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Investigation of Antagonistic effect of *Lactobacillus acidophilus* Isolated from the Gastrointestinal Tract of Oscar fish (*Astronotus Ocellatus*) on *Pseudomonas aeruginosa*

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ABSTRACT

Among bacteria, lactic acid bacteria are the most common type of bacteria that have been introduced as probiotics. These bacteria are effective in treating rotavirus diarrhea, reducing blood sugar, blood pressure, cholesterol, preventing colon and small bowel cancer, liver, intestinal inflammation, infections, acute diarrhea, and the growth and proliferation of harmful bacteria, as well as causes to strengthen the immune system, help digestion and absorption of minerals and vitamins. In this research, samples were taken from 60 Oscar fish. To achieve this goal, lactic acid bacteria were duplicated by specific primer pairs, 16s rDNA gene of Lactobacillus acidophilus bacteria using PCR; and Lactobacillus acidophilus was identified using a specific band, and its antagonistic effect on Pseudomonas aeruginosa was investigated by disk diffusion methods, so that halo diameter equal to 1.4mm was observed at a dose of 50 µl. Then the MIC and MBC of each metabolite were determined. It was found that increasing the dose of Metabolite Lactobacillus acidophilus increases the effect on Pseudomonas aeruginosa.

1. Introduction

The intestine of most animals are made up of bacteria called lactobacilli. Lactobacilli are living microorganisms that settle in the intestine environment and prevent the activity of non-beneficial microorganisms and pathogens (Andanil et al., 2012). Lactic acid bacteria are the most important probiotic microorganisms, which include a variety of bacteria such as lactobacilli and Bifidobacterium Lactobacilli are gram-positive, motionless; spore-free and negative- catalase bacilli; and convert various sugars to lactate and acetate. Their reduction of nitrate, catalase and oxidase are negative and their optimum growth temperature is 30-40°C (Forbes, 2013; Guy et al., 2014).

Lactobacillus also has a good antagonistic effect on Staphylococcus aureus, *Escherichia coli* and *Aeromonas hydrophila* (Salehi, 2013). Probiotics can increase microbial balance for their health and benefit by being settle in the intestinal environment. In addition, probiotics play an important role in maintaining the health of the consumer by synthesizing some essential substances (Jacobsen et al., 1999; Mohammadian et al., 2004).

Most lactobacilli are harmless and may be antagonist of pathogenic bacteria (Guy et al.,

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2014; Mohammadian et al., 2004; Mir Davoodi et al., 2004). The antimicrobial effects of some lactobacilli have been identified so far. Lactobacillus, for example, has the ability to inhibit the growth of *Helicobacter pylori*, which this bacteria can be used as a living probiotic organism in nutrition to reduce the risk of Helicobacter pylori pathogen. Lactobacillus acidophilus and Lactobacillus casei also have good antagonistic effect on *Staphylococcus aureus*, *Escherichia coli* and *Aeromonas hydrophila* (Salehi, 2013).

The most important activity of probiotics in fish digestive tract is through improving food absorption by producing extracellular enzymes and vitamins. The results of various experiments showed that growth, weight gain percentage, specific growth rate, feed efficiency, protein efficiency ratio and protein increase were higher in fishes fed with probiotics. Another important effect of probiotics is to reduce the incidence and outbreak of diseases, strengthen the immune system and antiviral activities (Behnsen et al., 2013).

As an opportunistic pathogen, *Pseudomonas aeruginosa* is more common in people who have a defective or weakened immune system, especially in patients with severe burns or people with cancer and AIDS whose immune systems have been weakened or suppressed (Palleroni, 2010).

Oscar fish, scientifically known as ASTRONOTUS OCELLATUS, is one of the most well-known carnivorous ornamental fishes from the Chichlidae family. This fish lives in freshwater and is native to the rivers Amazon, Paraguay and eastern Venezuela (Froese and Pauly, 2007).

