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ISOLATION AND CHARACTERIZATION OF *LACTOBACILLUS* SPECIES FROM FISH INTESTINE FOR PROBIOTIC PROPERTIES

YASHAB KUMAR[1], BENAZIR CHISTI [1], AJAY KUMAR SINGH[1], HARISON MASIH[1] AND SANTOSH KUMAR MISHRA*[2]

[1] *Department of Microbiology & Fermentation Technology, Sam Higginbottom Institute of Agriculture Technology & Sciences (Deemed University), Allahabad, INDIA*
[2] *Department of Biotechnology, IMS Engineering College, Ghaziabad, INDIA*

ABSTRACT

The aim of this research was to investigate the presence of *Lactobacillus* species in freshwater fish and screen for probiotic properties. For this purpose, intestinal contents (n=12) were collected from 5 different freshwater fish such as Rohu (*Labeo* spp.), Mangur (*Clarias* spp.), Tengar (*Mystus* sp.), Baikari (*Clupisoma* sp.) and Pungas (*Pangasius* spp.). Among the 120 isolates only 22 *Lactobacillus* isolates were selected for further study. Based on morphological, biochemical characteristics, the isolates were identified as *L.plantarum* (19 isolates), the predominant species isolated from 4 variety of fish (*Labeo* sp., *Clarias* sp., *Mystus* sp., *Clupisoma* sp.) and *L.casei* (3 isolates) found only in *Pangasius* sp. Identified species of *Lactobacillus* were assessed for *in vitro* probiotic properties such as tolerance to bile, resistance to low pH values, antagonistic activities and antibiotic susceptibility pattern. *In vitro* results showed that only 2 strains (1 of *L.plantarum* and 1 of *L.casei*) were able to meet the requirements. In the presence of relatively low pH (2) and high oxgall concentration (1%), *L.plantarum* showed higher resistance (pH 2: 7CFU/ml; 120min.) and tolerance at (1%: 31CFU/ml; 120min) than *L.casei*. Using agar well diffusion method, both the species elicited antagonism against gram-negative fish pathogens, *A.hydrophila* and *E.tarda*. *In vitro* experiment reveals stronger antimicrobial actions of *L.casei* against *A.hydrophila* (23.5 ± 0.5 mm at 0.2% glucose conc.) and *E.tarda* (22 ± 2 mm at 2% glucose conc.) than of *L.planatarum*. All the isolates of *Lactobacillus* when tested to 10 different types of antibiotics appeared more susceptible to most of the antibiotics and observed low resistance. However, the results obtained in this *in vitro* experiment can only give an indication of *in vivo* success, future research should include lactobacilli-based probiotic candidate from fish derived for large-scale *in vivo* experiments.

KEYWORDS: **Probiotic, *L.plantarum*, *L.casei*, *A.hydrophila*, *E.tarda*.**

SANTOSH KUMAR MISHRA
Department of Biotechnology, IMS Engineering College, Ghaziabad, INDIA

INTRODUCTION

Probiotic bacteria would be found to be useful not only as food but also serves as biological controllers of fish disease and activators of nutrient regeneration[1.] Pathogenic bacteria are the causative agents of bad water quality, stress and diseases as they act as primary and secondary pathogens [2] . Aquaculture is farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants, is one of the fastest growing food producing sectors globally. Disease outbreaks are being increasingly recognized as a significant constraint on aquaculture where fish are stocked at high intensities and subjected to stress, thus affecting the economic development. Current method of preventing infectious microbial diseases includes vaccinations and antibiotics or chemotherapeutics. However, the massive use of antimicrobials for disease control and growth promotion increases the selective pressure exerted on the microbial environment and encourages the natural emergence of bacterial resistance. In recent years, "Probiotics precisely mono or mixed cultures of live microorganisms is anticipated as an excellent strategy to substitute the existing contstraints. The term "Probiotics" refers to the Gram-positive bacteria associated with genus *Lactobacillus*, A range of bacteria has either been suggested and/or biological control agents in aquaculture. It has been described that the presence of LAB(Lactic Acid Bacteria), including *Lactobacillus* in the intestine of various fish species at larval, fry and fingerling stages inhibiting ponds in ukarine: *Vibrio alginolyticus* was found to reduce disease in Atlantic salmon (Salmosalar) caused by infection of pathogenic strains (*Aeromonas salmonicida, anguillarium*[3]. Antagonistic activity of *L.plantarum* 44 was reported against *Aeromonas hydrophila* at p4 levels of 3.5 and 4.5 [4]**.** Although some of lactic acid bacteria such as *Lactobacillus plantarum* isolated from soy milk, the antimicrobial activity of the bacteriocin produced by the isolate against the pathogenic bacteria and antibiotic resistance of these isolates[5] The present work was to isolate and identify probiotic bacteria from freshwater fishes and screen for their antagonistic activity against common fish pathogens, *in vitro.*

