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REVIEW

APPLICATION OF PROBIOTICS IN AQUACULTURE

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ABSTRACT
Average consumption of aquaculture products increases every year. To meet these needs, cultivation activities have been developed into intensified cultivation systems. Unfortunately, intensification of cultivation often causes a decline in environmental conditions which ultimately causes problems in the form of disease. Continuous use of antibiotics can cause pathogenic bacteria to become resistant. So now probiotics have been developed in the field of aquaculture. Probiotics are live microorganisms which, when given in adequate quantities, provide health benefits to the host. This review will discuss the application of probiotics in the field of aquaculture. Several studies have shown the benefits of probiotics, namely as a good growth promoter, inhibiting the growth of pathogens, increasing intestinal absorption and digestion, improving water quality, effects on fish reproduction.

Keywords: *Aquaculture, Probiotic, Microorganism, Fish*

INTRODUCTION

The Food and Agricultural Organization (FAO) reports that the average per capita consumption of aquaculture products increased from 14 percent in 1986 to 47 percent in 2006 and is expected to reach 50 percent in the next few years (**Desriac *et al*., 2010**). The World Aquaculture report (2012) shows that global production of fish from aquaculture grew by more than 30 percent between 2006 and 2011, from 47,300,000 tons to 63,600,000 tons. It is also estimated that by 2012 more than 50 percent of world food fish consumption will come from aquaculture, so it is expected to overtake capture fisheries as a source of edible fish (**Timmons *et al*., 2002; FAO, 2012**). Cultivation has been developed into an intensification cultivation system. Unfortunately, intensification of cultivation often causes a decline in environmental conditions which ultimately causes problems in the form of disease. Diseases caused by bacteria, apart from causing mass deaths, can also affect the quality of fish by reducing the quality of infected fish meat so that it is not liked by consumers. Several cases of disease outbreaks due to bacterial infections have caused cultivators to suffer major losses, therefore, disease management needs serious attention (**Gardenia *et al.,* 2010**).

Continuous use of antibiotics can cause pathogenic bacteria to become resistant. Apart from that, it is also possible for antibiotic residues to occur in the fish's body, thereby endangering consumers if consumed. Various antibiotics that are used on cultivars in the healing process incorrectly can harm the cultivars being kept therefore, one of the actions that can be taken to reduce the use of antibiotics in fish farming is the use of natural ingredients, so it is hoped that they will not leave residue in the fish's body and are safe. for the surrounding environment (**Setyowati, 2014**).

Prevention of disease and dependence on antibiotics is currently being overcome by developing probiotics in the field of aquaculture. Probiotics are live microorganisms which when given in adequate quantities, provide health benefits to the host (**Jorgen, 2012)**. The use of probiotics or beneficial bacteria, which control pathogens through various mechanisms, is increasingly seen as an alternative to antibiotic treatment and new demand for probiotics has increased in the environmentally friendly and sustainable aquaculture industry (**Mohapatra *et al*., 2012**). Several studies have shown the benefits of probiotics for farmed fish species, including being a good growth promoter, being able to produce compounds that inhibit the growth of pathogens, increasing intestinal absorption and digestibility of feed, improving air quality, stress tolerance and effects on fish reproduction (**Cruz *et al*., 2012**). The aim of this paper is to explain the application of probiotics (microbial strains) in the field of aquaculture and their ability to increase fish resistance to disease-causing pathogens.

METHOD

The method used in writing this review article is to review several journals and look for similarities to draw conclusions. Synthesize to combine information from various sources to form new ideas. Summarize to rewrite the information in your own words. The method in reviewing this journal focuses on conclusions and main points from the background, research objectives, methods, sample and population, tools and materials, research results, as well as discussions and conclusions from the journals reviewed.

RESULTS AND DISCUSSION

History of Probiotics

A Russian scientist named Elie Metchnikoff first suggested that it was possible to change the intestinal flora and replace harmful microbes with beneficial microbes, and introduced probiotics in 1907 (**Metchnikoff, 1907**). At that time, it was known that fermented milk products with lactic acid bacteria inhibited the growth of proteolytic bacteria due to the low pH produced by lactose fermentation. Metchnikoff also observed that certain rural populations in Europe, for example in Bulgaria and the Russian steppes, who mostly utilized milk fermented by lactic acid bacteria had long life spans. Based on these observations, Metchnikoff proposed that consumption of fermented milk with lactic acid bacteria was harmless and lowered intestinal pH, and that this would suppress the growth of proteolytic bacteria.

The empirical application of probiotics in aquaculture was first described by **Kozasa (1986)** due to the benefits provided by the use of probiotics in humans and poultry. **Kozasa (1986)**, used Bacillus toyoi spores as a feed additive to increase the growth rate of *Seriola quinqueradiata* fish. In 1991, **Porubcan (1991)** documented the use of *Bacillus* spp. to increase the productivity of *Penaeus monodon* cultivation and improve water quality by reducing ammonia and nitrite concentrations. Additionally, certain probiotics have the ability to inhibit the growth of pathogenic bacteria. Moriarty determined the ability of *Bacillus* spp. to reduce the proportion of *Vibrio* spp. in shrimp ponds, especially in sediments **(Moriarty, 1998).** Further research has emphasized the ability of probiotics to stimulate appetite, increase nutrient absorption, and strengthen the host immune system (**Wang *et al*., 2008**; **Irianto and Austin, 2002**).

Probiotik Definition

The term "probiotic" originally referred to microorganisms that have an effect on other microorganisms (**Lilly and Stillwell, 1965**). In 1974, **Parker (1974)** defined the concept of probiotics as, "organisms and substances that have a beneficial effect on an animal host by contributing to the balance of its intestinal microbes". Later, the definition was developed by **Fuller (1989)** whose explanation is very similar to the definition used today, namely "live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance". He emphasizes two important facts about probiotics: their viable properties and their capacity to help gut balance. The name "probiotic" consists of the Latin "pro" which means "for" and the Greek "βιός" which means "suitable for life, alive" (**Liddell *et al*., 2003**). Probiotics are live microorganisms which, when given in adequate amounts, provide health benefits to the host and maintain the microbial balance in the intestine (**Jorgen, 2012**).

