. The different strains of bacteria (photosynthetic and *Bacillus* sp.) used in their study as probiotics were effective in improving fish performance and a mixture of these bacteria produced the best results.

Juvenile European sea bass, *Dicentrachus labrax* fed a diet containing lactic acid bacteria (LAB), *Lactobacillus delbrueckii delbrueckii* showed 81% and 28 % higher body weight when they were treated for long (59 days) and short period (25 days) respectively as compared to untreated control diet. The use of *L. delbrueckii delbrueckii* as probiotic also had positive effects on health and welfare of sea bass. Probiotics decreased cortisol levels of treated animals and influenced the transcription of two genes involved in the regulation of body growth, IGF-I and MSTN. In particular, IGF-I transcription was increased and MSTN transcription was inhibited in treated groups. All the above mentioned results showed a rapid increase of body weight of treated animals. These results also show the potential of probiotics in the development of an environment-friendly marine aquaculture industry as well as to develop a valid alternative to the drugs and antibiotics (Carnevali *et al*., 2006).

The mixed culture of *Bacillus* KKU02 and *Bacillus* KKU03 which were previously isolated from the intestinal contents of shrimps showed significant improvement in the growth of the giant fresh water prawn, *Macrobranchium rosenbergii*. Inclusion of both isolates in feed also induced the production of amylase and protease enzymes (Deeseenthum *et al*., 2007).

The effect of probiotics on the growth performance and digestive enzyme activity of the shrimp, *Penaeus vannamei* was studied by Wang (2007). By the addition of photosynthetic bacteria and *Bacillus* sp. to shrimp diets, an increase of weight, daily weight gain and relative weight gain obtained, showing the potential of probiotics in increasing growth of shrimps. The same author also indicated that the enhanced growth performance of shrimp may be due to increase in the digestive enzyme activities induced by the probiotics. However, they could not distinguish between activity due to enzymes synthesized by shrimp and that induced by the probiotics. It appears that the exogenous enzymes produced by probiotics may represent a small contribution towards the total enzyme activity of the gut (Ding *et al*., 2004 and Ziaei-Nejad *et al*., 2006), and the presence of the probiotics is likely to stimulate the production of endogenous enzymes by the shrimp. In another study by Gomez *et al*. (2008), the influence of *Bacillus* probiotics on the digestive enzyme activity and the growth of White

Pacific shrimp, *Litopenaeus vannamei* was examined. The weight gain at the end of the study as well as protease and amylase of the midgut and the intestine were significantly higher in probiotic-treated shrimp as compared to the control containing no probiotics.

Isolation and enumeration of heterotrophic bacteria from the gastrointestinal tracts of rohu, *Labeo rohita* and mrigal, *Channa punctatus* have been conducted by Kar and Ghosh (2008) to study their importance in the nutrition of the host fish. They isolated all amylolytic, proteolytic and cellulolytic bacteria in the gut of rohu and mrigal. Among specific enzyme producing bacteria, proteolytic and cellulolytic bacteria were present at higher number within the gut of mrigal and rohu, respectively. Selected intestinal bacterial isolates were analyzed to determine the extracellular enzyme producing capacities. Protease and cellulase activities were exhibited by all bacterial isolates, while amylase production remained poorly detected by the strains isolated from mrigal. The authors demonstrated from their study that the bacteria present in the gut of *L. rohita* and *C. puntatus* were capable of producing various extracellular digestive enzymes which may contribute to the effective utilization of these extracellular enzyme-producing bacteria to improve the performance of these fish for commercial aquaculture.

The effects of commercial probiotic, *Lactobacillus* sp. supplementation in different three strategies; supplemented to live food (rotifer and *Artemia*), supplemented to live food and water together and supplemented to water only on growth and digestive enzymes activities in gilthead sea bream, *Sparus aurata* during their larval development were investigated by Suzer *et al.* (2008). They found that supplementation of probiotic to live food (rotifer and Artemia) is an effective mean to deliver the probiotic to the *S. aurata* larvae. The specific activities of pancreatic and intestinal enzymes were significantly higher in larvae to which the probiotic had been supplemented by live food and live food with water. However, addition of probiotic directly into the water of fish tanks did not significantly increase growth or digestive enzymes activities; it appears that administration of probiotics by this method may not be an effective method to utilize for improving fish growth.