MATERIALS AND METHODS

Collection of Fish intestine samples

In the present study 12 individuals were from 5 different varieties of fish (*Labeo* sp., *Clarius* sp., *Mystus* sp., *Clupisoma* sp., *Pangasius* sp.) were collected from the city, Allahabad. The fish was sacrificed by physical destruction of brain and the number of incidental organism was reduced by washing the fish skin with 70% ethanol. After dissecting the fish, 1 gm of the intestinal content of each fish was homogenized in 9 ml of sterile saline. After dilution, the sample was pour-plated on MRS agar plates, incubated an aerobically at 37°C for 48-72 hrs, identified strains were kept in MRS broth 10% (v/v) glycerol at -20°C.

Pathogens

To detect antagonistic activity of the isolated *Lactobacillus* sp., pure cultures of indicator bacteria *A.hydrophila* (MTCC No.1739)*, E.tarda* (MTCC No.2400) were obtained from the Microbial Type Culture Collection, IMTECH, Chandigarh.

Identification of the Lactobacillus species

The cultures were identified according to their morphological, cultural, physiological and biochemical characteristics [6]**.** The isolates were Gram stained, observed for motility, production of catalase and cytochrome oxidase. Tested for acid production from carbohydrates 1% (w/v) (i.e. L-Arabinose, Lactose, Glucose, Mannose, Maltose, Mannitol, Sorbitol, D-xylose, Trehalose, D-galactose, D-fructose, Sucrose, Salicin, Esculin, Melibiose, D-cellobiose, Raffinose, Ribose, Inulin), Hugh and Leifson's test in O/F/medium, production of indole from tryptone broth, H_2S from SIM and nitrate reduction in nitrate broth. Growth at 15 and 45 ºC on MRS medium for 1 week was recorded; ability to grow at different concentrations of NaCl (0.5%, 5.0%, 8.0%, 10% and 12%) was monitored at 600 nm after 24 hrs. of incubation.

Screening of the Isolated Lactobacillus species for Probiotic properties

Tolerance to acidic pH

Acidification was measured by change in pH (ΔpH) . Standardized assay was maintained with an inoculum amount to 10^5 CFU/ml [7]. The prepared inoculum was poured to previously adjusted MRS broth (pH values 2 and 4) with 1M HCl and 0.5M NaOH. Tubes were incubated at 37ºC and survival of *Lactobacilli* was observed for 30, 60, 90 and 120 minutes.

Tolerance to Bile salt

MRS broth tubes enriched with 0.0, 0.5, and 1% (w/v) of oxgall were cultivated with the standard inoculum prepared (10^5 CFU/ml) [8]. Viable cell counts were examined after 30, 60, 90 and 120 minutes of incubation at 37ºC by plate count method [7].

Antagonistic activity

Antagonistic activity of the selected *Lactobacillus* species against indicator bacteria (*A.hydrophila* MTCC No.1739, *E.tarda* MTCC No.2400) was determined by agar diffusion method [9]. The effect of the cell-free supernatant from the previously grown culture of *Lactobacillus* in modified MRS broth containing 0.0%, 0.2%, 2%, glucose was observed on nutrient agar plates. Inhibition was recorded after 24 hrs. of incubation by measuring the diameter of the absence of growth of pathogens around the wells.