Probiotic Microorganisms

Probiotic candidates must be microbes or combinations of microbes (genus, species, and strain level). Probiotics must be safe to use, the 2002 FAO/WHO guidelines recommend that, although bacteria are generally recognized as safe (GRAS), potential safety should be assessed by the minimum required tests:

1. Determination of antibiotic resistance patterns.
2. Assessment of specific metabolic activities (eg, D-lactate production, bile salt deconjugation).
3. Assessment of adverse events during host studies.
4. Adverse epidemiological surveillance.
5. If the strain is a toxin producing species, it should be tested for toxin production. One possible scheme for testing venom production has been recommended by the EU Scientific Committee on Animal Nutrition (2016).
6. If the strain has hemolytic potential, then determination of hemolytic activity is necessary.

In Europe, the European Food Safety Authority (EFSA) has adopted a premarket system for the safety assessment of microbial species used in food and feed production, setting priorities for risk assessment requirements. This assessment is made for a selected group of microorganisms, which if beneficial, leads to “Presumed Safety Qualified” status (**Machine, 2015**). In the field of aquaculture, several potential probiotic microorganisms mostly consist of Bacillus strains (**Panigrahi *et al*, 2007**), Carnobacterium (**Irianto & Austin, 2002; Kim & Austin, 2006a**) and Lactobacillus (**Nikoskelainen et al, 2001b; Panigrahi et al, 2004** ; **Vendrell *et al*., 2008**), although the use of other species such as Aeromonas and Vibrio has also been explored (**Irianto & Austin, 2002; Brunt and Austin, 2005**).

Probiotic Safety Considerations

Traditionally, probiotics used in the food industry have been considered safe; in fact, no risk to humans has been determined, remaining the best evidence of safety (**Saxelin *et al.*, 1996**). In practice, there are several reports of bacteremia in humans, where isolation of probiotic bacteria from infections appears to be the result of opportunistic infections caused by skin lesions, cancer, chronic diseases, or drug-induced disorders (**Cruz *et al.,* 2012**). This condition causes the intestinal barrier to decrease which promotes the passage of bacteria through the mucosal epithelium and subsequently, these microorganisms are transported to the mesenteric lymph nodes and other organs, causing bacteremia which can progress to septicemia (**Ishibashi and Yamazaki, 2001**). All reported cases of bacteremia occurred in patients with chronic diseases or a weakened immune system (**Soccol *et al*., 2010**). Regarding the safety of aquaculture products, in Asia and recently in Penaeus monodon cultivation in Latin America bacterial white spot syndrome (BWSS) has been reported, caused by repeated use of the probiotic Bacillus subtilis. The spots are similar to white spot viral syndrome (WSS), which is a deadly disease that spreads rapidly and causes mass mortality in shrimp (**Wang *et al*., 1999**). **Wang *et al.* (2000)** that BWSS is a non systemic infection in *P. monodon* and the lesions usually disappear after molting, under these conditions the culture is still active and grows normally without significant mortality.

In addition, because some aquaculture products are consumed raw or undercooked, the question has been raised whether residual probiotics can cause any infections in the final consumer (**Cruz *et al.,* 2012**). **Shakibazadeh *et al.* (2011)** assessed the potential risks to humans caused by the use of *probiotic Shewanella algae* in shrimp ponds. Studies conducted on mice given *S. algae* up to 1036 CFU to reach the LD50 value, prove the safety of using *S. algae* probiotics in mice. Based on these results, the author states that the use of *S. algae* is safe for shrimp consumers. While there is no international consensus to guarantee the efficiency and safety of probiotics, the FAO and WHO recognize the need to establish guidelines for a systematic approach to the evaluation of probiotics in foods, to substantiate their health claims. A working group with experts in the field was formed to recommend criteria and methodology for the evaluation of probiotics, based on scientific evidence (**Pineiro and Stanton, 2007**). So the "Guide to the Evaluation of Probiotics in Food" is presented, providing guidelines on the evaluation of the health and properties of probiotics in food. The working group states that no pathogenic or virulent properties have been found in lactobacilli, bifidobacteria, or lactococci, although they acknowledge that under certain conditions, some strains of lactobacilli have been associated with rare cases of bacteremia. However, the incidence did not increase with increasing use of lactobacillus in probiotics. It is also suggested that enterococci may have virulence characteristics; Therefore, it is not recommended as a probiotic for human consumption (FAO and WHO, 2006). Although the book is not focused on fishery products, it is necessary to conduct studies to evaluate the safety of probiotics in the field of aquaculture. To date, the use of animal models including mice and fish have not revealed specific determinants of virulence or pathogenicity of probiotic microorganisms, indicating the overall safety of them (**Lahtinen *et al.,* 2009**). However, it is important to continue research using three approaches: (i) analyzing the intrinsic properties of a probiotic strain, (ii) studying its pharmacokinetics (survival, activity in the gut, dose response, and recovery from the mucosa), and (iii) understanding the interactions between microorganisms. and host (**Soccol *et al*., 2010**).

Mechanisms of Probiotics

One of the most important properties of probiotics is the ability of bacterial strains to attach and reproduce in organism tissues and provide maximum benefit to the host species. Therefore, to achieve maximum benefits, probiotics must reach the tissues that need them. Some of the working mechanisms of probiotics are as follows.

