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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Usage of *Lactobacillus rhamnosus* as a Probiotic in Sea Bass (*Dicentrarchus labrax*)[*]

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Abstract: In this study, the effects of *Lactobacillus rhamnosus* (ATCC 53103) used as a probiotic supplement on the deformation rate, survival rate, weight gain, and intestinal microflora in sea bass (*Dicentrarchus labrax*) were investigated. For this purpose, the probiotic agent was incorporated into the rearing water and Artemia nauplii culture at concentrations of 10^6 CFU/mL and 10^8 CFU/mL, respectively. The incorporation of the probiotic was carried out until the 50th day. From 50 to 125 days post-hatching, 10^9 CFU/g of probiotic was incorporated into the diet of the both groups every day. The deformation rates of the larvae were recorded as 5% in the group received the probiotic via rearing water, 2% in the group received the probiotic via Artemia culture, and 7% in the control group ($P<0.01$). Survival rates of the experimental groups received probiotics were found significantly higher than the control group ($P<0.01$). No significant differences were found on the weight gain of sea bass individuals received probiotic ($P>0.01$). On the other hand, It was found that the probiotic addition to Artemia culture leads to a decrease in the number of Vibrio spp., ($P<0.01$). As a result, it has been determined that the addition of probiotics to rearing water and especially Artemia culture has positive effects on the rate of larval survival and deformation.

Keywords: Deformation, fish, microflora, probiotic, survival rate.

Levrek Balıklarında (*Dicentrarchus labrax*) Probiyotik Olarak *Lactobacillus rhamnosus* Kullanımı

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Öz: Bu çalışmada, levrek balıklarında (*Dicentrarchus labrax*) probiyotik olarak kullanılan *Lactobacillus rhamnosus* (ATCC 53103)'un balıkların deformasyon oranı, yaşama oranı, ağırlık artışı ve bağırsak mikroflorası üzerine etkisi araştırılmıştır. Bu amaçla, probiyotik ilavesi yetiştirme suyuna 10^6 kob/mL ve Artemia nauplii kültürüne 10^8 kob/mL düzeyinde yapılmış ve 50. güne kadar devam etmiştir. 50. günden sonra 125. güne kadar her gün, bu gruplardaki balıkların toz yemine 10^9 kob/g düzeyinde probiyotik ilavesine devam edilmiştir. Deneme sonunda larvalardaki deformasyon oranları; yetiştirme suyuna probiyotik ilave edilen grupta %5, Artemia kültürüne ilave edilen grupta %2 ve kontrol grubunda %7 olarak tespit edilmiştir ($P<0.01$). Probiyotik ilave edilen deneme gruplarının yaşama oranı kontrol grubuna kıyasla önemli düzeyde yüksek bulunmuştur ($P<0.01$). Probiyotik ilavesinin balıkların ağırlık artışı üzerinde önemli bir etkisinin olmadığı tespit edilmiştir ($P>0.01$). Ayrıca, Artemia kültürüne probiyotik ilavesinin Vibrio spp. sayısında önemli düzeyde azalmaya neden olduğu belirlenmiştir ($P<0.01$). Sonuç olarak yetiştime suyuna ve özellikle Artemia kültürüne probiyotik ilavesinin larval yaşama oranı ve deformasyon oranı üzerinde olumlu etki gösterdiği tespit edilmiştir.

Anahtar kelimeler: Balık, deformasyon, mikroflora, probiyotik, yaşama oranı.