To identify *Lactobacillus acidophilus*, DNA sequencing is performed using specified primers and by PCR. Molecular method is performed to identify bacteria that are used through primers designed from 16 S rRNA gene to perform PCR reaction and to detect the genera of lactobacilli (Noraphat, 2010). It will also use one of the primers special for lactobacilli genus after sequencing, which is registered in the NCBI gene bank. And then a primer special for bacteria is designed (Kwon et al., 2004)

2. Materials and Methods

A total of 60 samples were randomly collected from ornamental fish sales centers in different places. Then, fish intestines are sampled and cultured in MRS broth culture medium and placed in anaerobic jar with GaspackC (microaerophilic conditions), and after incubation time, we examine the bacteria for turbidity, which is re-sampled from the grown medium and cultured in MRS agar medium, after the incubation time, the desired colony is removed and Gram staining is performed (Silva et al., 2010). If it is positive, the sample is gram-positive bacillus and catalase, oxidase, motility test is performed, and if it is negative, it was removed from the desired colony and cultured again for PCR.

Identification of strains was performed using molecular methods. In this way that DNA extraction was isolated from all insulators and performed using C-TAB method (Cardinal and 1997) and lysis buffer. Lysis buffer compounds include 1 M tris with pH 5.7, 5 M sodium chloride, 0.5 M EDTA, 2% SDS and distilled water. To evaluate the quality of DNA extracted, 1% agarose gel was used for electrophoresis. After DNA extraction, specific primers were designed to reproduce the 16s rRNA fragment for Lactobacillus strains with 16s rRNA sequences available at the NCBI gene bank site. Specific primer from Cinnagen Company was used to reproduce the 16SrRNA gene fragment (Kwon et.al.2004). Validation of Lactobacillus Sp. using PCR In order to detect different Lactobacillus spp., specific primers were used to amplify 16s rRNA genes. The information is shown in Table 1 (Massi et al., 2004).

Table1. The sequences of primers used in this study

species	Primer sets (target site)	Sequence (5' →3')	Amplicon (bp)
Lactobacillus acidophilus	aci-ITS.F (16S) aci-ITS.R	CCTTTCTAAGGAAGCGAAGGAT AATTCTCTTCTCGGTCGCTCTA	199

After PCR operation and to confirm the gene, use electrophoresis to observe specific bands according to the marker. When the desired band is found according to the protocol, positive samples (which have the desired base pair or the specific gene) are specified and identified. Pseudomonas aeruginosa prepared in this study with ATCC code: 27853 was prepared from Pouya-Teb Laboratory Company.

2.1. Preparation of Lactobacillus acidophilus metabolites

To prepare the metabolite *Lactobacillus acidophilus*, it was placed in an MRS culture medium and the broth of those tubes in an anaerobic jar and a number of C-pack was placed with it, then it was placed in incubator for 48 to 72 hours, after this time, the tubes were removed from the jar and centrifuged in a centrifuge at 3000 rpm for 15 minutes. After centrifugation, the tubes and the deposition of bacteria using a 10 cc syringe of the desired metabolite using a 0.22-micron filter was sterilized next to a flame and filtered inside a sterile container. After completing these steps, all that was left in the container was the bacterial metabolite and there were no bacteria in it, and the metabolite was completely ready for Sustibility tests (Karimi Tarshizi et al, 2004).

2.2. Sensitivity test methods

2.2.1. Determination of Pseudomonas aeruginosa (ATCC: 27853) susceptibility to different amounts of metabolite prepared from Lactobacillus acidophilus by disk method.

importanbarium chloride and sulfuric acid, and by turbidity measurement based on optical absorption and bacteria were counted in CFU / ML standardly, and 10, 20, 30, 40, and 50 µl of the Lactobacillus acidophilus methabolite bacterium were added to the discs, which initially 10 µl was added to all discs and another 10 µl was added to the 20 µl disc, another 20 µl was added to the 30 µl disc, and 20 µl was added to the 40 and 50 µl discs. Because the volume of a disc is 30 µl, all discs should be placed in oven at a temperature of 40 degrees Celsius for 15 minutes to dry, then 10 and 20 µl were added to 40 and 50 µl disks, respectively, and put back in the oven to dry completely. In the next step, with the help of sterile swab and 10(µl) of microbial suspension, Pseudomonas aeruginosa was cultured on Müller Hinton agar medium. The Lactobacillus acidophilus-soaked discs were then placed at a certain distance from each other and from the edge of the plate on the surface of the Muller Hinton agar medium The plates were then incubated (at anaerobic jar) at 37 °C for 24 hours. After that, the halo diameter of the growth inhibition zone created by *Lactobacillus acidophilus* against *Pseudomonas aeruginosa* bacterium was measured in millimeters with a ruler. The steps were repeated three times (Campus et al., 2006).

