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Identification and Comparative Assessment of Microbial Load of Commercially Sold Frozen Fish at Bhilwara and Chittorgarh Markets

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Abstract: The objectives of this study are to comparatively assess and identify, enumerate the TVC, and Characterize the Microbial load of Bhilwara and Chittorgarh sold frozen fish. A total of 9 sample (*Cyprinus capio, Wallago atu* and *Pangasius bocourti)* each were collected and transported to laboratory for the study. The targeted organs of the sample (gills, skin and intestine) were homogenized and cultured on an agar (Nutrient, Mackonkey, EMB and PDA) for the isolation and characterization of the colonies present. Biochemical tests were done for the confirmation of each bacterial and fungal isolates obtained from the culture. The results show that Bhilwara samples has bacterial and fungi TVC of $4.9*10^4$, $10.3*10^4$, $2.7*10^4$ and $2.27*10^5$, $3.5*10^4$, $14.9*10^4$ while Chittorgarh sample has $9*10^3$, $5.8*10^4$, $7*10^3$ and $4.7*10^4$, $1.8*10^4$, $6*10^3$. Comparatively the organs that has the highest total viable count of the bacteria is Skin of Bhilwara sample with $10.3*10^4$ and the least bacterial count is the intestine of Bhilwara sample with $2.7*10^4$ respectively, while the highest viable fungi isolates is intestine of Bhilwara sample with $14.9*10^4$ nevertheless the least fungi count were from the Skin of Chittorgarh sample with $1.8*10^4$ respectively. The identified possible isolates bacteria were *Staphylococcus spp, Streptococcus sp, Escherichia coli, Chromobacterium sp, Shigella spp, Proteus spp, Enterobacter spp, Bacillus spp, Pseudomonas sp, Proviencia sp and Klebsiella sp* and fungi isolates were *Aspergillus spp, Mucor sp, Cladosporium sp, Fusarium, Rhizopus and Penicillium sp* are the isolates characterized and identified in this current study. Klebsiella spp, *Staphylococcus spp,. Aspergillus spp, Cladosporium sp* been the most prevailingisolates from the samples. The study underscores the significant bacterial and fungal contamination in frozen fish from Bhilwara and Chittorgarh, with *Staphylococcus aureus and Klebsiella pneumoniae* posing particular health risks due to their high prevalence and pathogenic potential. In conclusion, addressing the identified contamination issues is critical for ensuring the safety of frozen fish products and protecting public health. Through improved practices and regular monitoring, the risks associated with these contaminants can be significantly reduced.

Keywords: *Microbial Load, Frozen Fish, Bhilwara, Chittorgarh, Bacterial Contamination, Fungal Isolates*.

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I. INTRODUCTION

Fish in general is a largely perishable product because of high water exertion (Abbas et al., 2009). Fish, any of roughly 34,000 species of invertebrate creatures (phylum Chordata) set up in the fresh and swab waters of the world. Living species range from the primitive jawless lampreys and hagfishes through the cartilaginous harpies, skates, and shafts to the abundant and different bony fishes. Utmost fish species are cold- thoroughbred; still, one species, the opah (*Lampris guttatus*), is warm- thoroughbred. The term fish is applied to a variety of invertebrates of several evolutionary lines. It describes a life- form rather than a taxonomic group. As members of the phylum Chordata, fish share certain features with other invertebrates. These features are gill gashes at some point in the life cycle, a notochord, or cadaverous supporting rod, a rearward concave whim-whams cord, and a tail. Living fishes represent some five classes, which are as distinct from one another as are the four classes of familiar air- breathing creatures — amphibians, reptiles, catcalls, and mammals. For illustration, the jawless fishes (Agnatha) have gills in sacks and warrant branch cinctures. Extant agnathans are the lampreys and the hagfishes. As the name implies, the configurations of fishes of the class Chondrichthyes (from chondr, “cartilage,” and ichthyes, “fish”) are made entirely of cartilage (Stanley and Lynne, 2024). Maulu et al., 2020 in his study revealed that all the fishermen endured post-harvest fish losses at varying degrees with those losing up to 10 of the total catch being in the maturity. In discrepancy, monoculture directors did not report any post-harvest fish

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losses. utmost monoculture directors generally used chilling as preservation practice negative to artisanal and marketable fishermen who generally used smoking and sun sun-drying independently. likewise, fish product safety and quality control were inadequately rehearsed in the quarter. Lack of cold storehouse installations and shifting rainfall conditions were the major challenges impacting fish post-harvest conditioning. Fish is extremely susceptible to microbial impurity because of their soft apkins and the submarine terrain. Millions of bacteria are present in the face of slime, on the gills and in the bowel of live fish. numerous of them come implicit spoilers after the death of fish when the defense system breaks down and the bacteria multiply and foray the meat. One of the major factors contributing to poor quality of the fish in retail trade is hygienic running, indecorous storehouse, physical damage and come to communicate in dirty water and microorganisms. Bacterial corruption is characterized by softening of the muscle towel and the product of slime and obnoxious odors (Nur, et al., 2020).

There are also numerous multicellular organisms that are bitsy, videlicet micro-animals, some fungi, and some algae, but these are generally not considered microorganisms. Microorganisms can have veritably different territories, and live far and wide from the poles to the ambit, comeuppance, geysers, jewels, and the deep ocean. Some are acclimated to axes similar as veritably hot or veritably cold conditions, others to high pressure, and a many, similar as Deinococcus radiodurans, to high radiation surroundings. Microorganisms also make up the microbiota set up in and on all multicellular organisms. There's substantiation that 3.45- billion- time-old Australian jewels formerly contained microorganisms, the foremost direct substantiation of life on Earth (Schopf, 2017).