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INTRODUCTION

In parallel with the worldwide expansion of aquaculture, there has been a dramatic increase in microbial fish diseases. Several pathogenic and opportunistic bacteria have caused high mortality rates during larval first-feeding (Franke et al., 2017). Therefore, a wide variety of antibiotics have been used in order to prevent bacterial diseases in industrial animals (Gobi et al., 2018). However, using large amounts of antibiotics in farm animals have resulted in antibiotic residues in tissues, an imbalance of normal intestinal flora, a reduction in beneficial microbial populations in the intestines, and the generation of antibiotic-resistant bacteria (Byun et al., 1997; Lin et al., 2017; Marti et al., 2018; Preena et al., 2019). Consequently, alternative methods are required to support digestive system and immune system of fish larvae. Among these, one of the methods which has gained acceptance in the aquaculture industry is to control potential pathogens through the use of probiotic bacteria (Harzevili et al., 1998; Nikoskelainen et al., 2003; Arıg et al., 2013; Gumus et al., 2016; Goda et al., 2018).

Probiotics are defined as a viable pure or mixed culture of microorganisms which provides benefits to the host (Van Doan et al., 2018; Çelik et al., 2019). They prevent colonization of pathogenic microorganisms to the intestinal epithelial cells and avoid their propagation by producing antimicrobial metabolites. They also improve the properties of the indigenous microflora and thus improve the health status of the host and increase feed conversion (Byun et al., 1997; Nguyen et al., 2017). In aquaculture, probiotics are used to increase production, improve water quality, decrease the use of antibiotics and other chemicals, and increase resistance to diseases (Van Doan et al., 2018). They are added to the water and administered to artificial feeds and live foods, such as artemia and rotifer (Sutthi et al., 2018). The most widely used bacteria as probiotics are lactic acid bacteria (LAB) (Silvi et al., 2008; Lamari et al., 2013; Piccolo et al., 2015; Didinen et al., 2016; Ljubobratovic et al., 2017). The normal microflora of the gastrointestinal tract of healthy fish includes LAB. LAB inhibits the growth of various pathogens by producing substances like organic acids and bacteriocins (Byun et al., 1997; Jöborn et al., 1997).

In this study *Lactobacillus rhamnosus* (ATCC 53103) was added to the rearing water, live food cultures and artificial feeds of sea bass in order to study its effects on the deformation rate, survival rate, weight gain and intestinal microbial flora.

MATERIAL AND METHOD

Probiotic Strain and Their Preparation: In this study, *L. rhamnosus* ATCC 53103 strain which was obtained from a LGC Promochem Ltd. (Teddington, UK) was used as probiotic. In order to produce probiotics that would be used in the experiments, 200 mL Man, Rogosa and Sharpe Agar (MRS, Merck) was put into roux bottles and sterilized at 118°C for 15 min. in autoclave (Nüve, OT 90L). Then, the medium was hardened in roux bottles placed in a horizontal position. One mL of activated culture was aseptically transferred into a roux bottle, and incubated at 37°C for 18-24 hours. After the total number of bacteria was determined in the stock probiotic culture, required concentrations to be used in experimental groups were prepared by sterile physiological saline solution. The bacterial suspensions were kept at 4 °C until used within one week.

Experimental Design: In this research, it was carried out in the Hatko Sea Products Inc., Ören hatchery, Milas, Muğla, Turkey in 2005. Ethics committee approval was not required for fish at the time of the study.

Sea bass (*Dicentrarchus labrax*) larvae, average weight 0.21±0.01 mg, were taken in commercial marine hatcheries. The larvae were immediately transferred to the larvae tanks and trials were started. The probiotic bacteria was administered to sea bass for 125 days. The experiment was carried out in three groups; Following the hatching, the larvae were stocked in cylinders-conical tanks (2 m^3), with initial stocking density of 125 larvae /L.

Group 1: *L. rhamnosus* ATCC 53103 was added to the rearing water and diet. Taking the volume of the water in the rearing tanks into consideration, 10^{12} CFU/mL *L. rhamnosus* ATCC 53103 strain was added to the rearing water from 9 to 50 days post-hatching (DAH). In this way, the final concentration of bacteria in the tank became 10^6 CFU/mL during 50 DAH. Probiotics were added to the tanks twice a day at 08:00 and 16:00 hours, prior to Artemia nauplii addition. From 50 to 125 DAH, the fishes were given diet containing 10^9 CFU/g *L. rhamnosus* ATCC 53103. Probiotic was sprayed on fish diet and mixed by hand.