In an another study, the probiotic, *B. coagulans* SC8168 obtained from the pond sediment of shrimp was used as a water additive to study its effect on the survival and digestive enzymes activities of shrimp, *Penaeus vannamei* larvae (Zhou *et al.,* 2009). The authors concluded that probiotic, *B. coagulans* SC8168, supplemented as a water additive at specific concentration may significantly increase survival and activities of certain digestive enzymes of shrimp larvae. These results were based on a trial conducted under laboratory conditions and applications of these finding remains to be tested under commercial aquaculture conditions.

Yeasts as probiotics

Yeasts are ubiquitous microorganisms that disseminate with animals, air and water currents, and they can grow in various environments where organic substrates and nutrients are widely available. They have been detected in fish guts of wild and farmed animals, but their natural occurrence has been generally considered as incidental. Industrial yeasts are commonly used in aquaculture, either as feed supplement to feed live food organisms, or after processing as a feed ingredient (Stones and Mills, 2004). Some extracts containing β-glucans have been also used as immunostimulants, and more recently, living yeasts have been recommended as probiotics (Gatesoup, 2007).

Yeasts can be considered as useful probiotic species because they often produce important enzymes and also have a marked capacity to adhere to mucosal epithelia, thus competing for colonization of pathogens (Andlid *et al*., 1998 and 1999). Moreover, some strains such as *Debaryomyces hansenii* produce polyamines, such as spermine, spermidine and putrescine that exert a beneficial effect on mucosal cell differentiation and maturation of the mammalian gastrointestinal tract as well as modulate internal biological clock of fish (Dufour *et al*., 1988 and Peres *et al*., 1998). Several investigators have suggested that some yeasts are better growth promoters for development of young fish than bacteria (Vazquez-Juaez *et. al*., 1993 and Lara-Flores *et al*., 2003). In a recent study conducted by Laconi and Pompei (2007), it has been shown that several strains of yeasts isolated from 39 fish belonging to five different species of *Mugil* genus have amylase activity, but not protease or lipase activity. The production of extracellular enzymes is considered to be an important factor for utilization of feed by fish, because they are known to increase food digestibility higher than common probiotic bacteria (Buts *et al*., 1993; Gatesoupe, 1999 and Tovar- Ramı´reza *et al*., 2002). In fact, it has been demonstrated that incorporation of certain living strains of yeast in fish feed stimulate their growth, especially at the larval stage, through their better ability to adhere to the intestinal epithelia and the synthesis of polyamines which stimulate growth (Fouz *et. al*., 1991; Westerdahl *et al*.,1991; Alexander and Ingam,1992 and Bordas *et al*.,1996).

In a study conducted on tilapia fed a control diet and a diet containing yeast, survival and digestibility of fish were reduced by increasing the fish density in the control group; however this stress had no significant effects on the group fed diet treated with yeast (Lara-Flores *et al*., 2003).

In a study on European sea bass larvae, Tovar-Ramírez *et al*. (2002) compared the effects of two yeasts incorporated in their diet on performance of fish larvae. Diets containing *Debaryomyces hansenii* HF1 improved survival and vertebral development of larvae, most likely due to the acceleration of the digestive system maturation. These effects were not observed with *Saccharomyces cerevisiae* X2180, and the larvae grew better when they were fed a control diet which probably due to an improper application of yeasts on feeds after feed processing, because the physical properties of the pellets were affected and they disintegrated rapidly during the handling and feeding of fish. Tovar-Ramírez *et al.* (2004) resolved this problem by introducing *D. hansenii* HF1 in the diet prior to feed pelleting process. The application of this technique improved the growth of larval European sea bass fed diets containing probiotics, in addition to better maturation of the gastrointestinal tract, and survival of the larvae. These beneficial effects of probiotics were observed with a dose of 10^6 CFU g^{-1} of diet; however a higher dose of 6×10^6 CFU g^{-1} was less efficient.

