



Faculty of Science - University of Benghazi

Libyan Journal of Science & Technology

journal home page: www.sc.uob.edu.ly/pages/page/77

Isolation and Identification of Pathogenic Bacteria from Domestic Retail Fish Market at Zliten City, Libya

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Highlights

- The highest bacteria count was reported for the gills of *Euthynnus alletteratus* compared to *Sarpa salpa* and *Scomber japonicas*.
- The whole fish of *Euthynnus alletteratus* had also the highest bacterial count followed by the intestine of *Sarpa salpa* and *Scomber japonicas*.
- Identified bacteria include *Staphylococcus spp.*, *Staphylococcus aureus*, *Bacillus*, *Pseudomonas* and *Vibrio spp.*
- *Staphylococcus spp.* resisted 100% to Cephalexin and Rifampicin, where *Staphylococcus aureus* was resistance to Tetracycline and Vancomycin.

ARTICLE INFO

Article history:

Received 10 September 2018

Revised 08 October 2018

Accepted 15 October 2018

Available online 26 December 2018

Keywords:

Zliten fish market, Marine fishes, Bacterial isolation, Antibiotic resistant pattern.

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ABSTRACT

The microbial load of both fish and fish products has been a cause of concern to consumers, and the industry itself. The present study was focused on isolation and identification of pathogenic bacteria found in selected species of fish taken at the city market of Zliten, Libya. Thirty-five fish samples were analyzed; the bacterial counts and identification of potentially isolated-bacteria were carried out by using standard bacteriological procedures. The sensitivity test was determined by the disc-diffusion method. The results have shown the highest bacteria count was reported for the gills of *Euthynnus alletteratus* (7.61×10^6 cfu/mL) compared to *Sarpa salpa* and *Scomber japonicas* (1.82×10^6 cfu/mL and 4.45×10^6 cfu/mL) respectively. The whole fish of *Euthynnus alletteratus* had also the highest bacterial count (7.589×10^6 cfu/mL) followed by the intestine of *Sarpa salpa* (6.07×10^5 cfu/mL) and *Scomber japonicas* (2.22×10^6 cfu/mL). Bacteria identified from this study include *Staphylococcus spp.* and *Staphylococcus aureus*, *Bacillus*, *Pseudomonas* and *Vibrio spp.* The higher levels of *Staphylococcus spp.* was observed for *Euthynnus alletteratus* and *Scomber japonicas* ($P < 0.03$) compared to *Sarpa salpa*. The *Bacillus spp.* was higher in *Scomber japonicas* ($P < 0.004$) compared to *Euthynnus alletteratus*. No Significant differences were observed in the microbial load between the domestic and imported fish. However, the ratio of the isolated *Bacillus spp.* was higher ($P < 0.02$) in imported fish (*Scomber japonicas*) compared to domestic fish. Isolated bacteria from fish samples were tested for resistance against thirteen antibiotics (Penicillin, Vancomycin, Clindamycin, Cefoxitin, Oxacillin, Linezolid, Cephalexin, Rifampicin, Streptomycin, Tetracycline, Erythromycin, Gentamicin and Ampicillin). Among the isolated bacteria, the *Staphylococcus spp.* was resisted to different antibiotics whereas one hundred percent of (Cephalexin and Rifampicin). The *Staphylococcus aureus* was resistance to Tetracycline and Vancomycin. *Bacillus spp.* was resistance to Vancomycin whereas the *Pseudomonas* was resistance to Rifampicin. Even though, the isolated bacteria were sensitive to different antibiotic.

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1. Introduction

Aquatic animals had been a major source of food for many not only domestically but also globally because of its rich contents of nutrients that are essential to health and well-being of the human race (Hoyle and Merritt, 1994; Tacon and Metian, 2013). Numerous species of fishes, like marine fishes, had been considered an important ingredient of the menu of many people in all walks of life and that according to the statistics from FAO, fish and fish products constitute about 17% of the global animal protein intake (FAO, 2015).

