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SHORT COMMUNICATION KISA BİLGİLENDİRME

SCREENING FOR CANDIDATE PROBIOTIC BACTERIA FOR THE CONTROL OF *Vibrio anguillarum* IN RAINBOW TROUT (*Oncorhynchus mykiss*, Walbaum)

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Abstract: Candidate probiotic bacteria were searched for use as probiotic against vibriosis in rainbow trout in this study. A total of 143 bacterial strains were isolated from rainbow trout rearing water, gills and skin mucus and tested for antagonistic activity against *V. anguillarum*. Antagonistic strains were characterized for enzymatic activity (protease, lipase) and hydrophobicity. Bacteria belonging to the genus *Aeromonas* and *Pseudomonas* showed inhibitory activity against *V. anguillarum*. *Aeromonas* sp. HS23 showed protease and lipase activity, while *Aeromonas* sp. MS30 displayed only lipase activity. *Pseudomonas* spp. did not display enzymatic activity. On the other hand, all bacteria showed hydrophobicity indicating their adherence capability to the host. However, these strains should be further studied to explore their probiotic effects in vivo.

Keywords: *Aeromonas*, *Pseudomonas*, Hydrophobicity, Probiotic, Rainbow trout, Vibriosis

Özet: **Gökkuşağı Alabalıkları (*Oncorhynchus mykiss*, Walbaum)'nda *Vibrio anguillarum*'un Kontrolü İçin Aday Probiyotik Bakterilerin Aranması**

Bu çalışmada, gökkuşağı alabalıkları (*Oncorhynchus mykiss*, Walbaum)'nda vibriosise karşı probiyotik olarak kullanımı için aday probiyotik bakteriler araştırıldı. Toplam 143 bakteriyel suş gökkuşağı alabalıkları yetiştiricilik suyu, solungaçlar ve deri mukusundan izole edildi ve *Vibrio anguillarum*'a karşı antagonistik aktiviteleri test edildi. Antagonistik suşların enzimatik (proteaz ve lipaz) aktiviteleri ve hidrofobisite özellikleri belirlendi. *Aeromonas* ve *Pseudomonas* cinsine ait bakteriler *V. anguillarum*'a karşı inhibitör aktivite gösterdi. *Aeromonas* sp. HS23 proteaz ve lipaz aktivitesi gösterirken, *Aeromonas* sp. MS30 sadece lipaz aktivitesi gösterdi. *Pseudomonas* spp. enzimatik aktivite göstermedi. Diğer taraftan, tüm bakteriler konakçıya tutunma kabiliyetinin göstergesi olan hidrofobisite özelliği gösterdi. Bununla birlikte, bu suşların canlı üzerindeki probiyotik etkileri ileride yapılacak bir çalışmada araştırılmalıdır.

Anahtar Kelimeler: *Aeromonas*, *Pseudomonas*, Hidrofobisiti, Probiyotik, Gökkuşağı alabalığı, Vibriosis

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Introduction

Aquaculture has become an important economic activity in many countries. In large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines. However, the utility of antimicrobial agents as a preventive measure has been questioned, given extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria (Balcázar et al., 2006). Thus, the research into the use of probiotics for aquatic animals is increasing with the demand for environment-friendly sustainable aquaculture (Gatesoupe, 1999).

The term probiotics is generally used to denote bacteria that promote the health of other organisms. Probiotics have multiple mechanisms of action to inhibit pathogens which include competitive exclusion, production of substances that inhibit growth of opportunistic pathogens (antagonism), stimulation of the immune response, antiviral effects, increase of digestive function through production of enzymes, improved nutrition by providing essential nutrients, and improved water quality (Balcázar, 2002; Balcázar et al., 2006; Farzanfar, 2006).

The antagonistic effect of probiotic bacteria against specific pathogens can be a method of selection. In vitro evaluation prior to in vivo testing was a good indicator of a probiotic effective at controlling bacterial fish diseases (Brunt & Austin, 2005; Balcázar et al., 2006; Aly et al., 2008; Capkin and Altinok, 2009; Strom-Bestor and Wiklund 2011).

