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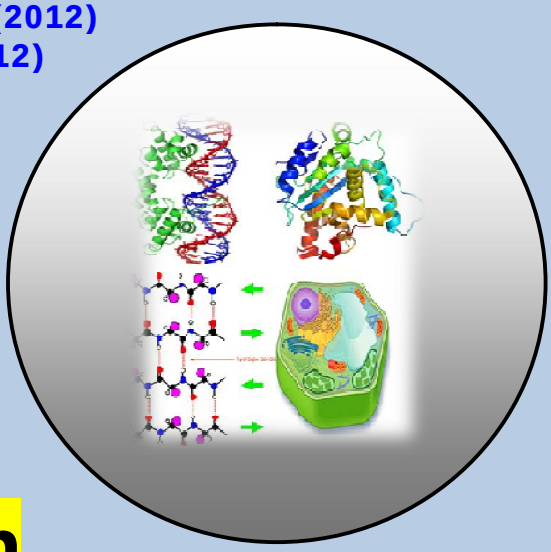

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Diversity of Bacterial Tissues in Fishes from two Traditional Fish Markets in Nagaon, Assam (India)

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ABSTRACT

In Assam, fishermen catch fish and sell their products in the open market. The unsanitary water used during the gutting of fish on the streets influenced the microbial levels in them. A study was undertaken with the objectives of identification of different type of bacteria's found in fresh fishes collected from different local fish markets. A total of 7 fresh individuals, each of Labeo roheta and Wallago attu were sampled from two local fish markets of Dhing and Nagaon respectively for the examination of presence of pathogenic bacteria in their tissues. Tissues from the gill, stomach and liver were excised under aseptic conditions. These were inoculated in Brain Heart Infusion broths (BHI) and incubated for 24 hours at 37°C then subsequently streaked on MacConkey Agars and Blood Agars. Bacteria colony was taken through a series of standardized test for bacterial identification. The tests used were gram staining and biochemical test such as Indole, Catalase, Oxidase, Citrate and Urease tests. Fourteen genera of bacteria were identified as associated with the fish tissues. The identified bacteria include eleven Gram negative and three Gram positive bacteria. Higher bacterial contamination was associated with the gills, gut and skin than the blood and muscle. The presence of high diversity of bacteria in the fish tissues presents health hazards to consumers.
Key words: Bacterial Contaminant Tissues, Fish market and Public Health.

INTRODUCTION

Aquaculture has become one of the fastest developing sources of animal protein to humans and animals due to dwindling wild fish stocks around the world. Fish is a perishable protein food. When fish is stored at <10° C, it remains for about 40 hours before it begins to spoil [**Huss HH, Pedersen A** and **DC Cann, 1974; Jay JM, 1992**]. Freezing does not prevent spoilage of fish because of autolytic activities and chemical changes occurring in fish after harvest [**Huss HH, Pedersen A** and **DC Cann, 1974; Jay JM, 1992**]. The degradation of fish is accelerated by microorganisms associated with aquatic environments as well as contaminants during post-harvest handling. When fish dies, microorganisms on the surface as well as gut and gills begin to utilize the fish protein and food nutrients resulting in loss of nutritional value. Microbial activities create undesirable changes like off-flavors, texture and appearance. Rate of bacterial spoilage is dependent on the initial microbial load, ambient temperature and improper handling. Therefore, proper storage is critical in maintaining a high standard of safety when processing fish [**Jay JM, 1992**]. Small-scale fishermen either use small engine boats, dugout canoes and nets or hooks for fishing in Assam. These fishing methods influence the microbiological and chemical quality of the fish that is brought to the markets. Hook fishing for instance keeps the earlier caught fish waiting, often ungutted and with no ice while the fisherman tries to accumulate a good number to trade. In net fishing, the nets are laid overnight or for long hours. In such cases, if fish dies under water with high ambient temperatures (water temperature 18-27° C) as is the case in Assam, the spoilage begins right under water. In addition to these problems faced by small scale fishermen in Assam, there is lack of investments in landing sites processing and selling sites, resulting in poor sanitation and hygiene **[Mmopelwa TC, 1990-1992**]. These problems lead to cross contamination and multiplication of microorganisms and hence poor quality of fish are presented to the consumers [**Mmopelwa TC, 1990-1992**].

