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Identification and antibiogram of aerobic and anaerobic bacteria isolated from frozen fish retailed at public markets in Misan city, Iraq

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Abstract
The aim of the current study was to identify the bacteria that can be isolated from various samples of frozen fish and to examine their antibiograms. 45 samples of three types of frozen imported fish (Zuri, filli fish, *Pangasius hypophthalmus*) (15 of each sample) were collected from different markets in Misan Governorate, during the periods from 1/12/2020 to 15/1/2021. The isolation and identification of bacteria as well as bacterial sensitivity test to antibiotics were performed. The results of this study revealed the presence of different bacterial types in frozen fish samples, the types of the detected bacteria involved the foolowing kinds: Coagulase-negative staphylococcus (2 isolates), Enterobacter aerogenes (1 isolate), and E. coli (1 isolate), but Leuconostoc citreum was not isolated. The amount of the isolation were different in amount depending on the tupes of fish studied. The presence of the detected bacteria was more prevalent in zuri than in white fish fillet and *Pangasius hypophthalmus* fish. The prevalence of both E.coli bacteria and Enterobacter aerogenes was the highest in zuri, while the contamination with Enterobacter aerogenes, Coagulase negative staphylococcus and Leuconostoc citreum were the lowest in both *Pangasius hypophthalmus* fish and white fish fillet. In conclusion, different types of bacteria that showed various degree of resistance to antibiotics were isolated from the three types of studied fish.

Keywords: Isolation contaminated bacteria, frozen fish, Food Safety, Antibiotic Sensitivity

1. Introduction

Fish is considered as an important food which has high nutritive value, as it is a source of low price animal protein as well as other important nutrients like calcium, phosphorus, and vitamins (Balami *et al*., 2019) [1]. The aquatic environment and delicate tissues of fish are extremely vulnerable to be invaded by microbes (Begum *et al*., 2021) [5]. During the past three decades, the eating habits of human were changed considerably, especially in East Asia particularly in China and East/North Africa region. Recently and according to a survey, 20 kgs of fish are consumed by the average person per year, which represents about 6.7% of all the protein that is ingested by people worldwide (FAO, 2016) [6]. Due to perishable nature of fish, its flavor and aroma are mainly affected by the temperature of preservation following being caught (Shikha *et al*., 2022) [2]. Approximately, bacterial counts of 10^7 cfu/g of the fish lead to the production of an off flavor and odor (Gelman *et al*., 2001) [3]. The majority of the transmitted diseases from fish to humans through zoonotic agents are bacterial, and infection is contracted when diseased fish or fomites are handled and result in cuts, abrasions and even puncture wounds to the skin (Boylan, 2011) [7]. Consumption of raw fish or fish with little to no processing results in bacterial contamination which either caused by direct contamination of fish by water or it may cause by secondary contamination during handling, processing, storage, distribution and preparation (Yemmen and Gargouri, 2022) [10]. Some bacterial species are able to cause disease in both people and fish. Without any clear signs of the disease, they may be separated from fish. There is a link between the following bacteria and human illnesses and poisonings: Aeromonas species, Salmonella species, Staphylococcus aureus, Clostridium botulinum, C. perfringens, Campylobacter jejuni, Delftia acidovorans, Edwardsiella tarda, Legionella pneumophila, and Plesiomonas shigellosis's are among the bacteria that have been identified. Streptococcus ini ae, Photo bacterium damsel, VI (Novotny *et al*., 2017) [9]. Pseudomonas, Aeromonas, and Enterobacter can be considered the most common bacterial species that are responsible for fish rotting(Anagnostopoulos *et al*., 2022) [4].

Based on the outcomes of various research, fish have bacterial colonies on their skin, gills, digestive tract and light-emitting organs (Zaccone *et al*., 2022) [8]. The direct transmission of fish-borne zoonotic illnesses results from consumption of raw or inadequately cooked fish meat. Indirect transmission is caused by presence of the contaminated water in the vicinity of diseased fish (Lehel *et al*., 2021) [11]. It is challenging mission to accomplish this goal due to a scarcity of information related to the microbiological quality of fish. There is trouble of processing frozen fish for export because of adhering to the HACCP protocol requirements. The goal of the current study goal was to ascertain the microbiological quality of fish following cooking and drying. Additionally, the study examined the isolated bacteria's unique pattern of antibiotic susceptibility. The current study was also conducted to isolate and detect bacterial infections from frozen fish samples and to raise awareness among local consumers and recipients.

2. Materials and Methods

Collection of fish samples

A total of 45 random samples of frozen fish of Zuri, filli fish and *Pangasius hypophthalmus* (15 of each) were collected from different fish markets at Misan Governorate. Each sample was kept individually in separated plastic bag and transferred directly to the laboratory in an insulated box under complete aseptic condition without any delay. The collected samples were examined bacteriologically as soon as possible.