Probiotics and the Immune system

Fish immune system

Like all vertebrates, fish possess a wide array of defense systems to protect themselves against disease-causing organisms. The immune system of fish is broadly categorized into specific or acquired immunity and nonspecific or innate immunity. Both systems use cellular and humoral mechanisms to provide protection against infection. Fish depend more heavily on nonspecific defense mechanisms than mammals. In particular, for certain cold water fish the rate of development of specific immune responses is temperature dependent. Immune functions of warm water fish are also dependent on water temperature (Lall and Olivier, 1993).

Nonspecific immune system

Nonspecific immunity includes the generalized host defences such as physical barriers (scales, skin and mucous), enzymes (lactoperoxidase in saliva, lysozyme in mucous and other secretion in gastric juice and tissues), phagocytic cells (macrophages and neutrophils) and blood proteins (interferon, C-reactive protein and complement) (Fletcher *et al.*, 1986).The most important nonspecific defense mechanisms in fish are the complement system and phagocytosis (Fletcher *et al.*, 1986).

The complement system

Complement, an important component of the non specific or innate immune system, is consisted of about 35 individual proteins. In mammals, activation of complement results in the generation of activated protein fragments that play a role in microbial killing, phagocytosis, inflammatory reactions, immune complex clearance, and antibody production. Fish appear to possess activation pathways similar to those in mammals. However the fish complement proteins identified thus far show many homologies to their mammalian counterparts. Complement is responsible for mediating inflammatory reactions, opsonization of foreign particles, and lysis of cell membranes. The information regarding complement proteins, regulatory proteins, and complement receptors in fish is not complete. It has been demonstrated that fish complement can lyses foreign cells and opsonise foreign organisms for destruction by phagocytes. There are also indications that complement fragments participate in inflammatory reactions. Fish possess multiple isoforms of several complement proteins, such as C3 and factor B. The proteins in complement system can be activated by two routes; the non-specific activation via the alternative pathway and this type can be achieved by various structures possessing repeating units such as lipopolysaccharide of Gram-negative bacteria. The other activation route is the specifically one by antibodies attached to the antigens. Activation of the complement system via both pathways results in the production of several biologically active compounds including anaphylotoxins, responsible for chemotactic attraction of leucocytes and opsonins which enhance ingestion of various particles by phagocytes (Manning, 1998).

Phagocytosis

Phagocytosis is a cellular process involving the ingestion of foreign materials by specialized cells. Phagocytic cells principally dedicated to the recognition and elimination of invading organisms and damaged tissue include granulocytes (particularly neutrophils) and mononuclear phagocytes (tissue macrophages and circulating monocytes). Their movement to sites of microbial invasion is an early event in the inflammatory response and the role of host-derived factors as attractants, such as eicosanoids. The protection offered by the phagocytes is their bactericidal larvacidal activity, which is closely associated with the production of oxygen free radicals. In addition to phagocytosis, macrophages are important in the initial processing of antigens for their recognition by T- and B- lymphocytes. Neutophils are present in the kidney, spleen and blood and in the inflammatory lesions, whereas macrophages are widely distributed in the reticuloendothelial system including kidney, spleen and gills (Lall and Olivier, 1993).

Specific immune system

Specific immunity is characterized by an initial interaction between antigens and lymphocytes (B- lymphocytes and T- lymphocytes), which are responsible for humoral and cellular immunity respectively. Humoral immunity is mediated by B-lymphocytes, these cells, in response to stimulation with a variety of antigens, are transformed into plasmocytes which will produce antibodies (immunoglobulins) specific for the stimulating antigens. Cell-mediated immunity can be passively transferred with T-lymphocytes only and not with serum. Cellular immunity is activated specifically by using T-dependent antigen which can stimulate the production of activated T-lymphocytes. Cellular immunity can also be stimulated non-specifically by using certain adjuvant and intracellular pathogens administered as an attenuated live vaccine (Lall and Olivier, 1993 and Manning, 1998).