Group 2: *L. rhamnosus* ATCC 53103 was supplied to larvae via Artemia nauplii and added to the diet. From 9 to 50 DAH, larvae were fed with Artemia nauplii to which 10^8 CFU/mL *L. rhamnosus* ATCC 53103 strain was added. After the 50 DAH, the bacterial administration was performed with diet. From 50 to 125 DAH, the fishes were given diet containing 10^9 CFU/g *L. rhamnosus* ATCC 53103. Probiotic was sprayed on fish diet and mixed by hand.

Control group: *L. rhamnosus* ATCC 53103 was not supplied to larvae. Between 9 and 50 DAH, larvae were fed with Artemia nauplii. From 50 to 125 DAH, the fishes were fed with microparticle diet (300 µ - 1.2 mm). Daily feeding rate was about 3.5% of total body weight.

***Determination of Intestinal Microflora of the Fish and Bacterial Load in Artemia Culture*:** After hatching, fish samples were taken on days 20, 27, 50, 58, 86, 104 and 121 and microbiological analyzes were done. In order to determine intestinal flora of the fish, during the larval period, about 20 larvae and 5-10 juveniles were put into sterile tubes (Muroga et al., 1987; Grisez et al., 1997). They were immersed in 0.1% benzalkonium chloride saline solution for 1 min; then, they were washed under sterile distilled water for 30 s. After that, these fish samples were homogenized using saline solution. Artemia nauplii used in feeding the fish larvae were taken 1 g and were homogenized in 9 mL saline solution (Muroga et al., 1987; Rollo et al., 2006). Homogenates were serially diluted and 0.1 mL of appropriate dilutions was spread onto plates. Man, Rogosa and Sharpe (MRS) Agar was used for lactic acid bacteria (LAB) and incubated anaerobically at 30°C for 48 hours, Violet Red Bile Agar (VRBA, Merck) was used for total enterobacteria (TEB) and incubated at 37°C for 24 hours, Plate Count Agar (PCA, Merck) was used for total aerobic mesophilic bacteria (TAMB) and incubated at 30°C for 48 hours, Blood Agar (BA, Merck) was used for total anaerobic bacteria (TAB) and incubated at 30°C for 24 hours in a steel jar, Thiosulfate-Citrate-Bile-Sucrose Agar (TCBSA, Merck) was used for Vibrio spp. and incubated at 30°C for 24 hours (Harrigan, 1998, Nikoskelainen et al., 2001; Villamil et al., 2010; Hamza et al., 2016).

Determination of Survival Rate, Deformation Rate, and Weight Gain: At the end of the experiment, survival rate of larvae was determined by counting the larvae remaining in the tanks. For determining the rate of deformation, at the end of the rearing period in the hatchery, the deformed larvae were separated from the healthy individuals. In order to do this, larvae were anesthetized with phenoxyethanol (200 ppm) (Crespel et al., 2017). Immobile larvae were placed on an illuminated glass table. The individuals who had spine, mouth, fin, and gill deformations were separated. Larvae weight was measured using the electronic balance (0,0001 g; Shimadzu ATX-224, Japan) at the beginning and end of the experiment. The weight gain of fish was calculated by following formula; Weight gain (g/fish) = Final weight – initial weight.

Statistical Analysis: Statistical analysis was carried out with SPSS 11.5 (Chicago, USA). The obtained data about intestinal flora and weight gain were analyzed using ANOVA (analysis of variance). The results were evaluated by Duncan Multiple Comparison Test. Chi-square analysis was used to compare the groups in terms of survival and deformation rates. Differences among treatments were considered significant at the P<0.01 level. All measurements were carried out in duplicate.