Bhilwara and Chittorgarh cities are District under State of Rajasthan, India. These 2 cities have significant consisting fish sellers, different kind of fish species are being sold, looking at market condition, biotic and abiotic factors concerned has been raised on the possible contamination and or bacterial growth on the frozen fishes. There is need to perform research for the examination of microbial loads of the fish sold at the markets, and associated health risk to the consumers.

II. METHODOLOGY

➢ *Sample Collection*

A total of 27 frozen Fish each of which are (*Wallago atu and Major carp)* from Bhilwara *and (Basa* fish) from Chittorgarh, were collected using sterile hand gloves and put in to sterile poly-ethene bags, after which it was then put in to a clean iced container/cooler for maintaining the ambient temperature and Transported to the Life Science Department Laboratory, for Examination and Identification of the possible microbial load of the fish's gills, skins and intestine by medium culture, isolation, characterization and identification of the microbes from the samples.

➢ *Sample Preparation*

The fish samples were imported in to Department of Life Science. 10g of the fish's skin, intestine and gills were weight and put into well cleaned sterile beaker and then macerated with 100ml of diluents. Each fish organ was weight and labeled categorically in replicates treatment, which then centrifuge at 10,000rpm for 10minutes in order to separate the supernatant from particles in both the treatment prepared for the culture, isolation and identification of microbial loads of each sample. A serial dilution method was done, the first dilution 1:10 were made following with subsequent one dilutions.

➢ *Agar Preparation*

5-15g of agar was added in to beaker or flask and mixed thoroughly with 100ml distilled water depending on the manufacturer's instruction in accordance with each agar, the solutions were heated in short intervals (30-45s) until the agar were fully dissolved and the solution is boiled. After boiling the agar solution, it was then autoclaved at 121^0C for 15mins, and then poured on to petri dishes and allowed to solidify at room temperature for it to be ready for the culture of the microbes. The agar prepared was used for both the two different markets samples, for targeting agar specific possible microbial loads.

- *Nutrient agar:* this was prepared by adding amount of distilled water following the manufacturer's guide, autoclaved for 15-20mins at 121^0C, cooled and solidified on agar plates for culture, its mainly used as universal agar for cultivation of any kind bacteria, yeast and mold.

- *EMB agar (Eosin Methylene Blue agar)*: this was prepared by adding amount of distilled water following the manufacturer's guide, autoclaved for 15-20mins at 121^0C, cooled and solidified on agar plates for culture, its mainly used for the isolation of gram negative bacteria.

- *Potato Dextrose Agar*: this was prepared by adding amount of distilled water following the manufacturer's guide, autoclaved for 15-20mins at 121^0C, cooled and solidified on agar plates for culture, its mainly used for the isolation of fungi.

➢ *Culture and Incubation of Micro-organism*

The stock samples were then inoculated on the solidified agar (Nutrient agar, Mackonkey agar, EMB, and Potato dextrose agar) depending on the target organism to culture, its then transferred in to incubator that was set at 35^0C and allow for a period of at least 18hrs-24hrs for bacteria and 48-72hrs for fungi depending on the targeted microorganism or results obtained. In this method; 1ml of each stock were inoculated on each targeted agar for the culture of bacteria and fungi respectively.

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Fig 1: Incubation and Culture of Micro Organism

➢ *Morphology Identification*

After the incubation the agar plates were brought out of the incubator for colony, texture, color, size, elevation identification and number of colonies count per plate. Colonies were counted using colony counter. Morphological features of each colony were noted for identification and classification of the microbes. The CFU (Colony-Forming Units) of each plate were counted and recorded.

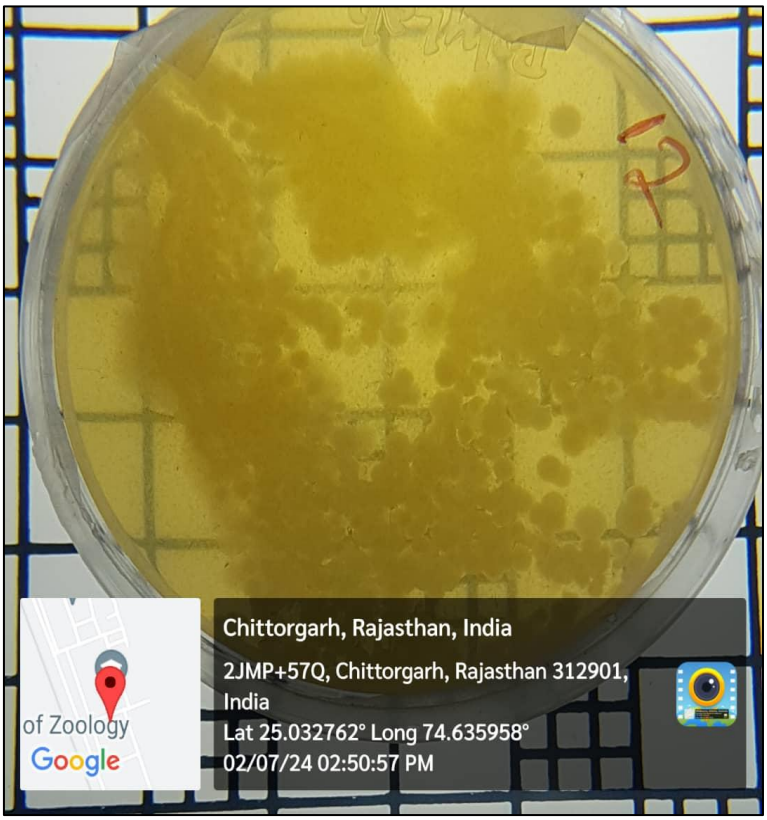

Fig 2: Microscopic View for Morphological Identification of Isolates

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➢ *Pure Culture*

A pure culture of each slide was made for further confirmatory and isolation of possible bacteria. A Universal and selective media were made for the isolation of the colonies based on the number of treatments. A streak plating method was adopted for the inoculation of the colonies in the agar plates. Its then incubated in an incubator for 24-48hrs for the culture of the colonies. After it was observed morphologically and subjected for confirmatory test i.e biochemical test.