Effect of probiotics on fish immune system

In recent years, rapid growth of intensive aquaculture has led to the outbreaks of infectious and nutritional fish diseases particularly when fish are stressed due to a variety of factors including high density, poor husbandry management and improper nutrition and feeding practices. (Smith *et al.*, 1994). Proper disease management is essential for the successful production of fish cultured in captivity. It is widely recognized that, high fish mortality causes considerable economic loss in commercial aquaculture operations (Amar *et al*., 2000). The extensive use of broad-spectrum antibiotics in fish farms has led to some problems like drug resistance (Smith *et al*., 1994). The control of infectious diseases with antibiotics and chemotherapeutics is widely practiced by the aquaculture industry (Amábile-Cuevas *et al*., 1995). However, antibiotic use can cause the development of drug-resistant microorganisms, accumulation of antibiotic residues in fish and discharge of these compound in effluents of fish farms into ponds, lakes and sea (FAO/WHO/OIE, 2006). These antibiotics may also cause inhibition of beneficial microbiota which is normally present in the digestive tract of the fish (Sugita *et al*., 1991a). Moreover, vaccines cannot be used alone as a universal disease control measure in aquaculture (Amábile-Cuevas *et al*., 1995) because of the limited availability of vaccines in few countries and their pathogen specific protective action for certain specific bacterial and viral diseases (FAO, 2006). Therefore, alternative strategies to antimicrobials (antibiotics, vaccines and chemotherapeutics) control agents have been proposed, such as the use of probiotics as biological control agents or as dietary live microbes supplements in commercial fish culture to improve growth and immune function of fish (Gatesoupe, 1999; Irianto and Austin, 2002a and Kesarcodi-Watson *et al*., 2008), particularly to promote an environment friendly aquaculture practice.

The use of probiotic for improving the immune system have been proved by several investigators, The immune system of black tiger *Penaeus monodon* was improved by the administration of probiotics in the diet (Rengpipat *et al*., 2000). Their survival and growth were higher in shrimp fed the *Bacillus* S11 than the non-treated group. It was also found that the *Bacillus* S11 administration increased the engulfment of foreign particles, increased phenoloxidase and antibacterial activities. After *Vibrio harveyi* challenge, both survival and phagocytic index were higher in shrimp treated with probiotic as compared with the untreated group. The mechanism of disease resistance by *Bacillus* S11 could be via the activation of both cellular and humoral immune defenses, as well as via a competitive exclusion in the digestive tract of shrimp.

It has been suggested that about 1-10% of intestinal bacteria isolated from both marine and freshwater fish exhibit antibacterial activity against fish pathogenic bacteria and they have the potential to be used in probiotic treatment of fish (Sugita *et al.,* 2002). The effect of different lactic acid bacterial strains on the immune response of turbot, *Scophthalmus maximus* was studied both *in vitro* and *in vivo*. It was found that out of six lactic acid bacterial strains tested, *Lactococcus lactis* was the most effective and strong stimulant and possess antibacterial properties. It was suggested that *L. lactis* was able to adhere to the intestinal mucus of turbot. The lactic acid bacteria appear to have the capacity to inhibit the pathogens. The extracellular products from strains of lactic acid bacteria tested had significantly decreased the detrimental effects of pathogenic *Vibrio anguillarum* (Villamil *et al.,* 2002). The number and phagocytic activity of circulating haemocytes were significantly higher in the abalone, *Halitois midae* fed diets containing a mixture of three putative probionts (isolated from the gastrointestinal tract of the abalone); one bacteria (SY9) and two yeasts (SS1 and AY1) compared to non-treated animals. In addition, the survival rate was 62% and 25% after challenge with the pathogenic bacterium, *Vibrio anguillarum* in the treated and non treated animals respectively (Macey and Coyne, 2005).

