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# Native Antimicrobials of Tilapia (*Oreochromis sp.*) Against Pathogenic Gram-negative Bacteria

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**Abstract:** Antimicrobials have many therapeutic, medicinal and industrial applications. However, restricted inhibitory spectrum of antimicrobials on distantly related species limits their wide applications. In the present study, authenticity of this contradiction had been investigated by evaluating amplitude of obstruction of indigenous antimicrobials of fish under different physical and cultural conditions against distantly related gram-negative pathogenic bacteria, *Aeromonas hydrophila* and *Pseudomonas aeruginosa.* Antimicrobials were examined for repression against pathogenic strains by agar well diffusion assay. Antimicrobials were more suppressive towards *Aeromonas hydrophila* than *Pseudomonas aeruginosa*. Antagonism by protein like thermolabile products was promising against *Aeromonas*. No inhibitions were outlined against gram-negative pathogens by protein like thermostable products. Native thermolabile proteinaceous antimicrobials of the present study are superior substitute for therapeutic applications over protective culture and antibiotics with least consequences on sensory, nutritional and microbial attributes of fish.

**Keywords:** Fish, Gram-negative pathogen, Indigenous antimicrobials, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*

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## Introduction

Bacteria harbor several functions and use different strategies to survive in microbial communities or to compete for nutrients and colonize space in their habitat (Hibbing *et al.,* 2010). One of the well-known strategies performed by bacteria to effectively inhibit the growth of other microorganisms is antagonism (Russel *et al.,* 2017). The production of antimicrobials like hydrogen peroxide, fatty acids, organic acids, ethanol, lytic enzymes, and bacteriocins is an indication of microbial antagonism (Roces *et al.,* 2012; Dobson *et al.,* 2012; Alvarez-Sieiro *et al.*, 2016). Out of the metabolites, bacteriocins, ribosomally synthesized cationic peptides, have attracted many researchers and have been studied extensively (Silva *et al.*, 2018). Unfortunately,

majority of bacteriocins are commonly known to inhibit closely related strains and have limited inhibitory effects against Gram-negative bacteria. Actually Gram-negative bacteria are resistant to many antimicrobial substances due to the impenetrability of their outer membrane (Christaki *et al.,* 2020). However, few studies have found that bacteriocins display a stronger antipathy to not only Gram-negative bacteria but also to certain yeasts, parasites, and cancer cells (Kaur and Kaur, 2015; Baindara *et al*., 2018). Thus, contradiction arises due to reported cases of narrow antimicrobial spectrum of bacteriocins and outcomes of other studies towards power of bacteriocins to kill different genera. In the present study, the reason behind this contradiction had been investigated by testing efficacy of bacteriocin-like substances from Gram-positive bacteria against two distantly related Gram-negative bacteria namely *Pseudomonas aeruginosa* and *Aeromonas hydrophila.*

In aquaculture *Aeromonas hydrophila* is considered as one of the foremost economically important bacterial pathogen because its infection leads to growth retardation and unmarketable appearance of infected fish. *Aeromonas* is an opportunistic pathogen and responsible for motile aeromonad septicemia (MAS) and aeromonad-associated diseases like epizootic ulcerative syndrome (EUS) in fish (Harikrishnan and Balasundaram, 2005). *Aeromonas* also increasingly being reported as important pathogens, since they have been associated with a broad spectrum of human diseases, including septicemia, meningitis, wound infections, and gastro-enteritis (Wadstrom and Ljungh, 1991). *Pseudomonas* species have been narrated as fresh water fish pathogens and affiliated with haemorrhage or skin and eye lesions under stressful ambience (Muhammed *et al*. 2021). *Pseudomonas aeruginosa*, a Gram-negative, multidrug resistant, opportunistic pathogen typically targets immune compromised people and is frequently responsible for hospital-acquired pneumonia and various sepsis syndromes (Yasmin *et al.,* 2006). Moreover, both *Aeromonas* and *Pseudomonas* are the two predominant spoilage floras of fish (Chinivasagam *et al.,* 1998; Pantazi *et al.,* 2008; Hozbor *et al*., 2012; Mace *et al.,* 2012; Zhu *et al.,* 2015; Zhu *et al*., 2016).

We undertook this *in vitro* study to assess antagonistic activities of indigenous antimicrobials of fish under different physical and cultural conditions against two distantly related Gram- negative indicator pathogenic strains. Intrinsic antimicrobials from intestinal lactic acid bacteria (LAB) were being surveyed.

