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Isolation of *Lactobacillus* spp. in African Catfish *Clarias gariepinus* as probable probiotics in aquaculture

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Abstract
This research sought to isolate probiotic bacteria from cultured African Catfish *Clarias gariepinus* to be used against bacterial fish pathogens. *Lactobacillus* spp. were successfully isolated from the intestine of *C. gariepinus*, identified using biochemical characters. Pathogenic bacteria were also isolated from the same species such as *Escherichia coli*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. The isolated *Lactobacillus* spp. was assessed *in vitro* for its antagonistic activity against isolated bacterial pathogens using disc diffusion method. Two impregnated paper discs of *Lactobacillus* spp., one antibiotic (Amoxicillin), and one blank disc were placed in each plate of cultured pathogenic bacteria. Results showed that *Lactobacillus* spp. is comparable to the antibiotic (Amoxicillin) on their antagonistic activity, quantified by measuring the zone of inhibition against *E. coli*, *V. parahaemolyticus* and *A. hydrophila*. The highest zone of inhibition of *Lactobacillus* spp. was shown against *A. hydrophilla* (8.5 mm) and lowest zone (7.25 mm) against *E. coli*. Antagonistic activity of antibiotic (Amoxicillin) showed highest zone of inhibition against *E. coli* 14 mm and the lowest was *V. parahaemolyticus* 7 mm. Based on the results of the study, *Lactobacillus* spp. was successfully isolated from the intestine of catfish. The present work highlights the isolated candidate can be a promising probiotic to be used in aquaculture feeding, because of its high potential activity against aquatic pathogenic bacteria *in vitro*.

Keywords: Susceptibility test, antagonistic activity, pathogenic bacteria, probiotics

Introduction
Probiotics in aquaculture are widely recognized as the major mean for controlling pathogens for environment friendly aquaculture [1-9]. Probiotics are defined as live microorganisms which are beneficial to the host when administered in appropriate and regular quantities. Once ingested, the probiotic microorganisms can modulate the balance and activities of the gastrointestinal microbiota, whose role is fundamental to gut homeostasis [2, 10, 11].
Aquaculture is farming of fish molluscs, crustaceans, and aquatics plants [12], and the fastest growing food-producing sector in the world with an average rate of 8.9% per year since 1970 [13-14]. Despite the fastest growing sector, the fish disease is still a major problem in the farming industry [15] where fish are stocked at high density to achieve high productivity. In this type of system, species are subjected to stress, so fish diseases tend to occur easily and spread rapidly, affecting the economic development [12,16]. Vaccinations and antibiotics or chemotherapeutics are the current methods to prevent infectious microbial diseases [12]. However, massive antibiotic usage for disease control and growth promotion posts significant risks due to the evolution of antibiotic- resistant microorganisms [17-19]. Thus, the need for alternative techniques is increasing and the contribution of probiotics is considered.
The role of probiotics and their mechanisms of action in aquaculture are continuously being researched and developed. This is due to their effectiveness to improve the quantity and quality of aquaculture production. The benefits of probiotics for aquaculture are improvements on the immune system [20], feed efficiency and protein retention [21], and growth rate [22]. Probiotics such as *Lactobacillus* sp. has been reported to have inhibitory activity against common human pathogens [23-24], fish pathogens [25-26] and produce antimicrobial substances such as bacteriocins used in the therapeutics [27].

Therefore, increased understanding of probiotic uses would lead to the development of natural antibiotic and reduce the dependency on chemical or drug uses in aquaculture [13]. In this regard, the research's goal is to isolate *Lactobacillus* spp. in order to exploit them for further uses in aquaculture. Specifically, this study isolated *Lactobacillus* spp. in African Catfish *Clarias gariepinus* as probable probiotic in aquaculture. Gram positive probiotic microorganisms in the intestine of African catfish were isolated and identified using biochemical techniques. Lastly, the antagonistic activity of isolated probiotics against pathogenic bacteria isolated from *C. gariepinus* were evaluated.

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Materials and Method

The experiment involved isolation and identification of probiotic microorganisms in the intestine of *C. gariepinus*. Measurement of inhibition zone was the unit of analysis in determining antagonistic activity of isolated against pathogenic bacteria.

Experimental Animal. African Catfish is a freshwater fish commonly cultured in Iloilo province. Fourteen (14) fresh African catfish were randomly collected from a commercial fish pond. The length and weight of fish samples ranged from 18-25 cm and 62-112 g, respectively [16]. Isolation and identification of probiotic microorganisms in the intestine of the catfish was done at Fish Health Laboratory, Iloilo State College of Fisheries.

