Antimicrobial Action and Biopreservation by Autochthonous Lactic Acid Bacteria of Fish

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Abstract The purpose of this research was to assess native Lactic Acid Bacteria (LAB) for antimicrobial activities in relation to fish preservation. LAB were isolated from intestine of *Oreochromis* sp. *Listeria monocytogenes*, being a big microbial hazard of fresh as well as preserved fish food, was chosen against LAB antimicrobials. In the present study sixteen LAB like isolates from fish intestine were judged for their anti-listeria activities by agar well diffusion assay. Thirteen of them were antagonized *Listeria monocytogenes* (ATCC 657) by creating inhibition zone in nutrient agar plate. Antagonistic activities with inhibitory zones but not with growth circles clearly marked that inhibition was due to antimicrobials but not for competitive exclusion. LAB like bacteria in different forms like live cells, cell-free supernatant, alkaline cell-free supernatant and heated cell-free supernatant conferred antibacterial activities. No acid and hydrogen peroxide inhibition was reported. Except few, most of the alkaline and catalase treated culture supernatant when being heated to 90°C exhibited strong antagonism against the pathogenic strain. Susceptibility of *Listeria monocytogenes* to LAB antimicrobials seemed to be caused by both thermostable and thermolabile compounds and could be practiced in fisheries industry for food hygiene and also as biopreservatives.

Keywords Antimicrobials, Biopreservatives, LAB, *Listeria monocytogenes*, Thermolabile, Thermostable

1. Introduction

Recently demand for fish food is very high because of high quality proteins, unsaturated fatty acids, vitamins and minerals [1]. Pathogen propagation through fish is a big threat [1]. Fresh fish is easily spoiled food compared to other food items. Owing to cross contamination by microbes from different sources, fishery products deteriorate very fast after cropping [2]. *Listeria monocytogenes* being food borne bacteria imposes a sententious health risk to human as a causative agent for listeriosis [2]. Fish could be infected by *Listeria* from its natural environments [2]. Processing and composing practices are the two main roots of *Listeria* entry in fish [1,3]. *Listeria monocytogenes* is considered as a hazard in food industry being pervasive, stress resistant, enduring, and psychro- and halotolerant [4] potentiality. Further, resistance to different antibiotics increases its threat [5,6]. Moreover, the situation becomes worst as *Listeria monocytogenes* remains alive and also flourish during handling and marketing of fish. [7] and [8] revealed low incidence of *Listeria* in freshwater fishes of India. [9] reported infrequent instances of Listeriosis in India. On contrary, [10-13] reported contamination of *Listeria monocytogenes* in fresh shell fishes as well as in fresh fin fishes from India. *Listeria monocytogenes* infected fish may impose a huge health risk to the Indian people as fish is an important part of diet for them.

Proper control measures of pathogenic microbes are important due to their role in health complications and food spoilage. Eradication and minimization of pathogenic and spoilage flora normally done by exercising different preservation techniques to obtain secure and

sound products. Currently preservation strategies of biological origin have been flourished. Unfortunately in fishery trading appliances of microbial cultures and their antimicrobials are very rare. After effects of extraneous protective cultures on intrinsic microbes as well as on discernible attributes of fish are the main reason behind these limitations [14]. Lethal peptides synthesised by bacterial ribosome, could be administered as unique antimicrobials in food security, preservation and also to avoid spoilage [15,16].

The aim of the present research paper was to place some basic data on the antimicrobial potentiality of the autochthonous microflora of fish to be applied in fisheries industry for food safety as well as for food preservatives. During the study fish intestine was screened for bacteriocins or killing peptides producing bacteria. Bacteriocins of LAB being GRAS (Generally Recognised as Safe) got the main focus in the present paper as natural food preservatives. In the present study, preliminary experiments were conducted to investigate possible inhibition of *Listeria monocytogenes* by culture and culture filtrates of LAB like bacteria.

2. Materials and Methods

2.1. Sample Preparation

Twenty live fish samples [Nile tilapia (*Oreochromis niloticus*)] were collected from local fish markets of Krishnagar and Santipur (bimonthly from March 2019 to February 2020). The collected samples were brought to the college laboratory in an ice box under full aseptic environment. Dissection, weighing, homogenization and serial dilutions of homogenates of the digestive tract contents of fish were done following [17,18]. Collection to investigation of fish was done within the time limit of 6 hrs.

2.2. Bacterial Culture and Isolation

Standard spread plate count method [19] at 35°C for 24 hrs on de Man, Rogosa, and Sharpe (MRS) Lactocilli agar plates was done for the determination of LAB like bacteria in anaerobic gas chamber. Bacteria with distinct morphologies from MRS agar plates were streaked aseptically several times on freshly prepared MRS Agar plates to obtain pure isolates.