## Materials and Methods

### *Isolation of LAB from fish intestine*:

Live tilapia fish (*Oreochromis sp.*) samples, five in numbers were collected monthly between February 2022-April 2022 from local fish markets of Krishnanagar and Santipur of Nadia districts, West Bengal, India. The reasons behind Tilapia had been chosen for the present research work was because of its easy availability and no concern related to conservation. The collected fish samples with average body weight of 100 g were immediately transported to the laboratory in an ice box under full aseptic environment. The exterior of the fish was wiped clean with 70% ethanol to eliminate the risk of contamination. The whole intestine was aseptically removed from abdominal cavity by dissecting abdomen at the ventral midline and then weighed. One gram of each intestine sample was homogenized and serially diluted in sterilized water (Buras *et al.*, 1987; Sanyal and Banerjee, 2013).

Spread plate count method (APHA, 1998) was done on de Man, Rogosa, and Sharpe (MRS) Lactobacilli agar plates at 35°C for 48 h anaerobically to obtain LAB like bacteria. Distinct colonies were then picked using a sterile wire loop and transferred onto freshly prepared MRS agar plates several times incubating at 35°C for 48 h until pure isolates were established and to be sure that dominant colonies were not contaminated.

The isolated strains on *Lactobacillus* selective media (MRS agar) were subjected to some lactobacillus specific biochemical tests like colony

characteristics, morphology of isolates, Gram stain reaction, oxidase test, catalase test, methyl red test corresponding to the method as delineated in literature (Menconi *et al*., 2014). The pure cultures were overlaid with glycerol and preserved at -20°C for further study.

*Extraction of Pathogenic bacteria*:

*Pseudomonas aeruginosa* and *Aeromonas hydrophila* were obtained from American Tissue Type Culture Collection (ATCC), Hi Media Laboratories Ltd., Mumbai, India. These pathogenic bacteria were then inoculated on non-selective media like nutrient agar plate and incubated at 35ºC for 24 h. Their growth was confirmed further on respective selective agar i.e. Aero Pseudo Selective Agar.

*Bacteriocinogenic Activity of LAB and its CFS*:

The isolated LABs were screened for antimicrobial activity against indicator strain, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* using agar well diffusion method on nutrient agar plates (Yaakoubi *et al.,* 2009). 50 µl indicator bacteria were cultured in nutrient broth for 24 h at 35°C and spread onto the surface of nutrient agar plates. Wells were created on the seeded nutrient agar plates and 50 µl of LAB culture was poured to each well (Balcazar *et al.*, 2008). The clear zone formed after incubation at 35°C for 24 h was measured as the activity of inhibiting lactic acid bacteria. $10^9$ CFU/ml (Složilová *et al*., 2014) (CFU= colony forming unit) of pathogenic as well as LAB bacteria were used for the study.

The cultures of LAB-like bacteria incubated at 35°C for 48 h in MRS broth were centrifuged (8000 ×g, 20 min, at 4°C) (Schelegueda *et al.,* 2015) and filter sterilized (0.20 mm pore size) and the antimicrobial properties of these cell-free supernatants (CFS) were tested to measure only the influence of extracellular metabolites of LAB. Filtered CFS (50 µl) (Jini *et al*., 2011) of different LAB like isolates were added in wells of approximately 0.5 mm diameter on nutrient agar plates which were formerly seeded with 50 µl of indicator strains. The nutrient agar plates were incubated for 24 h at 35°C. The presence of inhibition zone around the inoculated CFS was examined.

*Prohibitory Activity of differently Treated CFS*:

To investigate the chemical nature of the potential inhibitory substances of Lactic Acid Bacteria, filtered CFS were put through following treatments:

(a) To eliminate the effect of organic acids, pH of the filtered CFS was changed using 1(M) NaOH solution (Udhayashree *et al.,* 2012; Rammelsberg and Radler, 1990; Sanyal and Chatterjee, 2021) and then inhibitory activity of that alkaline CFS (ACFS, pH 7.5) was analyzed against the indicator strains by standard well diffusion method.

(b) The alkaline CFS was treated with catalase (2 mg/ml) (ACCFS) (Jini *et al.*, 2011; Sanyal and Chatterjee, 2021) and evaluated further for antimicrobial activity to get rid of the effect of $H_2O_2$.

(c) Effect of heat treatment: To examine the thermo stability of the inhibitory substances alkaline and catalase treated CFS was incubated at 90°C for 10 min (Složilová *et al*., 2014; Sanyal and Chatterjee, 2021) and then the residual antimicrobial activity of that CFS (HACCFS) was assayed by the well diffusion method against test pathogens.