Fig 3: Slide Showing a Pure Culture of Suspected Microorganism

➢ *Confirmatory Test (Biochemical Test)*

Biochemical test is done to confirm, identify and characterized bacteria based on its chemical and biological characteristics/features, a series of test were carried out to access each bacterium and fungus present, such as Catalase test, MR-VP test, Indole test, Biuret Test, Gelatin liquefaction Test, Amylase Test, Nitrate test and Urease Test.

➢ *Catalase Test.*

A swap of the pure culture was placed on a glass slide, a 1-2 drops of hydrogen peroxide (H_2O_2) solution was added to the culture and observed the reaction for 1-2 minutes. Presence of catalase enzyme is indicated by broken down of hydrogen from oxygen with formation of bubbles or gas. Positive results indicate the presence of catalase enzymes while a negative result indicates the absence catalase enzyme.

Fig 4: Showing a Catalase test of an isolate

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➢ *Indole Test*

The bacterial pure culture swap was inoculated in 3-5ml of either nutrient broth or tryptophan broth and incubated for 24-48hrs. After the incubation, 1-2ml of Kovac's reagent was then added to the culture, mixed well and observe the changes in color. Red or Pink color indicate the presence of indole otherwise it indicates negative results. Positive indole indicates the presence of tryptophanase enzyme and the production of indole while negative indole indicates the absence of tryptophanase enzyme or the inability to produce indole.

➢ *Methyl Red Test*

The pure bacterial culture was incubated in a glucose-phosphate broth for 24-48hrs. 3-4 drop of Methyl red indicator was then added to the broth culture and mix well. A color change was observed for (Red/Pink) indicates acidic metabolites i.e., MR positive while Yellow/Orange color indicates no acidic metabolites i.e., MR negative.

Fig 5: Showing Methyl Red Test Result

➢ *Biuret Test*

1-2drops of biuret reagent (copper sulfate solution) were added in to the bacterial broth samples. It was then mixed well and observed for the change in color. A purple or pink color indicated a positive result and that proteins or peptides are present in the sample Meanwhile no color change indicates no proteins or peptides present in. It can be done on both bacteria and or fungi.

➢ *Gelatin Liquefaction Test*

An amount of Gelatin agar or gelatin broth is inoculated with the test microorganism. The inoculated medium is incubated at 35^0C for 1-7days. The medium is then observed for signs of gelatin liquefaction, i.e., melting of the gelatin broth/agar or by formation of a clear or cloudy liquid around the inoculation site. Positive results indicate the presence of gelatinase enzymes otherwise it indicates absence of gelatinase enzymes.

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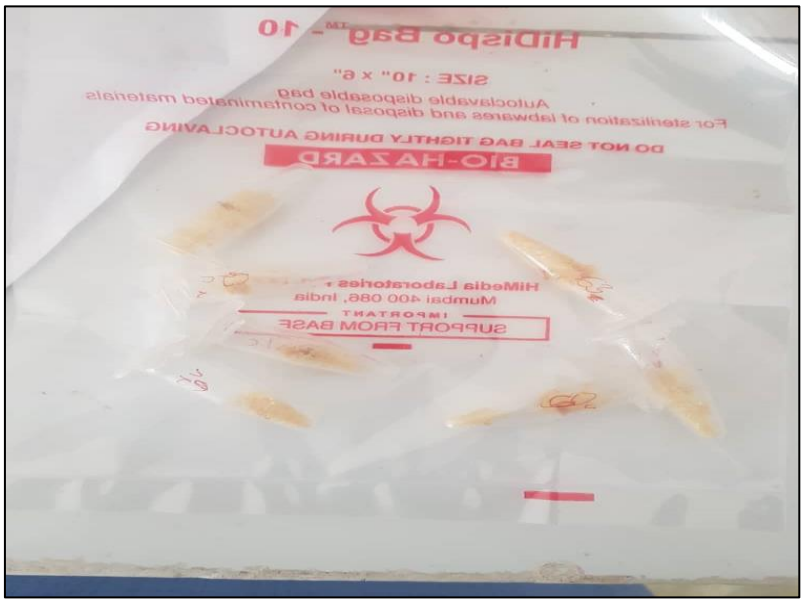

Fig 6: Gelatin Liquefaction Test

➢ *Amylase Test*

Substrate was prepared by the mixing of 1% Starch solution with 0.1% iodine solution. The culture was then added to the substrate mixture and incubated for at room temperature 25^0C for 5-10mins. Change in color was observed from blue-black to yellow or red, indicating amylase activity.

III. RESULTS

Table 1: Morphological Characteristics of Bacteria from Samples Collected from Bhilwara.