1. Production of inhibitory compounds
   Probiotics play a major role in preventing disease by producing certain inhibitory compounds that act antagonistically against pathogenic microbes and prevent their proliferation in the host body (**Tinh *et al*., 2007**). Anti-pathogenic activity by producing several compounds such as bacteriocins (**Tinh *et al.,* 2007**), siderophores, lysozymes, proteases, hydrogen peroxide and changes in pH values (**Sugita *et al.*, 2009**). Lactic acid bacteria can inhibit the growth of other bacteria by producing proteins called bacteriocins. One example of a widely known bacteriocin is nisin produced by *Lactobacillus lactis* ssp. (**Walstra *et al.*, 2005**). In addition, *Aeromonas* A199 bacteria are able to produce compounds that are useful in limiting pathogen activity which were later identified as indole (s,3-benzopyrrole) (**Lategan *et al.*, 2006**), and have anti-bacterial and anti-fungal activities (**Desriac *et al*., 2010)**.
2. Competition for adhesion sites
   Competition for space for adhesion and colonization on the gut and other tissue surfaces is another way that probiotic microorganisms fight pathogens (**Ringo *et al*., 2007**). Adhesion of probiotics is influenced by physico-chemical or specific factors, and is based on bacterial morphology and receptor molecules on epithelial cells (**Salminen *et al.,* 1996**).
3. Competition for available chemicals or energy
   The basis for the existence of any microbial population depends on its ability to compete with other microbes for the chemicals and energy available in the same ecosystem (**Verschuere *et al.,* 2000a**).

In one study, the removal of iron (Fe+3) is important for many pathogens such as Vibrio spp, (**Rorvik *et al.,* 1991)** by producing siderophore compounds by probiotic microbes that can remove Fe+3 in the environment.

4. Competition for nutrition
   Probiotics grow by adhering to mucus, digestive tract, epithelial cells and other tissues, and further contribute to the health or well-being of the host (**Farzanfar, 2006**). The proliferative ability of several bacteria has been tested in vitro and in vivo and the results show that pathogens will be eliminated in the presence of probiotics based on competition for important nutrients, space, etc. (**Verschuere *et al.*, 2000b**).

Probiotic Applied in Aquaculture

In many countries cultivation has become an important source of income. The development of intensive cultivation activities has resulted in a decline in environmental quality and the emergence of various diseases, which ultimately have an impact on the economy. Prevention of disease at the beginning of the development of aquaculture is by using drugs such as antibiotics, but through research activities it turns out that the use of antibiotics increases pathogen resistance and is dangerous for the health of humans who consume fish. Therefore, probiotics have recently been developed for disease prevention in the field of aquaculture. A more general concept regarding probiotics is "one or more microorganisms with beneficial effects on the host, able to survive in the digestive tract due to tolerance for acids and bile salts" (**Irianto and Austin, 2002**). Although the use of probiotics in aquaculture is relatively new, research interest has increased due to the potential of probiotics in disease control (**Wang and Lin, 2008**). Several applications of probiotics in aquaculture activities can be seen in table I (**Cruz *et al.,* 2012**).

Probiotic as Growth Promotor

Probiotics have been used in cultivation to increase the growth of cultivated species. Gupta et al, (2014) added probiotics *Bacillus coagulans* (MTCC 9872), *Bacillus licheniformis* (MTCC 6824) and *Paenibacillus polymyxa* (MTCC 122) in *Cyprinus carpio* fish feed and identified the effect of probiotics on fish growth performance, FCR (food conversion ratio), PER (protein efficiency ratio) and survival. Fish were fed for 80 days with a control basal diet (B0) and an experimental diet containing *B. coagulans* (B1), *B. licheniformis* (B2) and *P. polymyxa* (B3) at 109 CFU/g diet. The results of the study showed that treatment using *Paenibacillus polymyxa* (109 CFU/g diet) had significant growth, FCR, PER and survival rates (Table 1).

Table 1. Growth rate and feed utilization of *Cyprinus carpio* fed a basal diet and a diet containing probiotics for 80 days.

Growth Index	B0 (Control)	B1	B2	B3
Initial body weight (g)	0.329±0.01[a]	0.329±0.01 [a]	0.329±0.01 [a]	0.329±0.01 [a]
Final body weight (g)	0.803±0.027 [c]	1.066±0.043 [b]	1.046±0.049 [b]	1.346±0.039 [a]
Survival (%)	60±2.88 [b]	80.0±2.88 [a]	61.7±1.66 [b]	81.7±1.67 [a]
Relative percent survival	0	50.0 [a]	4.25	54.25
Specific growth rate (%/day)	1.12±0.27 [c]	1.47±0.29 [a]	1.45±0.22 [b]	1.76±0.31 [a]
Food conversion ratio	7.05±1.28 [a]	5.16±1.13 [a]	5.13±1.17 [b]	4.26±0.98 [b]
Protein efficiency ratio	0.35±0.09 [c]	0.58±0.07 [a]	0.51±0.08 [b]	0..70±0.07 [a]

The use of probiotics to increase growth by adding probiotics to the diet of rainbow trout has been reported by **Bagheri *et al.* (2008).** The results showed that the application of *B. subtilis* and *B. licheniformis* could significantly increase FCR, specific growth rate (SGR), body weight and protein efficiency ratio (PER) after 2 months of being fed feed containing 3.8A~109 CFU/g. The use of probiotics as a fish growth promoter has also been reported by **Lara *et al.*, (2003),** the addition of the probiotics *Streptococcus faecium* and *Lactobacillus acidophilus* to the diet of tilapia (*Oreochromis niloticus*) significantly increased the content of crude protein, crude fat and also the weight of the fish from 0.154 g to 6,164 g at 9 weeks of cultivation (**Lara *et al*., 2003**). In addition, rotifers are indispensable as the first live food for the larvae of most cultured aquatic species, because of their small size they are more accessible to the larvae, for example, sea shrimp nauplii, which are a very common live food. **Planas *et al.* (2004)** used lactic acid bacteria to increase the growth of the rotifer Brachionus plicatilis and obtained the best results with the addition of *Lactococcus casei* ssp. *casei, Pediococcus acidilactici*, and *Lactobacillus lactis* ssp. lactis

Increase Nutrient Digestion

A study has suggested that probiotics have beneficial effects on the digestive processes of aquatic animals because probiotic strains synthesize extracellular enzymes such as protease, amylase, and lipase and provide growth factors such as vitamins, fatty acids, and amino acids (**Balcázar *et al.,* 2006a**). Therefore, nutrients are absorbed more efficiently when feed is supplemented with probiotics (**Haroun *et al.,* 2006**). Probiotics have been used in fish feeds, as in the case of European bass (*Dicentrarchus labrax*) larvae. It has been reported that the probiotic yeast *Debaryomyces hansenii* HF1 has the ability to produce spermine and spermidine, in addition, this yeast secretes amylase and trypsin, digestive enzymes that are helpful in sea bass larvae. Studies in juvenile dentex *Dentex dentex* L. showed that when the diet was supplemented with 0.5 g of starin *Bacillus cereus* E kg-1, fish growth increased due to more efficient use of food (**Hidalgo *et al*., 2006**).

