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Inhibitory action of probiotic bacteria for some isolated and identified pathogenic bacteria from naturally infected Nile tilapia (Oreochromis niloticusis)

EL-Makhzangy Abeer Abd EL-Menem[1], Ghaly Mohamed Farouk[1] , Mahmoud Shahira Hanafy[2] and AwadSomayah mohamed[3]

[1]Botany and microbiology Department, Faculty of Sciences,Zagazig University,Zagazig, Egypt
[2]Chief Research of Pharmacology Animal Health Research Institute Zagazig Branch, Egypt
3Fish health and Management Department, Central laboratory for Aquaculture Research, Abassa, Abu-Hammad

ABSTRACT : **The high consumption of antibiotic in aquaculture for controlling fish disease lead to antibiotic resistance, so for improving this resistant against infectious disease it could be achieved by using of probiotics as an alternative strategy. Main objective of this study was to revealed the influence of some isolated probiotic bacteria as Bacillus spp and Lactobacillus spp on pathogenic bacteria which isolated from naturally infected Nile tilapia such as Aeromonas spp, Vibrio spp and Pseudomonas spp. Bioactivity of probiotic were measured by the diameter of inhibition zone on disc diffusion method, six probiotic were used for testing their ability to inhibited the growth of pathogenic bacteria, three of them were kindly provided and the other were isolated from pickles, fermentable milk and yogurt, respectively. The result indicates that Vibrio anguillrium recorded high prevalence pathogenic bacteria as 52% of isolates. The highest inhibition zone refers to Bacillus amyloliquefacien SW19 which range from (10-40 mm).**

Key words: Nile tilapia, pathogenic bacteria, probiotic bacteria.

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I. INTRODUCTION

Fish disease is considered to be one of the serious problems in aquaculture. In freshwater fish culture, disease outbreaks refer to the presence of pathogenic bacteria (Lim & Webster 2001). Fish have a susceptibility to a wide range of bacterial pathogens this occur when fish are physiologically unbalanced or nutritionally deficient or subjected to stressors such as overstock or poor water quality and the majority of bacterial infections are caused by Gram –negative bacteria (Aeromonas spp, Pseudomonas spp and Vibrosis spp), (Ashiru et al., 2011). Production of Nile tilapia in Egypt contributes to about 65.15% of Egyptian fish production (Gafrd, 2017). Nile tilapia is a highly popular freshwater fish due to growing fast and its ability to grow in extremely diverse or adverse conditions Egypt's. Nile tilapia production is considered as the first biggest producer in Africa and second producer in the world after China (FAO, 2010 and FAO, 2019). Bacterial diseases were the major cause of economic losses affecting fish farms, these bacteria influence the composition of gut biota and vice versa as the host and microorganism share the ecosystem (Verschuere et al., 2000 and Marzouk et al., 2008). Aeromonas hydrophila, Vibrio anguillarium and Pseudomonas fluorescens are recorded as the predominate bacteria which cause infection in fish farms and contributed to O.niloticus seasonal summer mortalities (Abd El–kader and Balabel, 2017).Symptoms in infected fish show loss of equilibrium , scales loss , sluggish swimming at the water surface, loss of appetite ,skin erosions and ulcer, exophthalmia, fin and tail rot, gills might be

congested or pale and anemic covered with excessive mucus (Hassan et al., 2011). The last decades, use of antibiotics in aquaculture system are considered as a traditional strategy for fish disease management, for disease prevention and control. Antibiotics have been used as traditional strategy during the past decades and also for fish growth as well as feed conversion efficacy. However, the evolution of antimicrobial resistant pathogens has been recognized but there is a high risk of transmission of the resistance bacteria from aquatic environment to humans (Prasad et al., 2012; Pandiyan et al., 2013). Anyway, the accumulation of residual antibiotics in aquaculture products may be detrimental to human health by killing beneficial micro -biota in the gastro intestinal (GI) ecosystem (Cabello, 2006). There has been heightened research in developing new dietary supplementation strategies by promoting various health and growth compounds like probiotics (Denev, 2008), probiotics offer alternative drugs to chemicals and antibiotic in aquatic animals (Rekiel et al., 2007, Hai, 2015). The application of probiotics in diets of Nile tilapia in low input ponds promotes growth and enhances body composition. Two probiotics, Saccharomyces cerevisiae and Bacillus subtilis, have got different effects depending on the level of application, best performance at 4g kg-1,10g kg-1 respectively (Opiyo et al., 2019). Lactobacillus bifidobacterium, Entrococcusspp, Streptococcus spp, Bacillus spp, Escherichia coli and Yeast are widely used as probiotics (Vigayetal., 2013, Indria et al., 2018). Addition of commercially or gut isolated prepared Bacillus spp to Oreochromus niloticus diet at a dose of 1x1010cfu/g had significant additive benefits in improved growth performance, immune status, serum antioxidant and functions of the liver and kidney (Mai, 2019).