In an attempt made by Gullian *et al*. (2004) to obtain probiotic bacterial strains with immunostimulatory effects on shrimp, *Penaeus vannamei*, three strains, *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64, exhibited inhibitory effects against *V. harveyi*. They investigated the colonization percentage in shrimp hepatopancreas and the competitive interaction with *V. harveyi* (S2) by using random amplified polymorphic DNA (RAPD). The colonization percentage of P62, *Vibrio* P63 and P64 were found to be 83 %, 60% and 58% respectively. The immunostimulatory effect of P62 and P64 was evaluated *in vivo* using several immune function tests. The total immunity index was significantly higher in the shrimp stimulated by P64 not P62. The isolated *Bacillus* strain P64 showed both probiotic and immunostimulatory features, while *Vibrio* P62 only showed good probiotic properties represented in the higher colonization percentage and also the higher weight compared to the control group.

The phagocytic activity of gilthead seabream was increased after two weeks of feeding the single bacteria-supplemented diets with *L. delbru¨eckii* or *B. subtilis,* whereas the combination of these two bacterial isolates resulted in an increment of phagocytic activity which persisted for as long as the bacteria were administered. Cytotoxic activity also exhibited a significant increase after three weeks of feeding the mixture of the two bacterial isolates. Nevertheless, the duration of these effects was always restricted to the period during which the fish were fed live bacteria, suggesting that the bacteria did not persist in the seabream gut (Salinas *et al*., 2005).

The dietary introduction of different *Lactobacillus* sp. has been applied in some investigations. It has been found that the addition of a probiotic bacteria *Lactobacillus rhamnosus* ATCC 53103 to rainbow trout *Oncorhyncus mykiss* diet could reduce mortality of fish challenged with a virulent strain of *Aeromonas salmonicida* (Nikoskelainen *et al*., 2001). The immune system response in rainbow trout, *Oncorhyncus mykiss* was also improved by the administration of probiotic bacteria *L. rhamnosus* JCM 1136 in feed, phagocytic activity of head kidney leucocytes, serum lysozyme and complement activities were significantly higher in fish fed the higher level (10^{11} CFU/g diet) of probiotic compared with the control fish (Panigrahy *et al*., 2004). The administration of probiotic in fish feed exhibited some influence on the immune response in rainbow trout. Therefore, the use of different forms of heat-killed, live-sprayed or freeze-dried *L. rhamnosus* JCM 1136, resulted in higher phagocytic and complement activity in the live spray or freeze-dried forms rather than the non-viable or the heat-killed form. Fish fed with the live probiotic had higher plasma immunoglobulin level (Panigrahi *et al*., 2005).

The addition of *Carnobacterium maltaromaticum* B26 and *Carnobacterium divergens* B33 in the diet of rainbow trout which were isolated from the intestine of healthy rainbow trout at a dose 10^7 cells g^{-1} resulted in protection against disease challenges conducted with virulent cultures of the pathogens, *Aeromonas salmonicida* and *Yersinia ruckeri*. These probiotics enhanced the cellular and humoral immune responses. Whereas, fish fed with B26 had significantly higher phagocytic activity of the head kidney macrophages, whereas the use of B33 led to significant increases in respiratory burst and serum lysozyme activities. In addition, the gut mucosal lysozyme activity in fish fed with both isolates was significantly higher as compared to the control group (Kim and Austin, 2006).

Another bacterial isolate, *Clostridium butyricum* was effective as a probiotic and as an immune stimulant for *Miichthys miiuy*. The humoral immune responses were mediated by supplementation of *C. butyricum* to the diet of *Miichthys miiuy*. Serum phenoloxidase, acid phosphatases and lysozyme activities were significantly increased by dietary supplementation of *Clostridium butyricum* (Zeng-fu *et al.*, 2006).