Antibiotic Susceptibility Test

Bacterial antibiotic resistance was determined on MRS using agar disc diffusion method [10] against 10 different types of antimicrobial agents (i.e. Amikacin: 30µg, Chloramphenicol: 30µg, Carbenicillin: 100µg, Cefuroxime: 30µg, Ciprofloxacin: 5µg, Erythromycin: 15 µg, Gentamicin: 10µg, Rifampicin: 5µg, Nalidixic acid: 30µg and PolymixinB: 300µg**)**. The resistance was those defined by National Committee for Clinical Laboratory Standards (NCCLS, 1999) for the isolated species of *Lactobacillus*.

Statistical Analysis

Statistical analysis using Chi-square test of independence of attributes was applied to find the incidence of *Lactobacillus* species. Analysis of Variance (two-way classification) was used to investigate significance of different probiotic attributes on the isolated *Lactobacillus* species.Antagonistic activity was interpreted using two-way ANOVA 'm' per cell. A significance level of $p<0.05$ was used.

RESULTS

In the present study, 12 intestinal samples of 5 different freshwater fish such as Rohu (*Labeo* sp.), Magur (*Clarias* sp.), Tengra (*Mystus* sp.), Baikari (*Clupisoma* sp.) and Pungas (*Pangasius* sp.) was collected, a total of 22 *Lactobacillus* isolates were isolated. The sampled fish ranged 27-38cm (avg. 33.61±3.3cm.) in length and 250-850gm (avg.487.5±180gm) in weight. (Table 1)
Based on morphological, cultural, physiological and biochemical characteristics, among 22 isolates of *Lactobacillus*, 19 isolates were of *L.plantarum* and 3 isolates of *L.casei*. The differentiating characteristics of *Lactobacillus* species are given in Table 2. All the isolates were observed Gram positive, catalase-negative, able to grow at 15 and 45ºC. Strains showed variation in their sugar fermentation pattern (Table 3). Among the biochemical tests such as motility, H_2S production, indole and nitrate reduction were found to be negative. The growth was optimum between 0.5-5 % (w/v) NaCl for the isolated species. Statistical evaluation revealed significant results for the incidence of

Lactobacillus species among five different varieties of fish.

Among 22 isolates of *Lactobacillus*, one strain each of *L. plantarum* and *L. casei* gave promising results to *in vitro* selection probiotic criteria such as acid, oxgall challenge and antagonistic activity. HCl proved to be more harmful than oxgall (Fig.1- 4). Acid challenge at pH 4 for 120 min. observed resistance for both the species, while at pH 2 for 120 min differs, as viable number of *L. plantarum* reached to 7 CFU/ml but *L. casei* was found to be sensitive (0 CFU/ml). Tolerence to detrimental actions of oxgall (0.5%; 120 min.) recorded for both the species of *Lactobacillus*. To 1% oxgall; *L.casei* was more sensitive (7 CFU/ml; 120 min.) than *L. plantarum* (31 CFU/ml; 120 min.).

The antagonistic activity at pH 6.5 was effective in all fractions of modified MRS (i.e. 0.0, 0.2, and 2.0%) against the indicator fish pathogen (Table 4). Stronger antibacterial action was observed for *L. casei* against *A. hydrophila* at 0.0% and 0.2% glucose, with a zone of inhibition of 20± 0 mm, 23.5±0.5 mm respectively. Moreover, inhibitory action at high glucose concentration (2%) was also shown by *L. casei* against *E. tarda* (22±2 mm) and 21±0 mm against *A .hydrophila* than of *L. plantarum.* The resistance pattern obtained with the isolated species of *Lactobacillus* against 10 antibiotics is shown in Table 5. Observations indicated that both the species of *Lactobacillus* were susceptible to most of the antibiotics and low resistance was found which can be considered a positive trait for bacteria employed in probiotics.