Bacterial diagnosis

Official methods (AOAC) were adopted to detect bacterial contamination. Following bringing of samples to the laboratory, they were collected at a weight of 20 grams of each fish and from different parts of the body and were placed in the center of the liquid media (nutrients broth). Other 20 grams samples were placed in the middle of a liquid media which contained 5 ml of nutrient broth, then the decimal dilution was made for each sample of fish in nutrient broth 10^{-2}, 10^{-3}, $10^{-}4$ the samples were later cultured in the nutrient broth agar and were incubated at of 37 °C m for one day for salmonella isolates were cultured on the special media.

Isolation and Identification of Bacteria

The morphology of bacterial colony was evaluated by assessment of colony size, shape, density, and color. Some biochemical tests were also used for isolation and identification of bacteria such as sugar test, Gram staining method, motility and indole test.

The precise species of bacteria were determined by subculture of the suspected colony in nutrient agar and nutrient broth media. Finally, the pure cultivation was performed by using a zigzag slant. The different biochemical tests such as gram staining and MR-VP tests, were accomplished in a completely sterile environment. Sensitivity test was used to determine sensitivity of the bacterial to antibiotics. Streaking on different solid agars was performed under laminar air flow.

Identification of isolates

According to manufacturer guidelines, the identification of all fish-isolated bacteria was achieved by using the Vitek equipment (bio Merieux Vitek Systems Inc., Hazelwood, Mo.).

Chemical analysis

The procedures used for calculation of the amounts of protein, fat, moisture and ash were recommended by the American Organization for Chemical Analysis (Association of Official Agricultural Chemists).

Statistical analysis

The average and standard deviation for the total number of aerobic plates were obtained. The Social Package of Social Sciences (SPSS) versiona22 was used to examine the data, and independent samples T-test was used to calculate statistical differences (Al-Rawi and Khalf Allahs, 2000).

3. Results and discussion

The tests results of microbial loads, staining, culture, biochemistry, antibiotic sensitivity pattern, and percentage of the isolated bacterial incidence were shown in distinct tables and discussed below under the following areas. The findings of this study revealed the presence of different bacterial kinds in frozen fish samples that were shown in Table 1, the types of the detected bacteria were as the following: Coagulase-negative staphylococcus (2 isolates), Enterobacter aerogenes (1 isolate), and E. coli (1 isolate), but Leuconostoc citreum was not detected. These isolations were in different amounts relying on the kinds of fish studied, the presence of the detected bacteria was more prevalent in zuri than in white fish fillet and *Pangasius hypophthalmus* fish. It was found that the presence of both E.coli bacteria and Enterobacter aerogenes was the highest in zuri, while the contamination with Enterobacter aerogenes, Coagulase negative staphylococcus and Leuconostoc citreum were the lowest in both *Pangasius hypophthalmus* fish and white fish fillet. These findings were similar to the study conducted by (Hussein *et al*., 2009) [24].

Table 1: Percentages of bacterial types isolated from samples of Sfrozen fish

Bacterial types	Total number of bacterial in frozen *Clupeonella cultriventris* (zuri) fish	Number of isolatets	Bacterial count	Total number of bacterial isolates in frozen *Pangasius hypophthalmus* fish	Bacterial count	Total number of bacterial isolates in frozen white fish fillet	Bacterial count
E.coli	14	1	$2x10^5$	6	$2.5x10^5$	7	$3x10^6$
Enterobacter aerogenes	10	1	$1x10^6$	2	$3x10^5$	7	$2x10^5$
Coagulase negative staphylococcus	7	2	$3x10^4$	2	$2x10^6$	3	$3x10^4$
Citrobacter freundii	5	1	$4x10^5$	2	$2x10^7$	2	$2x10^5$
Leuconostoc citratum	7	0		2	$2x10^6$	5	$3x10^5$

The same table showed that the rates of several microbe species in white fish fillet and *Clupeonella cultriventris* (zuri) fish. It was observed that the prevalence of E. coli and Leuconostoc citreum bacteria had increased which could be attributed to the very high temperatures in different central markets. These markets seem not to be adhered to the practice of storing and marketing fish by following particular cooling methods. Finally, these circumstances promote the growth of bacteria by contributing to the melting and warming of the outer surface, which allow the growth of a several germs and produce perishable and unfit materials for human use (hameed Albassam*et al*., 2021) [26]. The prevalence of variety coliforms, heterotrophic indicator microorganisms and pathogenic strains in the ice which is used to freeze fish and the other seafood has been studied by Falcao *et al*. (2002) [25], who suggests that the ice used to refrigerate seafood could be a source of coliform bacteria that leads to human infection. As a result, some of the contamination detected in the current investigation could be related to the ice that is used for freeze during processing.