. II.Materials and Methods

Naturally infected fish

Two hundred naturally infected Nile tilapia of different body weights and lengths were collected from private farms (Kanater, Kalubia Government, El-Behira, Sun El-Hagar), fish were transferred live in tanks containing pond water to the microbiological lab. All collected samples were subjected to clinical postmortem and bacteriological examination as described by (Austin and Austin, 2012; Hassan et al., 2020).

Isolation of pathogenic bacteria

The specimens of different tissues and organs such as (skin, ulcer, tail, gills liver, spleen and kidney) from diseased fish were taken under complete aseptic conditions and inoculated into tryptic soy broth (Difco), then incubated at 27 ˚c for 24 h. A loop full of broth were streaked onto tryptic soy agar plates (TSA),incubated at 27 ˚c for 48h for purification purposes ,pure colony was picked up and sub -cultured on a selective diagnostic agar media as Thiosulphate citrate bile salt agar (oxoid) (TCBS), Pseudomonas base agar media and Aeromonas base agar media supplemented with Ampicillin (5mg/l) at 27˚c for 48 h, finally pure colonies were transferred on TSA slant for identification according to(Quinn et al., 2002; Austin and Austin, 2012 and Hassan et al., 2020).

Identification of pathogenic bacteria

The obtained bacteria isolates were subjected to full identification by using colony characteristics, gram stain, and biochemical activities as described by (Austin and Austin, 2012).

Isolation and identification of probiotic bacteria

Five bacteria were used as probiotics, p1 kindly provided from mid gut of the larva, it was determined by (Gene bank accession No MK160141) Bacillus amyloliquefaciens SW19 (Shaimaa, 2019), p2 kindly provided from gut of CLarias griepinus, identified as Bacillus amyloliquefaciens7HN (KX015882) Reda et al., (2018), p3 commercial Baker's yeast Saccharomyces cerevisiae, p4 isolated from pickles, p5 from fermentable milk, last one p6 from yogurt. All isolates were inoculated on tryptic soy broth (TSB) and incubated at 27˚c for 24 h, followed by plating tryptic soy agar (TSA), then incubated at27˚c for 24-48 h (Apun-molina et al., 2009), in the case of lactic acid bacteria (LAB), bacterial isolates were enriched in De Man –Rogosa Sharpe Broth (MRSB) at 27˚c for 24 h , then streaking directly on De Man –Rogosa Sharpe agar (MRSA) incubated at 27˚c for 24-48h .the isolated probiotic bacteria p4, p5 and p6 were identified biochemically, Gram stain and colonies morphology .

Sensitivity of the pathogenic bacterial isolates to probiotic bacterial strains

Pathogenic bacteria which isolated from naturally infected fish and biochemically identified to determine the antibacterial effect of six probiotics by disc diffusion techniques .Pathogenic bacteria were cultured in TSB media then incubated for 24h at 30˚c, then 30µl of cultures with 103 CFU/ml were spread on (TSA) media by sterile cotton swab, on other hand selected probiotic were cultured in MRS broth at 30˚c for 72 h. Bacterium cells were harvested by centrifugation at 5000 rpm and 4˚ c for 15 min. In sterile condition, their supernatants were used for antibacterial tests using disc diffusion methods (Balcāzar et al., 2008, Allamesh et al., 2012, Muthukumar and Kandeepan , 2015).

III.RESLUTS And DISCUSSIONS

Clinical Examination of naturally diseased fish

The external examination of fish exhibited discoloration of skin associated with the development of different patches of ulcerative and hemorrhagic skin. The apical caudal fin rays appeared necrotic, reddish mouth, swollen abdomen, opercula around the anal opening, darkness of skin showed as shown in fig (1).

