



## Study of Antibiotic Resistance in the Fish Gut and Antibacterial Potential of Lactic Acid Bacteria Against the Isolated Multiple Drug-Resistant Bacteria

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### ABSTRACT

The one health approach integrates human, animal, and environmental health to address emerging diseases. Antibiotic resistance spread among microorganisms has been a concern in all ecosystems, including aquatic environments. This resistance is spreading horizontally in ways that are both hidden and unfathomable. The search for alternatives is the most important need for combating the spread of antibiotic resistance. This work aims to study the spread of antibiotic resistance through horizontal transmission *via* plasmids and the ability of bacteria to form biofilms. In this study, a total of 31 isolates were obtained from different fishes. Cultural, microscopic, and biochemical tests were performed to identify them. The antibiotic sensitivity was performed using the disc diffusion method with oxytetracycline, linezolid, vancomycin, imipenem, cefoxitin, ampicillin, and amoxicillin. Plasmid extraction and biofilm-producing capacity of the resistant isolates were studied. 67% of isolates were found to be gram-negative and 23% gram-positive. Antibiotic resistance profiling showed a higher percentage of resistance in gram-negative organisms with maximum resistance to oxytetracycline. Plasmids were found in 43% of the gram-negative isolates and out of these, 83% were able to form biofilms. To screen for alternatives to antibiotics, cell-free extracts of Lactic acid bacteria were tested by the agar diffusion method, and a zone of inhibition was noted. This study highlights the challenging trend of antibiotic resistance spread in aquatic ecosystems and the need for antibiotic substitutes.

**Keywords:** Fish, Gut, Antibiotics, Multidrug resistance, Lactic acid bacteria

### INTRODUCTION

Antibiotic-resistant bacteria (ARB) have become a global concern due to the massive use or misuse of antibiotics to prevent possible diseases and overcome major production problems. If left unchecked, antibiotic microbial resistance (AMR) can become a major threat to health and could trigger another pandemic worldwide<sup>[1]</sup>. The consumption of antibiotics in the country is complicated by over-the-counter sales of most antibiotics, the production and marketing of numerous fixed-dose combinations (FDC), and the overlap in regulatory authorities between federal and state-level agencies. India's total defined daily dose of antibiotics was 5071 million in 2019,<sup>[2]</sup> which reflects the massive load of antibiotics being used. When populations are isolated, the use of antibiotics by one population does not affect others, however, if the populations are interacting even for 1%, then the transmission is tremendous.<sup>[3]</sup>

The term antibiotic pollution has been used to address the problems of humans as well as the ecosystem as a whole. The ecosystem involves soil, water, and health environment (one health). Antibiotics are used in crops, aquaculture, and also in animal

husbandry. The spread of antibiotics in aquatic systems occurs through wastewater treatment plants, agricultural water runoffs, industrial wastewater, livestock, and aquaculture. The drug-resistant bacteria can transfer the genes by vertical and horizontal transfer. The uptake of these genes leads to the expression of various resistance mechanisms like biofilm formation, quorum sensing, membrane modifications, and neutralization of antibiotics, to name a few<sup>[4]</sup>.

The Prime Minister's Science, Technology, and Innovation Advisory Council (PM-STIAC) has approved the establishment of the National One Health Mission. The focus of this mission is to integrate, coordinate, and support One Health activities in the country. The mission looks into screening and surveillance programs, and database collection of resistant organisms at the national and global level. The effort is comprehensive and considers all aspects of human, animal, and environment to bring together a holistic approach to preparedness for pandemics that would occur in the future. Globally, food security, antimicrobial problems, and water quality have been handled by the G20 Forum<sup>[5]</sup>.

Studies show that by the year 2050, India will be heading to a situation where common infections and minor injuries can kill due to the spread of antibiotic resistance<sup>[6]</sup>. It has been reported that the majority of river systems have very high levels of ARBs (up to 98% of all bacteria found), with lakes having the highest levels (up to 77% of all bacteria found), while dams, ponds, and springs have the lowest levels (<1%)<sup>[7]</sup>. The presence of vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and extended-spectrum beta-lactamase-producing Enterobacteriaceae—which generate highly epidemic infections—is the main cause for concern. A large number of metagenomic studies have revealed the presence of antibiotic resistance genes (ARG) in surface, stored, and flowing waters. All of these studies indicate that aquatic systems are the major reservoirs for the spread of antibiotic resistance<sup>[8]</sup>.