The consequences of fish spoilage are far reaching, and more than just the loss of protein. There have been great economic losses reported due to food borne illness as the result of consuming contaminated fish. The microbial association with fish compromises safety and the quality for human consumption; particularly critical is when the microorganisms are opportunistic and/or pathogenic in nature [**World Health Organization, 1996**]. Considering the problems relating to poor handling and insufficient and improper storage facilities on the streets, the risks of contracting food borne diseases by consumers may be high. These circumstances and the growing demands for fish prompted this research to look into the safety and quality of fish sold.

[**Ahmed, G. U., Akter, M. N., Nipa, S. A. and Hossain, M. M., 2009**] found that seasonal variations in pH, temperature and dissolved oxygen play important roles in the multiplication of pathogens thus leading to diseases in fish. Low or rapid changes in water temperature, rapid or prolonged depression of pH, low alkalinity and low dissolved oxygen are seasonal aggregations of fish diseases [**Lilley, J. H., Philips, M. J. and Tonguthai, K., 1992**].

Majority of water borne pathogenic microorganisms enter water courses as a result of faecal and waste water contamination [**Adewoye, S. O. and Adegunlola, G. A., 2010**]. Bacteria present in the aquatic environment influences the composition of the gut microbiota and at the same time, those in the gut influence the environment microbiota. High proportion of *Bacillus* sps. in the intestinal of fish species may show that intestinal environment is suitable for the given probiotic to settle and grow and also lead to harbour a great number of microbial cells of host intestine [**Bagheri, T., Hedayati, S. A., Yavari, V., Alizade, M. and Farzanfar, A., 2008**]. The types of bacteria contaminants in the feed given to the cultured animal also influence the diversity of bacteria species found in guts of the fish. Bacterial species isolated from fish could have serious health issues for humans who consume or ingest them. Some of the bacteria species that are isolated from the gut of fish are faecal coliforms; and this is especially characteristic of farms where there is little or no biosecurity. Contamination of hands and surfaces during cleaning and evisceration of fish is a common route for pathogenic infection in humans [**Adedeji, O. B., Emikpe, B. O. and Adebisi, T., 2011**].

Many studies have shown that bacteria belonging mostly to the genera *Aeromonas, Corynebacterium, Myxobacterium, Pseudomonas* and *Vibrio* cause infectious diseases in fish. The infections begin on the mucus membranes. To initiate infection the bacteria must reach susceptible organ or adhere to the epithelial cells. In certain diseases they relocalized at the mucosal surface and cause damage by liberating toxins. In most cases, however, infection is caused by the pathogen penetrating the epithelium and then growing in the sub-mucosa or spreading even further. Successful invasion depends on the ability of the pathogen to out compete the normal microflora for nutrients. Small breaks or lesions in the mucosal membrane also facilitate spread of pathogens.

MATERIAL AND METHODS

In the laboratory each fish was rinsed with de-ionized water and the surface of the fish decontaminated by dipping it in ethyl alcohol and lightly flames. The fish was aseptically dissected and parts of the gills, gut and muscle were taken for analysis. Blood sample was also collected using a sterile syringe. Each tissue was homogenized separately in a blender in sterile phosphate buffered saline PBS of pH 7.2 to achieve a 10% w/v suspension of fish. The fish sps collected for bacterial identification was done as per bacteriological techniques describe by methodology and guide book of W.Vishwanath and Rennoh (2002). Bacterial culture was done as per technique given by Bullock (1961 & 1971). The isolation and identification of bacteria was done at laboratory of Biotech Hub, ***Dhing College and ADP College*** respectively.

EXPERIMENTAL DESIGN AND FISH SPECIES

Dhing Fish Market (**Site A**) (26.47°N 92.47°E) is located in Nagaon district of Assam. Nagaon Fish Market (**Site B**) (26.35°N 92.68°E) is situated 120 km east of Guwahati. It is the 4th largest city of Assam. The research was carried out on to two fish sps. ***Wallago attu*** *and* ***Labeo rohita****.* Sampling was done from July 2013 to December 2013. The samples were categorized into two groups:

Group 1- comprised of seven (7) frozen rohu and fresh water shark (Bhorali) from the Dhing fish markets; **Group 2**- comprised of seven (7) catfish from Nagaon fish market.