It has been demonstrated that the beneficial bacterium *Arthrobacter* XE-7 isolated from the natural microflora of *Penaeus chinensis* can be used as a probiotic for shrimp post-larvae by adding into the water maintained in fish aquarium (Li *et al*., 2006). *In vitro* studies showed that the pathogenic vibrio (*Vibrio. parahaemolyticus, V. anguillarum* and *V. neeir*) were inhibited by *Arthrobacter* XE-7. In addition to its ability to antagonize the pathogenic vibrios, *Arthrobacter* XE-7 was effective in reducing the concentration of NH_3–N. It appears that the probiotic nature of *Arthrobacter* XE- 7 is based on both the competitive exclusion of the pathogen and the water quality improvement.

Studies conducted on white shrimp, *Litopenaeus vannamei* have shown that *L. plantarum* is beneficial to improve immune system of this shrimp sp (Chiu *et al.*, 2007). Administration of *L. plantarum* in the diet at 10^{10} CFU (kg diet)$^{-1}$ induced immune modulation and enhanced the immune function and resistance to *V. alginolyticus* infection in shrimp. In addition to improvement in survival rate and disease resistance after the *Vibrio alginolyticus* challenge, immune response such as phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, and clearance efficiency against *V. alginolyticus* were all significantly increased. On the other hand, the administration of *L. plantarum* (906) and *L. fructivorans* (AS17B) to the larval gilthead seabream, *Sparus aurata* resulted in a significant increase in the density of immunoglobulin (Ig) and acidophilic granulocytes, in addition to the presence of heterogeneous populations of lymphocytes and granulocytes in the developing intestinal mucosa (Picchietti *et al*., 2007). In another study, the survival and immune parameters of shrimp, *Litopenaeus vannamei* fed with *Bacillus* OJ were increased when challenged by white spot syndrome virus (Li *et al*., 2009).

A study designed to investigate the combined effects of *B. subtilis* and vitamin C on the immune response of Indian major carp, *Labio rohita* exhibited that the significantly highest antibody level and lowest mortality were in fish fed *B. subtilis* followed by the combined probiotic and vitamin C treatment with (Nayak *et al.*, 2007). The total leukocyte count was also significantly higher in *B. subtilis* and *B. subtilis* combined with vitamin C than control group.

The positive response of tilapia immune system to probiotic application has been outlined by some investigators. The protective mechanisms associated with *L. rhamnosus* against *Edwardsiella tarda* infection in tilapia were examined by Pirarat *et al*. (2006). They found that the mortality was significantly lower and the alternative complement activity was higher in fish fed the probiotic-supplemented diet than the control groups. These results suggest that *L. rhamnosus* enhanced the alternative complement system of the fish, enabling phagocytic cell aggregation, increasing phagocytic activity and subsequently protecting the fish from acute septicemic death by *E. tarda* infection. Prevention of thymic necrosis by the probiotic supplement seems to minimize immunosuppression as well as initiates an immune response against edwardsiellosis.