Treatment	Organ	Treatment	Serial dilution	Total colony	Colony color	Colony shape and form
Treatment 1	Gills	WG1	10^{-1}	18	Creamy yellow	Bunches and round
		WG2	10^{-2}	12	Creamy milk	Irregular round with serrate margin
		WG3	10^{-3}	07	Dark creamy milk	Irregular with undulate margin
	Skin	WS1	10^{-1}	11	Creamy milk	Filamentous rodlike
		WS2	10^{-2}	33	Light yellow	Punctiform
		WS3	10^{-3}	53	Yellow	Bunches of round shape
	Intestine	WI1	10^{-1}	02	Milky	Spindle shape
		WI2	10^{-2}	03	Whitish	Long curve
		WI3	10^{-3}	05	Brownish	Draughtsman lobate
Treatment 2	Gills	CG1	10^{-1}	02	Greenish	Spot-like
		CG2	10^{-2}	04	Greenish	Irregular round
		CG3	10^{-3}	06	Blueish green	Irregular round
	Skin	CS1	10^{-1}	02	Purple	Rhizoid
		CS2	10^{-2}	01	Bluish green	.....
		CS3	10^{-3}	03	Pink	Filamentous
	Intestine	CI1	10^{-1}	9	Yellow	Round
		CI2	10^{-2}	3	Reddish	Spongelike
		CI3	10^{-3}	5	greenish	Undulate

Note: W= *Wallago atu , C= Major carp, G= gills, S= Skin and I=Intestine*

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Table 2a: Morphological Characteristics of Fungi from Sample collected from Bhilwara

Treatment	Organ	Replicate	Serial dilution	Total Colony	Colony color	Colony shape
TREATMENT 1	Gills	WG1	10^{-1}	02	Yellow	Irregular with scalloped margin
		WG2	10^{-2}	01	Whitish	Round
		WG3	10^{-3}	01	Whitish yellow	Round and umbonate
	Skin	WS1	10^{-1}	03	White	Cottony and ribbonlike
		WS2	10^{-2}	02	Milky brown	Hexagonal
		WS3	10^{-3}	01	……..	Filamentous
	Intestine	WI1	10^{-1}	01	Black and white hairy	Umbonate round
		WI2	10^{-2}	03	Brownish	Elongated branches
		WI3	10^{-3}	04	Milky	Umbonate

Table 2b: Morphological Characteristics of Fungi from Sample collected from Bhilwara

Treatment	Organ	Replicate	Serial dilution	Total Colony	Colony color	Colony shape
TREATMENT 2	Gills	CG1	10^{-1}	53	Yellow	Irregular with scalloped margin
		CG2	10^{-2}	102	Whitish	Round
		CG3	10^{-3}	68	Whitish yellow	Umbonate
	Skin	CS1	10^{-1}	6	White	Cottony
		CS2	10^{-2}	9	Milky brown	Round form
		CS3	10^{-3}	14	……..	Filamentous
	Intestine	CI1	10^{-1}	76	Black and white hairy	Round
		CI2	10^{-2}	42	Brownish	Elongated branches
		CI3	10^{-3}	23	Milky	Undulate

Table 3: Morphological Characteristics of Bacteria from Sample collected from Chittorgarh.

Organ	Treatment	Serial dilution	Total colony	Colony color	Colony shape and form
Gills	PG1	10^{-1}	04	Whitish	Filamentous
	PG2	10^{-2}	5	Pink	Round irregular
	PG3	10^{-3}	2	Dark milky yellow	Curled, large and irregular
Skin	PS1	10^{-1}	32	Yellowish	Elongated rodlike
	PS2	10^{-2}	05	Dark brown	Round irregular
	PS3	10^{-3}	21	Greenish yellow	Warm-like
Intestine	PI1	10^{-1}	01	Yellow	Filamentous
	PI2	10^{-2}	03	Whitish yellow	Round irregular
	PI3	10^{-3}	03	Whitish	Filamentous

Note: P=*Pangasius spp*, G= gills, S=Skin and I= Intestine

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Table 4: Morphological Characteristics of Fungi Isolated from Chittorgarh Sample.

Organ	Treatment/Replicate	Serial Dilution	Total colony	Colony Color	Colony shape and form
Gills	PG1	10^{-}	14	Whitish	Filamentous and Rhizoid
	PG2	10^{-2}	18	Light brown	Punctiform
	PG3	10^{-3}	15	Brownish	Undulate
Skin	PS1	10^{-1}	6	Whitish yellow	Round form
	PS2	10^{-2}	5	Whitish yellow	Round form
	PS3	10^{-3}	7	Milky	Punctiform
Intestine	PI1	10^{-1}	02	Whitish yellow	Round
	PI2	10^{-2}	3	Grey	Undulating round
	PI3	10^{-3}	1	White and Grey	Cottonlike hairy and round

Table 5: Isolated and Characterized Bacteria Bhilwara Sample

Sample	Organ	Biochemical Test					Gram Staining	Possible Bacteria
		Catalase	Methyl Red	Biuret Test	Indole Test	V-P Test		
01	Gills	+ve	+ve	+ve	-ve	+ve	+ve	*Staphylococcus aureus*
		+ve	-ve	+ve	+ve	-ve	-ve	*Proteus mirabilis*
		+ve	+ve	+ve	-ve	-ve	-ve	*Klebsiella pneumoniae*
		+ve	+ve	+ve	-ve	-ve	-ve	*Klebsiella pneumoniae*
	Skin	+ve	+ve	+ve	-ve	-ve	+ve	*Staphylococcus epidermidis*
		+ve	+ve	+ve	-ve	-ve	-ve	*Klebsiella pneumoniae*
		+ve	+ve	+ve	-ve	+ve	+ve	*Staphylococcus aureus*
		+ve	-ve	+ve	+ve	+ve	-ve	*Enterobacter cloacae*
	Intestine	+ve	-ve	+ve	+ve	+ve	-ve	*Providencia rettgeri*
		+ve	+ve	-ve	-ve	-ve	+ve	*Bacillus anthratis*
		+ve	+ve	+ve	-ve	-ve	+ve	*Staphylococcus epidermis*
		+ve	+ve	-ve	+ve	+ve	-ve	*Escherichia coli*
02	Gills	-ve	+ve	-ve	-ve	-ve	-ve	*Shigella dysenteriae*
		+ve	-ve	+ve	-ve	-ve	-ve	*Pseudomonas spp*
	Skin	+ve	-ve	-ve	+ve	+ve	-ve	*Chromobacterium violaceum*
		+ve	-ve	+ve	-ve	-ve	-ve	*Pseudomonas spp*
	Intestine	+ve	+ve	+ve	-ve	-ve	+ve	*Staphylococcus epidermis*
		+ve	+ve	-ve	+ve	+ve	-ve	*Escherichia coli*