Listeria monocytogenes (ATCC 657) was obtained from American Tissue Type Culture Collection (ATCC), Hi Media Laboratories Ltd., Mumbai, India. The food spoilage bacteria was then inoculated on non selective media like nutrient agar plate and incubated at 35°C for 24 hrs. Its growth was confirmed further on respective selective agar i.e. *Listeria* Selective Agar Base with *Listeria* Selective Supplement for *Listeria*. Agar well diffusion method [20,21] was used to observe inhibitory

actions of different LAB like isolates against *Listeria monocytogenes* (ATCC 657).

2.3. Antibacterial Inhibition VS. Incubation Time

The effect of incubation time versus antibacterial activity was studied by inoculating MRS broth with LAB like bacteria from MRS agar plates. Broth cultures were incubated separately at 35°C for 24 hrs and 48 hrs in anaerobic condition. 50µl (10^9 CFU/ml, CFU= colony forming unit) of indicator pathogenic bacteria [22], from 24 hrs nutrient broth, were spread on nutrient agar plates. Nutrient agar plates were then pierced with wells of approximately 0.5mm diameter. 50µl [23] concentrations of LAB like bacteria containing 10^9 CFU/ml [22] from respective hrs of MRS broth cultures were poured into the well and then incubated at 35°C for 24 hrs.

2.4. Bacteriocinogenic Activity of CFS

LAB like bacterial isolates from 48 hrs MRS Broth cultures, after incubation, were subjected to centrifugation at 8000 g for 10 min at 4°C [24] to eliminate cells from the growth medium and to obtain unrefined cell-free supernatant (CFS). Filtration [24] of the unprocessed supernatants was performed through syringe-driven filter membranes [0.20 mm pore size] to eradicate any remaining bacterial cell. The bacteriocin-like compound's activity was carried out by standard well diffusion method for filtered CFS. Cell free supernatants (50 µl) of different LAB like isolates [23] were added in wells on nutrient agar plates which were formerly spread with 50 µL of indicator strain. The nutrient agar plates were incubated for 24 hrs at 35°C.

2.5. Antimicrobial Activity of Various Treated CFS

2.5.1. Removal of Organic Acid

To get rid of the effect of organic acids, pH of the filtered CFS was calibrated to 9 using 1(M) NaOH solution [25] and then inhibitory activity of that alkaline CFS (ACCFS) was analysed against the indicator strain, *Listeria monocytogenes* (ATCC 657) by standard well diffusion method.

2.5.2. H₂O₂ Elimination

The alkaline CFS was treated with catalase (ACCFS) [24] and evaluated further for antimicrobial activity.

2.5.3. Heat Sensitivity Testing

Alkaline and catalase treated CFS was incubated at 90°C for 10min [22], and then inhibitory activity of that CFS (HACCFS) was inspected against the indicator strain by standard well diffusion method.

The diameters of the inhibition zones were measured following [26].

3. Results

Round, small non pigmented and white to cream coloured colonies were primarily chosen from MRS agar surface. Isolates with LAB attributes like Gram-positive, rod shaped structure, catalase-positive were selected. Sixteen distinct LAB like isolates were maintained in 5 sets (A Series, E Series, F Series, G Series and H Series). Combative activities of sixteen LAB like isolates were represented through Table 1. Agar spot evaluation

exhibited inhibitory zones relating antimicrobial compounds, no growth circles were detected (Figure 1,2,3). Furthermore, anti-listeria action was found to be greater at 48 hours than 24 hrs respectively (Figure 2). The relationship of the inhibitory activity with the incubation time clearly showed that most of the isolates effectively had reached their log phase of growth at 48 hrs and inevitably produced optimum antimicrobials.

Table 1. Antibigram of LAB like isolates of fish against the tested indicator strain *Listeria monocytogenes* (ATCC 657)

LAB	<i>Listeria monocytogenes</i>						
	Effect of 24 hrs incubated LAB culture	Effect of 48 hrs incubated LAB culture					
	Antimicrobial effect of cell	Antimicrobial effect of cell	Crude CFS	Filtered CFS	Neutralized CFS	Neutralized CFS+ catalase	Neutralized CFS+catalase+ heat treatment
A1	-	+	+	+	+	+	-
A2	-	+	+	+	+	+	+
A3	-	-	-	-	-	-	-
A4	-	-	-	-	-	-	-
A5	-	+	+	+	+	+	-
E1	+	+	+	+	+	+	+
E2	-	+	+	+	+	+	+
E3	-	+	-	-	-	-	-
F1	-	+	+	+	+	+	+
F2	-	+	-	-	-	-	-
F3	-	+	+	+	+	+	+
G1	-	+	+	+	+	+	+
G2	-	+	+	+	+	+	+
H1	+	+	+	+	+	+	+
H2	-	-	-	-	-	-	-
H3	+	+	+	+	+	+	+