Vibrio anguillarum as the causative agent of vibriosis isolated from a lot of marine fishes, mainly sea bream and sea bass (Çağırgan, 1993; Balebona et al., 1998). Recently, the diseases has been reported that it also caused important losses in rainbow trout (*Oncorhynchus mykiss*), cultured in freshwater in Turkey (Timur & Korun, 2004; Tanrıkul, 2007).

The aims of this study were to isolate probiotic bacteria from indigenous and exogenous micro biota of rainbow trout, screen all isolates in vitro antagonistic effects to *V. anguillarum*, and determine enzymatic activity and hydrophobicity of the isolates for the control of vibriosis.

Materials and Methods

Isolation and identification of candidate probiotic bacteria

Bacteria were isolated from rearing water, gills and skin mucus of rainbow trout by spread plate method at 25 °C for 2 days. Representative colonies from these plates were subcultured onto fresh medium for purity, and identified by gram staining, catalase reaction, (3% H_2O_2), motility in TSB, oxsidase reaction, ability to metabolise glucose by oxidation and/or fermentation in OF basal medium supplemented with 1.5 % glucose and API 20 E rapid identification system (BioMe´rieux SA, Marcy l'Etoile, France).

Inhibitory activity of the isolates

All bacteria isolated from the samples were tested for antagonistic activity by a well diffusion agar assay (WDAA) against fish pathogen *Vibrio anguillarum.* *Vibrio anguillarum* was grown in 4mL TSB for 1 d at 25 °C, and 10 µL of each culture was mixed into 10 mL of melted TSA (43.5-44°C). After solidifying and drying for 15–20 minute, wells were punched (diameter = 3 mm) and 10 µL of 2 days old candidate probiont culture (approx. 10^8–10^9 CFU mL^{-1}) grown in TSB at 25°C was added to wells in triplicates. Plates were incubated at 25°C for one day and observed for clearing zones around the wells. Strains causing clearing zones in the WDAA were tested once more in TSA to ensure that the antagonistic activity was stable after storage and sub-culture (Hjelm et al., 2004).

Hydrophobicity test using Congo Red Stain(CRS)

Hydrophobicity test was conducted using Congo Red Stain (CRS) to determine the hydrophobicity of the bacteria with petri plates prepared with Triptic Soy Agar with 0.03% Congo Red Sigma-Aldrich). Congo Red was added after sterilization of TSA. Each isolate was spread on plates by the cross-streak method and incubated at 30 °C for 24 h. Red colonies were considered positive (hydrophobic) and white or colorless colonies were considered negative (non-hydrophobic) (Sharma et al., 2006).

Extracellular enzymatic activity

Lipase and protease production of candidate probiotics were tested using tributyrin agar and skim milk agar, respectively. A clear zone surrounding the colonies indicated lipolytic and proteolytic activity.

Results and Discussion

Bacteria isolation and characterization

Antagonistic activities against *Vibrio anguillarum* of 143 bacterial isolates were tested by the well diffusion agar assay. *Aeromonas* sp. HS23 isolated from rearing water of rainbow trout and *Aeromonas* sp. MS30, *Pseudomonas* sp. MS3 and *Pseudomonas* sp. MS16 isolated from skin mucus of rainbow trout displayed inhibition zones (12, 26, 17 and 12mm, respectively) against *Vibrio anguillarum*. These bacteria were also tested for enzymatic activity and hydrophobicity. *Aeromonas* sp. HS23 showed protease and lipase activity. *Aeromonas* sp. MS30 displayed lipase activity. On the other hand, all bacteria displayed hydrophobicity that indicate their adherence capability to the host (table1). Phenotypic characteristics of candidate probiotic bacteria were given in table 2.

In this study, *Aeromonas* sp. HS23, *Aeromonas* sp. MS30, *Pseudomonas* sp. MS3 and *Pseudomonas* sp. MS16 showed inhibitory activity against *V. anguillarum*. These results supports previous studies which demonstrated in vitro antagonism of antibacterial strain *Pseudomonas fluorescens* strain AH2 isolated from iced freshwater fish (*Lates niloticus*) (Gram et al., 1999) and *P. fluorescens* and *P. libaniensis* isolated from rainbow trout (Spanggaard et al., 2001) against the fish-pathogenic bacterium *Vibrio anguillarum.* Gibson (1999) also demonstrated *Aeromonas media* displayed antagonistic activity against eight species of *Vibrio*. Likewise, Lategan et al. (2006) reported the inhibitory activity of *Aeromonas media* in vitro against *Vibrio anguillarum*.