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Table 6: Isolated and Characterized Bacteria Obtained from Chittorgarh Sample

Organ	Biochemical Test					Gram Staining	Possible bacteria
	Catalase	Methyl Red	Biuret Test	Indole Test	V-P Test		
Gills	+ve	+ve	-ve	-ve	+ve	+ve	*Bacillus subtilis.*
	+ve	-ve	+ve	+ve	-ve	-ve	*Proteus mirabilis*
	+ve	+ve	+ve	+ve	-ve	-ve	*Proteus vulgaris*
	+ve	+ve	-ve	+ve	+ve	-ve	*Escherichia coli*
Skin	+ve	+ve	+ve	-ve	-ve	-ve	*Klebsiella pneumoniae*
	+ve	-ve	-ve	-ve	-ve	-ve	*Shigella spp*
	-ve	+ve	-ve	-ve	-ve	+ve	*Streptococcus faecalis*
	+ve	+ve	+ve	-ve	+ve	-ve	*Pseudomonas aeruginosa*
Intestine	+ve	+ve	-ve	-ve	+ve	+ve	*Bacillus cereus*
	+ve	+ve	+ve	-ve	+ve	+ve	*Staphylococcus aureus*
	+ve	+ve	+ve	-ve	-ve	-ve	*Klebsiella pneumoniae*
	+ve	+ve	-ve	-ve	-ve	-ve	*Enterobacter aerogenes*

Table 5 and 6 above shows confirmatory results of Biochemical test (Biuret, Catalase, MR-VP, and Indole) and microscopic test (Gram staining) for the Identification and characterization of each bacterium from the isolated colonies obtained from the samples of each treatment and organ (gills, skin and intestine), from the examined results a variety of possible bacteria species were detected.

Table 7: Isolated and Characterized Fungi of Bhilwara Sample

Sample	Organ	Biochemical Test					Possible Fungi
		Biuret Test	Gelatin Liquefaction	Nitrate Test	Catalase	Amylase	
01	Gills	-ve	+ve	+ve	+ve	+ve	*Aspergillus niger*
	Skin	-ve	+ve	+ve	+ve	+ve	*Mucor spp.*
	Intestine	-ve	-ve	+ve	+ve	+ve	*Aspergillus fumigatus*
02	Gills	+ve	+ve	+ve	+ve	+ve	*Aspergillus terreus*
	Skin	-ve	-ve	+ve	+ve	+ve	*Aspergillus flavus*
		-ve	+ve	…	+ve	+ve	*Fusarium spp.*
	Intestine	-ve	+ve	+ve	+ve	+ve	*Aspergillus niger*

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Table 8: Isolated and Characterized Fungi obtained from Chittorgarh Sample.

Organ	Biochemical Test					Possible fungi
	Biuret test	Gelatin Liquefaction	Nitrate test	Catalase	Amylase	
Gills	-ve	+ve	-ve	+ve	+ve	*Rhizopus stolonifera*
	-ve	-ve	-ve	+ve	+ve	*Cladosporium spp*
Skin	-ve	+ve	+ve	+ve	+ve	*Aspergillus niger*
	-ve	-ve	+ve	+ve	+ve	*Aspergillus fumigatus*
Intestine	-ve	-ve	+ve	+ve	+ve	*Aspergillus flavus*
	-ve	+ve	-ve	+ve	+ve	*Penicillium candidum*

Table 7 and 8 above shows confirmatory test, biochemical test (Gelatin liquefaction, Amylase, Nitrate, Biuret, and Catalase) results for the identification and characterization of the isolated fungi colonies from each sample and organs

Table 9: Showing the Descriptive and Analysis of Variance results of the Both the Bhilwara and Chittorgarh Bacterial Isolates from the Treatment

TREATMENT	GILLS	SKIN	INTESTINE
C	$4.0 \times 10^{-2} \pm 2.00$	$2.00 \times 10^{-2} \pm 1.00$	$5.67 \times 10^{-2} \pm 3.06$
W	$12.33 \times 10^{-2} \pm 5.51$	$32.3 \times 10^{-2} \pm 13.58$	$3.33 \times 10^{-2} \pm 1.53$
P	$3.67 \times 10^{-2} \pm 1.528$	$19.33 \times 10^{-2} \pm 21.00$	$2.33 \times 10^{-2} \pm 1.16$

NOTE: Treatment C= *Common carp*, W= *Wallago atu* and P= *Pangasius spp*
ANOVA Gills p-value= 0.038 at 1%, Skin = 0.107 at 1% and Intestine = 0.213 at 1%

The above table indicates Descriptive value and analysis of variance results, where the there is no significance difference between both the treatment at different organs, whereas gills treatment p=value is 0.038 at 1%, skin treatment p=value is 0.107 at 1% and intestines p=value is 0.213 at 1%, therefore there is no significant differences between the samples on bacterial isolates.