Table 1
Incidence of Lactobacillus species from different varieties of fish

S.no.	Fish variety	No. of samples	Positive isolates	Incidence	
				L.plantarum	*L.casei*
1.	*Labeo* sp.	04	05	05	0
2.	*Clarias* sp.	02	08	08	0
3.	*Mystus* sp.	02	04	04	0
4.	*Clupisoma* sp.	02	02	02	0
5.	*Pangasius* sp.	02	03	0	03

Table 2
Differentiating characteristics of Lactobacillus species

Characteristics	Isolate 1	Isolate 2
No of Isolates	19	03
Cultural characteristics		
Colour on MRS	Off white	Off white
Growth	Luxuriant	Luxuriant
Texture	Smooth	Smooth
Edge	Entire	Entire
Elevation	Raised	Raised
Morphological characteristics		
Shape	Short rods	Short rods
Gram stain	+	+
Physiological characteristics		
Catalase activity	-	-
Oxidase Test	+	+

Growth at 15°C and 45°C	+	+
Motility test	-	-
H_2S production	-	-
Indole production	-	-
Nitrate reduction	-	-
Hugh and Leifson's test	+	+

(+) positive reaction (-) negative reaction, Numbers are the positive isolate.

Table 3
Carbohydrate fermentation pattern

Substrate	Strains	
	Isolate 1	**Isolate 2**
Glucose	+ A	+ A
Galactose	+ A,G	+ A
Arabinose	+ A	-
Mannose	+ A,G	-
Mannitol	+ A,G	+ A
Maltose	+ A,G	-
Lactose	+ A	-
D-Sorbitol	+ A,G	+ A
D(+) Xylose	+ A	-
Trehalose	+ A	+ A
D(-)Fructose	+ A	+ A
Sucrose	+ A,G	+ A
Salicin	+ A,G	+ A
Melibiose	+ A,G	-
D-Cellobiose	+ A,G	+ A
Raffinose	+ A	-
Ribose	+ A	+ A
Inulin	-	-
Identified Isolates	***L.plantarum***	***L.casei***

(+) positive reaction,(-) negative reaction, (A) acid, (G) gas

Table 4
Antagonistic activity of Lactobacillus species against common fish pathogen

S.no.	Isolates	Glucose(%)	Zone of inhibition (mm)	
			A.hydrophila	*E.tarda*
1.	*L.plantarum*	0.0	15.5 ± 3.5	12.5 ± 2.5
		0.2	20 ± 5	21 ± 1
		2.0	16.5 ± 3.5	19.5 ± 0.5
2.	*L.casei*	0.0	20 ± 0	14 ± 1
		0.2	23.5 ± 0.5	19 ± 2
		2.0	21 ± 0	22 ± 2

Table 5
Antibiotic Susceptibility profile of the isolated Lactobacillus strains

S.no.	Antibiotic	(µg/disc)	Isolates	
			L.plantarum	*L.casei*
1.	Amikacin	30	+++	+++
2.	Chloramphenicol	30	+++	+++
3.	Carbenicillin	100	+++	+++
4.	Cefuroxime	30	+++	++
5.	Ciprofloxacin	5	+++	+++
6.	Erythromycin	15	++	+++
7.	Gentamicin	10	+++	+++
8.	Rifampicin	5	-	++
9.	Nalidixic acid	30	++	-
10.	PolymixinB	300	+++	++

Sensitive (+++), Intermediate (++), Resistant (-)

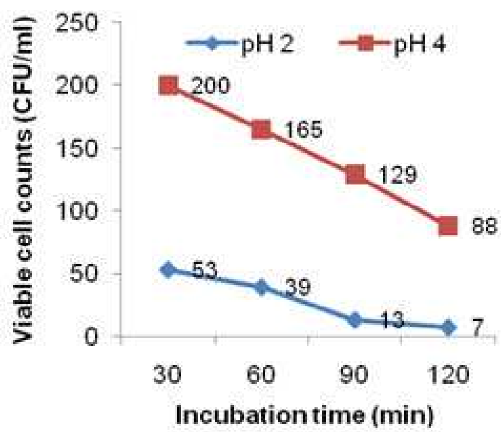

Figure. 1
Effect of different pH level on the growth of L.plantarum.