Economic losses due to fish diseases in aquaculture are great. In survey<sup>[9]</sup>, an estimated loss of fish production (as a percentage of the total output) and total economic loss (in INR) is 10.8% & 67,500 per farm (30,770 per ha) in Cachar and 5% & 55,300.0 per farm (14,900 per ha) in Nagaon district of Assam. A survey of 23 coastal districts on the economic losses of shrimp farming, was found to be in crores. Therefore, in aquaculture, antibiotics are used to treat diseases or manage infectious diseases in breeding regions<sup>[10]</sup>. The most commonly used antibiotics in aquaculture worldwide are tetracycline, oxytetracycline (tetracyclines), oxolinic acid, flumequine, sarafloxacin, enrofloxacin (quinolones), amoxicillin ( $\beta$ -lactams), Erythromycin (macrolides), sulfadimethoxine (sulfonamides), ormetoprim (diaminopyrimidines) and florfenicol (amphenicols)<sup>[11]</sup>.

The challenge of antibiotic resistance is further accentuated by the ability of aquatic bacteria to make biofilms. The most prevalent and well-known aquatic pathogens that cause infections of vibriosis in aquatic animals are *Vibrio* spp. They naturally live along coasts and estuaries and coexist with other aquatic creatures. Aquaculture faces serious concerns due to the emergence of antibiotic resistance caused by biofilm-forming *Vibrio* spp.<sup>[12]</sup>. *Pseudomonas* is another pathogen which infects fishes and humans. The work of<sup>[13]</sup> indicated how aquaculture mediates infections of *Pseudomonas* because of its ability to form biofilms in aquaculture systems

With no regulations in place for the use of antibiotics and antifungals in animal husbandry, poultry, and aquaculture, antibiotic pollution control strategies need to be developed. More studies concerning surveillance and trends are required for a better understanding of antimicrobial resistant (AMR) bacteria, antibiotic resistant genes (ARG), and their interactions in the environment.

Although Maharashtra is one of the major suppliers of aquatic food and well established in aquaculture<sup>[14]</sup>, there have been no studies taken up on the aspect of antibiotic resistance in aquatic fauna and data obtained from this study revealed the status of antibiotic-resistant bacteria in this region. Also, an attempt has been made to check the antimicrobial effect of cell-free extract of lactic acid bacteria on the AMR strains.

## MATERIAL AND METHODS

### Sample collection and processing

Fish samples from various sources were collected and carried to the lab for processing and analysis within 12 to 14 hours. The fishes

collected were *Labeo rohita* (Rohu), *Lactarius lactarius* (Saundale), *Rastrelliger kanagurta* (Bangda), and *Oreochromis* (Tilapia).<sup>[15]</sup> The sample collection from the fish gut was carried out by guidelines according to Ribeiro et al., 2012. 1 gm of the fish intestine was weighed and a 1:10 dilution was used for Total heterotrophic count<sup>[16]</sup>.

### Total Heterotrophic bacterial count

The total heterotrophic bacterial count was determined by the spread plate method with ten-fold dilutions of the intestinal exudate on nutrient agar. Counts were taken after 24 to 48 hours of incubation at room temperature.<sup>[17]</sup>

### Biochemical Identification of Isolates

The obtained isolates were inoculated on selective media such as MacConkey's agar, cysteine lactose electrolyte deficient agar CLED, Salmonella shigella agar, salt mannitol agar, xylose lysine deoxycholate agar. Thereafter, biochemical tests were performed for the fermentation of sugars, the presence of enzymes such as catalase and oxidase, etc., to identify the bacteria presumptively<sup>[18]</sup>. The identification was done according to *Bergey's Manual of Systematic Bacteriology*, n.d<sup>[19]</sup>.

### Antimicrobial susceptibility of the isolates

The bacterial isolates were selected for antimicrobial susceptibility testing according to Clinical and Laboratory Standards Institutes (CLSI) diffusion techniques on Mueller Hinton agar using the following antibiotic discs: tetracycline (TE) 30  $\mu$ g, ampicillin (AMP) 10  $\mu$ g, imipenem (IPM) 10  $\mu$ g, amikacin (AN) 30  $\mu$ g, amoxicillin (AMX) 30  $\mu$ g and vancomycin. The zone of inhibition was interpreted according to the Clinical Laboratory Standard Institute (CLSI) and Kirby Bauer chart. The antibiotics were selected keeping in mind the antibiotic resistance literature available<sup>[20]</sup>.