Table 10: Showing the Descriptive and Analysis of Variance Statistics results of the Both the Bhilwara and Chittorgarh fungal Isolates from the Treatment

TREATMENT	GILLS	SKIN	INTESTINE
C	$74.3 \times 10^{-2} \pm 25.1$	$9.67 \times 10^{-2} \pm 4.04$	$47.00 \times 10^{-2} \pm 26.90$
W	$1.33 \times 10^{-2} \pm 0.577$	$2.00 \times 10^{-2} \pm 1.00$	$2.67 \times 10^{-2} \pm 1.00$
P	$15.67 \times 10^{-2} \pm 1.20$	$6.00 \times 10^{-2} \pm 1.00$	$2.00 \times 10^{-2} \pm 1.53$

NOTE: Treatment C= *Common carp*, W= *Wallago atu* and P= *Pangasius spp.*
ANOVA Gills p-value= 0.002 at 1%, Skin = 0.025 at 1% and Intestine = 0.019 at 1%

The above table indicates Descriptive value and analysis of variance results, where the there is significance difference at gills treatment which the p value is 0.002 at 1%, and there are no significance differences between the treatment of skin with p value 0.025 at 1% and intestine with 0.019 at 1%.

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Table 11: Shows the TVC of Bacteria and fungi on each Organs

Isolates	Gills	Skin	Intestine
Bhilwara			
Bacteria	$4.9*10^4$	$10.3*10^4$	$2.7*10^4$
Fungi	$2.27*10^5$	$3.5*10^4$	$14.9*10^4$
Chittorgarh			
Bacteria	$9*10^3$	$5.8*10^4$	$7*10^3$
Fungi	$4.7*10^4$	$1.8*10^4$	$6*10^3$

Table 11 above shows the Total Viable Count of each Isolates from each organ of the samples according to sample collection area.

IV. DISCUSSION

Fish is a good source of protein, minerals, its highly perishable in nature, fish after harvest it undergoes series of microbial activities that may lead to its deterioration and spoilage if not preserved well. Freezing is a method of preserving fish against spoilage, though there are microorganism that can thrive in cool environment (Psychrophiles), mode of handling and storage facilities also results in contamination of the preserved fish. As a result of this the need to access and examine the microbial load of frozen fish sold at Bhilwara and Chittorgarh become of paramount important to the consumers of sold fish. The bacteria isolated from gills, skin and intestine of frozen fish bought from Bhilwara; Major carp (*Cyprinus capio*) were gills (*Staphylococcus aureus, Proteus mirabilis and Klebsiella pneumoniae*), while on skin (*Staphylococcus epidermidis, Klebsiella pneumoniae, Staphylococcus aureus* and *Enterobacter cloacae*) and also in intestine were (*Providencia rettgeri* and *Bacillus anthratis*) respectively. Meanwhile Boal fish (*Wallago atu*) isolated bacteria were; gills (*Shigella dysenteriae* and *Pseudomonas spp*) respectively, while on Skin and (*Chromobacterium violaceum* and *Pseudomonas spp*) and also in intestine were (*Staphylococcus epidermis* and *Escherichia coli*) respectively.

The fungi isolated from gills, skin and intestine of frozen fish bought from Bhilwara; on Major carp sample were *Aspergillus niger, Mucor spp and Aspergillus fumigatus* respectively while on Boal fish sample were*; Aspergillus terreus, Aspergillus flavus, Fusarium spp and Aspergillus niger* respectively. The average Total Viable Count "TVC" of Bacteria and fungi on the Bhilwara samples gills, skin and intestine were $5.96*10^4$ and $6.89*10^4$ respectively.

The bacteria isolated from Chittorgarh fish sample organs were; Basa fish "*Pangasius bocourti*" based on agar plates the gills isolates were; *Bacillus subtilis, Proteus mirabilis* and *Proteus vulgaris, Escherichia coli,* Skin isolates were *Klebsiella pneumoniae, Shigella spp and Streptococcus faecalis, Pseudomonas aeruginosa* while isolates from intestine were *Bacillus cereus, Staphylococcus aureus and Klebsiella pneumoniae, Enterobacter aerogenes* respectively. Meanwhile the fungi isolate from the organs were; *Rhizopus stolonifera, Cladosporium spp* from the gills, *Aspergillus niger, Aspergillus fumigatus* from the Skin and *Aspergillus flavus, Penicillium candidum* from the intestine respectively.

The study shows that both *Staphylococcus spp, Klebsiella sp, Proteus spp, Bacillus spp and E. coli* were found in the gills of both *Cyprinus capio, Wallago atu, and Pangasius bacourti"* but there may be competing scenario between the isolates of the bacteria due to antagonist behavior, on skin bacteria like *Chromobacterium sp, Streptococcus sp, Shigella and Pseudomonas sp* were found on the skin of the fish samples, also from the intestine the following isolate was obtained *Enterobacter cloacae* as the non-common isolates from the others. Ibraq *et al.,* 2020 reported that the presence of, *Pseudomonas aeruginosa, Escherichia coli, Enterobacter cloacae, Bacillus spp, Klebsiella spp, Proteus spp, Salmonella spp, Streptococcus spp, Bacillus spp, Staphylococcus sp., and Enterobacter aeruginosa* from two fish species and it is anticipated that bacterial contamination potentially will add a risk for fish food safety in aquaculture.