2.2.2. Determination of the minimum inhibitory concentration (MIC) of a metabolite prepared from Lactobacillus acidophilus on Pseudomonas aeruginosa

After performing the disk method, the tubular dilution method of MIC is used. In this method, 1 cc of broth nutrient medium was poured into 11 sterile tubes with lids and 1 cc of the Lactobacillus acidophilus methabolite was added to the first tube, and then mixed the contents of the tubes (with the help of the sampler) and 1 cc of the first tube was added to the second tube with the help of a sampler and was mixed again. This operation was performed up to tube number 10 and 1 cc was taken out of tube number 10 and was placed in tube number 11 (the control tube). The metabolite values of Lactobacillus acidophilus bacterium in tubes 500 to 0.9 were 0.9, 1.9, 3.9, 7/81, 15/62, 31/25, 62/5, 125, 250,500, µl/m. Then, 10 µl of microbial suspension equivalent to half McFarland tube of Pseudomonas aeruginosa was added to all 11 tubes containing bacterial and control metabolites and all tubes were placed in incubator for 24 hours at 37°C. After incubation, it was time to read the MIC. Thus the turbidity rate from the first tube to the control tube was compared with the turbidity of the control tube. The amount of substance in the last tube without turbidity was determined as the MIC of the metabolite. This test was repeated three times (Anders, 2001; Touring, 2003; Davison et al., 2000).

2.2.3. Determination of the minimum bacterial concentration (MBC) of the metabolite prepared from Lactobacillus acidophilus on Pseudomonas aeruginosa

To determine the MBC, metabolites were prepared from non-turbidity tubes in nutrient agar in the amount of a standard ring with standard fieldoplatin and after incubation for 24 to 48 hours at 37°C, count the number of colonies per plate with culture site, and from the lowest concentration of the first tube in which less than 10 colonies are formed, the amount of tube material will be MBC. This test was repeated three times (French, 2006).

3. Results

3.1. Antibacterial function against pathogenic bacteria

The result of determining the mean susceptibility of pseudomonas aeruginosa to different amounts of metabolites prepared from Lactobacillus acidophilus by disk method was obtained, which in the disk method after three repetitions, metabolites isolated from Lactobacillus acidophilus was recorded in 10, 20, 30, 40, 50 µl discs in the following diagram. *L. acidophilus* had the maximum inhibitory zone on *P. aeruginosa* (14 mm), (Table 2).

Determination of the L. acidophilus Minimum Inhibitory Concentrations (MIC):

The metabolite prepared Lactobacillus acidophilus on Pseudomonas aeruginosa was determined after three time repeating the test, the mean MIC of the metabolite prepared from Lactobacillus acidophilus on Pseudomonas aeruginosa and its analysis on culture medium tube No. 3.

Determination of the L. acidophilus Minimum bactericidal concentration:

The metabolite prepared Lactobacillus acidophilus on Pseudomonas aeruginosa, after three time repeating the test, the mean MBC metabolite prepared from Lactobacillus acidophilus was recorded on Pseudomonas aeruginosa tube No. 2

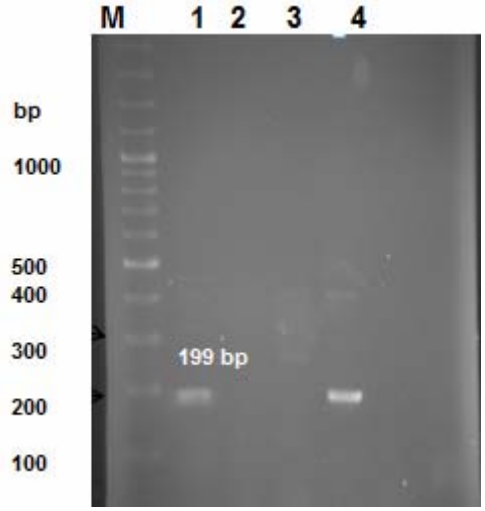

Figure1. Specific electrophoresis of *L. acidophilus*

Table 2. Inhibitory activity of *L. acidophilus* on *pseudomonas aeruginosa*

Lactobacillus acidophilus metabolites	Diameter of inhibition zone (mm) on *pseudomonas aeruginosa*
10	0
20	8
30	10
40	12
50	14

