Isolation and Identification of major bacteria from three Ethiopian rift valley lakes live and processed fish, and water samples: Implications in sanitary system of fish products

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Abstract

Bacterial pathogens are a great threat to fish production. Gram-negative bacteria are among the major bacterial fish pathogens and zoonotic with the potential to infect humans. This cross-sectional study was conducted to isolate and identify major gram-negative bacteria from live and processed fish, and water samples from Lakes Hawassa, Langanoo and Ziway. A total of 674 different types of samples: 630 tissue samples (210 samples for each intestine, Kidney and liver collected from 210 live fish (*Oreochromis niloticus, Cyprinus carpio* and *Clarias gariepinus*), 20 processed fish samples from lake Ziway fish processing center and 24 lake water samples were included in the study from each lake. The mean values of pH, temperature, dissolved oxygen and nitrate in all water samples were within the normal range at which most freshwater fish species become non-stressed. Of a total of 674 samples included in the study, the bacteria were isolated from 154 (22.8%) samples with significant difference (P<0.05) observed in some isolates with respect to sample origin. Of these 154 isolates, 103(66.8%) isolates were gram-negative bacteria consisting of 15 species based on morphology and a range of biochemical tests. From live fish samples, *Escherichia coli* was the dominant species with 15 isolates followed by *Edwardsiella tarda* (12), *Salmonella Paratyphi* (10), *Salmonella Typhi* (9), *Shigella dysenteriae*(7), *Shigella flexneri* (7), *Klebsiella pneumonia* (7), *Enterobacter aerogenes* (6), *Enterobacter cloacae* (5), *Pseudomonas aeruginosa* (5), *Vibrio parahemolyticus* (5), *Aeromonas sobria* (4), *Citrobacter freundii* (4), *Citrobacter koseri* (4) and *Plesiomonas shigelloides*(3). Detection of common fecal coliforms (*E. coli, K. pneumoniae* and *E. aerogenes*) and *Salmonella* spp. in processed fish indicates the potential danger of passage of pathogenic bacteria and/or their poisons to humans via infected and/or contaminated fish products. Human infection by pathogenic fish bacteria and food poisoning is possible through contamination of fish product in fish production chain due to inadequate handling, poor hygiene and contact with contaminated water. Therefore, producers, consumers and all other stakeholders need to be cautious during handling, processing and consumption of fish harvested from the study lakes.

1. Introduction

Fish plays an important role in the human diet with an ever-growing need globally. World fish production has increased dramatically during the past 60 years, to around 179 million tons in 2018 with a value of $401 billion and global fish consumption also increased from 9.0 kg per capita in 1961 to 20.5 kg in 2018 (FAO, 2020, Ibrahim et al., 2020). Ethiopia depends on its inland lakes and rivers for its fish production with an estimate of more than 200 edible fish species and its annual fish production potential about 94,500 thousand tons with significant contribution to the country's economy and food supply (Abdulhakim and Alemayehu, 2020).

However, fish consumption is associated with some serious bacterial infections mainly due to poor sanitary facilities and practices around water systems, unhygienic conditions in fishing and fish production chain. Environmental dynamics in freshwater ecosystems are fundamental in the development of pathogenic fish bacteria and there has been a steady increase in the numbers of bacterial species associated with fish diseases (Pękala-Safińska, 2018). Gram-negative bacteria like Aeromonas spp., Flavobacterium spp., Pseudomonas spp., Edwardsiella spp., Vibrio spp., Acinetobacter spp. and Plesiomonas shigelloides are a great threat to freshwater fish production (Pękala-Safińska, 2018, Kerie et al., 2019, Ayoub et al., 2021). Most of these bacterial fish pathogens are zoonotic with the potential to infect humans and some are serious (Haenen et al., 2021, Hashish et al., 2018).