Despite the high nutritional value and the advantages of fish protein people get from different aquatic animals, the consumption of fishes can also be an agent or host for different pathogenic microorganisms to enter into the system of the human body that may alter the equilibrium in the body. Food

poisoning had become now a major concern of the health sector because of the high incidence of food poisoning. Some of the diseases that affect the people can be caused by pathogenic microorganisms harboured by the fishes that when they are being ingested will harm the body. These pathogenic microorganisms can gain entry into the system of the fishes by many ways that may start from the very time they are caught, disposed in the market until the cooking process by the consumers themselves (Hastein et al., 2006). Improper handling, preservation and lack of cleanliness in the preparation and cooking of these fishes can pose a risk of harbouring opportunistic microorganisms. The most common microorganisms found in fishes that may cause diseases are staphylococcus and Salmonella. To minimize contamination of the fishes, certain measures should be observed. Fishes should

be preserved at low temperature or frozen to prevent rotting and dried fishes should undergo complete drying. Good cooling process helps reduce or minimize the microbial load from 80 to 90% (Stratev et al., 2015).

Based on studies, visual inspection of the fishes are mostly used by consumers to determine the freshness of the fish they are buying which maybe sometimes deceiving because the freshness can be masked by the freezing process or when fishes are being frozen. As mentioned earlier, the pathogenic microorganisms cannot be seen readily by the naked eye but instead can only be seen microscopically. Fishes taken from polluted water caused by environmental hazards like sewage disposal and other industrial waste may accumulate in their tissues hazardous chemicals and heavy metals like lead, cadmium, arsenic, and mercury which when ingested can cause detrimental effect to the human body. It is because of the alarming concern of food poisoning that this study is undertaken. It aimed to identify the microbial contamination of some selected species of fish sold at the domestic market of the city of Zliten. The study would focus on the following parameter; a total number of bacteria, isolation and identification of pathogenic bacteria, the percentage of antibiotic resistance, patterns of contamination and measures of treatment and prevention.

2. Materials and Methods

2.1. Fish Sample

The fish samples involved in this study were three species; a) Alkowsaly (family: Scombroideae; 13) and *Scomber japonicus*, a second-class fish species which are being imported for the whole year round, b) Alersam; *Euthynnus alletteratus* (family: Scombroideae; 11) which are locally produced, and c) Chelba *Sarpa salpa* (family: Sparidae; 11) second class fish species, which are also locally found and is considered as one of the pelagic seasonal migrants that only exist in certain time of the year.

2.2. Sampling Method

The samples for this study were collected from the fish market of the City of Zliten using the selective sampling method. The study was undertaken from 09/15/2015 to 15/03/2016. The samples were placed in sterile plastic bags and properly labeled to prevent contamination. After collection samples were transported to the laboratory of the Faculty of Marine Resources in a cool container. The microbiological tests were analyzed within the two hours span.

2.3. Sample Preparation

Sample preparation of all fish body was made using the method described by Eze et al. (2011). About 10 g of the fish sample was cut from the head, middle and tail regions with a sterile knife. The cut samples were crushed into small pieces in a sterile mortar with about 10 ml sterile water. From the crushed sample, 1 mL aliquot volume was measured out and homogenized in a clean, dry sterile beaker containing 9 mL of distilled water giving a 1:10 dilution. The samples of Intestines, Gills were prepared as the previous sample by using 1 g instated of 10 g.

2.4. Reagents and Nutrient Culture Media

Oxidases, Catalase and Cocalase reagents were used in this study (Oxide Ltd., Basingstoke, Hampshire, England). Blood agar (Base) (Fluka India), MacConkey agar (Oxide Ltd, UK), Plate count agar (Oxide Ltd, UK), Nutrient broth (Fluka, India), Peptone (Fisher Scientific, USA), SS- agar (Fluka, India), Columbia Blood agar base (Oxide Ltd, UK), TCBS Cholera Medium (Oxide Ltd, UK), Cled Medium With Andrade Indicator (Oxide Ltd, UK), Mueller-Hinton agar (Oxide Ltd, UK), and Brian heart infusion media (Oxide Ltd, UK) and Gram stain were used

in this study. The API 20E bar was used to define negative gram bacteria. Farm bacterial 24 hours old and alcohol concentration of 95% were used in this study.