RESULTS AND DISCUSSION

Intestinal Microflora: In the inoculations made until the 50th day, the number of TAMB were not different between Group 1 and Group 2, but later their numbers substantially decreased in Group 2 (P<0.01). Between 20 and 58 days, it was found that the number of TEB were higher both in Groups 1 and 2, than the control group during the experiment period (P<0.01). The number of Vibrio spp. were lower in the experimental groups, to which probiotics were added, than the control group. The lowest number Vibrio spp. was detected in the fish in Group 2 (P<0.01) (Figure 1a, 1b, 1c, 1d).

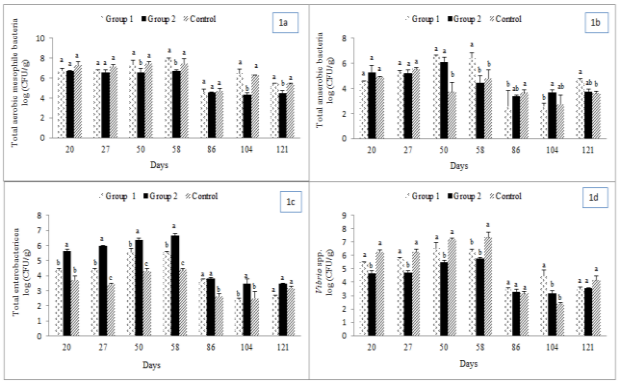

Figure 1. Changes in the number of TAMB (a), TAB (b), Enterobacteriaceae (c), and Vibrio spp. (d) in the intestinal flora of the sea bass.

In this research, after the larvae in the experimental groups started to receive feed, the number of Enterobacteriaceae in their intestines increased, while the number of Vibrio spp. decreased. The situation was just the opposite in the control group. When experimental groups are compared, the number of TEB in Group 2 was higher than Group 1. The number of *Vibrio* spp. was lower. The tendency of a decline in the number of Vibrio spp. indicates that they are inhibited by the probiotic bacteria. It was observed that more successful results would be obtained by adding probiotic bacteria to Artemia culture. These results were similar to many studies. For instance, in the research conducted by Byun et al., (1997), it was determined that the number of aerobic and anaerobic bacteria in the intestinal flora of flounders decreased by using *Lactobacillus* spp. whereas the number of Enterobacteriaceae increased. Gatesoupe, (1991) showed that in rotifer culture, the use of *Lactobacillus plantarum* decreased the number of aerobic bacteria. Rollo et al., (2006) that the use of *Lactobacillus fructivorans* and *L. plantarum* in artemia and rotifer cultures decreased the number of aerobic and anaerobic bacteria as well as Enterobacteriaceae and *Staphylococcus* spp. in sea bream. Hamza et al., (2006) reported that the number of total bacteria in the digestive system of sea bass applied

probiotic (*Virgibacillus proomii and Bacillus mojavensis*) decreased significantly. In contrast, Jöborn et al., (1997), stated that when *Carnobacterium* spp. was used as a probiotic, total number of aerobic bacteria increased in the intestinal flora of rainbow trout. Similarly, Silvi et al., (2008) also reported that *Lactobacillus delbrueckii subsp delbrueckii* strain on the intestinal microbiota of sea bass was effective. It has been stated that total intestinal microbiota dramatically increases except for day 22, in particular the number of Enterobacteriaceae is affected by probiotic therapy.

In this study, the number of Vibrio spp. in the intestines of the fish in Group 1 and Group 2 was found to be lower than the control group. The lowest number of bacteria was observed in Group 2. Not all of the probiotic bacteria added to rearing water could be taken by the fish; however, the bacteria added to Artemia culture was filtered by Artemia and more bacteria could be taken by the fish. As determined in the study conducted by Reitan et al., (1993), it is estimated that the reason of a decrease in the number of Vibrio spp. is that probiotic bacteria are bioencapsulated by live feed organisms. Also, Villamil et al., (2010) reported that *Pediococcus acidilactici* transmitted via live feed inhibited *Vibrio splendidus* in turbot larvae. Goda et al., (2018) reported that the Bactozyme, a commercial product containing probiotic, cause decrease the number of Vibrio spp. in the intestinal flora of the sea bass. In conclusion, in this study, it was determined that the use of *L. rhamnosus*, especially in Artemia culture, prevented the colonization and proliferation of some opportunistic pathogens.