Reagents. Nutrient agar (HIMEDIA, Vadhani India), Mueller-Hinton Agar (MHA), Brain Heart Infusion Agar (BHIA) and Nutrient Broth (NB) were the media used to grow all coliform bacteria. To isolate pathogenic bacteria, specific agar were used: Thiosulfate Citrate Bile Salt-Sucrose Agar, TCBS, (TM MEDIA, Rajasthan, India) specific for the growth of *Vibrio* spp., MacConkey Agar (TM MEDIA, Rajasthan, India) selective for the growth of *Escherichia coli* and Lactobacillus Selection Agar Base, selective for the growth of *Lactobacillus* spp. Further, media for biochemical test reactions for carbohydrate test were Kligler Iron Agar, KIA (CONDA PRONADISA, Consing S.A.), Simmon Citrate Agar, SCA (HIMEDIA, Vadhani, India), Catalase, Hydrogen Sulfide Test, Sulfur-Motility, SIM (SCHARLAU, Spain), were also prepared. Mueller-Hinton Agar was used to culture pathogenic bacteria for antagonistic activity.

Isolation of Bacteria. Fish were bought from a local aquaculture farm and brought to the laboratory. The abdominal surfaces were scrubbed thoroughly with 70% ethanol and aseptically dissected to remove the intestines [28]. Fourteen (14) pieces of fresh African catfish were dissected ventrally from the anus to operculum using sterilized scissors, and intestines were removed using sterilized forceps. At each time, 10 g of intestine was separately floated in 90 ml of sterile saline solution and homogenized for 20 minutes using a blender, then subsequently centrifuged. After centrifugation, the supernatant was collected and serially diluted (10^1, 10^2, and 10^3) in Nutrient Broth and were incubated for 24h at 37°C [12, 29, 30] and aseptically plated by the spread plate technique into Nutrient Agar, Mac Conkey, Thiosulfate Citrate Bile Salt-Sucrose Agar (TCBS), Brain Heart Infusion Agar (BHIA), and *Lactobacillus* Selection Agar Base in an inverted plate in duplicate, respectively. Identification of bacteria isolated were based on morphological characteristics described by MacFaddin (1980), biochemical characteristics (Kligler Iron Agar, Carbohydrate Fermentation, Citrate Test and Catalase) and morphological test (gram staining, motility test).

Preparation of McFarland Nephelometer Standards. Bacterial suspension of 15 x 10^8 CFU/ml was compared to 0.5 McFarland standard; that was prepared by mixing 0.5 ml of 0.048 M barium chloride dehydrate ($BaCl_2$ $2H_2O$) and 9.5 ml of 0.36 N sulfuric acid (H_2SO_4) in a test tube with a constant stirring in the vortex mixer [31-32].

Susceptibility test. Antagonistic activity was determined using the paper-disk diffusion method [33] against three bacterial pathogens (*Escherichia coli*, *Vibrio parahaemolyticus*, and *Aeromonas hydrophila*) isolated from the intestines of catfish. The *Lactobacillus* spp. strains were inoculated on Nutrient Broth and incubated for 24 hours at 37°C. A bacterial culture of 15 x 10^8 based on 0.5 McFarland Standard in replicate was used to spread in 20 ml Mueller Hinton Agar (MHA) plates evenly using a sterile cotton swab. Pathogenic bacterial strains were spread on the surface of the agar and dried for 10 minutes. Two impregnated paper discs of *Lactobacillus* spp., one antibiotic (Amoxicillin), and one blank disc were placed in each plate of cultured pathogenic bacteria. The plates were then incubated at 37 °C for 24h. The zone of inhibition formed in the surrounding discs were observed and measured using a ruler and express in millimeter.

Results and Discussion

A probiotic bacterium *Lactobacillus* spp. was successfully isolated from from *C. gariepinus.* Three gram-negative pathogens, *E. coli*, *V. parahaemolyticus*, and *A. hydrophila* was isolated and identified using morphological and biochemical analysis. Table 1 presents the morphological characteristics of isolated *Lactobacillus spp. and pathogenic bacteria* observed which had the similar characters according to previous studies [29-30]. The biochemical reactions of the *Lactobacillus* spp. and the three pathogenic bacteria are presented in Table 2 using MacFaddin's scheme. These were similar identified as *E. coli*, *V. parahaemolyticus*, and *A. hydrophila.*

Table 1: Morphological characters of isolated *Lactobacillus* spp. and pathogenic bacteria

Characters	*Lactobacillus* spp.	*E. coli*	*V. Parahaemolyticus*	*A. hydrophila*
Colour	cream- white	pink	green	cream- white
Texture	smooth	shiny; smooth	smooth	shiny; smooth
Elevation	raised	slightly raised	slightly raised	convex
Shape	circular; rods	rods	curved; rods	rounded; rod
Gram Stain	+	-	-	-

Table 2: Biochemical reactions and confirmatory tests on bacteria isolated from intestine of catfish *Clarias gariepinus*

Media	Kligler Iron Agar (KIA)				Carbohydrate Fermentation				Indole Motility Citrate	
	Slant	Butt	Gas	H_2S	Glu	Lac	Sac	Cit	M	C
LSAB										
Lactobacillus spp.	K	A	-	-	+	+	+	+	-	-
MacConkey										
Escherichia coli	A	A	+	-	+	+	+	-	+	+
TCBS										
Vibrio parahaemolyticus	K	A	+	-	+	-	-	+	+	+
BHIA										
Aeromonas hydrophila	K	A	+	-	+	-	+	+	+	+

Legend: K- Alkaline (-) negative M- Motility A- Acid (+) positive CT- Catalase

***Lactobacillus* spp.**

Biochemical test reaction of *Lactobacillus* spp. isolated from *Lactobacillus* Selection Agar Base in the intestine of Catfish revealed the following characters: on KIA, it is alkaline on slant, acid on butt, and negative for H_2S production and gas formation. It also fermented glucose, lactose, and saccharose. Reaction to citrate was positive, negative to motility and catalase [34].