(d) Estimation of proteinaceous nature of inhibitory substance: Reactivity of refined cell free supernatants was examined against proteinase K (Hi Media, India) and trypsin (Hi Media, India) (Sahnouni *et al.,* 2014). About 5 ml CFS was taken in test tubes and treated with proteinase K (2 mg/ml) and trypsin (2mg/ml) at pH 7. The test tubes with the enzyme were incubated at 37°C for 2 h. Then samples were assayed for antimicrobial activity by using well diffusion method.

*Statistical Analysis*:

Diameters of inhibition zones were expressed as mean± standard error (SE).

## Results

Bacterial isolates screened from tilapia intestine named as TI. Fifteen isolates with typical morphological and biochemical characteristics of lactic acid bacteria were preferred for further investigation. Small, milky white, circular, convex, elevated and non- pigmented, Gram-positive, catalase negative, oxidase test negative and Methyl red test positive cells were only considered.

Agar well diffusion assay displayed inhibitory zones relating to inevitable production of antimicrobials by most of the isolates (Fig. 1). Antipathy towards *Pseudomonas aeruginosa* was more evident at 48 h (Fig. 1). Conversely, inhibition against *Aeromonas hydrophila* was mostly prominent at 24 h (Fig. 1). *In vitro* antagonism against *Aeromonas hydrophila* and *Pseudomonas aeruginosa* was mainly sketched by live cells (Fig. 1).

CFS from LAB like bacteria was more effective against *Aeromonas hydrophila* than *Pseudomonas aeruginosa* (Table 1). Loss of inhibition of alkaline CFS manifested acid inhibition against *Aeromonas* and *Pseudomonas* (Table 1). Furthermore, the fact that activity of some of the alkaline CFS vanished when treated with catalase advocates association of hydrogen peroxide in some cases (Table 1).

Alkaline and catalase treated CFS (ACCFS) of only two isolates were promising against *Aeromonas hydrophila* (Table 1; Fig. 2). The loss of inhibitory activity from ACCFS after incubation with proteolytic enzymes supports its proteinaceous nature. On the other hand, CFS (HACCFS ) of the same two LAB isolates were not victorious against *Aeromonas hydrophila* (Table 1) after being heated at 90°C for 10 min. This stipulates thermolabile antagonism of LAB against *Aeromonas hydrophila*. Thermolabile anti aeromonas products of TI3 and TI7 were not worthwhile against *Pseudomonas* (Fig. 2). Filtered CFS after alkali and catalase treatment (ACCFS) manifested no achievement against *Pseudomonas aeruginosa* (Fig. 2). Antagonism against *Pseudomonas aeruginosa* with protein like thermolabile products ((Table 1) was not confirmed. No thermo-stable inhibitions were outlined against the two tested gram-negative pathogenic bacteria.

## Discussion

Limited anti-aeromonas protein like thermolabile inhibitions was declared. Furthermore, potent isolates with thermolabile bacteriocin-like anti-aeromonas products were not functional against *Pseudomonas*. This authenticates elevated counterforce of *Pseudomonas* as Gram-negative bacteria to LAB bacteriocin. The bacteriocin-like substances of tested LAB bacteria primarily may act on the permeability barrier of the cytoplasmic membrane of *Aeromonas hydrophila* and able to induce lysis and show inhibition ultimately (Bierbaum and Sahl, 2009; Pérez-Ramos *et al*., 2021). The susceptibility of bacteria to bacteriocins confines on the definite receptors existing on bacteria (Yang *et al*., 2014). Low levels of inhibition we observed in case of *Pseudomonas aeruginosa* can occur as isolates have evolved resistance to local bacteriocin producers (Kerr *et al.*, 2002; Ghoul *et al.*, 2015). *Pseudomonas aeruginosa* is known for its capacity to acquire new resistance mechanisms to antimicrobials and/or production of biofilm at high rate serving as a diffusion barrier to limit antimicrobial access to the bacterial cells (Lambert, 2002; Breidenstein *et al.*, 2011; Ferreira *et al.*, 2014; Hong *et al.*, 2015). Degradation of bacteriocins by proteolytic enzymes, presence of efflux systems and production of antibiotic-inactivating enzymes such as β-lactamases (Breidenstein *et al.*, 2011) might be additional possible reasons of this difference in inhibitory activity. Bacteria are typically relentless to the compounds that they generate by themselves (de Freire Bastos *et al*., 2015; Bountra *et al*., 2017). When a bacterial isolate is ungovernable to an antimicrobial generated by another strain, both are expected to yield the same compound (Hatakka and Saxelin, 2008). Bacteriocins usually inhibit species intimately connected to the producers (Hatakka and Saxelin, 2008; Prieto *et al.*, 2012). Present work again