The Number of Bacteria in Artemia Culture: It was found that in the inoculations made from Artemia nauplii, the number of Vibrio spp. was 7.01±0.55, TEB was 4.56±0.82 log CFU/g, and TAMB was 8.00±0.43 log CFU/g. After the Artemia culture was incubated with probiotic bacteria for 2 hours, 10^2 CFU/g lactic acid bacteria were detected in MRS Agar.

The larval form of most fish is released in an early ontogenetic phase. They start feeding although neither the digestive tract nor the immune system are still fully developed, therefore, these larvae are exposed to gastrointestinal microbiota-associated diseases (Silvi et al., 2008). During first feeding, the microorganisms found in feed and water goe to larval intestine. The first colonization in fish intestine is of great importance in terms of host health and development. For the formation of a balanced intestinal microflora, probiotic bacteria must be used soon after hatching (Hamza et al., 2016).

It is a well-known fact that in the formation of the fish intestinal flora, rearing water and the species of bacteria on the live feed are effective. Artemia is one of the best food sources for many species of animals which were cultured. However, Artemia nauplii have heavy bacterial loads and therefore they are considered as the most important vector which is responsible for carrying pathogenic bacteria into rearing systems (Reitan et al., 1993). In this research, the number of Vibrio spp. and TAMB were 7.01 log CFU/nauplii and 8.00 log CFU/nauplii, respectively. Hameed and Balasubramanian, (2000) stated that the number of TAMB was $3.8x10^3$-$8.1x10^3$ CFU/nauplii and the number of Vibrio spp. was $9.4x10^2$-$4.3x10^3$ CFU/nauplii. Olsen et al., (2000) also stated that total number of bacteria was $2.4x10^4$ CFU/nauplii and that 58% of the total bacteria consisted of Vibrio spp. It is thought that this difference comes from the fact that Artemia is a biological product and they are obtained from different regions. In addition, during the study period in which Artemia was used, the numbers of bacteria found in the intestinal flora of the fish were higher than the period during which commercial diet was used. Therefore, it is concluded that the high bacterial load in the larvae resulted from Artemia culture.

Survival Rate, Deformation Rate, and Weight Gain: At the end of the experiment, the survival rates of Group 1, Group 2, and the control group were determined as 27.4%, 34.8% and 18.9%, respectively. The survival rates of the experimental groups that received probiotic bacteria were significantly higher than the control group ($P<0.01$) (Figure 2a). Deformation rates of the experimental groups are shown in Figure 2b. The differences between the experimental groups and the control group were regarded as significant ($P<0.01$). The average weights of the fish in experimental groups are shown in Figure 2c. In the 20th day of experiment, the average weights of the experimental groups were 0.21±0.01 mg while at the end of the experiment, the average weights of the Group 1, Group 2, and the control group were determined as 2.82±0.08 g, 2.83±0.03 g, and 2.85±0.1 g, respectively. The average weight gains were compared between experimental groups, and no significant differences were observed ($P>0.01$).