Fig (1): Skin ulcerated with hemorrhagic patches on different parts of body surface in naturally infected Nile tilapia (Oreochromis niloticusis)

On post mortem examination, the most common internal changes were congestion and enlargement of internal organs, congested liver with distended gall bladder, dark congested kidney and yellow ascetic fluid in distended cases were also recognized in fig (2).

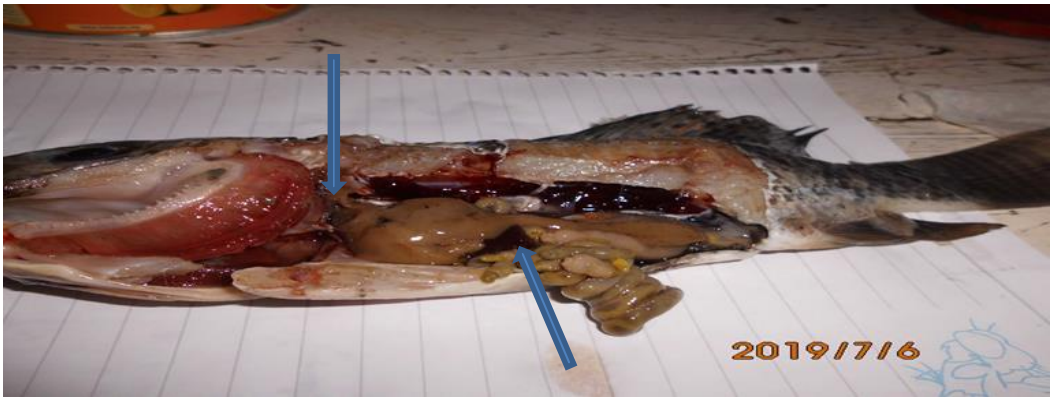

Fig (2): Paleness and anemic liver with necrosis with distended gall bladder, (arrow) and gills Congested

Isolation and identification of pathogenic bacteria

One hundred and twenty–three bacterial isolates were isolated from naturally infected fish (Nile tilapia) from six farms of private fish farm, all isolates were phenotypically identified following standard protocol, 104 isolates (84.55%) were recorded as Gram negative bacteria as Aeromonas spp, Vibrio spp and pseudomonas spp, the other isolates 19 at rate of (15.4%) of total isolates strain were Gram positive bacteria which identified as staphylococcus spp and bacillus spp.

Pseudomonas spp was identified microscopically and bio-chemically to Ps. medocina & Ps. Aeruginosa. These isolates Gram negative, long curved rods, motile and oxidase positive, it represented 16.3% as shown in table (1). Vibrio spp recorded 52.3%. It identified microscopically and bio-chemically as V. anguillarum, V. alginolyticus and V. paraheamolyticus, all vibrio spp were Gram negative, curved rods, fermentative and positive for oxidase, catalase and methyl red V. anguillarum and V. alginolyticus produce yellow colonies on TCBS media while V. paraheamolyticus produced green colonies, as represented in table (2). In Aeromonas spp (42.3%) they were identified microscopically and bio-chemically as A. hydrophila, A. cavaei, A. jandaei, A. sobria and finally A. veronii, all these spp were Gram –negative short rods, motile, fermentable, catalase and oxidase positive and the complete characterization show in table (3).

Table (1): Morphological and biochemical characters of suspected Pseudomonas sppisolates from naturally infected Nile tilapia (oreochromis niloticus)

Biochemical tests	Pseudomonas spp	
	Pseudomonas mendocina	Pseudomonas aeruginosa
Gram-stain	-ve	-ve
Shape	Long curved rods	Long curved rods
Motility	+	+
Cytochrom oxidase	+	+
O/F	o	o/-
Growth at 4oC	-	-
Growth at25oC	+	+
Growth at 35oC	+	+
Growth at 42oC	-	+
Growth on 0. 0% NaCl	+	+
Arginine dehydrogenase	v	+
Catalase	+	+
H2S (TSI)	+	-
Indole	-	-
Lysine decarboxylase	-	-
Gelatin degradation	-	v
Ornithen decarboxylase	-	-
Urease test	v	v
Acid production from:		
Glucose	+	+