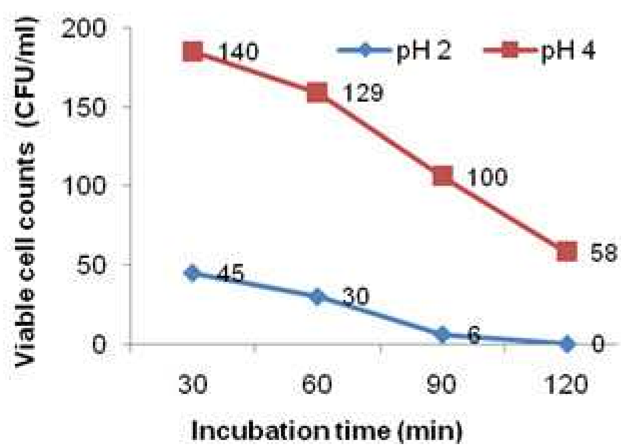

Figure. 2
Effect of different pH level on the growth of L.casei.

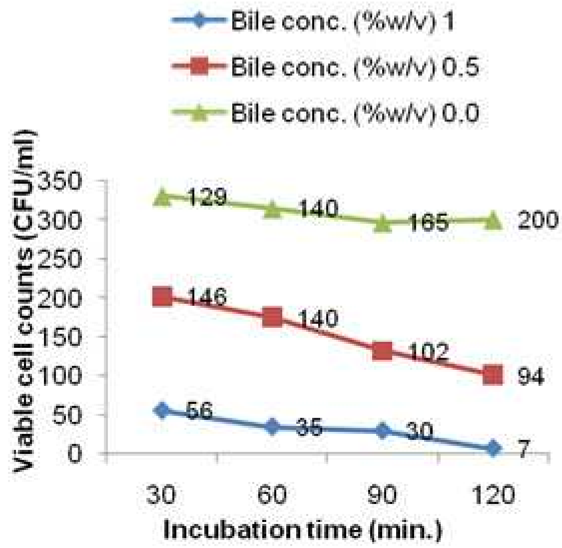

Figure. 3
Effect of different Bile concentrations (%w/v) on the growth of L.plantarum.

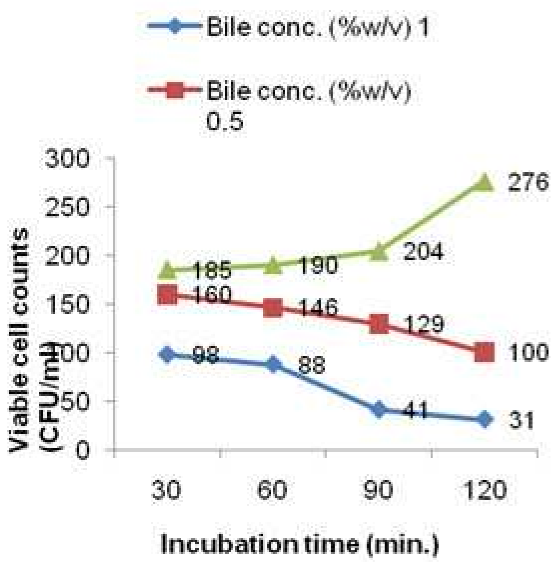

Figure. 4
Effect of different Bile concentrations (%w/v) on the growth of L.casei.