BIOLOGICAL SAMPLING AND ANALYSIS

Seven *Labeo rohita and Wallago attu* each were randomly selected at each site and sampling time. The length and weight were measured with measuring board and electronic balance respectively. After which they were placed in a waterproof plastic bag and transported at 4°C in an ice chest to the laboratory. Tissues from gills, stomach and liver were sampled aseptically within six (6) hours of fish sampling. The excised tissues were placed into Brain Heart Infusion broths (BHI) and then incubated for 24 hrs at 37°C. These were inoculated unto sterile MacConkey (MA) and Blood agars (BA) and incubated for 24 hrs at 37°C with control media. Bacteria colonies and cells were identified by physical characterization and staining. Pure colonies were then taken through a series of standardized biochemical tests including citrate, urease, indole, oxidase and catalase.

BACTERIAL IDENTIFICATION

The blended fish samples (25g) were added to 225ml of lactose broth (Oxoid, CM137) and homogenized in a stomacher for 2 minutes. The mixture was aseptically transferred into a wide-mouth screw-cap bottle and left to stand for 60 minutes at room temperature in order to enrich the population of recovering bacteria from stressful conditions. Aliquots (1ml) from the enrichment broth were added to 10 ml of selenite cystine broth (Oxoid, CM699) supplemented with 4% sodium biselenite (Oxoid ECC No. 2319663). These were incubated at 35° C for 24 hours. After gentle mixing, a loopful of culture was streaked on to xylose lysine desocholate (XLD, Oxoid, CM469), agar and incubated at 35° C for 24 – 48 hours. Typical Salmonella colonies were isolated. Two or more colonies from XLD were streaked and stabbed into the butt of triple iron sugar (TSI, Himedia M021) slants. These were incubated at 35° C for 24 hours. The test tubes that had alkaline (red) slants and acidic (yellow) butts, with or without the production of H_2S (blackening) were treated as suspects of salmonellae [**Andrew W, 1992; FDA,1992**]. From each presumptive TSI slant, a loopful was inoculated on urea broth (Himedia, M112) supplemented with 40% urea. These were incubated at 35° C for 24 hours. All test tubes that were urease negative were treated as suspect *Salmonella* and confirmed by using API 20E system [**Andrew W, 1992**]. Species were isolated from fish samples (25g) homogenized in 225ml of 0.1% sterile peptone water. Further dilutions were made as deemed necessary. Aliquots (1ml) were spread plated over the surface of Baird-Parker agar (Oxoid, CM275), supplemented with egg yolk emulsion (Oxoid, SR047C). The plates were incubated at 35° C for 24-48 hours. Black, circular colonies with off-white margins were presumed to be *Staphylococcus* isolates. A colony of bacteria was collected with a sterile inoculating loop (of plastic or platinum) and applied on a microscope slide. One drop of 3% H_2O_2 was added to the bacteria. Gas formation (O_2) in the form av bubbles shows that the bacterium has a catalase.

For isolation of *Vibrio*, *Pseudomonas* and other Gram-negative bacteria, the blended fish samples (25g) were added to 225ml of alkaline peptone water (APW) with 1% NaCl at pH 8.6 for enrichment.

This was incubated at 37° C for 8 and 24 hours. Thiosulphate citrate bile salts sucrose agar plates were streaked with a loopful of enrichment broth after 8 hours and another after 24 hours. This was done to allow for the growth of *Vibrio* species, which might be overtaken by the overgrowth of enteric bacteria after long hours of incubation. The plates were incubated at 37° C for 24 hours. Small green and yellow colonies from plates inoculated after 8 hours of incubation of enrichment broth were presumed *Vibrio* isolates. Typical yellow, green, blue-green and black colonies which appeared after 24 hours of incubation were purified and isolates grown on TSI agar slants and subjected salt tolerance test and Gram stain. The isolated colonies were further identified by API 20E system [**FDA,1992**].

RESULT AND DISCUSSION

Rohu (*labeo rohita)* and *(Wallago attu) bhorali* collected from local fish markets were found to harbor bacteria belonging to different five genera of bacteria (***Table 2, 3***), in various tissues of the fish, but at different magnitudes. The bacteria isolated from the various tissues of the fish from both the markets included *Aeromonas* sp., *Bacillus* sp., *Citrobacter* sp., *Corynebacterium* sp., *Enterobacter* sp., *Escherichia* sp., *Flavobacterium* sp., *Klebsiella* sp., *Micrococcus* sp., *Pseudomonas* sp., *Salmonella* sp., *Staphylococcus* sp., *Streptococcus* sp., *Vibrio* sp. Bacterial genera isolated from the blood were between 4-6, and 2-4 genera in the muscle of fish. Between 4-6 genera were isolated from the skin, 2-4 genera from the gills and 6-8 genera from the gut (**Table 2, 3**).