Sucrose	-	-
Salicin	-	-
Lactose	-	-
Maltose	-	+
Inosition	+	-
fructose	-	-
Nitrate reduction	+	-
Esculin hydrolysis	-	-

O/F oxidation fermentation reaction F: Fermentation O: Oxidation V: Variable +: Positive -: Negative

Table (2): Morphological and biochemical characters of suspected Vibrio spp isolated from naturally infected Nile tilapia (oreochromis niloticus)

Biochemical tests	Vibrio spp		
	Vibrio alginolticus	Vibrio anguillarum	Vibrio paraheamolytica
Gram-stain	-ve	-ve	-ve
Shape	Curved rods (comma) shape	Short rods	Curved rods
Motility	+	+	+
Cytochrom oxidase	+	+	+
O/F	F	F	F
Catalase	+	+	+
Growth on TCBS	Yellow colonies +	Yellow colonies+	Green colonies
Methyl red	+	+	+
Vogausproskauer	+	+	+
Arginine dehydrogenase	-	-	-
Indole	+	+	+
Citrate utilization	+	+	+
Growth on 0. 0% NaCl	-	-	-
Growth on 2. 0%NaCl	+	+	+
Growth on 5. 0% NaCl	+	+	+
Growth on 7. 0% NaCl	+	+	-

Growth on 10. 0% NaCl	+	-	-
Marg reaction	Evitage N	Evitage N	Evitage N

Table (3): Morphological and biochemical characters of suspected Aeromonasspp isolates from naturally infected Nile tilapia (oreochromisniloticus)

Biochemical tests	Aeromonasspp				
	A. sobria	A. caviae	A. jandaei	A. veronii	A.hydrophyila
Gram stain	-ve	-ve	-ve	-ve	-ve
shape	Short rod	Short rod	Short rod	Short rod	Short rod
Motility	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+
O/F	F	F	F	F	F
Growth at 5oc	+	+	-	-	+
Growth on 0 %Nacl	+	+	+	+	+
Growth on 3. 5% Nacl	+	+	+	+	+
Growth on 6% Nacl	+	-	-	-	-
Catalase test	+	+	+	+	+
H2s(TSI)	+	-	-	-	+
Indole	+	+	+	+	+
Starch hydrolysis	+	+	-	-	-
Methyl red	+	+	-	-	+
Vogausproskauer	+	-	+	+	+
Citrate	+	+	+	+	+
Gelatin liquefaction	+	+	-	-	+
Heamolysis	β	-	γ	-	β
Acid production from:					
Arabinose	-	+	-	-	+
Salicin	-	-	-	+	v
Sucrose	+	+	-	+	+
inositol	-	-	-	-	-
Maltose	+	+	+	+	+
Mannitol	+	+	+	+	+

Tween 80	+	+	+	+	+
Glucose	+	-	+	+	+
Ornithen decarboxylase	+	-	+	+	-
Lysine decarboxylase	+	-	+	+	+
Arginine dehydrolase	-	+	-	-	+

The staphylococcus spp (17.38%) was identified as Staphylococcus aureus. It was Gram positive, cocci, of oxidative, oxidase negative, catalase positive, also positive for methyl red and vogous proskauer, they utilize citrate as sole carbon source, finally can grow in media with 3.5, 6 and 8% NaCl. While bacillus spp (5%) recorded rod shape, motile with peritrichous flagella, good growth at 7% NaCl, no growth at 10%, also growing well at 30-40˚c not at 50˚c as present in table (4).

Table (4): Morphological and biochemical characters of suspected Staphylococcus & Bacillus spp isolates from naturally infected Nile tilapia (oreochromis niloticus)

Biochemical test	Staphylococcus spp	Bacillus spp
	Staphylococcus aureus	Bacillus subtilis
Gram-stain	+	+
Shape	Large yellow colony on rich media	circular colony, rough, white or slightly yellow
Growth temperature	15-45ºC	25-35 ºC
Catalase	+	+
Cytochrom oxidase	-	+
D – mannitol fermentation	+	+
Hemolysis on blood agar	Hemolysisβ	Hemolysisβ
Nitrate reduction	+	+
Lactose	+	v
Mannitol	+	+

Isolation and identification of probiotic bacteria

As present in table (5), the isolated bacteria were characterized by morphological and biochemical; p4 which was isolated from pickles, recognized as Bacillus anthracis, rod shape, Gram stain positive, non -motile ,catalase ,oxidase positive and urease negative, while p5 and p6 which isolated from fermentable cow milk and yogurt respectively, recorded as Lactobacillus bulgaricus, rod shape, gram stain positive, KOH negative, catalase and oxidase negative, citrate negative, indole positive and no growth at 45˚c.