In several experiments, European sea bass (*Dicentrarchus labrax*) larval diets were supplemented with yeast probiotics (*Saccharomyces cerevisiae* strain fish treated with probiotics were significantly higher than those without probiotics, while the activity and expression patterns of enzyme genes were significantly higher in control fish than in fish treated with probiotics (**Tovar *el al.,* 2010**). *Bacillus subtilis* isolated from the intestines of *Cirrhinus mrigala* into the diet of guppy (*Poecilia reticulata*, *P. sphenops*), and swordtail (*Xiphophorus helleri,* digestion (**Gómez *et al.,* 2007**). According to Moriarty **(Moriarty, 1996),** *Bacillus subtilis* secretes various exoenzymes that improve enzymatic digestion. In fact, bacteria isolated from the digestive tracts of aquatic animals have demonstrated chitinase, protease, cellulase, lipase, and trypsin (**Burr, 2005**).

Improve Water Quality

Several studies in water quality was noted during the addition of probiotic strains mainly from the gram-positive Bacillus genus. In one study, fish farming produced high nitrogen concentrations ranging from 0.05 to 3.3 mg L-1 and up to 6.4 mg L-1 after 7 months of monitoring (Maillard et al., 2005). For tilapia production in the circulating system, the total ammonia concentration (NH4 + NH3) increased by 4.73-14.87 mg L-1 in the 21 days of the experiment, while the nitrite concentration increased by 3.75-9.77 mg L-1 (Rafiee and Saad, 2005). Due to the high concentration of nitrogen compounds produced, especially total ammonia, which is very toxic, the use of probiotics is recommended, because it can improve water quality. Haroun et al. feeding tilapia fish *Oreochromis niloticus* L. containing commercial probiotics, consisting of *Bacillus licheniformis* and *B. subtilis* for 17 weeks. Assessment of water quality parameters shows acceptable ranges for fish farming: 5.7-6.3 mg L-1 for dissolved oxygen concentration, 0.36-0.42 mg L-1 for ammonia concentration, and pH between 6.3 and 8.2 **(Wang *et al.,* 2005).**

Lalloo *et al*., (2007) isolated several strains of Bacillus from *Cyprinus carpio* and carried out tests to improve water quality in ornamental fish cultivation and to inhibit the growth of *Aeromonas hydrophila*. Three of the nine isolates showed a high capacity to inhibit pathogens. Additionally, the concentrations of ammonia, nitrate and phosphate showed a decrease at levels of 74%, 76% and 72% respectively. **Taoka *et al.* (2006)** The effects of a commercial probiotic formulated from a mixed culture of bacteria and yeast on the survival of Japanese flounder *Paralichthys olivaceus*, and water quality in a closed circulation system were studied. The probiotic-treated group showed a significantly greater survival rate compared with the control group at the end of the rearing experiment (50 days of culture), and water quality parameters were significantly lower in the dietary probiotic group (from 0.24 ± 0.22 - 0, 12 ± 0.10 mg L-1 NH4, from 0.15 ± 0.08 - 0.08 ± 0.08 mg L-1 of NO2, and from 13.0 ± 3.9 - 10.2 ± 3.0 mg L-1 of PO4). Meanwhile, ***Wang et al*. 2005** showed that commercial products made from *Bacillus* sp., *Saccharomyces cerevisiae*, *Nitrosomonas* sp., and *Nitrobacter* sp. has the ability to increase the microbiota of beneficial bacteria in *Penaeus vannamei* shrimp cultivation, further reducing the concentration of inorganic nitrogen from 3.74 to 1.79 mg L-1 and phosphate from 0.1105 to 0.0364 mg L-1 (**Jiqiu *et al.,* 2006**)

Increase Stress Tolerant

Intensive farming practices cause stress in farmed fish species. One study that shows the ability of *Lactobacillus delbrueckii* ssp. Supplementation of *L. delbrueckii* in the diet *of Dicentrarchus labrax* on the level of stress tolerance, at a time interval of 25 to 59 days. In the study, in addition to evaluating increased growth, the hormone cortisol was quantified in fish tissue as a stress marker, because it is directly involved in the animal's response to stress. Cortisol levels obtained in treated fish were significantly lower than in controls (3.6 ± 0.36 ng g-1 and 5.1 ± 0.47 ng g-1, resp.) and fish treated with probiotics showed higher growth rates. well (Figures 1 and 2) (**Carnevali *et al.,* 2006**).