The potential use of some bacterial isolates as probiotics isolated from Nile tilapia was studied by Aly *et al.* (2008a). *B. pumilus, B. firmus* and *Citrobacter freundii* isolates showed inhibitory effects against *Aeromonas hydrophila in vitro* and no signs of disease or mortalities were observed in fish injected with *B. pumilus* and *B. firmus*. Fish fed diets supplemented with these probiotics showed no evidence of disease after they were challenged against pathogenic *A. hydrophila* infection. The survival rate was increased in fish fed diets supplemented with *B. pumilus*. These authors considered *B. pumilus* as a promising probiotic for controlling *A. hydrophila* infections in Nile tilapia, *O. niloticus.* Another investigation was conducted also by Aly *et al*. (2008b) on Nile tilapia. The authors tested *B. subtilis* and *L. acidophilus* as potential probiotics on the immune response of Nile tilapia. The survival rate and hematocrit values were significantly higher in fish fed the mixture of *B. subtilis* and *L. acidophilus* than the control group. They also found that the lysozyme activity increased significantly in all the probiotic-treated groups after one and two months of feeding compared to the untreated control group. A recent study conducted has indicated that the complement component 3 content, myeloperoxidase (MPO) activity and the respiratory burst activity of blood phagocytes of Nile tilapia were higher in tilapias treated with *Enterococcus faecium* (10^7 CFU ml^{-1}) than the control group (Wang *et al*., 2008). In addition, Zhou *et al.* (2010) found that the immune response of Nile tilapia was improved by the addition of *B. subtilis* B10, *B. coagulans* B16 and *Rhodopseudomonas palustris* G06 to the rearing water at a concentration of 10^7 CFU ml^{-1} in a trial of 40 days duration. Both of superoxide dismutase and catalase activities as well as the respiratory burst activity showed an improvement over the control group, while serum lysozyme content was not affected by the probiotic supplementation in any of the treated groups.

It is clear from the previous investigations focused on probiotics that immune response, disease resistance and survival of different fish and shrimp species can be stimulated by the application of various probiotic bacteria or yeast to the diet or water. While the response of these aquatic species to probiotics may to great extend depend on probiotic dose.

Effects of yeast on immune system of fish

Yeasts are particularly interesting because they contain β-glucans, nucleotides and mannan oligosaccharides that stimulate the immune system of fish (Siwicki *et al*., 1994; Anderson *et al*., 1995; Sahoo and Mukherjee, 2001; Ortuño *et al*., 2002 and Li *et al*., 2004). Total mortality of Nile tilapia fry fed diet containing different levels of commercial live bakers' yeast, *Saccharomyces cerevisiae* and challenged by injection with *Aeromonas hydrophila,* decreased with the increase in the level of yeast in experimental fish diets. The lowest bacterial counts and fish mortality were observed in fish fed 5.0 g yeast/kg. These results indicate that the supplementation of bakers' yeast in tilapia diet prevents certain diseases and it has potential to reduce the dependence on antibiotics used to control infectious diseases in tilapia aquaculture operations (Abdel-Tawwab *et al*., 2008). The juvenile leopard groupers, *Mycteroperca rosacea* that received the live yeast *Debaryomyces hansenii* (CBS 8339) supplemented diet showed an enhanced immune response reflected in IgM and superoxide dismutase activity as compared to control group (Reyes-Becerril *et al*., 2008). It appears that diets supplemented with *D. hansenii* stimulates the immune function of juvenile *M. rosacea* and improves disease resistance against *Amyloodinium. ocellatum.*

Probiotics and water quality

In intensive fish culture systems, uneaten feed, feces and plankton die-offs causes accumulation of high load of organic material in the bottom of ponds. Therefore, water quality in intensive aquaculture systems to a large extent can be affected by the microbial biodegradation of organic residues (Avnimelech *et al*., 1995). The water quality is known to be affected by microbial processes via utilization of oxygen, regeneration of inorganic nutrients and production of toxic metabolites like ammonia, nitrite and sulphide (Moriarty, 1996).

Information on the beneficial effects of the application of bacterial products in aquaculture systems is limited (Querroz and Boyd, 1998). According to Boyd (1995) bacterial extracellular enzymes which solubilize organic matter, are unlikely to improve pond soil and water quality conditions. In addition, without proper environmental condition, applications of bacterial amendments or enzymes neither enhance bacterial activity nor improve water quality (Boyd 1995 and Shariff *et al*., 2001). Early work of Boyd *et al*. (1984) did not show a significant effect of different doses of bacterial suspension on the nutrient concentration of water in fish ponds. Likewise, Timmerson and Gerard (1990) reported the same results after using two commercial bacterial suspensions under laboratory condition.