### Characterization of Multidrug-resistant isolates

#### Plasmid profile of the resistant isolates

Plasmid DNA from high MAR (multi-antibiotic resistance) isolates was extracted by standard protocol by the alkaline lysis method<sup>[21]</sup> and resolved on 1.2% agarose gel to obtain the suspected R-plasmid (resistant) profile of the isolates.<sup>[15]</sup> The plasmid profiling of antibiotic-resistant organisms to ampicillin was performed.

#### Biofilm-producing ability

Biofilm formation by antibiotic resistance organisms was determined qualitatively by the congo red agar method (CRA medium).<sup>[22]</sup> The potential isolates were inoculated in trypticase soy broth. Further estimated quantitatively by the Microtitre plate method and staining with 0.1% crystal violet<sup>[23]</sup>. The dye bound to the cells was then extracted in 33% glacial acetic acid for quantification using an ELISA auto reader (Merilizer/EIAQuant).

### Antimicrobial effect of lactic acid bacteria on drug-resistant isolates

Antibiotic-resistant bacterial cultures (0.5 mL) with Absorbance of 0.1 at 620 nm were inoculated into sterile molten Nutrient agar (20 mL) cooled to  $45 \pm 2^\circ\text{C}$ . Lactic acid bacteria isolated from different curd samples were grown in De Man–Rogosa–Sharpe

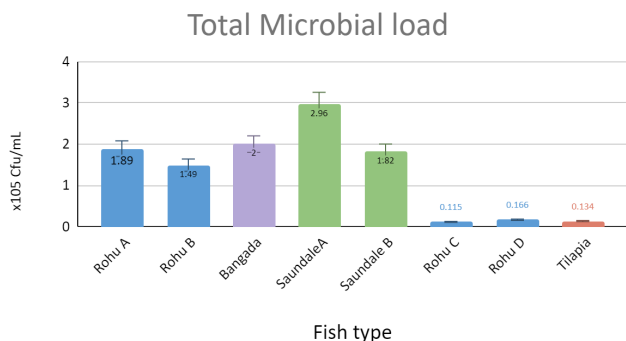


Fig.1: Total hetero-trophic bacterial count

(MRS) broth overnight under microaerophilic conditions. The isolates were then centrifuged at 10,000 rpm for 10 minutes, and the supernatant was collected under sterile conditions. The pH of the cell-free extract was adjusted to neutral, membrane filtered, and used to test the antimicrobial substances using the agar well diffusion method. The plates were incubated at 37°C for 24 hours. The zone of inhibition (in mm) surrounding the wells was measured [24].

## RESULTS AND DISCUSSION

### Total microbial load (Heterophilic count)

The results of the total microbial load showed that the fish samples that were tested carried a high microbial count of about 10<sup>4</sup> to 10<sup>5</sup> cfu/mL (Fig 1). A large microbial community at one site, increases the probability of the exchange of genes, not only for antibiotic resistance but also virulence, causing the spread of pathogenicity. Similar counts of about 10<sup>5</sup> cfu/ml from a variety of fish have been reported by V. Thangamani and N. Rajendran, 2016 [25]. A similar study on the microbial load of crabs by Imefon et al., 2022 showed counts of 10<sup>5</sup> cfu/mL [26]. The microbial load of the fish depends on many factors. The time of the sample collection, type of fish, diet, and a large number of diverse environmental factors are key factors that dictate the type of microbiota in the fish gut [27].

### Biochemical Identification of Isolates

From the total of 31 isolates, 67% were found to be gram-negative and 23% gram-positive [28]. Amongst the gram positives, 8% belong

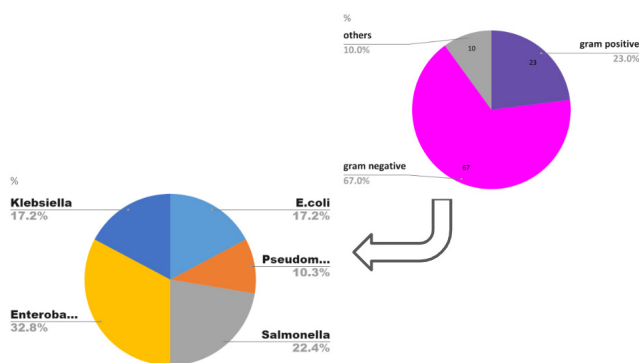


Fig. 2: Percentage of gram-positive and gram-negative isolates from fish gut samples

to *Bacillus* species and 92% were cocci. Among the gram-negative isolates, 88% belonged to the Family Enterobacteriaceae and included *Escherichia coli* (17%), *Enterobacter* species (33%), *Klebsiella* species (17%), and *Pseudomonas* species (10%), whereas 10% remained unidentified (Fig 2). Gram-negative bacteria dominate the fish gut [29] as fish liver releases bile and the LPS in gram-negative bacteria makes them bile tolerant. However, it is also a known fact that the type of bacteria largely depends on the competitive /non-competitive fish in the aquaculture and the type of environment.