The study indicated that certain species seemed to be associated more with certain tissues than with others. For example *salmonella* sp. was abundant in muscle of fish, while the blood contained higher presence of *Pseudomonas* sp. For the gills, gut and skin, the respective dominant genera were *Bacillus* sp., *Pseudomonas* sp. and *Salmonella* sp. respectively. Bacteria colonies and cells were identified by physical characterization and staining. Pure colonies were then taken through a series of standardized biochemical tests including citrate, urease, indole, oxidase and catalase.

Table 1. Gram +ve and Gram –ve bacteria.

Gram positive bacteria	Gram negative bacteria
Cornybacterium	*Salmonella*
Bacillus	*Flavobacterium*
Staphylococcus	*Pseudomonas*
	Vibrio
	Pasteurella
	Escherichia
	Micrococcus
	Aeromonas
	Citrobacter
	Enterobacter
	Klebsiella

Table 2. Bacteria diversity in the tissues of *Wallago attu.*

Bacterial species	Dhing					Nagaon				
	Gill	Muscle	Blood	Gut	Skin	Gill	Muscle	Blood	Gut	Skin
Salmonella	3	10		4	9	4	11		5	
Cornybacterium			2					3		
Flavobacterium			3		7			4		8
Pseudomonas			13	5						
Escherichia		9		3			10		4	
Micrococcus			1	1				2	2	
Aeromonas			1	1				2	2	
Bacillus	3				7					8
Citrobacter					1					2
Enterobacter						4				
Klebsiella									3	
Staphylococcus				2						
Vibrio	2			2	8	3			3	9
Pasteurella		6					7			
Total	8	25	20	18	32	11	28	11	19	27

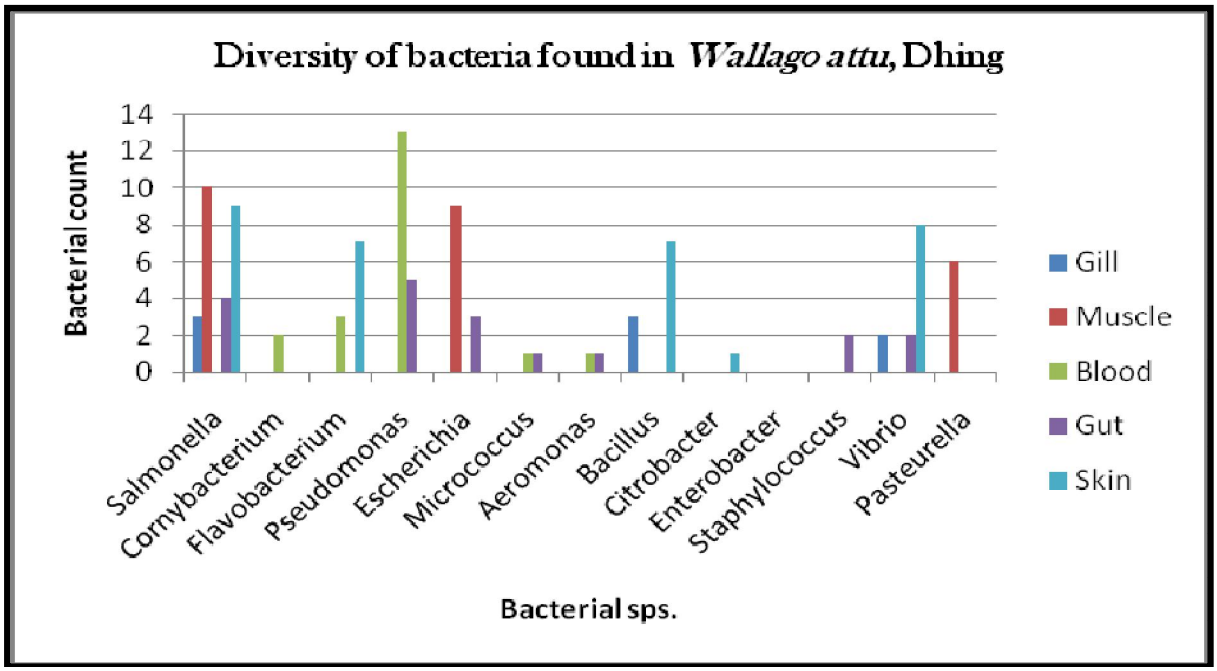

Figure 1. Bacteria diversity in the tissues of Wallago attu.

