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Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections

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Abstract The probiotic activity of two bacteria (*Bacillus subtilis* and *Lactobacillus acidophilus*) was evaluated by its effect on the immune response of Nile tilapia (*Oreochromis niloticus*), beside its protective effect against challenge infections. Furthermore, their in-vitro inhibitory activity was evaluated.

The in-vitro antimicrobial assay showed that *Bacillus subtilis* and *Lactobacillus acidophilus* inhibited the growth of *A. hydrophila*. The *B. subtilis* inhibited the development of *P. fluorescens* while *L. acidophilus* inhibited the growth of *Strept. iniae*. The *B. subtilis* and *L. acidophilus* proved harmless when injected in the *O. niloticus*.

The feed, containing a mixture of *B. subtilis* and *L. acidophilus* or *B. subtilis* alone, showed significantly greater numbers of viable cells than feed containing *L. acidophilus* only after 1, 2, 3 and 4 weeks of storage at 4 °C and 25 °C. The survival rate and the body-weight gain were significantly increased in the fish given *B. subtilis* and *L. acidophilus* for one and two months after application.

The hematocrit values showed a significant increase in the group that received the mixture of *B. subtilis* and *L. acidophilus* compared with the control group. The nitroblue tetrazolium (NBT) assay, neutrophil adherence and lysozyme activity, showed a significant increase in all the probiotic-treated groups after 1 and 2 months of feeding, when compared with the untreated control group. The serum bactericidal activity was high in the group that was given a mixture of the two bacteria.

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The relative level of protection (RLP) was significantly higher against *A. hydrophila*, in the bacterial mixture treated group and against *P. fluorescens* in the *L. acidophilus* treated group, after one month of the feeding trial. A significantly higher RLP, against A. *hydrophila* or *P. fluorescens*, was noticed after 2 months of the feeding trial in the group given a mixture of the two bacteria, and against *Strept. iniae* in the group fed a diet containing *L. acidophilus*.

Introduction

Aquatic animals in large-scale production facilities are exposed to stress conditions, diseases and deterioration of the environmental conditions, leading to serious economic losses [1,2]. The prevention and treatment of the infectious aquatic animal-diseases, in Egypt, include a limited number of Government-approved antibiotics and chemotherapeutics, beside limited vaccines that can be used to assist the environmental management. However, the use of antibiotics can lead to the development of antibiotic-resistant bacterial strains [3] and may modulate the immune response [4].

A promising alternative approach for controlling fish diseases is the use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms. The use of probiotics, in human and animal nutrition, is well documented [5] and recently, have been applied to aquaculture [6,7]. *Bacillus subtilis* (*B. subtilis*) has been shown to possess antitumor and immunomodulatory activities [8]. Some studies have demonstrated that *B. subtilis* and spores of *B. subtilis* act as probiotics by promoting the growth and viability of the beneficial lactic acid bacteria in the intestinal tracts of humans and some animals [9]. The *Lactobacillus acidophilus* (*L. acidophilus*) has been considered to be the predominant lactobacillus in the intestinal tract of healthy humans [10]. *L. acidophilus* strains have been widely utilized as a dairy starter culture for their therapeutic activities associated with an intestinal microbial balance. Probiotics are defined as cultures of live microorganisms that benefit the host (humans and animals) by improving the properties of the indigenous microflora [11]. Such effects have been attributed to biochemical, physiological, and antimicrobial effects, as well as competitive exclusion in the intestinal tract [12].

The present study aimed to evaluate the efficiency of using two bacteria (*Bacillus subtilis* and *Lactobacillus acidophilus*) as a potential probiotic in the farming of Nile tilapia (*Oreochromis niloticus*). The evaluation was based upon their safety, in-vitro inhibitory activity, and effects on the immune response. Moreover, the survival rate and growth performance were considered, besides the possible protective effects against a challenge infection.

Material and methods

Fish

Two-thousand and one hundred apparently healthy, Nile tilapia (*O. niloticus*) of both sexes were collected from the WorldFish Center, Abbassa, Egypt. One hundred and eighty *O. niloticus* (65 ± 5 g) were used to test the safety of the used probiotic strains. The remaining 1920 *O. niloticus* (5 ± 1.3 g) were used for the feeding experiment. They were kept for 2 weeks under observation for acclimatization in glass aquaria ($60 \times 50 \times 70$ cm). The water of the aquaria was renewed daily, and its temperature was maintained at 26 ± 1 °C.

Bacterial strains

The probiotic, *Bacillus subtilis* (*B. subtilis*) (ATCC 6633) was obtained as lyophilized cells from Sigma. *Lactobacillus acidophilus* (*L. acidophilus*) was kindly supplied as a reference strain from the Animal Health Research Institute, Dokki, Egypt. The pathogenic strains, *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Streptococcus iniae* were obtained, as reference strains, from the Fish Health Laboratory at The WorldFish Center, Abbassa, Egypt.

In-vitro antimicrobial activity assay (Agar spot assay)

The probiotic strains (*B. sublitis* and *L. acidophilus*) were cultured in Trypticase soya broth for 24 h at 30 °C. Spots were then made by pouring 10 μl of overnight cultures of *B. sublitis* and *L. acidophilus*, each on one side of the trypticase soya agar plates. The plates were incubated overnight at 30 °C and the growth of the strains was checked the following day. After the spot development, a soft agar (composed of Tryptone Soya Broth +0.7% bacteriological agar, containing 5% of overnight cultures of the pathogenic strain from each of *A. hydrophila*, *P. fluorescens* and *Strept. iniae* in tryptone soya broth) was poured on the plates. The inhibition was recorded by measuring the absence of pathogen growth around the spots. All tests were performed in duplicate [13].

Safety of probiotic strains

One hundred and eighty tilapia (65 ± 5 g) were divided into 3 equal groups (60 fish) in three replicates (each of 20 fish) and distributed randomly among 9 aquaria. The first group was intraperitoneally (I/P) injected with 0.5 ml *L. acidophilus* fresh culture suspension containing 10^7 bacteria ml^{-1} while the second group was I/P injected with 0.5 ml *B. subtilis* fresh culture suspension containing 10^7 bacteria ml^{-1}. The third group served as a control and I/P injected with 0.5 ml sterile saline (0.85% NaCl). Both the test and control groups of fish were observed and fed on a basal diet containing 30% protein and water temperature was 26 ± 1 °C throughout the experiment. The mortality rate was recorded daily for 15 days.