DISCUSSION

The use of probiotics for disease control in aquaculture is an area of interest, as the use of antibiotics is causing concern over the possible development of antibiotic-resistant bacteria. Probiotics have been defined by the World Health Organization-Food and Agriculture Organization, as "live microorganisms" which when administered in adequate amounts, confer a health benefit on the host. In the past decade, several gram-negative and gram-positive bacteria have been evaluated in the *in vitro* or *in vivo* for their potential to inhibitpathogenic organisms and overcome infections in fish and larvae in aquaculture[11]. In the present study 5 different freshwater fish, such as Rohu (*Labeo* sp.), Magur (*Clarias* sp.), Tengra (*Mystus* sp.), Baikari (*Clupisoma* sp.) and Pungas (*Pangasius* sp.) were collected and screened for *Lactobacillus* species. Among 5 different varieties of fish, the presence of *Lactobacillus* in *Clarias* sp. and *Labeo* sp. Shows the resemblance with the findings of other researcher who reported maximum population of *Lactobacillus* in catfish (*Clarias orientalis*) and 10 isolates in Rohu (*Labeo rohita*) [12]. The isolation of 44 strains of *Lactobacillus* from the gut contents of African catfish (*Clarias gariepinus*), further supports the presents study[13]. The isolates of *Lactobacillus* were culturally, morphologically and biochemically characterized in two groups; *Lactobacillus plantarum* (19 isolates) and *Lactobacillus casei* (3 isolates). It is interesting to note that majority of the *Lactobacillus* species that have been isolated from adult fish were those species, which were commonly found on meat, animals and human [14]. *Lactobacillus* species found in this study were entirely different to the species described by already reported species. The author found most frequently occurring isolates: *L.plantarum*, *L.casei*, *L.leichmannii*, *L.acidophilus*, *L.fermenti*, *L.cellobiosus and L.buchneri* [3]. However, in the present study only the species *L.plantarum* and *L.casei* were similar. This may be the result of different hosts, different habitats, or totally different identification procedures. *Invitro* tolerance to acidic pH, both the species showed resistance at pH 4 for120 min. This finding fell close to the observations in which 100% survival of 43 strain of *L.plantarum* isolated from Thai fermented food products containing fish or pork at pH 4 after 2 hrs. of incubation [15]. Similar results were reported that shows the adaptation of *L.casei, L.acidophilus*, *L.delbrueckii* subsp*. lactis*, *L.plantarum* and *L.rhamnosus* at pH 4[16]. Acid challenge at pH 2 for 120 min. reflects a substantial decrease in the number of survivors as the exposure time progress. *L.casei* was found to be more sensitive (pH 2; 120 min.) as its viable number reached zero. This has been previously recorded viable cells of 7 strains of *L.casei* at pH 2 and 3 hrs. Comparing to this, results of *L.casei* does not agree. These observations indicate that the ability of probiotic strains to survive acidic conditions varies widely among strains and species [17].

The sensitivity of both species of *Lactobacillus* to oxgall stress revealed survival at all concentrations (i.e. 0.0%, 0.5% and 1%) for 120 min. To 1% oxgall for the studied period of incubation demonstrated variable susceptibility; *L.casei* exhibited more sensitivity (7 CFU/ml; 120 min.) than *L.plantarum* (31CFU/ml; 120 min.). The resistance ability to bile salts varies a lot among the lactic acid bacterial species and even between strains themselves [18,19]. Many authors investigated the effect of bile on survival of LAB. Researchers demonstrated improved viability of strains of *L.plantarum* and *L.fermentum* (origin: feaces of 3-6 months old breast fed infants) when exposed to oxgall 0.4% for 3 hrs.[20]. In a similar study shows observation of improved viability of *L.plantarum* at 1% bile salt after 4 hrs. than *L.rhamnosus* [21]. Comparing the above findings with the present study, the results are much more promising with *L.plantarum* while observations of *L.casei* are in partial satisfaction.