However, there are some benefits of using bacterial products in aquaculture, some of which are the reduction of blue green algal populations which prevent off-flavor and the levels of nitrate, nitrite, ammonia and phosphate, increase dissolved oxygen concentrations and promotion of organic matter decomposition in water (Boyd, 1995). The use of microbial biotechnology in pond aquaculture has been strongly advocated by Moriarty (1996) and he recommended that it is important for the successful use of probiotics in aquaculture to understand the nature of competition between species and strains of bacteria in the aquatic environment. The water quality parameters have significantly improved by the routine use of commercial probiotics in farmed shrimp. This includes reduced organic matter accumulation, improved water quality and increased shrimp size and total production (Suhendra *et al*., 1997). Probiotics may be involved in the turnover of the organic nutrients which in turn results in an improvement of water quality in the aquaculture ponds (Moriarity, 1997). To support this view, there have been some applications of the useful use of bacteria; *Nitrosomonas* bacteria have the ability to convert ammonia into nitrite. However, *Nitrobacter* bacteria have an additional ability to mineralize nitrite to nitrate and it is known that ammonia compounds are highly toxic to fish but Nitrate is significantly less toxic. Nitrifying bacteria excrete polymers allowing them to associate with surfaces and form biofilms (Hagopian and Riley, 1998). Recirculation systems used for fish culture should use biofilters to remove ammonia, Skjolstrup *et al.* (1998) found that there was about 50% reduction in both ammonia and nitrite levels by using an experimental bio-filter in the recirculation unit of rainbow trout. Another example is sulfur-reducing bacteria that oxidize organic carbon using sulfur as a source of molecular oxygen. The hydrogen ion is released when organic carbon fragments are oxidized to combine with sulfate to form sulfide which is less toxic to the aquatic animals. Methane-reducing bacteria use carbon dioxide as a source of molecular oxygen. Methane diffuses into the air and thereby improves the water quality (Sahu *et al*., 2008).

Probiotic bacterial strains may have a significant algaecide effect on many species of micro-algae, particularly red tide plankton (Fukami *et al*., 1997). Antagonistic bacteria towards algae would be considered undesirable in green water used for larval rearing in hatcheries where unicellular algae are cultured. However, it would be advantageous when undesirable algae species grow in the culture pond. Therefore, microorganisms are important and they play a critical role in aquaculture systems at the hatchery and the grow out stages, because water quality and disease control are closely related and directly affected by microbial activity (Jory, 1998). Probiotics play a very important role in the degradation of organic matter thereby significantly reducing the sludge and slime formation. As a result, water quality will improved by reducing the disease (caused by *Vibrio* sp., *Aeromonas* sp. and viruses) incidences, enhancing the zooplankton population, reducing odors and ultimately enhancing aquacultural production. By speeding up the rate of organic matter breakdown, free amino acids and glucose are also released providing food sources for the beneficial microorganisms. Inorganic forms of nitrogen, such as ammonia, nitrate and nitrite are also reduced. By improving water quality and FCR in aquaculture operations, the overall health and immune function of the shrimp and fish could be markedly improved (Green and Green, 2003).

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Aim of work

The present study was designed in two experimental studies; the first was conducted to study the effect of different probiotics, *Bacillus subtilis* NIOFSD017, *Lactobacillus plantarum* NIOFSD018, a mixture containing both bacterial isolates (*B. subtilis* NIOFSD017, *L. plantarum* NIOFSD018), and yeast, *Saccharomyces cerevisiae* NIOFSD019 supplements in diets for Nile tilapia, *Oreochromis niloticus*, in order to evaluate their effect on growth performance, feed utilization, activity of digestive enzymes and the immune response. A second experimental study was designed to determine the optimum probiotic doses of our specific bacterial isolate which was found to be highly effective and exhibited best results. The objective of this research was to develop an environmental friendly probiotic by NIOF (National Institute of Oceanography and Fisheries), which is based on bacterial species isolates from our local environmental conditions and for its specific use to promote growth and feed utilization of tilapia.