Numerous enteric bacteria were detected, which indicated the level of handler cross contamination. Their presence is of possible risk to people, especially those suffering from weakened immune systems, such as those with HIV/AIDS. To guarantee the safety of fish consumption, strict rules must be followed which are related to the registration of fishermen, traders, education and the necessity of monitoring sanitation at trading locations and using ice properly (Mhango *et al*., 2010) [29]. In this study, the results related to the test of antibiotic resistance in bacterial isolated in *Pangasius hypophthalmus* revealed that that most of the E. colis isolates were resistant to Ciprofloxacin (100%) as shown in Table 2. The resistance to Oxacillin (40%) and Erythromycin (40%) was found to be lower. Enterobacter aerogenes showed resistance to Gentamycin (85.7%), Erythromycin (85.7%), Ampicillin (71.5%) and Ciprofloxacin (70%). Whereas the resistance to Oxacillin (57.1%), Tetracyclinine (57.1%) and Ciproflxacin (50%) was lower. Coagulase negative staphylococcus, Citrobacter freundii and Leuconostoc citreum were lower resistant to all antibiotics.

Table 2: Percentages of antibiotic resistance in bacterial isolated in *Pangasius hypophthalmus*.

Bacterial type	Ampicillin	Oxacillin	Gentamycin	Erythromycin	Ciprofloxacin	Tetracyclinine	Ciproflxacin
E.coli	0	40	0	40	100	0	0
Enterobacter aerogenes	71.5	57.1	85.7	85.7	70	57.1	50
Coagulase negative staphylococcus	50	50	0	50	50	0	50
Citrobacter freundii	0	0	0	0	30	0	25
Leuconostoc citreum	34	30	40	0	25	0	25

There were various gram-positive and gram-negative bacteria in the intestine of fish such as Listeria, Bacillus, and Staphylococcus. Gram-negative bacteria included certai species like Aeromonas hydrophila, Citrobacter freundii, Escherichia coli, Enterobacter aerogenes, Vibrio anguillarum, Klebsiella and Pseudomonas (Ngoc *et al*., 2022) [19]. The presence of these bacteria in large concentrated levels in the liver, kidney, intestine, and other organs of fish could be a significant source of cross-contamination during processing procedure (Woraprayote *et al*., 2018) [20].

Table (2) showed that the coli was only resistant to ampicillin in *Pangasius hypophthalmus*. These results were in agreement with recent research which reported that TEM-type b-lactamases are the most common plasmid-mediated b-lactamases in gram-negative bacteria and the most common in ampicillin-resistant E. coli isolates from foods and animals (Gonçalves *et al*., 2013; Van *et al*., 2008) [14, 15]. According to a study, the majority of the E. coli isolates showed resistance to oxytetracycline (97%), lincomycin (98%) and oxacillin (99%). Resistance to ciprofloxacin (59%) was more common than that to ampicillin (51%) and chloramphenicol (73%) (Abo-Amer *et al*., 2018). E. colis exhibited the highest resistance toward streptomycin, whis is followed closely by cloxacillin, oxacillin and erythromycin as reported by (Ayandele *et al*., 2020) [17]. Another investigation reported that E. coli isolates was at high level of ampicillin resistance (63.6%), that is followed by ceftazidime (69.09%), nalidixic acid (78.1%), ciprofloxacin (45.5%–49.1%), kanamycin (36.4%–41.8%), chloramphenicol, cefotaxime, meropenem, tetracycline, and colistin (Ngoc *et al*., 2022) [19]. In a previous study reported by Salako *et al*., (2020), it was found that 61-69% of E. coli isolates from Pangasiusa freezing processes at two factories in the Mekong Deltaa of Vietnam showed resistance to ampicillin (43-47%), followed by cefotaxime (33-40%). It was mentioned that the isolates prevalence of multi-drug resistance was evident. The development of antibiotic resistance bacteria was observed discovered from samples collected from Pangasius fish during farming could be interpreted by the buildup of antibiotics in the water, surroundings and ponds. The negative coagulase a level 1 score is given to this parameter related to Staphylococcus strains as hospital studies revealed increasing both incidences and infection rate with the types of bacteria, many of which are being antibiotic resistant, and because of unknown state where these Staphylococci strains originated or what the risky these strains are (Noseda *et al*., 2013) [21].

In general, starving Pangasiusa fish prior to killing them could be efficient in decreasing the cross-contamination of fish fillet with a harmful, spoilage-causing, and bacteria that are antibiotic-resistant during processing (Ngoc *et al*., 2022) [19].