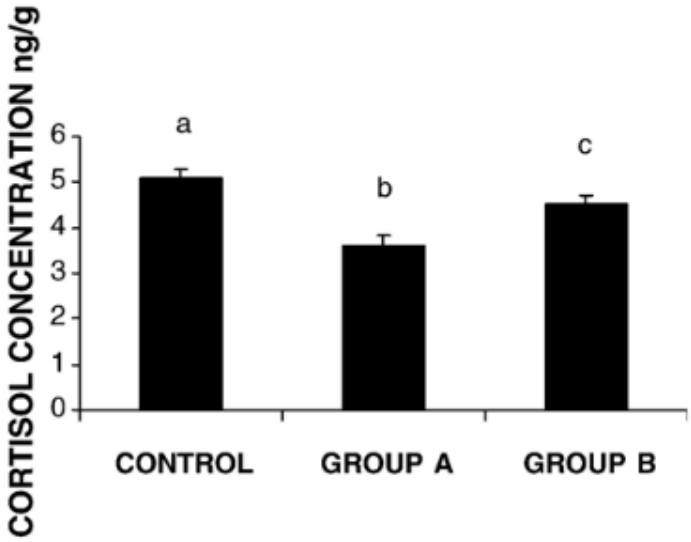

Figure 1. Tingkat kortisol dalam jaringan. Grup A menerima probiotik dari hari 11 ke 70. Grup B menerima probiotik mulai dari hari 30 sampai 70.

Another way to assess stress is to involve heat shock in fish, as in the case of Japanese flounder (*Paralichthys olivaceus*) which is cultured in a recirculating system (**Taoka *et al.,* 2006**). The stress test was carried out until ½ of the treated fish died, thus calculating the lethal time (LT50) for fish without the addition of probiotics and with the addition of commercial probiotics containing *Bacillus subtilis*, *Lactobacillus acidophilus*, *Clostridium butyricum,* and *Saccharomyces cerevisiae*. The probiotic treatment group showed greater tolerance in the stress test than the control group, LT50 of 40 and 25 minutes, respectively.

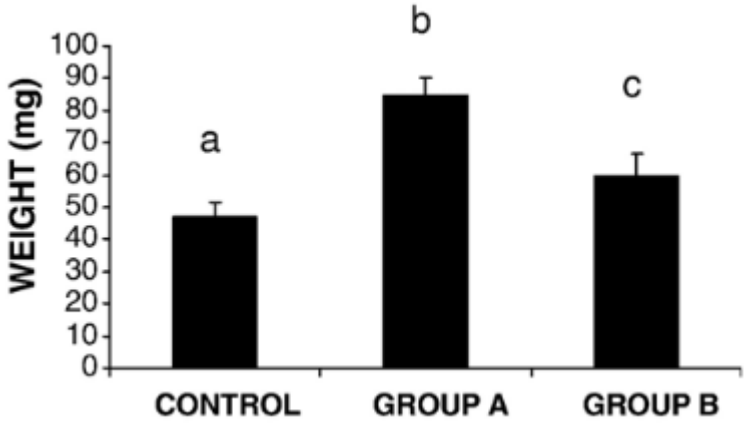

Figure 2. Berat diukur pada ke 70 hari pasca perlakukan. Grup A menerima probiotik dari hari 11 ke 70. Grup B menerima probiotik mulai dari hari 30 ke 70.

Probiotic Effect on Fish Reproduction

Cultured fish have high nutritional requirements, so reproductive capacity depends on the appropriate concentration of lipids, proteins, fatty acids, vitamins C and E, and carotenoids **Izquierdo *et al.* (2001).** Furthermore, the relationship of these components influences reproduction in various processes such as fertility, fertilization, birth and larval development. In practice, many fish hatcheries improve the nutrition of their broodstock by feeding them only fresh fish by-products or in combination with commercial diets. The most common fresh organisms used to feed brood fish include squid, cuttlefish, clams, krill, and small crustaceans. The use of processed fish products often does not provide adequate nutritional levels required by parent fish, increasing the risk of pathogen transmission to parents and fry, including parasites, bacteria, and viruses. Therefore, probiotics are added to food or water used to prevent infections and to explore their effects on reproduction.

A pioneering study on the effect of probiotic supplementation on fish reproductive performance was carried out by **Ghosh *et al.* (2007)** used *B. subtilis* strain isolated from the intestines of *Cirrhinus mrigala*, given to four species of ornamental fish: *Poecilia reticulata*, *P. sphenops, Xiphophorus helleri and X. maculates* with different doses in a one year experiment (5×108 cells g-1 (T1), 5 × 107 cells g-1 (T2), 5 × 106 cells g-1 (T3) and 5 × 105 cells g-1 (T4) control. The results showed that using *B. subtilis* at a dose of 106 - 108 cells g-1 from feed, showed an increase in gonadosomatic index production, fecundity, survival, and seed

production from females of all four species (Tables 2, 3, 4, 5). especially thiamine (vitamin B1) and vitamin B12, contribute to reducing the number of dead or deformed alevins.

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Abasali and Mohamad (2010) conducted similar research with *X. helleri*, using commercial probiotics containing *Lactobacillus acidophilus, L. casei, Enterococcus faecium, and Bifidobacterium thermophilum*. Total alevin production per female and relative fecundity were evaluated. Their results showed significant differences between the control and probiotic groups; in the first parameter the average of alevins is found, namely; 105 and 150, respectively. Meanwhile in the second parameter, the number of females that have fecundity, namely; 28 females for control and 41 for treatment with probiotics.

Table 2. Reproduction Performance of *Poecilia reticulata*

Experiment groups	R_1	R_2	R_3	R_4	R_0
Fecundity	20.23±10.29^{a}	19.9±9.46^{a}	18±9.39^{ab}	17.81±9.5^{b}	15.23±7.39^{a}
Fry survival	19.19±9.39^{a}	18.66±8.94^{ab}	16.77±9.62b	16.45±9.24^{b}	12.91±5.15^{c}
Dead fry	1.04±1.49^{a}	1.24±1.72^{a}	1.23±1.76^{a}	1.36±1.81^{a}	2.32±2.69^{b}
Deformed fry	0.1±0.31^{a}	0.15±0.36^{ab}	0.24±0.53^{ab}	0.21±0.54^{ab}	0.32±0.79^{b}
Weight	0.74±0.07^{a}	0.70±0.10^{ab}	0.79±0.14c	0.68±0.11b	0.71±0.07^{ab}
Length	39.56±1.1^{a}	39.53±1.15^{a}	41.31±1.88b	39.17±1.22^{a}	39.68±1.05^{a}
Fry weight (g)	0.0024±0.0004^{a}	0.0023±0.0003^{a}	0.0022±0.002^{a}	0.0024±0.0003^{a}	0.0019±0.0002^{b}
Fry length (mm)	6.36±0.54^{a}	6.29±0.58^{ab}	6.08±0.52^{c}	6.16±0.68^{bc}	5.82±0.76^{d}
GSI (%)	9.25±0.56^{a}	9.24±0.5^{a}	9.1±0.39^{a}	8.84±0.36^{ab}	8.13±0.44^{b}