2.5. Estimation of Bacteria Load

The fish samples were taken from three parts of the fish: One gram of gills, intestines, and fish body were taken and each part was suspended in 9 ml of physiological solution and then grind well by a glass full swing. After good grinding, the mixture was left for 10 minutes before it was diluted from one to the sixth dilution of the sample. A 0.1 mL of each dilution was placed in petri dishes and was injected with agar counting and left for a quarter of an hour. Then the dishes were incubated upside down in the incubator at 37 °C for 18-24 h. The results were recorded and the dishes that did not give positive results were incubated at 37 °C for 18- 24 hours (Slaby et al., 1981).

2.6. Isolation of Bacterial Species

The bacterial species isolated in this study were mainly *Staphylococcus*, *Streptococcus* and *Bacillus* gram-positive mainly *Escherichia coli*, *Aeromonas* and *Vibrio*. Two of Loop full from each sample was suspended in two tubes of a nutrient broth and alkaline peptone water. Loop full from each suspension of a nutrient broth were inoculated onto plates of MacConkey and Shigella–Salmonella agar (SSA) to isolate members of family Enterobacteriaceae and other gram-negative bacilli; onto blood agar to isolate gram-positive cocci. Whereas the loop full of the suspension of alkaline peptone water were inoculated onto plates of Columbia blood agar to isolate *Aeromonas* and onto TCBS agar (Thiosulfate Citrate Bile Salts Sucrose Agar) to isolate *Vibrio* bacteria. All of the dishes were incubated in the incubator at a temperature of 37°C for 24 hours. Colonies from the agar plates were identified using standard bacteriological procedures (Collee et al., 1989; Koneman et al., 1997) and, whenever appropriate, the API 20E system (Biomerieux, Marcy. L'Etoile, France) was used.

2.7. Antibiotics Test

Susceptibility of the isolated bacteria to antimicrobial agents was determined according to the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute/NCCLS, 2005). The antibiotics were used in this study are shown in Table 1.

Table 1

Antibiotics used in the sensitivity tests.

Antibiotic	Abbreviations	Concentration (μg/disc)
Penicillin- G	P	10
Vancomycin	VA	5
Cefoxitin	FOX	30
Oxacillin	OX	1
Clindamycin	DA	2
Linezolid	LNZ	30
Aztreonam	ATM	30
Cephalothin	KF	30
Ceftazidime	CAZ	30
Ceftriaxone	CRO	30
Polymyxin-B	PB	30
Nalidixic acid	NA	30
Nitrofurantoin	F	300
Meropenem	MEM	10
Cefuroxime	CXM	30
Cephalexin	CL	30
Rifampicin	RD	5
Streptomycin	S	10
Tetracycline	TE	30
Erythromycin	E	15
Gentamicin	CN	10
Ampicillin	AMP	10

3. Statistical Analysis

For all determinations, statistical analyses were carried out including the calculation of the *P*-value according to Chi-Squares test. The significance of the measured data was considered as follows: Not significance (N.S) when $P > 0.05$; whereas $P < 0.05$ is considered as significance (S).

4. Results and Discussion

The bacteria load were done for all the studied samples of the fishes. The total number of isolated bacteria were 76 isolates. The isolates were included gram-positive and gram-negative bacteria. Multiple species (mainly two species were detected in 24 samples out of 35 samples (68.57%).

The bacteria load ranged from 2×10^7 cfu/mL to 3.35×10^6 cfu/mL for the whole fish, from 3.00×10^7 cfu/mL to 6.67×10^6

cfu/mL for the gills of fish, and from 10.00×10^8 cfu/mL to 13.34×10^7 cfu/mL for the intestine of the fishes (Table 2). The gills of locally Alersam and Chelba was reported the highest of the bacteria load compared to the imported Alkowaly. This was agreed with findings of Yagoub (2009) who observed that the mean bacteria load of fresh fishes sold in Khartoum market was ranged from 3×10^7 cfu/mL to 4×10^9 cfu/mL in the skin, from 3×10^7 cfu/mL to 7×10^9 cfu/mL in the gills and from 1.6×10^8 cfu/mL to 1.5×10^5 cfu/mL in the intestine. However, in another study observed that the bacteria load in catfish was bigger in the gills 83×10^5 cfu/mL and was less in the skin 53×10^5 cfu/mL (Ibrahim and Adetyi, 2013). Previous reports suggest that several factors including the method of isolation, season of isolation, type of culture and size of fishes used in the study affect the bacteria load (Novotny et al., 2004).