Table (5): Morphological and biochemical test of probiotic bacteria isolated from different sources (p4: pickles, p5: fermentable cow milk and p6: yogurt)

Biochemical tests	Bacterial isolates		
	P4	P5	P6
Gram stain	+	+	+
KOH test	-	-	-
shape	Rod	Rod	rod
Motility	-	-	-
Growth at 10	-	+	+
Growth at 45	+	-	-
Catalase test	+	-	-
Oxidase	+	-	-
Urease	-	-	-
Citrate	v	-	-
Indole	-	+	+
Starch	+	-	-
Methyl red	-	-	-
Voges-proskauer	+	-	-
Nitrate reduction	+	-	-
Fermented sugar			
Maltose	+	+	+
Lactose	-	+	+
Sucrose	+	+	+
Arabinose	-	+	+
Salicin	-	+	+

P4: Bacillus anthracis, p5 and p6: Lactobacillus bulgaricus

Antimicrobial activity of probiotic bacterial strains against pathogenic bacterial isolates

In the current study showed that high inhibition zones were recorded in Bacillus amyloliquefaciens SW19 (No MK160141) 40 mm in diameter with v. anguillarum, then followed by Bacillus amyloliquefaciens 7HN (KX015882), A.veronii were more resistant strain towards probiotics bacteria, lowest inhibition zone recorded in Ps.medocina, as show in table (6) and fig (3).

Table (6): Antimicrobial activity of different probiotic bacteria isolated from different source against pathogenic isolated bacteria from naturally infected Nile tilapia (Oreochromis niloticus)

Pathogenic bacteria	Diameter of inhibition zones (mm)					
	P1	P2	P3	P4	P5	P6
A.jandiae	15	25	13	15	20	18
A.caviae	10	15	15	15	-	10
A. hydrophila	-	20	16	10	20	15
A.sobria	20	25	13	-	10	18
A.veronii	-	20	15	-	-	-
V .anguillarum	20	40	15	15	20	20
V.alginolyticus,	10	35	-	-	15	15
V.paraheamolyticus	15	30	-	10	15	18
P .aeroginosa	15	20	10	-	-	16
Ps.medocina	10	15	-	-	-	10

P1:7HN Bacillus amyloliquefaciens (KX015882), p2: Bacillus amyloliquefaciens SW19 (No MK160141) p3: Bacillus anthracis, p4&p5: Lactobacillus bulgaricus and p6: commercial Baker's yeast Saccharomyces cerevisiae

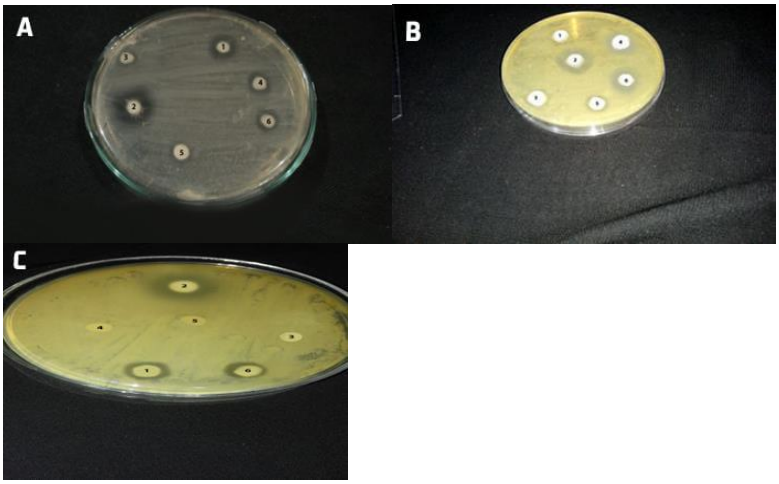

Fig (3): Antibacterial activity of isolated probiotic bacterial strain against V.anguillarium (A), A.sobria (B)and Ps.medocina (C).

