AGRISCIENCE AND TECHNOLOGY
Armenian National Agrarian University
ԱԳՐՈԳԻՏՈՒԹՅՈՒՆ ԵՎ ՏԵԽՆՈԼՈԳԻԱ АГРОНАУКА И ТЕХНОЛОГИЯ
International Scientific Journal
ISSN 2579-2822

Journal homepage: anau.am/scientific-journal

UDC 639.3.043

Novel Potential Feed Probiotics for Fish: *Lactobacillus rhamnosus Vahe*

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ARTICLE INFO

Keywords:
aquaculture,
fish,
probiotic,
L. rhamnosus Vahe,
viability

ABSTRACT

The article considers the possible role of lactobacilli probiotics in protection of salmon against fish pathogens. The aim of this work is to study the viability of the potential probiotic *Lactobacillus rhamnosus Vahe* immobilized in salmon feed. Lactobacilli strains of human origin might have good probiotic efficacy in animals, including fish. According to this study, a decrease in viability (from 100 % to 78.00 ± 3.14 %) of the probiotic in feed biofilms was detected over a 2-month period. A simple procedure has been recommended that ensures viability of probiotics and can be applied for the evaluation of probiotic candidates in the future.

Introduction

The main trend in the development of aquaculture in Armenia is pond fish farming. Its effectiveness largely depends on the quality and quantity of used feed. Reduction of feed cost is one of the main economic factors, which can increase the profitability of fish farming. Probiotics used in the fish feed significantly influence feed consumption per unit of fish growth, since they increase the rate of feed assimilation, neutralize mycotoxins, displace pathogenic microflora and strengthen the general resistance of fish (Mitropoulou, et al., 2013, Ridha and Azad, 2016, Van Doan, et al., 2018). Therefore, probiotics come forth as an alternative for disease prophylaxis and treatment, particularly from the prospect of emerging antibiotic resistance in aquatic environments (Carvalho and Santos, 2016, O'Flaherty and Cummins, 2017) and in aquacultural sites (Smith, 2008, Suzuki, et al., 2017). Lactobacilli probiotics might colonize the fish-gut and also fight against gram-negative fish/human pathogens in fish tanks (Ring and Gatesoupe, 1998, Munoz-Atienza, et al., 2013).

The aim of the current study is the investigation of viability of the potential probiotic *Lactobacillus rhamnosus Vahe* in immobilized salmon feed, demonstrating high antagonistic activity against several multi-resistant hospital pathogenic isolates of *Acinetobacter baumanii, Enterobacter gergoviae, Klebsiella pneumonae* and *Staphylococcus*

aureus, and possibly against other fish bacterial pathogens belonging to *Edwardsiella* and *Pseudomonas* species.

Materials and methods

Probiotic strain *L. rhamnosus Vahe* isolated from the feces of the healthy infant was used in this study (Pepoyan, et al., 2018b, Pepoyan, et al., 2020, Balayan, et al., 2019). Bacterial strains were cultured in De Man, Rogosa and Sharpe (MRS) broth and on MRS agar (Thermo Scientific™, UK). When required, Oxoid™ Endo Agar (Thermo Scientific™, UK) and VITEK® 2 compact (BioMerieux, France) were used for the identification of bacterial cells (Holt, et al., 1994).

To obtain the biofilms and to assess the viability of the bacterial cells, the bacteria were grown for 24 hours in MRS medium, then the fish feed granules (Nuti 0, ALLER PERFORMA) were added to the bacterial suspension, left for 1 hour, and then transferred to sterile saline and stored in a refrigerator at 2-8 ^{0}C. For viability analyses 100 mg of obtained probiotic supplement was added to 0.3 ml saline, mixed, left for 2-3 minutes and then 0.1 ml of the suspension was transferred on MRS agar; viability was checked 24 hours after incubation at 37 ^{0}C. The viability of the bacteria was assessed on the 5th, 10th, 20th, 30th, 40th, 50th and 60th days.

To investigate the changes in antibacterial activities of the probiotic strain after immobilization, a colony of *K. pneumonae* was dissolved in 1 ml of MRS, then 0.1 ml of the mixture was added to 0.9 ml of MRS as a *K. pneumoniae* control, and 0.1 ml was added to 0.9 ml of MRS containing 0.1 ml of probiotic suspension. A suspension of probiotics was prepared in the following way: 3 feed pellets with probiotic biofilms were added to 0.3 ml of saline solution, mixed for 10-15 seconds and 0.1 ml of supernatant was used for the experiment. To compare the anti-*Edwardsiella* activity, the probiotic on the feed granules was added to 10 ml of the tank water containing 104 CFU/ml *Edwardsiella* sp. The titer of bacteria was compared with its control suspension without feed granules after 2 days of incubation at 22-25 ^{0}C.

Results and discussions

Probiotics can be selected based on the production of antimicrobial compounds such as bacteriocins, siderophores, or the presence of competition for nutrients. In addition, such properties as antioxidant and antimutagenic activity, and the ability to form biofilms are important for the host organism.