Table 2

Pathogenic and potentially pathogenic bacteria isolated from fish samples from domestic retail fish market at Zliten city, Libya.

Bacteria type G (+)	No (%) detected			Total = 35
	Fish types			
	<i>Scomber japonicas</i> n = (11)	<i>Sarpa salpa</i> n = (11)	<i>Euthynnus alletteratus</i> n = (11)	
<i>Staphylococcus aureus</i>	5 (45.45)	11 (31.42)	5 (38.4)	1 (9.09)
<i>Staphylococcus</i> spp.	(0)0	9 (25.71)	5 (38.45)	4 (36.36)
<i>Bacillus</i> spp.	(0)0	10 (28.57)	7 (53.84)	3 (27.27)
G (-)				
<i>Enterobacter asslomerans</i>	0 (0)	1 (9.09)	0 (0)	1 (2.85)
<i>Enterobacter intermedius</i>	0 (0)	1(9.09)	0 (0)	1 (2.85)
<i>Enterobacter asslomerans</i>	0 (0)	0 (0)	1 (7.69)	1 (2.85)
<i>Enterobacter sakazakii</i>	1 (9.09)	0 (0)	0 (0)	1 (2.85)
<i>Enterobacter cloacae</i>	0 (0)	1 (9.09)	0 (0)	1 (2.85)
<i>Enterobacter</i> spp.	0 (0)	0 (0)	1 (7.69)	1 (2.85)
<i>Pseudomonas cepacia</i>	0 (0)	0 (0)	1 (7.69)	1 (2.85)
<i>Pseudomonas paucimobilis</i>	1 (9.09)	0 (0)	0 (0)	1 (2.85)
<i>Pseudomonas</i> spp.	2 (18.18)	2 (18.18)	2 (15.38)	6 (17.14)
<i>Citrobacter freundii</i>	0 (0)	5 (45.45)	1 (7.69)	6 (17.14)
<i>Serratia plymuthica</i>	0 (0)	0 (0)	1 (7.69)	1 (2.85)
<i>Klebsiella pneumoniae</i>	0 (0)	0 (0)	1 (7.69)	1 (2.85)
<i>K. pneumonia</i> spp. <i>ozaenae</i>	(0)0	(0)0	(7.69)1	1 (2.85)
<i>Pasteurella</i> spp.	1 (9.09)	1 (9.09)	0 (0)	2 (5.71)
<i>Moraxella</i> spp.	0 (0)	1 (9.09)	1 (7.69)	2 (5.71)
CDc Group	7 (63.63)	1 (9.09)	4 (30.76)	12 (34.28)
<i>Aeromonas calco. var anitrat</i>	1 (9.09)	0 (0)	1 (7.69)	2 (5.71)
<i>Vibrio fluvialis</i>	0 (0)	0 (0)	1 (7.69)	1 (2.85)
<i>Chromobacterium violaceum</i>	0 (0)	0 (0)	1 (7.69)	1 (2.85)
<i>Acinetobacter calcoaceticus</i> var. <i>lwoffii</i>	2 (18.18)	0 (0)	1 (7.69)	3 (8.57)

Our study agreed with Ayulo et al. (1994) and Saavedra et al. (2004) who found that the most causes for the studied fish's diseases were from the common types related to *staphylococcus spp.* However, in a study by Abraham et al. (2010) who found that the percentage of isolated *staphylococcus spp.* was 27%. The different result was reported by Saito et al. (2011) who found that the perecentage of isolated *Staphylococcus aureus* was 19.6%, which was less than our finding (31.42%). The variation in the perecentage of isolated *Staphylococcus aureus* may be related to the difference of level of contamination of the studied fish's samples from one environment to another. In another study was done by Ali (2014) who found that *staphylococcus* species are one of the most identified bacteria whereas five species of *staphylococcus* isolated from skin, muscle, intestine, liver of *Silurus glanis* and *Cyprinus carpio*: *S. saprophyticus*, *S. epidermidis*, *S. hyicus*, *S. aureus*, and *S. intermedius*.