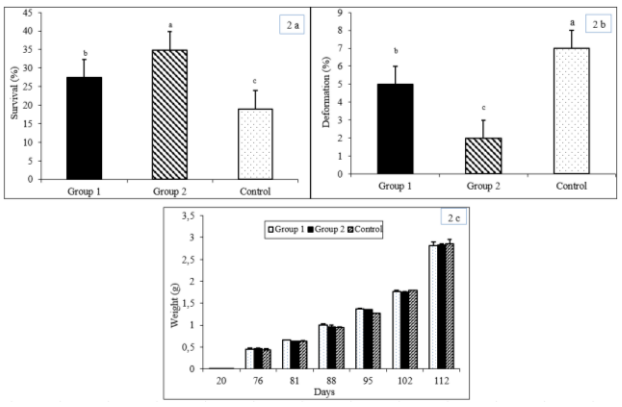

Figure 2. The survival, deformation rate, and weight of the sea bass larvae.

In this study, the rate of larval survival in the experimental groups with probiotic bacteria was higher than the control group. The application of probiotics via Artemia gave better survival rates. Similar results were obtained by many researchers. For example, Silvi et al., (2008) and stated that the *L. delbrueckii subsp. delbrueckii* strain influenced the survival rate of sea bass. Piccolo et al., (2015) reported that the *L. plantarum* strain increased survival of sea bass. In the study carried out by Hamza et al., (2016) two separate probiotics (*Virgibacillus proomii* and *Bacillus mojavensis*) were mixed with the diet of sea bass, and the survival rate was found to be significantly higher.

Developmental conformation is a critical issue for fish hatcheries. The skeletal deformities develop during especially ontogenesis as far as metamorphosis (Lamari et al., 2013). In many studies it was stated that these deformities could be reduced by using probiotics (Lamari et al., 2013; Aubin et al., 2005; Lin et al., 2017). In this research, the deformation rate in larvae is 5% in Group 1, 2% in Group 2 and 7% in the Control Group. Tovar-Ramirez et al., (2004) reported that the addition of *Debaryomyces hansenii* to the feed of sea bass larvae decreased deformation rates significantly. It has been reported that skeletal deformation significantly decreased in the pike-perch where *Lactobacillus* strains were used (Ljubobratovic et al., 2017). Lamari et al., (2013) investigated the effects of lactic acid bacteria on the histological development of the sea bass and indicated that the spine deformities had the highest incidence in the *Lactobacillus casei* group. In another study, it was reported that the incidence of common vertebral deformities decreased when *P. acidilactici* was used in trout (Aubin et al., 2005). Consequently, It was determined that vertebral malformations could be prevented by the addition of probiotic in the first stages of larval development. But, the interaction between probiotic microorganisms, especially lactic acid bacteria and bone conformation has not been clarified, it probably has indirect effect and may be associated with the inhibition of pathogenic bacteria (Villamil et al., 2010). In addition, probiotics may stimulate the immune system and reduce inflammation in fish larvae, local inflammation can affect the integrity of the spine and in this case, it can causes a risk of deformation (Gil-Martens, 2010).

In this research, it was observed that *L. rhamnosus* ATCC 53103 strain, which was added to Artemia culture or rearing water, did not increase weight gain in sea bass larvae. Nikoskelainen et al., (2001), reported similar results with the use of *L. rhamnosus* as probiotics in rainbow trouts. The studies carried out regarding the use of *Leuconostoc mesenteroides* and *L. plantarum* (Vendrell et al., 2008) in rainbow trout and the use of *Lactobacillus lactis* (Harzevili et al., 1998) in rotifer cultures also revealed that probiotic bacteria supplements did not have a significant effect on growth performance. Unlike these studies, It has been reported that the body weight of fish is increased in some studies where lactic acid bacteria are used (Carnevali et al., 2006; Lin et al., 2017; Nguyen et al., 2017; Goda et al., 2018). The differences in the results obtained in these studies may be due to fish species used, the concentration of bacteria and the mode of administration.

As a result, the addition of probiotic bacteria to Artemia cultures used as live feeds in larvae was found to be more effective. The use of *L. rhamnosus* ATCC 53103 strain as a probiotic increased survival rate, decreased deformation rate and significantly inhibited Vibrio spp. the potential pathogenic agent for sea bass.

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