### Antibiotic Sensitivity tests

As seen from the graph, 62% gram gram-positive isolates showed resistance to cephoxitin. In the case of gram-negative isolates, the resistant forms against oxytetracycline were found to be 88%. On comparison of the obtained results the percentage resistance of gram-negative organisms was found to be higher than the gram-positive organisms. (Fig 3)

### Characterization of Multidrug-resistant isolates

#### Plasmid profile

As is seen in Fig. 4, 43% of gram-negative resistant isolates were harboring plasmid DNA of approx. 10,000 base pairs M.wt. [30] for ampicillin. In a study performed by M. Fujita and Suzuki, 45 antibiotic-resistant bacteria and 10 dominant bacteria were isolated

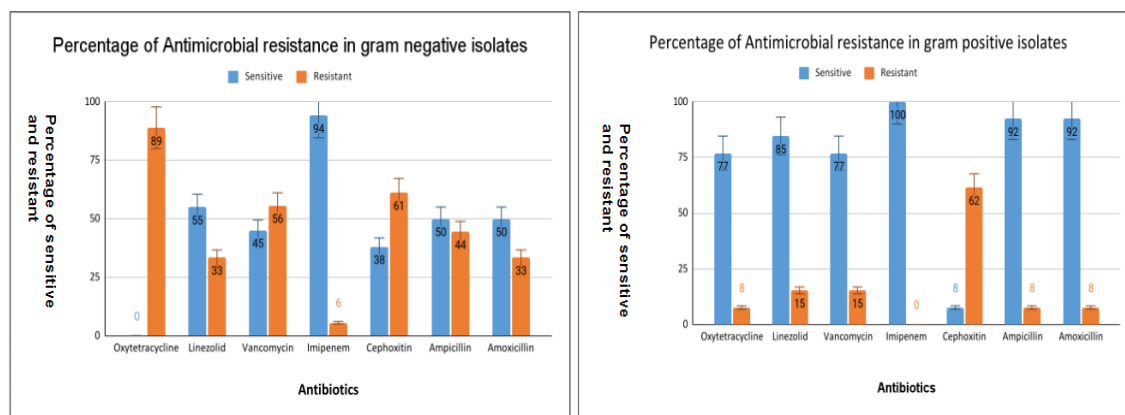


Fig. 3: percentage of antibiotic-resistant isolates



Fig. 4: Plasmid profiling from antimicrobial (ampicillin) resistant organisms

from wastewater samples, and their plasmid contents were examined. Plasmid sizes of 10kb or less were found to be dominant and linked to the transfer of antimicrobial resistance.<sup>[31]</sup> These bacteria were capable of conjugation and, thus, horizontal transfer of these plasmids. Recent research has shown that these aquatic bacteria harbor plasmids, which is an indication that resistance can be horizontally transferred among bacterial communities. For example, in a study by Capkin and co-workers, in coliform bacteria, ampC was the most often occurring antibiotic resistance gene. 78% of the bacteria contained at least one resistance gene, and 66% of the bacteria had two or more resistance genes. The percentage of plasmid-containing bacteria that could transfer them to other bacteria was about 36.54%. These findings suggest that the development of antibiotic resistance and the spread of resistance genes among bacteria may be significantly influenced by the aquatic environment.<sup>[32]</sup>

**Biofilm determination**

Of the total gram-negative resistant isolates, 85% could make biofilms, as observed by the CRA plate method (Fig 5 and 6). The quantitative estimation of biofilm formation showed that four isolates were able to make the maximum amount of biofilm.<sup>[33]</sup>

Thus, there is a strong possibility that this ampicillin-resistant isolate could transfer its DNA through transformation/conjugation. Also, the isolates are biofilm producers, increasing the intensity of resistant characters. The plasmid to other bacteria. The three-faceted