P1: 7HN Bacillus amyloliquefaciens(KX015882,P2:Bacillus amyloliquefaciens SW19(No MK160141),P3:Bacillus anthracis,P4&P5: Lactobacillus bulgaricus and p6: commercial Baker's yeast Saccharomyces cerevisiae

Aquaculture industry provides abundant and high-quality animal protein for human's requirement, Nile tilapia is the main cultured fish species in Egypt. Tilapia fish are susceptible to several bacterial diseases under stressed conditions (Dong et al., 2017; Hassan et al., 2020). Bacterioses is considered as the most important agents related to large mortalities and severe economic losses in world-wide production (Sirimanapong et al., 2018).

Over the years, probiotics gained prominence to be an alternative method to the use of chemotherapeutics for the treatment of diseases in aquaculture. Probiotics are defined as living

microorganisms which act beneficially on host physiology when added to the diet, they can cause′s improving the absorption and digestion of nutrients, so cause improvements in the immune system, generating a positive effect on the host health and greater resistance to diseases (Dawood and Koshio 2016).

In this study, naturally infected Nile tilapia showed hemorrhagic skin, frayed fins and tail body, corneal opacity, body ulceration, detachment of scales, hemorrhages over the fish body, especially and swelling of the abdomen. Post mortem examination showing congestion of the internal organs and presence of ascetic fluid. Our result in the same direction with El-Son, (2016) who observed signs such as anorexia, skin alterations, an extension of the visceral cavity, exophthalmia, corneal opacity, bleeding, and abdominal inflammation, splenomegaly and hepatomegaly. The bacteriological examination of infected fishes in this study showed that Gram negative bacteria like Aeromonas spp, Vibrio spp and pseudomonas spp were presented as (84.55%), vibrio spp were the predominant isolates as (52.3%), the other isolates Gram positive bacteria at rate of (15.4%) which identified as staphylococcus spp and bacillus spp this result in agreement with Najiah et al., (2012), El-Gamal et al., (2018) and Hassan et al., (2020) which reported that three Gram-negative bacteria at 86.26% of the total isolated strains with the predominance of Aeromonas spp. at 46.70 %. Besides, two Gram-positive bacteria at 13.74% were isolated with the dominance of Streptococcus spp.at 8.79 %.

The present study cleared that V.anguillarum had the highest prevalence rate of isolated bacteria, the obtained result agreed with Frans et al., (2011) they stated that V. anguillarum is widely found in various cultured and wild fish as well as in salt or brackish water causing a fatal hemorrhagic septicemic disease, called vibriosis.

The present study cleared that the three probiotic isolates were gram positive, rod shaped, KOH negative and non-motile, and identified as Bacillus anthracis and Lactobacillus bulgaricus. This result agrees with Mithun et al., (2015) and El Kahlout et al., (2018) who indicated that the isolated strains from yoghurt appeared in blue-purple color proving that the isolates are Gram-positive bacteria. The biochemical tests included catalase and gas production from glucose fermentation. The Catalase test was negative (no babbles). Additionally, when isolates were examined for gas production by glucose fermentation, no gas accumulation was seen in the tubes even after five days. In our study, the antimicrobial activity assay of probiotics was performed by agar disc diffusion method. Inhibition zones of isolated probiotics range from 10 and 40 mm against pathogenic isolates Aeromonas spp, Vibrio spp and Pseudomonas spp. Related results were reported by Aly et al., (2008) who mentioned that B. Subtilis and L. acidophilus inhibited the growth of A. hydrophila in vitro. Bacillus amyloliquefaciens showed higher antimicrobial activity towards all isolated pathogenic bacteria and the highest inhibition zone was recorded in V. anguillarumas 40 mm, these result similar to those obtained by Zhang et al., (2022) and Pereira et al., (2019) who found that Bacillus spp had the ability to produce antimicrobial substances with diverse structural forms. The present investigation indicated that V. paraheamolyticus and V. anguillarum were more sensitive to probiotic bacterial strains while A. veronii and Ps. medocina were more resistance. Our result is inconsistent with the result obtained by Yang et al., (2022) they stated that the supernatant of B. subtilis CK3 exhibit a significant bactericidal effect on A. veronii, and the size of inhibition zone increased with the increase in CK3 supernatant loading.

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