+ = Growth occurred

- = No Growth

Figure 1.a)

Figure 1.b)

Figure 1.c)

Figure 1. [a+b+c] Inhibitory Zones of LAB like bacteria in Agar Diffusion Method

68.75% CFS from LAB like bacteria was found to be effective (Table 1) against *Listeria monocytogenes* (ATCC 657). Filtered CFS after being alkaline with NaOH and treated with catalase remained successful (Table 1) against *Listeria monocytogenes* (ATCC 657). The data advocated that organic acids and hydrogen peroxide were not liable for inhibition of *Listeria*

monocytogenes(ATCC 657) (Table 1 and Figure 3). Activities of major LAB CFS remained durable at 90°C after 10min of incubation. However, limited decrease in inhibition zone was detected after being heated at 90°C for 10 min as compared to inhibition zone of cell-culture or alkaline CFS (Figure 4a to 4e).

Figure 2. Effect of Incubation time on Inhibitory activities of LAB

Figure 3. Diverse mode of Antilisterial Activities of LAB

Figure 4. Antilisterial activity exhibited by inhibitory zones of different diameters (mean $\pm$ SE, $n = 5$) of individual LAB strains against *Listeria monocytogenes* (ATCC 657) (a), A series(b), E Series (c), F Series (d), G Series (e), and H Series

Thus thermostable products, probably proteins were reported as most potent inhibitory agent against *Listeria monocytogenes*. Few thermolabile products were also found to be impressive against *Listeria monocytogenes*. Our results further revealed that antimicrobials produced by nine LAB isolates (Table 1 and Figure 3) furnished thermotolerant and alkaline pH secured attributes. Two LAB like bacteria generated antagonistic activity towards *Listeria monocytogenes* exclusively in the form of live cells (Table 1 and Figure 3). Moreover, thermolabile inhibition against *Listeria* was also proclaimed by two intestinal LAB isolates (Table 1 and Figure 3).

4. Discussion

The study culminated extraction and execution of antibacterial activity of LAB like bacteria from Tilapia. The present paper reported divergent susceptibility of a single strain of *Listeria monocytogenes* (ATCC 657) to antilisterial compounds produced by different LAB like bacteria isolated from fish intestine. Agar spot method with clean inhibitory zones of different diameters indicated synthesis of antimicrobials but not competitive exclusion as reason of restrains actions. Impact of growth conditions on inhibitory activity revealed that, time played an important role in cell growth as well as in antimicrobial activity. Most of the antimicrobials of LAB were sufficiently opposed gram positive *Listeria monocytogenes*. This further confirmed the view of [27] that bacteriocins wipe out closely related bacteria. Detention of the strength of both thermostable and thermolabile antimicrobials at alkaline pH could not approve the antilisterial compounds of the present study as

niscin[28] or enterocin [29] like bacteriocins. Moreover, heat resistant and high pH tolerance capacity stipulated the unspecified thermostable products as pediocin [30]. Furthermore, some undefined thermolabile compounds with high pH resilience elicited the inhibition to *Listeria monocytogenes*. Some of the antilisterial effect seemed to be caused by substances connected with live cells. These strains or their antimicrobials thus show some aptitude as fish preservatives in the fishery industry. Biopreservation with bacteriocins are always preferred as chance of entry of immune genes of bacteriocins from protective strains to pathogenic bacteria is high through conjugation or transposon as genes and immune gene of bacteriocins exist on the same plasmid or in adjacent regions of a chromosome [31].

5. Conclusions

The concept of the research is aspiring as autochthonous LAB like strains or their bacteriocins are enforced to prolong storage life of fish which could depreciate the proportion of change of microbial ecosystem as well as organoleptic modifications of the fish product. Isolated LAB like strains being probiotics would perform as curative, contraceptive and additives in buyer's fitness. However, LAB like isolates should be investigated for biogenic amines production and for multiple antibiotic resistances. The paper further emphasized application of protein like thermolabile and thermostable products over protective culture for storage quality improvement of fish. Protein like thermolabile and thermostable products of the research work could be a replacement to antibiotics in fish industry. However, investigation into resistance of protein

like antilisterial compounds is needed. Moreover, being proteins these antimicrobials are safe food supplements as digested by proteases of the gastrointestinal system. Finally in vivo application of antimicrobials in fish is required for inspecting the consequences of chemical and physical conditions of food on efficacies of antilisterial products.

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