Comparatively, Total Viable Count of Bacterial isolates from the samples of Bhilwara and Chittorgarh in the gills $4.9*10^4$-$9*10^3$, in skin were $10.3*10^4$-$5.8*10^4$, and intestine were $2.7*10^4$-$7*10^3$. This signifies that Skin of Chittorgarh sample has the high Count of bacterial isolate than the skin of Bhilwara, and Intestine of Bhilwara sample has the least bacterial count compared to the other spots. Ibraq et al., 2020 in their research shows that viable bacteria count (cfu/ml) in fish samples, *Cyprinus carpio* and *S. plagiostomus* ranged 2.0×10^3 -2.90×10^4 and 1.6×10^3 -2.78×10^4 in the skin, 1.64×10^4 -2.99×10^4 and 1.5×10^3 -2.95×10^4 in the buccal cavity and 1.1×10^3 -2.56×10^4 and 1.1×10^3 -2.71×10^4 in the gills respectively. The skin of *Cyprinus carpio* had the greatest bacteria count range of 2.0×10^3 -2.90×10^4.

In comparison of the Total Fungi isolates from Bhilwara and Chittorgarh in the gills were; *Aspergillus niger, Aspergillus terreus* and *Rhizopus stolonifera, Cladosporium spp,* on the Skin isolates were; *Mucor spp., Aspergillus flavus, Fusarium spp* and *Aspergillus niger, Aspergillus fumigatus,* while isolates from the intestine were; also *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus flavus, Penicillium candidum,* this indicates that the contaminant is more of Aspergillus species than any other from all the samples. The Total Viable Count of the Fungi on (gills, skin, and intestine) from Bhilwara and Chittorgarh were; $2.27*10^5$-$4.7*10^4$, $3.5*10^4$-$1.8*10^5$ and $14.9*10^4$-$6*10^3$ respectively,

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from this it was observed that Bhilwara sample intestine has high index of fungi while gills of Bhilwara and Skin of Chittorgarh have the least count of fungi. According to **Nader et al. (2019).** In the tested *Tilapia nilotica* and *Mugil cephalus*, the mean total mould counts were 3.63 $x10^2$±8.75x10 and 1.65 $x10^2$±4.78x10 CFU/g, respectively. This could be linked to *Tilapia nilotica's* higher moisture content than *Mugil cephalus*, which could lead to a higher mould contamination.

The prevalence of bacteria isolates from Bhilwara sample shows that *Staphylococcus species* has (56.8%), *Klebsiella spp* (30.77%), *Enterobacter sp, and Pseudomonas* (2.96%), *Bacillus sp* (1.78%), *Escherichia coli and Proteus sp* (1.45%) and *Chromobacterium sp, Shigella sp and Providencia sp* (1.18%), which signifies that *Staphylococcus spp* has high prevalence and *Chromobacterium sp, Shigella sp, and Providencia sp* has least % Occurrences. (Ibraq et al., 2020) reported that the prevalence of bacterial isolates during his investigation is presented as (30%) *Bacillus* sp, (25%) *E. coli*, (20%) *Enterobacter aeruginosa*, (19%) *Staphylococcus* species, (10%) *Enterobacter cloacae*, (10%) *Klebsiella pneumonia*, (8%) *Proteus vulgaris*, (7%) *Pseudomonas aeruginosa*, (5%) *Shigella* sp, (4%) A*eromonas hydrophila*, and (2%) *Salmonella* species. While, as in *S. plagiostomus* fish species were 10% *Bacillus* sp, 10% *E. coli*, 10% *Enterobacter aeruginosa*, 4% *Staphylococcus* species, 5% *Enterobacter cloacae*, 2% *Klebsiella pneumonia*, 1% *Proteus vulgari* and 2% *Pseudomonas aeruginosa.* The % Occurrences of fungi isolates were *Mucor sp* 23.08%, *Fusarium sp* 7.69% and *Aspergillus spp* 69.23%, therefore this shows that the highest of fungi is *Aspergillus spp* and the lowest is *Fusarium sp.* Junaid et al. (2010) found that all stockfish samples were contaminated with fungi. Seven distinct fungal species were identified in the stockfish samples from four different markets: Mucor spp., Aspergillus flavus, Trichophyton verrucosum, Aspergillus niger, Aspergillus fumigatus, Penicillium spp., and Rhizopus spp. Among these, Mucor spp. was the most frequently occurring. Additionally, A. niger, A. flavus, A. versicolor, A. parasiticus, Rhizopus spp., Mucor spp., Phoma herbarum, and Trichoderma hamatum were isolated from Tilapia nilotica.

In the Chittorgarh samples, bacterial isolates revealed that Klebsiella spp. had a prevalence of 20.83%, followed by Streptococcus spp. at 12.5%, Pseudomonas spp. at 4.76%, Proteus spp. at 4.17%, Bacillus spp., Shigella spp., and E. coli at 2.98%, Staphylococcus spp. at 1.79%, and Enterobacter spp. at 1.19%. Klebsiella spp. exhibited the highest occurrence, while Enterobacter spp. showed the lowest percentage. Ibraq et al., 2020 reported in their study that *Enterobacter cloacae* were found 10% and 5% in cultured and wild fish, *Enterobacter aeruginosa as 2*0% and 10% in cultured and wild fish, and *Proteus vulgaris* as 8% and 1% in cultured and wild fish respectively. 10% and 2% of *Klebsiella pneumoniae* were found in cultured and wild fish respectively and *Shigella* sp. as 5% only in cultured fish. Meanwhile the prevalence of fungi in the sample were *Cladosporium sp* 57.61%, *Aspergillus spp 26.09%, Rhizopus stolonifer 15.22%* and *Penicillium sp* with 1.09%. *Cladosporium* with highest count and *Penicillium* with the least count. (Darwish et al., 2014). In his report observed six fungal species were isolated from Tilapia fish and fillet of Tilapia. Tilapia nilotica fish had the highest value of isolation percentage of *Aspergillus niger* (27.52%) followed by *A. flavus* (22.94 %), however, the lowest values were obtained for *A. terreus, A. westerdijkiae*, and *A. pseudocaelatus* 2.75, 2.75 and 1.83, respectively. While the values were obtained for *Cladosporium cladosporidiae* and *Alternaria alternate* 11.93%, and 4.59%, respectively. Fillet of Tilapia had *A. flavus* as the highest value of isolation percentage (24.4%) followed by *A. niger* (21.95%) while the lowest values were obtained for *P. citrinum* (4.88%), *P. corylophilium* (2.44%) and *P. griseofulvum* (2.44%), respectively.