Table 1: Antipathy of LAB metabolites against pathogenic bacteria

| LAB | *Aeromonas hydrophila* | | | | *Pseudomonas aeruginosa* | | | |
|---|---|---|---|---|---|---|---|---|
| | Filtered CFS | Alkaline CFS | Alkaline CFS+ catalase | Alkaline CFS+catalase+ heat treatment | Filtered CFS | Alkaline CFS | Alkaline CFS+ catalase | Alkaline CFS+catalase+ heat treatment |
| TI1 | + | - | - | - | - | - | - | - |
| TI 2 | - | - | - | - | - | - | - | - |
| TI 3 | + | + | + | - | + | - | - | - |
| TI 4 | - | - | - | - | + | - | - | - |
| TI 5 | + | - | - | - | - | - | - | - |
| TI 6 | + | - | - | - | - | - | - | - |
| TI 7 | + | + | + | - | - | - | - | - |
| TI 8 | - | - | - | - | - | - | - | - |
| TI 9 | - | - | - | - | - | - | - | - |
| TI 10 | - | - | - | - | - | - | - | - |
| TI 11 | - | - | - | - | - | - | - | - |
| TI 12 | - | - | - | - | - | - | - | - |
| TI 13 | - | - | - | - | - | - | - | - |
| TI 14 | - | - | - | - | - | - | - | - |
| TI 15 | - | - | - | - | + | + | + | - |

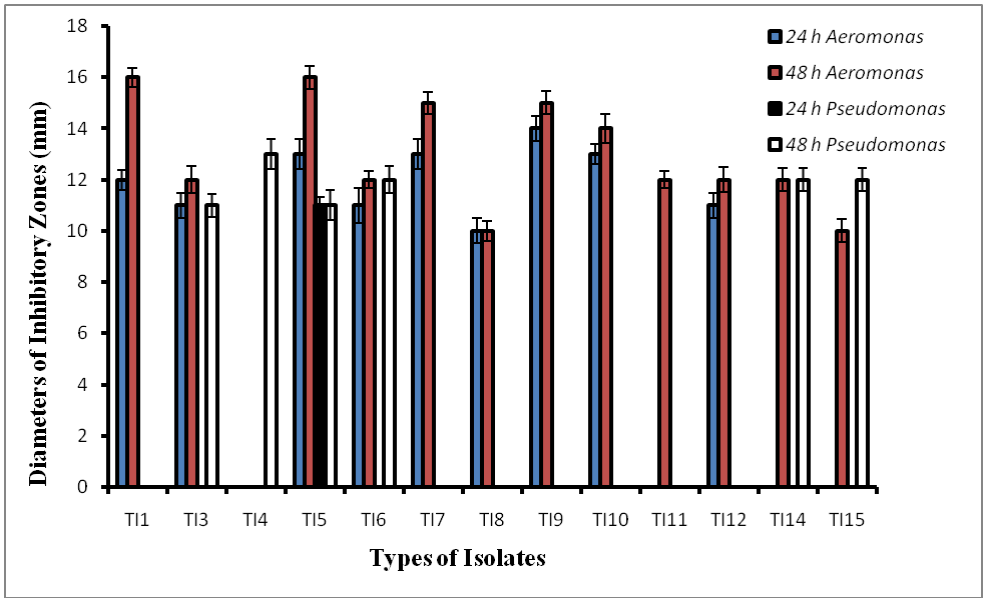


Fig. 1: Incubation time vs antagonism in LAB isolates.