4. Discussion

In the study, after molecular isolation of Lactobacillus acidophilus from the intestine of Oscar fish, its antagonistic effects on Pseudomonas aeruginosa were investigated. Then, after performing laboratory tests of disk, MIC and MBC, it was found that Pseudomonas aeruginosa was sensitive to the metabolites of Lactobacillus acidophilus, which this sensitivity was observed in the disk method with a concentration of 50 µl of growth inhibition halo 14 mm. It was also observed that with increasing the concentration of Lactobacillus acidophilus, the effectiveness and inhibition of Pseudomonas growth was increased. The levels of MIC and MBC of Lactobacillus acidophilus metabolites were equal to tubes No. 2 and 3.

In a study by *Saranai & Hemashenpagam (*2011), the antimicrobial activity of lactic acid bacteria against standard strains of Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae and Proteus was investigated. The largest halo diameter of growth inhibition with 100 µl metabolite was observed on Pseudomonas aeruginosa, which was 14 mm for Lactobacillus brevis, 15 mm for Lactobacillus fermentum and 25 mm for Lactobacillus plantarum with halo diameter of growth inhibition of 25 mm against Pseudomonas aeruginosa respectively (Saranai and Hamshenpagam, 2011).

In a study conducted by Keramatollah Dori et al., the antimicrobial effect of Lactobacillus salivarius on Escherichia coli was investigated, which reveals the beneficial antagonistic effect of this lactic acid bacterium on the digestive tract of humans and animals. (Allah Dori et al., 1392).The study of Kazemi Darsenki et al., which examined the antimicrobial effect of various lactic acid bacteria against *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, Shigella dysentery* and *Bacillus cereus* strains, growth inhibition halos of 9 to 14 mm were indicated by lactic acid bacteria (Kazemi Darsenki et al., 1389) .In a study conducted by Diaz et al., (2013), they studied and identified the probiotic species of lactobacillus from dolphin ,and separated it from the dolphin's digestive tract. And found that there is a symbiosis between lactobacilli and dolphin gastrointestinal tract.

Achagarovskii and Zholkevskaya studied the antimicrobial activity and production of bacteriocins by Lactobacillus in 2003. After isolating Lactobacillus bacteria from MRS medium during three days at 30 ° C using the well method, they showed that Lactobacillus including *Lactobacillus Brevis* inhibits the growth of *Escherichia coli* with a diameter of 12 to 14 mm, *Bacillus cereus* with a diameter of 14 mm and Enterococcus faecalis with a diameter of 18 mm. Also, *Lactobacillus plantarum* inhibited 24 out of 32 cases and the most halos created by it on *Escherichia coli, Micrococcus luteus* and *Clostridium sporangia* were 18 mm in diameter (Achagarovskii, & Zholkevskaya, 2003). Emami et al., also showed that Lactobacillus casei and acidophilus strains are highly resistant to common oral antibiotics and their supernatant culture solution has significant activity against nosocomial pathogens such as *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli*. (Emami et al., 2010) which is consistent with our results. Conkner et al., reported that consumption of the culture supernatant of Bacillus fermentum, casei, and acidophilus creates an antibacterial effect on a wide range of gram-positive and gram-negative pathogenic bacteria (Coconnier et al, 1998). Also, Alavijeh et al., (2012) studied the effect of Lactobacillus acidophilus isolated from yogurt on Pseudomonas aeruginosa, which was 12 mm growth inhibition, and is consistent with the present study.

Conclusion

All of this evidence suggests that lactobacilli species are common in different societies. Regarding that there is numerous evidences that lactobacilli play a vital role in promoting animal health by preventing various diseases and increasing survival efficiency. These beneficial bacteria can be used to treat infections caused by *Pseudomonas aeruginosa.*

Refereces

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