Escherichia coli

E. *coli* isolated from intestines and plated on MacConkey had the follows: for KIA, it showed an alkaline reaction on slant, acid on butt, negative for gas formation, and positive for H_2S production. For carbohydrate fermentation (OF Media), glucose, lactose and saccharose showed a positive reaction. Citrate utilization was negative, positive for motility and catalase [34].

Vibrio parahaemolyticus

Biochemical test reaction of *V. parahaemolyticus* isolated from TCBS showed the following: on KIA, was alkaline for slant, acid on butt, positive for gas formation, and negative H_2S production. Reaction to carbohydrate fermentation was positive for glucose and lactose, but negative for saccharose. Citrate reaction was found positive and motility and catalase tests [34-35].

Aeromonas hydrophila

Biochemical test reaction of *A. hydrophila* isolated on BHIA from the intestine of catfish revealed the following results on KIA: alkaline for slant, acid on butt, gas formation but negative for H_2S. It ferments glucose and saccharose but not lactose; utilizes citrate, and is positive for catalase and motility [34].

Antagonistic Activity

By disc diffusion assay, *Lactobacillus* spp. showed a clear bactericidal effect against gram-negative pathogens, *V. parahaemolyticus, A. hydrophila,* and *E. coli.* Table 3 shows the susceptibility of the isolated pathogenic bacteria to the antibiotic (Amoxicillin) and the blank disc. The antagonistic activity ascertained using supernatant solutions in *E. coli, V. parahaemolyticus,* and *A. hydrophila* showed variable inhibition size ranging from 5.5 to 11.25 mm. The inhibition of *Lactobacillus* spp. against *E. coli* (7.25mm) was lower compared previous studies with 8-9.3 [36] and 13.7 mm [37]. Against *V. parahaemolyticus*, *Lactobacillus* spp. earlier study [37] showed a zone of inhibition of 11.6mm; in the present study inhibition was of 7.5mm. The inhibition zone of *Lactobacillus* spp. against *Aeromonas hydrophila* in prior reports [12] was 15.5-20mm; in present study the inhibition zone was 8.5mm. The antibiotic susceptibility test with Amoxicillin (positive control) with *E. coli* resulted in an inhibition of *E. coli* 14 mm, *V. parahaemolyticus* 7 mm, and *A. hydrophila* 13.5mm. No inhibitions were found on blank discs (negative control).

Table 3: Mean zone of inhibition against Common Fish Pathogens

Pathogenic Bacteria	*Inhibition Zone*		
	***Lactobacillus* spp.**	***Amoxicillin* (Positive Control)**	**Blank Disc (Negative control)**
E. coli	7.25	14.0	0
V. parahaemolyticus	7.5	7.0	0
A. hydrophila	8.5	13.5	0

Lactobacillus spp. is often antagonistic against other freshwater fish pathogenic bacteria [2, 36] obtained in many countries. It was also indicated that some of the *Lactobacilli* used on probiotics capable of stimulating the immune system [39]. Studies were also carried out to enhance the use of *Lactobacillus* as a probiotic in aquaculture. For instance, the presence of *Lactobacillus* spp. were shown to promote the growth and survival rate of rotifers [40], marine larvae [41], Atlantic cod fry [42], *Portunus trituberculatus* larvae [43-44] and inhibit the fish pathogen, *A. hydrophila* in tilapia [45]. They can target fish eggs and larvae, fish juveniles and adults, crustaceans, bivalve molluscs, and also live food such as rotifers, Artemia, and unicellular algae [46].

Overall, this antagonistic activity of Lactobacillus against fish pathogens indicates possible use as probiotic in aquaculture. This can be enhanced and used as probiotic supplement in aquaculture feeds to enhance the growth of cultured fish. While this study provides valuable information *in vitro*, future studies should be aimed at revealing the factors that determine the occurrence of *Lactobacillus* spp. in fish along with the ability to adhere to host cells, mechanism of antagonistic action between the probiont and the pathogen, its competitive exclusion stability *in vivo*. Close network of aquaculture experts, fish nutritionists and microbiologists are necessary to develop such aquatic foods.

Conclusion

Lactobacillus spp. was successfully isolated from the intestine of catfish. The antagonistic activity of *Lactobacillus* spp. against *Escherichia coli*, *Vibrio parahaemolyticus*, and *Aeromonas hydrophila* are comparable to the antibiotic (Amoxicillin). The present work shows that the isolate can be a promising probiotic to be used in aquaculture feeding, and to its high potential against aquatic pathogenic bacteria *in vitro.* This research will be beneficial to promote the use of probiotic as an alternative way to control disease and enhance water quality.

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