Legends:*Average per female, Mean ±SD values with different letters in each row are significantly (*P<0.05*) different, GSI: gonadosomatic index

Table 3. Reproduction Performance of *Poecilia sphenops*

Experiment groups	R_1	R_2	R_3	R_4	R_0
Fecundity	43.29±10.29^{a}	42.68±19.52^{a}	40.98±18.67^{ab}	39.36±17.14 ^{ab}	37.28±19.38^{b}
Fry survival	41.54±9.39^{a}	41.02±18.35^{a}	39.12±17.56 ^{a}	37.64±16.02^{ab}	34.60±16.37^{b}
Dead fry	1.75±1.49^{a}	1.66±1.92^{a}	1.86±2.34^{a}	1.72±1.96^{a}	2.68±3.04^{b}
Deformed fry	0.15±0.31^{a}	0.33±0.58^{a}	0.48±0.78^{ab}	0.17±0.38^{a}	0.88±1.17^{b}
Weight	1.44±0.07^{a}	1.57±0.56^{a}	1.45±0.42^{a}	1.38±0.4b ^{a}	1.48±0.65^{a}
Length	49.63±1.1^{a}	50.28±4.92^{a}	49.19±4.63b^{a}	47.36±4.23^{a}	49.38±5.35^{a}
Fry weight (g)	0.00052±0.0004^{a}	0.0051±0.0015^{a}	0.005±0.0017^{a}	0.0049±0.0016^{a}	0.0045±0.0018^{b}
Fry length (mm)	7.44±0.54^{a}	7.38±0.26^{a}	7.28±0.36^{ab}	7.28±0.26^{ab}	7.11±0.32^{b}
GSI (%)	9.09±0.46^{a}	8.92±0.62^{a}	9.36±0.39^{a}	8.67±0.56^{ab}	7.96±0.41^{b}

Legends:*Average per female, Mean ±SD values with different letters in each row are significantly (*P<0.05*) different, GSI: gonadosomatic index

Table 4. Reproduction Performance of *Xiphophorus helleri*

Experiment groups	R_1	R_2	R_3	R_4	R_0
Fecundity	74.36±25.29^{a}	63.46±22.09^{b}	62±26.39^{b}	68.46±19.23 ^{ab}	54.92±24.21^{c}
Fry survival	71.77±26.32^{a}	60.74±26.15^{b}	59.22±24.95^{b}	65.85±21.57^{ab}	51.06±27.91^{c}
Dead fry	2.72±2.75^{a}	2.59±2.58^{a}	2.78±2.44^{a}	2.61±2.69^{a}	3.86±3.83^{b}
Deformed fry	0.53±0.93^{a}	0.32±0.59^{a}	0.39±0.69^{a}	0.52±0.91^{a}	1.21±1.92^{b}
Weight	2.65±0.68^{a}	2.31±0.59^{a}	2.49±0.62^{a}	2.61±0.83b ^{a}	2.29±0.73^{a}
Length	59.58±5.05^{a}	54.29±6.23^{b}	57.24±4.21b^{ab}	59.44±6.92^{a}	54.21±4.97^{b}
Fry weight (g)	0.0056±0.0019^{ab}	0.0053±0.0011^{a}	0.006±0.0019^{b}	0.0056±0.0015^{ab}	0.0048±0.0021^{c}
Fry length (mm)	7.72±0.35^{a}	7.68±0.24^{a}	7.76±0.32^{a}	7.62±0.39^{ab}	7.32±0.42^{b}
GSI (%)	10.84±0.51^{a}	10.49±0.49^{ab}	10.74±0.58^{ab}	10.26±0.45^{ab}	9.92±0.37^{b}

Legends:*Average per female, Mean ±SD values with different letters in each row are significantly (*P<0.05*) different, GSI: gonadosomatic index

Table 5. Reproduction Performance of *Xiphophorus maculatus*

Experiment groups	R_1	R_2	R_3	R_4	R_0
Fecundity	38.23±17.29^{a}	37.94±16.19^{a}	34.34±17.53^{b}	36.13±17.45 ^{ab}	33.42±18.49^{b}
Fry survival	36.55±17.23^{a}	36.35±16.39^{a}	32.6±15.56^{b}	34.3±16.39^{ab}	31.62±14.92^{b}
Dead fry	1.68±2.29^{a}	1.59±1.88^{a}	1.74±2.25^{a}	1.83±2.44^{a}	1.8±2.32^{a}
Deformed fry	0.23±0.55^{ab}	0.62±1.08^{c}	0.15±0.36^{a}	0.53±0.98^{bc}	1.24±1.66^{d}
Weight	0.73±0.1^{a}	0.71±0.09^{a}	0.73±0.15^{a}	0.74±0.06 ^{a}	0.78±0.16^{a}
Length	39.52±3.12^{a}	39.47±2.27^{a}	38.84±4.06^{a}	39.35±2.06^{a}	39.64±4.92^{a}
Fry weight (g)	0.0027±0.0004^{a}	0.0026±0.0003^{ab}	0.0026±0.00^{ab}	0.0026±0.0004^{ab}	0.0024±0.0003^{b}
Fry length (mm)	6.89±0.32^{a}	6.66±0.4^{b}	6.68±0.35^{b}	6.69±0.39^{b}	6.44±0.34^{c}
GSI (%)	7.92±0.36^{a}	7.73±0.52^{ab}	7.91±0.33^{a}	7.18±0.25^{bc}	6.73±0.41^{c}