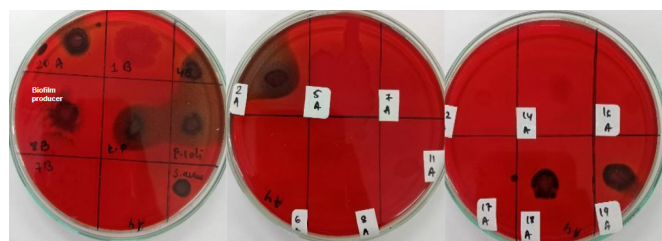


Fig. 5: Detection of Biofilm formation by Congo red agar method

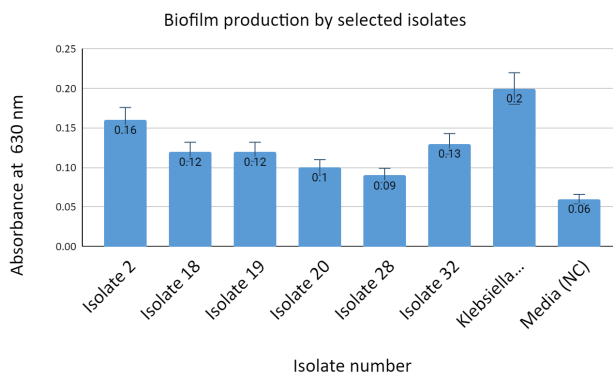
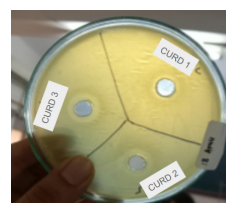


Fig. 6: Quantitative estimation of biofilm formation from selected isolates

ability to be Antibiotic-resistant, bearing a plasmid, and the ability to make biofilms is a dangerous trend towards the spread of antibiotic resistance in a bacterium<sup>[34]</sup>.

**Antimicrobial effect of lactic acid bacteria on drug-resistant isolates**

The effect of lactic acid bacteria cell-free extract on antimicrobial resistance organisms was studied and the zone of inhibition surrounding the wells containing probiotic supernatant was measured (Fig 7). It was observed that the probiotic was effective in inhibiting the growth of antibiotic-resistant organisms<sup>[35]</sup>. In their work, they studied that the production of multiple metabolites has shown antagonistic effects on MDR pathogens. Probiotics exhibit a multitude of anti-MRSA actions (antibacterial, anti-biofilm, anti-virulence, anti-drug resistance, co-aggregation, and anti-quorum sensing) through the production of numerous antagonistic compounds such as organic acids, hydrogen peroxide, low molecular weight compounds, biosurfactants, bacteriocins, and bacteriocins like inhibitory substances. Probiotics may make pathogens susceptible to



Antimicrobial effect of lactic acid bacteria on drug-resistant isolates

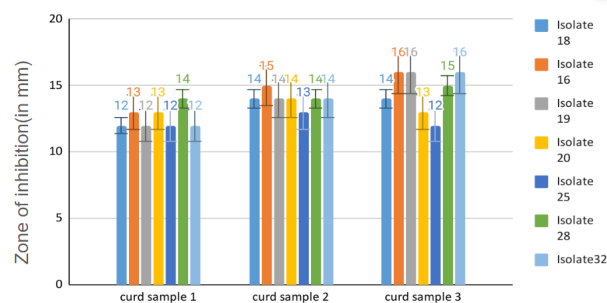


Fig. 7: Antimicrobial effect of lactic acid bacteria on drug-resistant isolates

lactic acid, secrete antimicrobial peptides or proteins, and disrupt the ability of pathogens to form biofilms.

## CONCLUSION

The heterophilic count of the gut of all the fishes showed a high value of culturable bacteria, indicating the increased propensity of growing together for survival benefits. A high load of bacteria also increases the probability of high contact among bacteria, a factor adding to the exchange of genetic elements. The increased amount of surface area of the fish gut gives a good habitat for the bacteria to grow in symbiosis. The bacteria were predominantly gram-negative, with most of them belonging to the Enterobacteriaceae family. However, gram-positive includes both coagulase-positive cocci and non-coagulase producers. Many of these culturable bacteria are known pathogens to humans, including *Staphylococci*, *Salmonella*, *E.coli*, *Pseudomonas* etc. The antibiotic resistance patterns showed an increased% of resistance against cephoxitin, oxytetracycline, and ampicillin. Oxytetracycline and amoxicillin are antibiotics given to fish to prevent fish diseases. The high percentage of ampicillin resistance is an indication that there has been development of antibiotic resistance through the aquatic ecosystems, with fish acting as a reservoir. The detection plasmid in 43% of the ampicillin-resistant isolates and amongst 85% with biofilm-making capacity further adds to the probability of the resistance being horizontally transferred. This is a clear indication of the future trend of the spread of antibiotic resistance through aquatic ecosystems..An alternative to the use of antibiotics is the need of the hour. The use of lactic acid bacterial metabolites against isolates shows promising results.

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## CONFLICTS

There were no conflicts of interest in the work

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