This study demonstrated that the fish's samples were not contaminated with faecal contaminated resource, whereas the *Escherichia. coli* was not isolated from the studied fish's samples, our findings were not agreed with Lindberg et al. (1998) who found that the fish's samples were contaminated with Coliform and *E. coli* and by Thampuran et al. (2005) who reported that the microbial quality of the tilapia indicated that all tissue samples except muscle tissues were contaminated with faecal coliform were *E. coli* is the most common contaminant and is often presented in high numbers.

According to Elsherief et al. (2014), who found that the coliform represents 94% from the total isolated bacteria, which reflect contamination of the resources of the studied samples by the animal and human wastes. Another study by Gillespie and Macrae (1975); and Boukaker et al. (2013) studied the isolation of different types of psychrophilic bacteria including *Pseudomonas* that was responsible for damaged meat fish

through conservation or damage during transportation and handling. However, our results indicated that the percentage of isolated coliform was less than the previous studies.

Our study showed that the pseudomonas bacteria was the most widespread after *Staphylococcus*, *Bacillus* whereas they isolated from 8/35 (22.86%). Our finding was not agreed with Yagoub (2009), who found the percentage of isolated *Pseudomonas* was (63%).

Bacillus spp. is the most bacteria responsible for the contamination of food status and medical equipment from the air and the dust rounded environments (Anihouvi et al., 2007; and Ali et al., 2014). The *Bacillus* spp. followed by *Staphylococcus* spp. and *Enterobacter* spp. whereas it isolated by 28.57%.

In this study, there were significant differences between rates of isolation of different type of bacteria. The isolation of *Staphylococcus* spp. was a higher in Alkowaly and Alersam than Chelba ($P<0.03$). However, The *Bacillus* spp. was a higher in Alkowaly than Alersam ($P<0.004$). No significant difference was found between domestic fishes and imported fishes except *Bacillus* spp., which the isolation of it was a higher in imported

Alkowaly than domestic fishes ($P<0.03$). This is due to the difference in the environment and its contamination.

The sensitivity to antibiotics was done on seventy-six bacteria, which isolated from thirty-five fish samples. Our data showed that *Staphylococcus* spp. were resistance to Cefoxitin and Oxacillin 100% and 77% to Penicillin-G, Clindamycin, and Streptomycin whereas 66.6%, 77.7% and 55.5% were susceptible to Cephalexin, Rifampicin and Vancomycin, respectively. *Staphylococcus aureus* was sensitive to Tetracycline (63.3%) and Vancomycin 54.5%. However, the resistance was 100% to each of antibiotic Cefoxitin, Gentamicin, Oxacillin and Streptomycin. Higher resistance rates to several antimicrobials were observed among *Bacillus* spp. Antimicrobials resistance profiles of gram-positive strains isolated from the fish samples are shown in Table 3.

Results of antibiotic sensitivity test of gram-negative bacteria were varied. Higher resistance rates (100%) to at least three antimicrobial tested such as Cephalothin, Meropenem, Erythromycin and Ceftriaxone. among all strains, while varied in their sensitivity to other antibiotics (Table 4).

Table 3

Resistance to antibiotics of Gram-positive bacteria isolated from fish samples from domestic retail fish market at Zliten city, Libya.

Antibiotic	No. (%) resistant		
	<i>Staphylococcus aureus</i> n=(11)	<i>Staphylococcus</i> Spp. n=(9)	<i>Bacillus</i> Spp. n=(10)
Penicillin-G	9 (81.8)	7 (77.7)	10 (100)
Vancomycin	5 (45.4)	4 (44.4)	4 (40)
Clindamycin	10 (90)	7 (77.7)	10 (100)
Cefoxitin	11 (100)	9 (100)	10 (100)
Oxacillin	11 (100)	9 (100)	10 (100)
Linezolid	7 (63.6)	4 (44.4)	9 (90)
Cephalexin	5 (45.4)	1 (11.1)	3 (30)
Rifampicin	8 (72.7)	2 (22.2)	10 (100)
Streptomycin	11 (100)	7 (77.7)	(80)8
Tetracycline	3 (27.7)	3 (33.3)	6 (60)
Erythromycin	9 (81.8)	5 (55.5)	9 (90)
Gentamicin	11 (100)	4 (44.4)	9 (90)
Ampicillin	9 (81.8)	3 (33.3)	6 (60)

Table 4

Resistance to antibiotics of Gram-negative bacteria isolated from fish samples from domestic retail fish market at Zliten city, Libya.