Aeromonas sp. HS23 showed protease and lipase activity, while *Aeromonas* sp. MS30 displayed lipase activity in the present study. Some authors claim that the production of extracellular enzymes such as proteases and lipases help the nutrition of the host (Balcázar et al., 2006; Farzanfar, 2006), whereas others believe that the overproduction of these enzymes is a virulence factor, since pathogenic strains have high proteolytic, extracellular lipolytic activity (Quesada-Herrera et al., 2004). However, Leyva-Madrigal et al. (2011), selected lactic acid bacteria exhibiting extracellular enzymatic activity from shrimp gut for vivo experiment and noted this lactic acid bacteria mixtures have a beneficial effect against white spot syndrome virus (WSSV) in whiteleg shrimp (*Litopenaeus vannamei*).

Adhesion to the intestinal mucosa is considered an important selection criterion for persistent beneficial effects of probiotics (Ouwehand et al. 1999). When probiotic bacteria colonize the gastrointestinal tract for antagonism against pathogens, modulation of the immune system, and healing of the damaged gastric mucosa, adhesion is recognized as an prerequisite (Rinkinen et al., 2000). The evaluation of probiotic adhesion may be performed by hydrophobicity test using congo red stain (Sharma et al., 2006; Leyva-Madrigal et al., 2011). A positive hydrophobicity result indicates that the bacteria have the ability to bind nonspecifically to the epithelium of intestine by hydrophobic interactions. In the absence of hydrophobic molecules on the surface of the bacteria and the epithelium, they would repel, as both have negative charge (An and Friedman, 2000; Rinkinen, 2004; Leyva-Madrigal et al., 2011). In this study, all candidate probiotic bacteria were displayed hydrophobicity indicate their adherence capability to the host. However, there are no studies published on the adherence ability of the candidate probiotic bacteria belong to the genus *Aeromonas* and *Pseudomonas*.

Table 1. Inhibition zones (mm) against *V. anguiallarum*, enzimatic activities and hydrophobicity of candidate probiotic bacteria

	Inhibition zone (mm) against *V. anguiallarum*	Proteaz activity	Lipaz activity	Hydrophobicity
Aeromonas sp. HS23	12	+	+	+
Aeromonas sp. MS30	26	-	+	+
Pseudomonas sp. MS3	17	-	-	+
Pseudomonas sp. MS16	12	-	-	+

Table 2. Phenotypic characterization of candidate probiotic bacteria

	Aeromonas sp. HS23	*Aeromonas* sp. MS30	*Pseudomonas* sp. MS3	*Pseudomonas* sp. MS16
Gr staining	-	-	-	-
Motility	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
O/F Test	Fermentative	Fermentative	Oxidative	Oxidative
β-alactosidase(ONPG)[a]	+	-	-	-
Arginine dihydrolase [a]	+	+	+	+
Lysine decarboxylase[a]	+	-	-	-
Ornithine decarboxylase[a]	-	-	-	-
Citrat utilization[a]	+	+	+	+
Production of H_2S[a]	-	-	-	-
Urease [a]	-	-	-	-
TDA[a]	-	-	+	-
Production of indole[a]	+	-	-	-
VP[a]	-	-	-	-
Gelatinase [a]	+	-	-	-
Acid production from:				
Glucose[a]	+	+	-	-
Mannitol[a]	+	-	-	-
Inositol[a]	-	-	-	-
Sorbitol[a]	-	-	-	-
Rhamnose[a]	-	-	-	-
Saccharose[a]	+	-	-	-
Mellibiose[a]	-	-	-	-
Amygdalin[a]	+	-	-	-
Arabinose[a]	-	-	-	-

[a] Performed to API 20 E

Conclusions

As a result of this study, we found candidate probiotic bacteria could be used in the control of vibriosis. However, these strains should be further studied to explore their probiotic effects on in vivo.

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