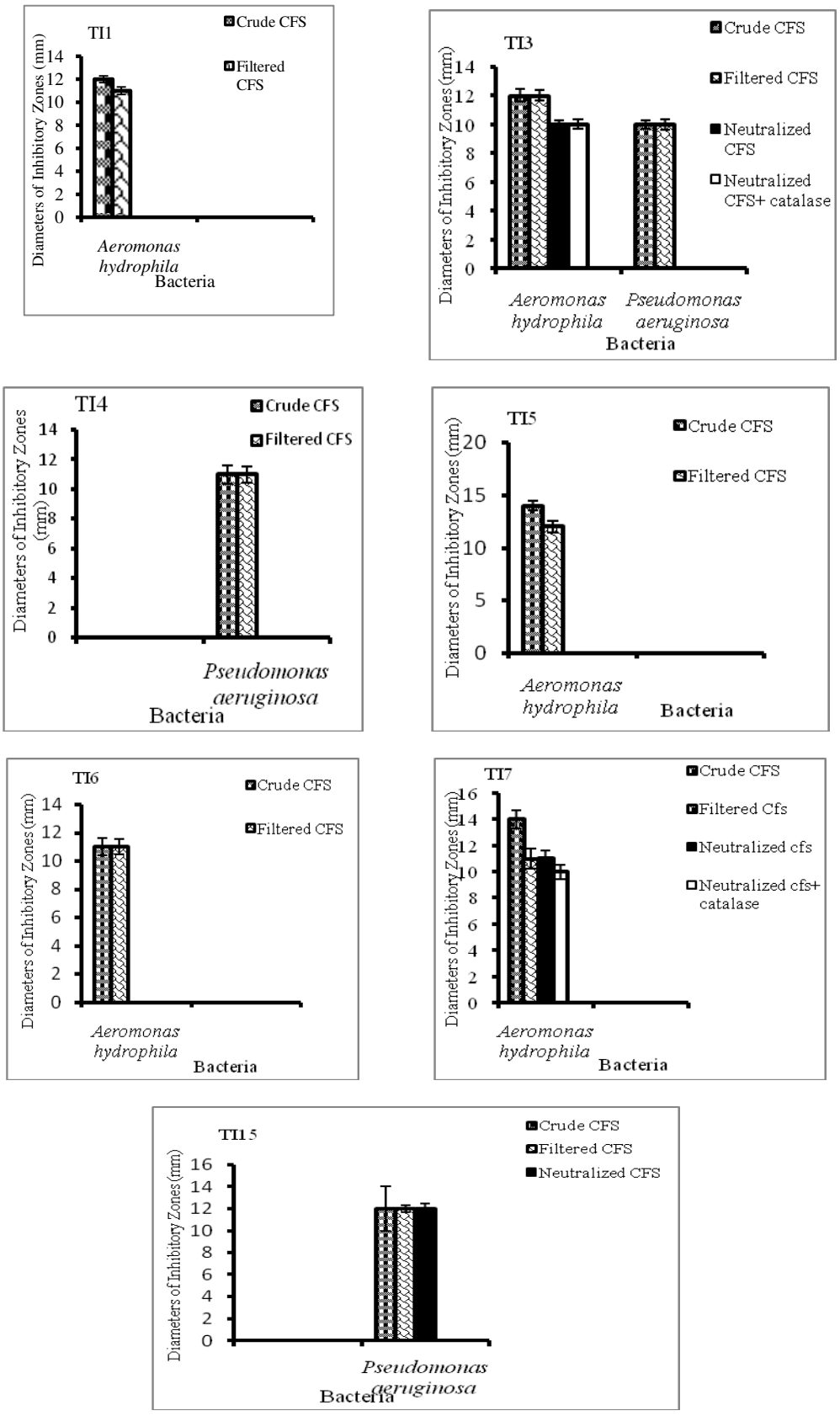


Fig. 2: Inhibitory Zones Specified by different forms of CFS of LAB (mean ± SE, n =5).

delineates that bacteriocins of Gram-positive bacteria have stunted inhibitory activity. In fact anti-Gram-negative antagonism was also perceived, even though not in the CFS, is another attractive but hidden facet of the study. The secondary metabolites of *Pseudomonas* such as 2,4-diacetylphloroglucinol, phenazines, pyrrolnitrin, pyoluteorin, lipopeptides (Gross and Loper, 2009) and Pyocins are reported to be toxic against phylogenetically distant rivals so that they might affect the growth and bacteriocin secretion of tested LAB strains (Li *et al*., 2011) and thus low/no inhibition observed.

## Conclusion

The present research persuades therapeutic application of bacteriocins of native LAB like strains to be used on pathogenic flora of fish. Intrinsic bacteria definitely will have minimum consequences on microbial system, nutritional properties and organoleptic aspects of fish. Being probiotic LAB like strains might add curative and safety outcome on public health. Nevertheless, LAB like isolates should be appraised for biogenic amines production and antibiotic resistances. The paper further communicates aptitude of thermolabile proteinaceous products over protective culture to remove pathogenic and spoilage flora of fish. Genes and immune genes of bacteriocins reside on same plasmid or chromosome of bacteria. It is therefore feasible that immune genes from protective strains can comfortably enter to pathogenic bacteria through conjugation or transposon (Yang *et al.,* 2014). Bacteriocin of the present work could be a substitution to antibiotics. In some cases, protein like thermolabile antimicrobials could be used in live feed and fed to fish to protect them from pathogenic bacteria. Therefore, protein like anti-aeromonas products may act as prophylaxis against diseases caused by bacteria, especially *Aeromonas hydrophila*, in fish. Hence, consequences of *in situ* application of unknown thermolabile high pH tolerent anti-aeromonas products require more investigations.

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