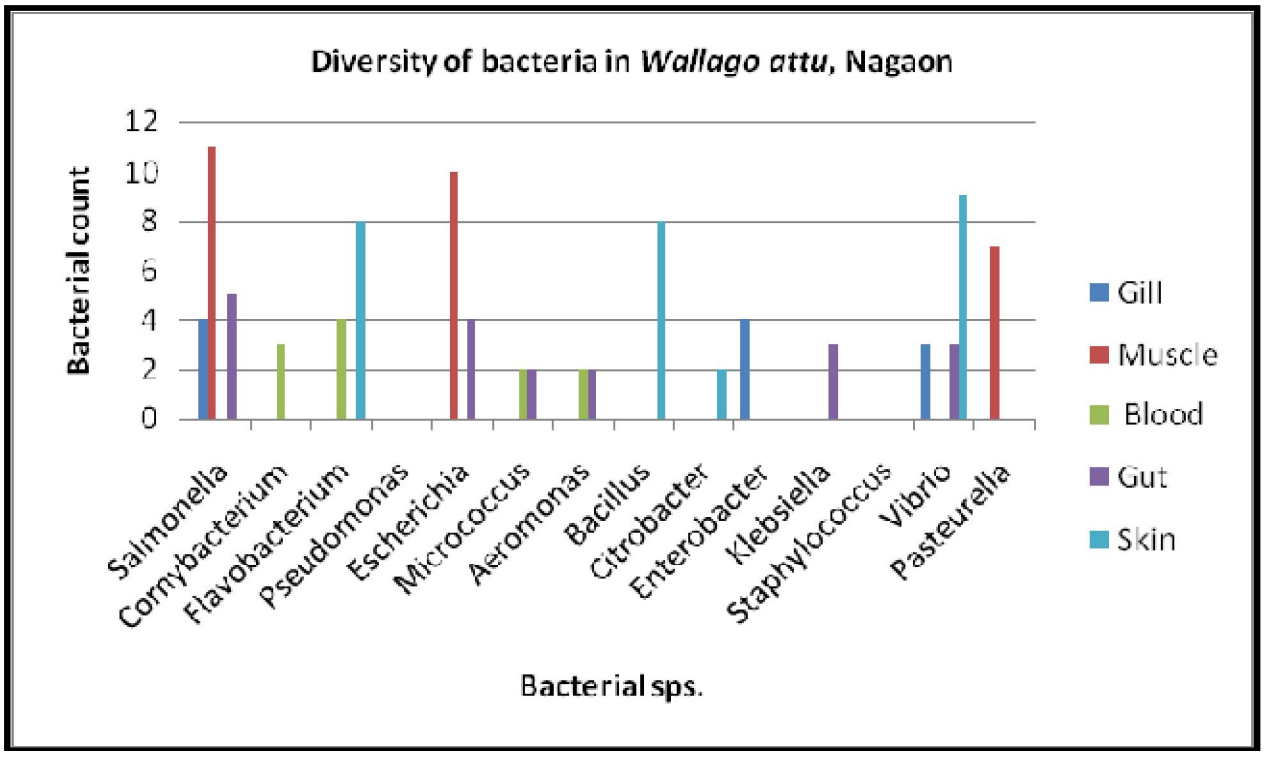

Figure 2. Bacteria diversity in the tissues of Wallago attu.