Legends:*Average per female, Mean ±SD values with different letters in each row are significantly (*P<0.05*) different, GSI: gonadosomatic index

Probiotic Effect on Enzyme

Digestive Enzyme

The digestive organs are very sensitive to food composition and cause changes in digestive enzyme activities, which are ultimately reflected in fish health and growth **(Shan *et al.* (2008)**. Changes in microbial metabolism affect enzymatic activity either by increasing or decreasing. Amylase and lipase are the main enzymes associated with carbohydrate and fat digestion, respectively. **Tovar *et al.* (2002)** reported increased secretion of amylase and trypsin in *Dicentrarchus labrax* larvae after feeding with live yeast *Debaryomyces hansenii*. Additionally, **Mohapatra *et al.* (2012)** noted higher levels of digestive enzyme activity (protease, amylase and lipase) in *Labeo rohita* when fed with a mixture of *Bacillus subtilis*, *Lactococcus lactis* and *Saccharomyces cerevisiae*. Bacteria also secrete proteases to digest peptide bonds in proteins and therefore break down proteins into their constituent monomers and free amino acids, which can benefit the nutritional status of animals. Phosphatase activity was observed in tilapia (*Oreochromis niloticus*) fed probiotic supplements, thus reflecting the possible development of enterocyte brush border membranes, and therefore, indicating increased absorption of carbohydrates and lipids (**Lara-Flores and Aguirre-Guzman, 2009**). *Bacillus* sp. isolated from *Cyprinus carpio* has extracellular amylolytic, cellulolytic, proteolytic and lipolytic activities (**Bairagi *et al.,* 2002**). Probiotics also play a very positive effect on the digestive process as well as the assimilation of food components (**Irianto and Austin, 2002**).

Metabolic Enzyme

Bacteria, especially members of the genus Bacillus, secrete various exo-enzymes (**Moriarty, 1998**). Exogenous enzymes produced by probiotics represent only a small contribution to total intestinal enzyme activity (**Ziaei-Nejad *et al.,* 2006**), and the presence of probiotics may stimulate endogenous enzyme production by shrimp. *Saccharomyces cerevisiae* var boulardii administered to trout showed higher activity of three types of enzymes in the brush border membrane of enterocytes: alkaline phosphatase (ALP), g-glutamyl-transpeptidase (GGT) and leucine-amino-peptidase N (LAP) (**Wache *et al.,* 2006**). Decreased activity of enzymes, such as amino aspartate transferase (AST), alanine amino transferase (ALT) and lactate dehydrogenase (LDH) was observed in *Oreochromis niloticus* after feeding with a diet containing *Pseudomonas* spp. and a mixture of *Micrococcus luteus* and *Pseudomonas* spp. Yeasts are well known in the field of animal nutrition because they can act as producers of polyamines, which promote intestinal maturation (**Peulen *et al.*, 2000**).

Probiotic Roles in Improving Fish Immune System

The fish immune system has two inseparable components: (1) an innate, natural or nonspecific defense system formed by a series of cellular and humoral components, and (2) an adaptive, acquired or specific immune system characterized by a humoral immune response through the production of antibodies and by the cellular immune response, mediated by T-lymphocytes, is capable of reacting specifically with antigens. The innate immune system consists of cellular and humoral components. The humoral component consists of, 1. Antimicrobial peptides, 2. Lysozyme, 3. Complement, 4. Transferrin, 5. Interferon, 6. Pentraxins, 7. Lectins, 8. Antiproteases and natural antibodies. while specific cytotoxic cells and phagocytes (monocytes/macrophages and neutrophils) are innate cellular immune effectors (Gómez and Balcázar, 2008). Apart from this, cytokines are an integral component of adaptive and innate immune responses, especially IL-1β, interferon, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and several chemokines that regulate innate immunity (**Gómez and Balcázar, 2008**).

The innate immune system, unlike the specific immune system, does not have the ability to acquire memory cells after eliminating pathogenic antigens. However, this system is quite important in fish due to the relatively slow production of antibodies in comparison. The adaptive immune response in ectothermic vertebrates requires considerable time (e.g., antibody production in salmonids takes at least 4–6 weeks) to

respond and is highly dependent on temperature (**Ellis, 2001**) Probiotics help in increasing the innate immune response and survival of fish from pathogen infections. Probiotics provide protection by enhancing the immune response of fish against pathogens by overcoming the adverse consequences of antibiotics and chemotherapeutic agents. Probiotics have been defined by the World Health Organization-Food and Agriculture Organization as live microorganisms which, when administered in adequate amounts, provide health benefits to the host (**FAO/WHO, 2001**). Many studies have proven that probiotics increase host resistance to pathogenic microbes (Table 6) (**Mohapatra *et al.,* 2012**) and by enhancing the immune response of fish (Table 7) (**Gómez and Balcázar, 2008**).