Antibiotics	No. (%) resistant			
	<i>Enterobacter</i> spp. n=(17)	<i>Pseudomonas</i> spp. n=(8)	CDC group n=(12)	Other gram-negative spp. n=(9)
Aztreonam	7 (41.17)	5 (62.5)	11 (91.6)	1 (11.11)
Cephalothin	17 (100)	8 (100)	12 (100)	9 (100)
Ceftazidime	7 (41.17)	6 (75)	10 (83.3)	1 (11.11)
Ceftriaxone	6 (35.1)	5 (62.5)	6 (50)	2 (22.22)
Polymyxin-B	10 (58.8)	6 (75)	10 (83.3)	3 (33.33)
Nalidixicacid	15 (88.2)	8 (100)	11 (91.6)	2 (22.22)
Nitrofurantoin	12 (70.5)	5 (62.5)	8 (66.6)	8 (88.89)
Meropenem	17 (100)	8 (100)	12 (100)	8 (88.89)
Cefuroxime	15 (88.2)	8 (100)	12 (100)	8 (88.89)
Cephalexin	12 (70.5)	7 (87.5)	7 (58.3)	8 (88.89)
Rifampicin	17 (100)	3 (37.5)	8 (66.6)	5 (55.56)
Streptomycin	13 (76.4)	5 (62.5)	11 (91.6)	7 (77.78)
Tetracycline	12 (70.5)	5 (62.5)	7 (58.3)	4 (44.44)
Erythromycin	17 (100)	7 (87.5)	11 (91.6)	8 (88.89)
Gentamicin	13 (76.4)	6 (75)	9 (75)	4 (44.44)
Ampicillin	15 (88.2)	5 (62.5)	9 (75)	8 (88.89)

Other gram-negative spp. include: *Pasteurella* spp (5.71)2, *Moraxella* spp. (5.71)2, *Klebsiella pneumonia* (2.85)1, *Chromobacterium violaceum* (2.85)1.

The *Enterobacter* spp and another gram negative isolated bacteria was sensitive to Aztreonam and Ceftazidime 10 (55.5%). However, the resistance was 100% to all of antibiotics including Cephalothin, Meropenem and Erythromycin. Different

rates of multidrug resistance (MDR) was detected among gram-positive and negative bacteria. In general, the isolated bacteria in this study are responsible to cause several types of diseases including diarrhea, urinary tract infection, and wound infection

etc.; their resistance to antibiotics is considered an extra burden, especially if we look at the different rates of MDR that we observed in our study among the type of microorganisms. However, high rates of antibiotics resistance and sometimes MDR among different bacterial species isolated from different fish samples were reported previously (Grema *et al.*, 2015; Miranda and Zemelman, 2001; Chelossi *et al.*, 2003; and Smaldone *et al.*, 2014). In this study, most species of isolated gram-negative and gram-positive bacteria were resistant to most studied antibiotics and some of them were multidrug resistant for more than 2-3 different antibiotics. These findings indicate that fish as a communal food source may play a role in the spread of multidrug-resistant bacteria in the community and pose a serious health risk to a human being particularly the workers in this field. In addition to, this is an indicator to take a series of steps to reduce the increase of resistance of antibiotics. The difference in the resistance for the antibiotics reflects the type of resistance in different regions around the world. On the other hand, our findings showed that a reasonable sensitivity rate to some of the antibiotics was detected. To our knowledge, this is the first report of microbial quality of fish in this region.

In conclusion, fish and fishery products could be the major source and act as a host to some of the microbial contaminants that may be found in water, post-harvesting, marketing, dealing, fish handling. Mis-control of these factors could lead to loss of fish meat quality. However, the sanitation and principles of prevention of foodborne disease should be applied to protect our consumers against the public health hazard. Finally, we hope that this study may encourage other investigators for further study, which in turn may provide a better idea of the quality of the local and imported fish.

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