➢ *Comparative Views*

This study shows that Bhilwara frozen fish were highly exposed in to bacterial contamination *Staphylococcus* Species; *Staphylococcus epidermis, Staphylococcus aureus* having the highest number of colonies count and prevalence index, *Staphylococcus* species are versatile and adaptable pathogen capable of causing a wide range of infections. while frozen fish sold at Chittorgarh has bacterial high prevalence of *Klebsiella pneumoniae*¸ and it's associated with high morbidity and mortality due to its ability to cause severe infections and its high level of antibiotic resistance. Moreover, frozen fish from Bhilwara have *Providencia sp* as the least prevailing isolate and *Enterobacter cloacae* has the least prevailing count from Chittorgarh sample. Furthermore, the fungi isolate obtained from Bhilwara frozen sample shows that *Aspergillus spp; Aspergillus flavus, Aspergillus fumigatus, Aspergillus terreus and Aspergillus niger* as the high prevailing fungi isolate and *Fusarium sp* with the least count whilst the most prevailing Chittorgarh isolated fungi is *Cladosporium sp* and the least is *Penicillium sp*. This indicates that the nature of contamination, storage facilities, differences in fish species and site of sample collection has also significant factor in variation of the type and degree of the isolate's contaminations.

➢ *General Health Risk and Concerned*

In general, Understanding the various *Staphylococcus* species, their pathogenic mechanisms, and their clinical implications is crucial in the field of microbiology and infectious diseases. Continued research and vigilance in antibiotic stewardship are vital in combating infections caused by these adaptable and often resistant bacteria. *Proteus mirabilis* is a significant pathogen in the context of urinary tract infections and catheter-associated complications. Its ability to produce urease and form biofilms contributes to its pathogenicity and resistance to treatment. *Klebsiella pneumoniae* is a significant pathogen in healthcare settings, associated with high morbidity and mortality due to its ability to cause severe infections and its high level of antibiotic resistance. *Pseudomonas, E. coli, Bacillus, and Providencia* has distinct characteristics and pathogenic potential. Their ability to cause disease ranges from opportunistic infections in vulnerable individuals to severe and sometimes life-threatening conditions. *Chromobacterium violaceum, Shigella dysenteriae*, and *Enterobacter cloacae* are significant pathogens with distinct characteristics and pathogenic mechanisms. Each poses

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unique challenges in terms of infection control and treatment, particularly in the context of rising antibiotic resistance. *Aspergillus* is a genus of fungi with several species capable of causing a wide range of diseases, particularly in immunocompromised individuals. *Rhizopus*, *Cladosporium*, and *Penicillium* are diverse fungi with varying pathogenic potentials. *Rhizopus* is known for causing severe mucormycosis in vulnerable individuals. *Cladosporium*, although mainly associated with allergies, can cause opportunistic infections. *Penicillium* is notable for its role in antibiotic production but can also cause opportunistic infections and allergic reactions. Both *Fusarium* and *Mucor* fungi are important from an environmental, agricultural, and medical perspective. While *Fusarium* is primarily known for its impact on crops and its production of harmful mycotoxins, it can also cause severe infections in immunocompromised individuals. *Mucor*, on the other hand, is a key decomposer in ecosystems but is also the cause of mucormycosis, a life-threatening infection that requires urgent medical attention.

V. CONCLUSION

Conclusively, the study underscores the significant bacterial and fungal contamination in frozen fish from Bhilwara and Chittorgarh, with *Staphylococcus aureus and Klebsiella pneumoniae* posing particular health risks due to their high prevalence and pathogenic potential. The findings suggest that the contamination could be attributed to inadequate hygiene practices during handling and storage, environmental factors, and differences in fish species. The presence of antibiotic-resistant bacteria like *Klebsiella pneumoniae* and toxin-producing fungi such as *Aspergillus* species raises serious public health concerns.

VI. RECOMMENDATIONS

- *Improved Hygiene and Handling Practices:*

Implement stringent hygiene protocols during the processing, storage, and transportation of frozen fish to minimize bacterial and fungal contamination. Regular training for handlers on best practices in food safety.

- *Enhanced Storage Facilities:*

Upgrading storage facilities to ensure consistent and appropriate temperatures are maintained, reducing the risk of microbial growth. Regular monitoring of storage conditions to ensure compliance with safety standards.

- *Routine Microbial Testing:*

Establishing routine microbial testing for both bacterial and fungal contaminants in frozen fish products to ensure safety before reaching consumers. Implement corrective actions when contamination levels exceed safety thresholds.

- *Public Awareness and Consumer Education:*

Educating consumers on the importance of proper storage and cooking of frozen fish to reduce the risk of foodborne illnesses. Raise awareness about the potential risks associated with contaminated fish products.

- *Further Research:*

Conducting further research to understand the sources of contamination and the effectiveness of different interventions in reducing microbial load in frozen fish. Explore the impact of different fish species and environmental conditions on contamination levels to develop targeted strategies for mitigation.

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