In a study no inhibitions were found by normal cell free supernatant of *L.plantarum* against *A.hydrophila* and *E.tarda* [22]. In a Similar study in *L. plantarum* 44a exhibited inhibition zones of 12.4±1.3mm at pH 3.7 and 7.3±0.90mm at pH 4.5 against *A.hydrophila* [4]. These observations contradict with the results of present study, where the normal cell free supernatant of *L.plantarum* has shown inhibition of 15.5±3.5mm and 12.5±2.5mm against *A.hydrophila* and *E.tarda* respectively at pH 6.5. Microbial inhibition under limitation of glucose is a rather unique property displayed by *Lactobacillus* strains. In general, the antimicrobial activity of lactobacilli may be due to organic acids [23] & hydrogen peroxide bacteriocins [24]. Other inhibitory substances from metabolites, was evaluated the inhibitory potential of 55 strains of *Lactobacillus* at 0.0%, 0.2% and 2% glucose in MRS against *A.hydrophila*, *E.tarda* and *S.aureus,* reported highest antimicrobial potency at 2.0% glucose followed by 0.2% and no inhibition at 0.0% glucose concentration in the medium [21, 25]. Comparing to this study, our experiment showed greater levels of inhibition at 0.0% and 0.2% glucose concentration. It was observed that *L.plantarum* has shown maximum inhibition at low (0.2%) concentration of glucose against *E.tarda* (21±1 mm) than *A.hydrophila* (20±5 mm). Inhibition of these pathogens by selected lactobacilli at high and low glucose concentration is a desirable characteristic of probiotic candidate as it increases the chance of inhibition within the gastrointestinal tract where carbohydrate availability is fluctuating.

However, in the present study it was found that the normal cell free supernatant i.e. without any glucose supplement (0.0%) and supernatant with 0.2% glucose of *L.casei* reports stronger antibacterial effect than *L.plantarum*. Moreover, stronger antibacterial actions at high (2.0%) glucose concentration was also shown by *L.casei*, 22±2 mm against *E.tarda* and 21±0 mm against *A.hydrophila* than of *L.plantarum*.

From the safety point of view, one of the crucial criteria is the knowledge of antibiotic susceptibility pattern of the isolated *Lactobacillus* strains. Since bacteria used as potential probiotic may serve as host of antibiotic resistance genes, which can be transferred to pathogenic bacteria. To date, knowledge on the susceptibility of LAB to antimicrobial agents is rather limited in view of large numbers of genera and species encountered in this group of bacteria apart from variances in the resistance spectra [26]**.**Tolerance of LAB to antibiotics is of interest due to their possible use of reconstitute the intestinal microflora. On the other hand, transmission of antibiotic resistance genes to unrelated pathogens or potentially pathogenic bacteria in the gut is a major health concern, with obvious ramification for the selection and safety of probiotic strains. From the 10 antimicrobial agents tested against 2 species of *Lactobacillus*, *L.plantarum* was susceptible to Amikacin, Chloramphenicol, Carbenicillin, Cefuroxime, Ciprofloxacin, Gentamicin and PolymixinB, while *L.casei* was reported susceptible to Amikacin, Chloramphenicol, Carbenicillin, Ciprofloxacin, Erythromycin and Gentamicin. These results were in support of a study undertaken to establish the levels of susceptibility of *Lactobacillus* spp. to various antimicrobial[27] & resistant to gram-negative spectrum antibiotic (nalidixic acid) and aminoglycoside antibiotics (amikacin, kanamycin, neomycin and streptomycin)[28]. This is in partial resemblance with the present study, *L.casei* showed resistance to nalidixic acid and *L.plantarum* to a protein synthesis inhibitor, rifamicin.

CONCLUSION

According to SCAN all bacterial products intended for use as feed additives must be examined to establish the susceptibility of the component strains to a relevant range of antibiotics. Since food chain can be considered as the main route of transmission of antibiotic resistant bacteria between animal and human [29]**.** However, the results observed offer a positive trait for the studied organism with low antibiotic resistance and thus maintains the probiotic selection criteria for food/feed productions. The present work highlights the isolated candidate can be a promising probiotic to be used in aquaculture, with regard to its high potential against aquatic pathogenic bacteria *in vitro.* However, future studies should be aimed at revealing the factors that determine the occurrence of *Lactobacilli* in fish along with the ability to adhere to host cells, mechanism of antagonistic action between the probiont and the pathogen, their competitive exclusion stability *in vivo*, is further needed.

CONFLICT OF INTEREST: Conflict of interest declared none

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