Table 6. Application of Probiotics in Increasing Fish Immune Response

Probiotic strain	Host Species	Effect
Vibrio fluviales A3 47S *Aeromonas hydrophila* A3 51, *Carnobacterium* sp. BA211, *Micrococcus luteus* A1 6	*Oncorhynchus mykiss*	Immune stimulation and improved survival after challenge with *Aeromonas salmonicid*
Lactobacillus rhamnosus ATCC 53103	*Oncorhynchus mykiss*	Immune stimulation and improved survival after challenge with *Aeromonas salmonicida*
Lactococcus lactis CECT 539	*Scophthalmus maximus*	Immune stimulation
Lactobacillus rhamnosus JCM 1136	*Oncorhynchus mykiss*	Immune stimulation
Lactobacillus delbriieckii CECT 287 *Bacillus subtilis* CECT 35	*Sparus aurata*	Immune stimulation
Aeromonas sobria GC2	*Oncorhynchus mykiss*	Immune stimulation and improved survival after challenge with *Lactococcus garvieae* and *Streptococcus iniae*
Bacillus subtilis *Lactobacillus acidophilus* *Clostridium butyricum* *Saccharomyces cerevisiae*	*Paralichthys olivaceus*	Immune stimulation and improved survival after challenge with *Vibrio anguillarum*
Carnobacterium maltaromaticum B26 *Carnobacterium divergens* B33	*Oncorhynchus mykiss*	Immune stimulation and improved survival after challenge with *Aeromonas salmonicida* and *Yersinia ruckeri*. Expression of cytokine genes
Lactobacillus rhamnosus ATCC 53103	*Oreochromis niloticus*	Immune stimulation and improved survival after challenge with *Edwardsiella tarda*
Lactobacillus rhamnosus ATCC 53103 *Bacillus subtilis* *Enterococcus faecium*	*Oncorhynchus mykiss*	Immune stimulation and expression of cytokine genes
Lactobacillus sakei CLFP 202 *Lactococcus lactis* CLFP 100 *Leuconostoc mesenteroides* CLFP 196	*Oncorhynchus mykiss*, *Salmo trutta*	Immune stimulation and improved survival after challenge with *Aeromonas salmonicida*
Lactobacillus plantarum CLFP 238 *Leuconostoc mesenteroides* CLFP 196	*Oncorhynchus mykiss*	Competitive exclusion and improved survival after challenge with *Lactococcus garvieae*

Table 7. Effect of different probiotic supplement to immune response in fish

Probiotic strain	Form of probiotics	Mode of supplementation	Immunological effect
Bacillus subtilis *Lactobacillus acidophilus*	Viable	Individual and combination	Increased RB, SBA, NA, lysozyme
Lactobacillus sakei	*Viable*	Individual	Increased RB, IG, CA, PA, and decreased lysozyme
Lactococcus lectis	Viable	Individual	Increased RB, IG, PA, lysozyme, CA

Probiotic strain	Form of probiotics	Mode of supplementation	Immunological effect
Lenuconostoc mesenteroides	*Viable*	Individual	Increased RB, IG, PA, lysozyme, CA
Aeromonas sorbia	*Viable*	individual	Increased RB, PA, leucocytes and decreased serum lysozyme and AP activity. Brunt and Austin, 2005; Brunt et al.,
Pdp11,51M6	*Heat killed*	Individual and combination	Incrased PA, RB
Shewanella putrefaciens S. baltica	*Inactivated*	Individual and combination	Increased PA, CA, AP, RB
Vibrio fluvialis Micrococcus luteus, Aeromonas hydrophilla	*Viable*	Indidividual and combination	Increased RB
Gram-positive coccus	*Viable*	Individual	Increased bloodlets and lysozyme activity
Vibrio fulvialis	*Viable*	Individual	Increased bloodlets, lysozyme activity and PB
A. hydrophilla	*Viable*	Individual	Increased bloodlets, lysozyme activity and PB
Carnobacteium maltaromaticum	*Viable*	Individual	Increased RB, lysozyme, serum mucus, PB
Carnobacterium divergens	*Viable*	Individual	Increased RB, lysozyme, serum mucus, PB
Bacillus subtilis	*Viable*	Individual	Increased RB, SBA, IG, PB, AP, CA, lysozyme, gut mucus
Lactobacillus rhamnosus	*Viable*	Individual	IG, RB, CA, PA
Saccharomyces cerevisiae	*Viable*	Individual	PA, RB, CA, Myeloperoxidase
Clostridium butyricum	Viable and inactivated	Individual	Increased Lysozyme, PA, CA, IG, RB
Lactobacillus rhamnosus, Bacillus subtilis Enterococcus facium	Viable (freeze dired)	Individual	Tissue and strain dependent modulation
Lactobacillus delbrueckii	*Viable*	Individual	Increased IG and related genes
Aeromonas sorbia	*Viable*	Individual	CA, PA, lysozyme, bloodlets, IG
Brochothrix thermosphacta	*Viable*	Individual	Increased PA and decreased CA, RB, Lysozyme, IG
Lactobacillus delbrueckii ssp. *lactis, Bacillus subtilis*	*Viable*	Individual and combination	Increased PA, Cytotoxic activity
Lactobacillus delbrueckii Bacillus subtilis Pdp11, 51M6	Inactivated	Individual	Increased RB, cytotoxic activity
Lactobacillus delbrueckii Bacillus subtilis	Heat killed	Individual/Combination	Increased RB, AP, PA, CA, IG depending on mixing of probiont
Kocuria spp.	*Viable*	Individual	Increased PA, AP, RB, Lysozyme
Lactobacillus plantarum	*Viable*	Individual	Lysozyme, PA, AP, CA
Bacillus subtilis Lactobacillus acidophilus Clostridium butyrium Saccharomyces cerevisiae	*Viable*	Combination	Increased neutrophil migration, Lysoyme, RB, bacteriocidal activity
Lactococcus lactis, Leuconostoc mesenteroides	Viable, inactibated	Individual	Increased PA, nitric oxide
Enterococcus faecium	*Viable*	Individual	Increase CA, RB, MPO and lysozyme
Bacillus coagulans,	*Viable*	Individual	Increased RB, SOD, Catalase, MPO

Probiotic strain	Form of probiotics	Mode of supplementation	Immunological effect
B. subtilis, Rhodopseudomonas palustris			
Zooshikella spp.	*Viable*	Individual	IG, disease resistance

CONCLUSION

1. Application of probiotics in the field of cultivation, namely, as a growth promoter, increases nutrient digestibility in the intestine, improves water quality, increases stress tolerance and innate immune response and is able to inhibit the growth of pathogens.
2. Probiotic microorganisms are able to increase fish resistance to pathogen attacks, such as; bacteria, viruses and fungi with high survival. This is because probiotic microorganisms can improve the fish's immune system and produce compounds that can inhibit the growth of pathogens, such as; bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide and changes in pH and indole (s, 3-benzopyrrole) values.

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