Table 3: Percentages of antibiotic resistance in bacterial isolated in Zuri

Bacterial type	Ampicillin	Oxacillin	Gentamicin	Erythromycin	Ciprofloxacin	Tetracycline	Cefoxitin
E.coli	0	40	0	40	100	0	0
Enterobacter aerogenes	71.5	57.1	85.7	85.7	70	57.1	50
Coagulase negative staphylococcus	25	50	0	100	100	0	50
Citrobacter braaki	75	25	75	33	0	25	50
Leuconostoc citreum	50	50	0	50	25	50	50

The Black, Azov, and Caspian Seas' native cultriventris fish is named in Russia as "Zuri fish" or "Kilka fish." The both two species of anchovy and big eye kilka, C. engrauliformis (Borodin, 1904) and C. grimmi, are considered as the subspecies of this fish, which is regarded as a member of the Clapeidae subfamily of fish (Kessler, 1877) [22]. The Caspian Sea coast is a place where the common Kilka, C. cultriventris caspi (Svetovidov, 1941), can live. In comparison to other years, the harvest of this species of fish is increased due to the changing of the catch (Soleimani *et al*., 2016) [23]. It was discovered that percentages of resistance to antibiotic in E.coli isolated in Zuri was (100%) to Ciprofloxacin and showed the lower resistant to (Oxacillin and Erthromycin), whereas there was no reported resistance to (Ampicillin, Gentamicin, Tetraccycline and Cefoxitin). On the other hand, Enterobacter aerogenes was resistant to Gentamicin (85.7%) and Erthromycin (85.7%), Ampicillin (71. 5%) and Ciprofloxacin (70%), but revealed low resistance to Oxacillin, Tetracycline and Cefoxitin. Coagulase negative staphylococcus was of high resistance to Erythromycin (100%) and Ciprofloxacin (100%) while there no resistance to Gentamicin and Tetracycline was preported and low resistance to Ampicillin (25%), Oxacillin (50%) and Cefoxitin (50%) was observed. Citrobacter braaki was noticed to be resistant to Ampicillin (75%) and Gentamicin (75%) with showed low resistance to Oxacillin, Erythromycin, Ciprofloxacin, tetracycline and Cefoxitin. Leuconostoc citreum was of low resistance to all antibiotics table 3. This report could be considered the first study on zuri fish in Iraq.

Table 4: Percentages of antibiotic resistance in bacterial isolated in fillet fish

Bacterial type	Ampicillin	Oxacillin	Gentamicin	Erythromycin	Ciprofloxacin	tetracycline	Cefoxitin
E.coli	50	50	0	25	0	0	0
Enterobacter aerogenes	50	60	72	50	25	30	40
Coagulase negative staphylococcus	40	50	100	50	50	0	50
Citrobacter braaki	50	30	50	50	25	50	40
Leuconostoc citreum	45	30	0	50	25	100	50

Table (4) showed the Percentages of antibiotic resistance in bacterial isolated from fillet fish. E.coli exhibited low resistance to (Ampicillin, Oxacillin and Erthromycin) without resistance to (Gentamicin, Ciprofloxacin, tetracycline and Cefoxitin). Enterobacter aerogenes revealed low resistance to all antibiotics. While Coagulase negative staphylococcus was found to be resistant to Gentamicin (100%) without resistance to tetracycline (0%) and was of lower resistance to Ampicillin (40%), Oxacillin (50%), Erthromycin (50%), Ciprofloxacin (50%) and Cefoxitin (50%). Citrobacter braaki showed lower resistance to all antibiotic, but Leuconostoc citreum was observed to be high resistant to tetracycline (100%) with no resistance to Gentamicin, and of lower resistance to other antibiotics.

Coliforms, which involve E. coli, are microorganisms that may infect food and sicken people since they affect the digestive system. This is inescapable without following the right sterilization methods. In their study, Ellis *et al*. (2020) [27] noticed that the majority (60%) of the MDR isolates of E. coli showed co-resistance to following antibiotics such as ampicillin, ciprofloxacin, tetracycline, and trimethoprim in addition to resistance to other drugs. The all MDR isolates exhibited ciprofloxacin resistance without nalidixic acid resistance was reported in any of them. Prior to the national action plan which was implemented into place in 2016, tetracycline and other antimicrobial products were usually used in Vietnam, which could represent the high levels of tetracycline resistance (Longsand Lua, 2017) [28].

4. Conclusions and Recommendations

In conclusion, the findings of this study showed that a high-quality supply of animal protein may not be healthy and fit for human consumption because of sanitation lack, filthy equipment, and unhygienic cooking practices. Various species of bacteria isolates were found in samples collected from certain species of fish. These isolated bacteria showed different levels of resistance to various antibiotics. Good hygiene standards should be followed and recommended at the time of manufacturing fish, freezing, cooking. Furthermore, conservation procedures should be devised to decrease food contamination by bacteria.

Conflict of Interest

Not available

Financial Support

Not available

5. References

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