In the framework of previous studies we characterized probiotic strain *L. rhamnosus* Vahe with antagonistic activity against *A. baumannii*, *S. aureus, K. pneumoniae* and Enterobacter gergoviae (Pepoyan, et al., 2018 b). The radioprotective ability, biofilm forming ability, cell surface hydrophobicity and the effectiveness of using the probiotic strain were demonstrated. In addition, preliminary studies have disclosed the possibility to use the strain for aquaculture (Pepoyan, et al., 2018 a, b).

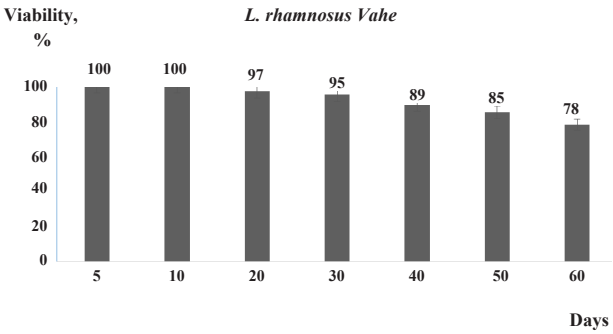

Figure 1. The viability of *L. rhamnosus Vahe* in probiotic feed granules during 2-month storage (*composed by the authors*).

For successful application of probiotic strains as microbial ingredients for fish, other characteristics seem to be essential, such as high viability during processing, throughout storage and after gastro-intestinal transit. Figure 1 relates to changes in viability of *L. rhamnosus Vahe* in probiotic fish feed granules during 2-month storage. These investigations showed that non-lyophilized cells of *L. rhamnosus Vahe* remain viable for a long time (Figure 1). A decrease in bacterial titer was recorded only after 20 days of storage, and the rate of the decrease was very slow. According to this study, a decrease in viability (from 100 % to 78.00 ± 3.14 %) of the probiotic in feed biofilms was detected over a 2-month period.

The antagonistic potential of the biofilm forming strain against *K. pneumoniae* was also investigated. The study showed that the biofilm forming strain is able to maintain antagonistic potential against the pathogen during 60 days of storage. It is shown that the number of the pathogenic cells has sharply dropped when the pathogen is grown together with the probiotic strain. The results (Figure 2) show that the titer of *Klebsiella* cells after incubation with

the probiotic is only 2 × 104 CFU/ml, while in the control tube, the titer of *Klebsiella* reaches 4 × 1011 CFU/ml.

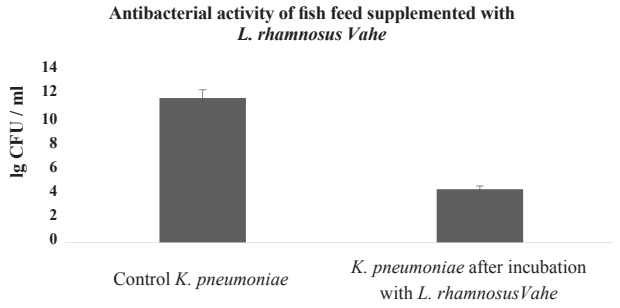

Figure 2. The effect of *L. rhamnosus Vahe* on *K. pneumoniae* cells (*composed by the authors*).

The preliminary study of the effect of probiotic feed on 104 CFU/ml of *Edwardsiella sp.* in tank water showed a suppression of pathogenic bacteria, in contrast to the identified positive effect of feed alone on the viability of *Edwardsiella spp*.

Several demanding strategies such as micro- and bio-encapsulation of the probiotics have been described (Martínez, et al., 2012, Rosas-Ledesma, et al., 2012); we have also presented a simple and cost-effective method that affected only the integrity of the pellets to an acceptable degree.

When supplementing experimental diets with probiotics the need arises to up-scale cell cultivation. The experimental evaluation confirms that expensive and complex probiotic administration processes using technologies such as drum or vacuum coating systems are not required to ensure the high survival rate of the probiotic during preparation and long-term storage. After almost eight weeks of the storage, the viable colony forming units did not decrease even by a tenth power. It is important that bacterial cells should be harvested at the end of the logarithmic growth phase, because a delayed harvesting could cause a self-inhibition of bacteria or the production of unwanted secondary metabolites.

Conclusion

It has been found out that fish feed supplemented with probiotic *L. rhamnosus Vahe* could be stored at 4 ^{0}C for 60 days, without a major decrease in the viability of the probiotic cells. The study of the antagonistic potential of the biofilm forming *L. rhamnosus Vahe* strain showed that the probiotic strain is able to maintain antagonistic potential against *K. pneumoniae* during 60 days of storage and suppress the growth of *Edwardsiella sp.* in tank water.

Thus, the recommended simple immobilization procedure of biofilm forming probiotic cells on the surfaces of feed granules assures viability of the probiotic and can be applied for the evaluation of probiotic candidates in the future.

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Accepted on 02.07.2020
Reviewed on 01.09.2020