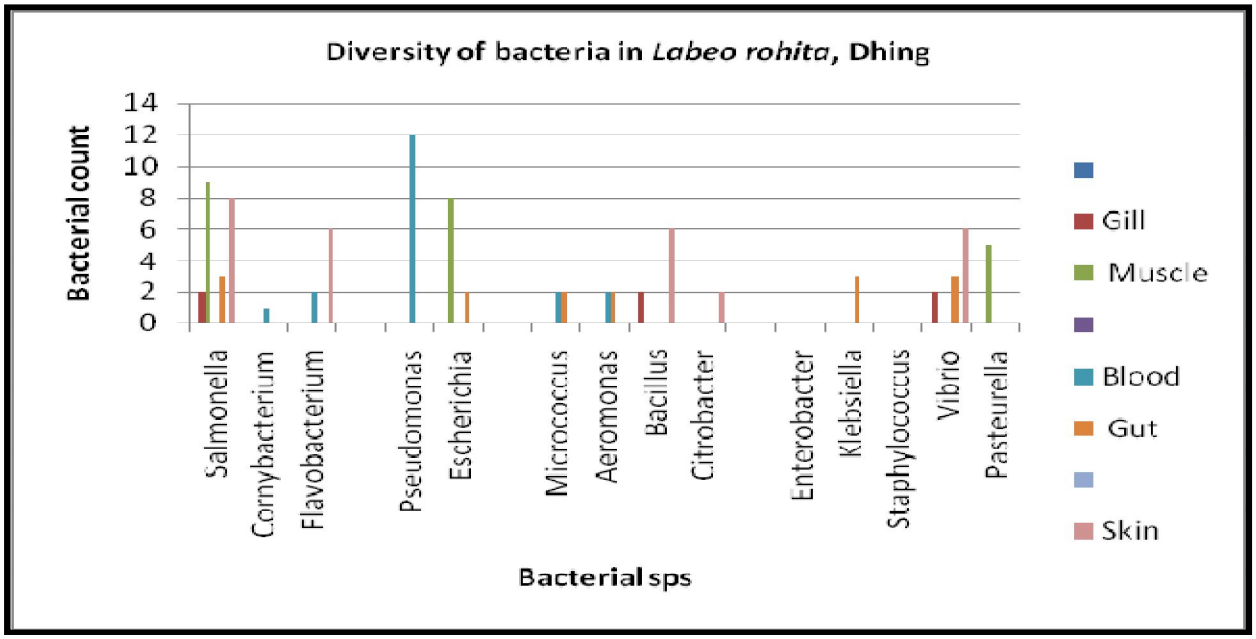

Figure 3. Bacteria diversity in the tissues of *Labeo rohita*.

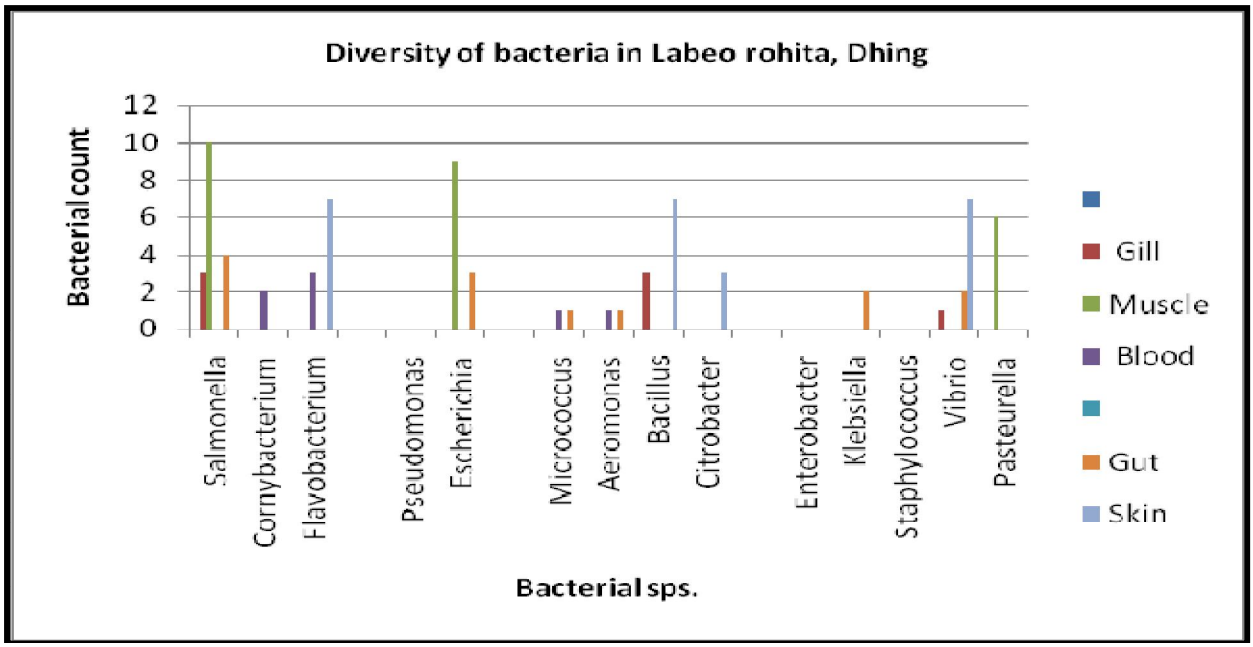

Figure 4. Bacteria diversity in the tissues of *Labeo rohita.*

Table 3. Bacteria diversity in the tissues of *Labeo rohita.*

Bacterial species	Dhing					Nagaon				
	Gill	Muscle	Blood	Gut	Skin	Gill	Muscle	Blood	Gut	Skin
Salmonella	2	9		3	8	3	10		4	
Cornybacterium			1					2		
Flavobacterium			2		6			3		7
Pseudomonas			12	4						
Escherichia		8		2			9		3	
Micrococcus			2	2				1	1	
Aeromonas			2	2				1	1	
Bacillus	2				6	3				7
Citrobacter					2					3
Enterobacter										
Klebsiella				3					2	
Staphylococcus										
Vibrio	2			3	6	1			2	7
Pasteurella		5					6			
Total	6	22	19	19	28	7	25	7	13	24

The result reveals that different bacterial species have different levels of biochemical reactions as given in **Table 4.** Moreover from the study it was found that *Salmonella* sps bacteria have highest degree of diversity in muscles and *Pseudomonas* sps bacteria have highest degree in blood. Vice versa *Cornybacterium* sps bacteria was found lowest in blood in both local fish market (***Figure 1, 2, 3 and* 4**). The identified bacteria included eleven Gram negative and three Gram positive bacterias (**Table- 1**). The presence of high diversity of bacteria in the fish tissues presents health hazards to consumers.

Table 4. Biochemical tests.

Sl.No.	Bacteria	Catalase test	Oxidase test	Urease test	Citrate test	Indole test
1.	*Salmonella*	-	-	-	+	-
2.	*Cornybacterium*	+	-	-	-	-
3.	*Flavobacterium*	+	+	-	-	-
4.	*Pseudomonas*	-	+	-	-	-
5.	*Escherichia*	+	-	-	-	+
6.	*Micrococcus*	+	+	-	-	-
7.	*Aeromonas*	+	-	-	-	-
8.	*Bacillus*	+	-	-	-	-
9.	*Citrobacter*	-	-	-	+	-
10.	*Enterobacter*	+	-	+	+	-
11.	*Klebsiella*	-	-	+	+	-
12.	*Staphylococcus*	+	-	+	-	-
13.	*Vibrio*	-	+	-	-	-
14.	*Pasteurella*	+	+	-	-	-

CONCLUSION

Salmonella sp. was most commonly identified with most of the tissues, and was detected in the muscles, on the gills and skin etc. The occurrence of disease is a complex interaction between host species pathogens and the environment. Most aqua cultural practices that favour disease occurrence include high stocking densities which increase stress in the stocks, intensive feeding which provides abundant substrate for microbial growth and sub-optimal environment of inadequate water exchange. Disease outbreaks would thus be relatively less common in the non-fertilized pond as it has less stressful environments, even though the pathogens and host species may be present. The presence of the coliform group of bacteria, mainly *Citrobacter, Enterobacter, Escherichia* and *Klebsiella.* In fish and fish products presents a health hazard to humans the presence of faecal coliform in fish intended for human consumption may constitute a potential danger not only in causing disease, but also because of the possible transfer of antibiotic resistance from aquatic bacteria to human infecting bacteria from non aquatic sources.

Some human pathogens such as *Aeromonas, Escherichia, Klebsiella, Pseudomonas, Salmonella* and *Vibrio* have been found to survive and multiply in the gut, mucus and tissues of fish and thus render fish a potential vector. The digestive tract and intraperitoneal fluid of fish in this stud y showed concentrations of pathogens. Handling and cleaning of such contaminated fish can result in contamination of the hands of farm workers and through them to their family members and others.

The occurrence of *Salmonella* in fish suggests a temperature effect in pond water, which promoted the growth as well as the cross contamination from viscera to flesh during processing. Similar situations existed here where ambient temperature could be 25-35° C during day time might have contributed to the growth in the flesh. But unhygienic processing conditions and absence of clean water during degutting might have contributed to the contamination followed by growth of the bacteria. Provision of low temperature storage is therefore indicated.

The high prevalence of *Staphylococcus* in the fish samples indicated the unhygienic handling of the fish since these species are found on human skin. The diversity of potential pathogens from the samples of fish is of concern particularly at a time when many in our communities are immunologically compromised as a result of various illnesses. These opportunistic and pathogenic bacteria were also previously isolated by several other researchers from fish.

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