Assessment of bacterial community in live and processed fish, and the source natural habitat is essential to monitor fish health, product quality and related potential public health risk of fish-borne bacterial illnesses. Reports on bacterial pathogens in freshwater fish, water physicochemical parameters and bacteriological quality of Ethiopian rift valley lakes are scant. Hence, this study was aimed to assess the occurrence, distribution and prevalence of major gram-negative bacteria from live (apparently healthy and clinically diseased) and processed fish of three common fish species - Nile tilapia(*Oreochromis niloticus*),common carp (*Cyprinus carpio*)and catfish(*Clarias gariepinus*) - and water samples from three rift valley (inland) Lakes Hawassa, Langanoo and Ziway in Ethiopia. The implication of the findings in sanitary system and safety of fish products was discussed.

2. Methods

2.1. The Study area

The study was conducted at selected rift valley lakes (Fig. 1) of Ethiopia (lake Hawassa, lake Langanoo and lake Ziway) which are well known for their common fish catches. Lake Hawassa is located at an altitude of 1680 m in the central part of the Ethiopian rift valley ($6^0$33' – $7^0$33'N and $38^0$29'E) 275 km south of the capital, Addis Ababa. It has a surface area of 90 km^2 and catchment area of 1250 km^2 while the mean depth is 11 m, the maximum depth being 22 m (UNESCO, WHO and UNEP, 1996). Lake Langanoo is located 200 km South of Addis Ababa lies between 7°36' N;38°45'E. It is 18 km long and 16 km wide with an open water area of 230 km^2, 7.5 km shoreline and 1600 km^2 catchments area (Mepham et al., 1992). Lake Ziway is located on the Eastern side of Ziway town, 163 km south east of Addis Ababa. It lies in northern part of the rift valley between 7°51N to 8°7'N and 38'43' E 38°57' E with an open water area of 422 km^2 and shoreline length of 137 km(Legesse *et al.*, 2001). The geographic locations and other physical parameters of the study lakes are given below (Figure 1).

2.2. Sample size

The size of the sample was determined using the formula by Naing et al. (2006) $n = Z^2P(1 - P)) / d^2$, where 'n' is the sample size, 'Z' is the confidence interval (1.96), 'P' is the disease prevalence of 9.67% as previously reported by Eshetu (2000) in Ethiopia and 'd' is the expected error (0.05).

2.3. Study design and sampling strategy

The study design was cross-sectional and its period was from February to August 2021. For sampling, the three water bodies (lakes) were selected purposively that the lakes are known for their common fish catches and from which fish are continuously harvested for subsistence as well as for cash (Eyob, 2021). Different sites of each lake were used for fish and water sampling. A total of twelve (12) sampling sites, four from each lake, were selected based on

information available about the sampling sites as they were considered to be major fishing grounds. Accordingly, *Amora Gedel* (S1), *Haile resort area* (S2), *Inlet of Tikurwuha River* (S3) and *Referal hospital area* (S4) were sites of lake Hawassa. *Dole* (S1), *O'etu* (S2), *Wabishebelle* (S3) and *Yakona* (S4) were sites of lake Langanoo and *Abosa* (S1), *Bochessa* (S2), *Cafeteria* (S3) and *Wasiko* (S4) were sites of lake Ziway. These sites were located at different directions of the lakes and relatively far apart from each other's. The intervals between these site ranges from 3 km to 7 km distance. Thus, these sampling sites were considered to represent each lake. A random sampling technique was applied to include each fish species whereas in case of disease occurrence, diseased fish were purposively selected after fish were drawn from the peripheral, mid and central regions of sampling site water using fishing boats and fishing gears of the local fishers. Bacteria isolated and identified using a range of biochemical tests and morphological characteristics.

2.4. Water Physicochemical Parameters Analyses

Physicochemical parameters of the water samples collected from four different sites of each lake which were considered to be major fishing grounds were analyzed using routine methods. Temperature (Temp.), electrical conductivity (EC) and dissolved oxygen (DO) of the water samples were processed and measured using salinity-conductivity, temperature meter (YSI model 33 S-C-T meter) and (YSI model 51 B dissolved oxygen meter, USA) respectively. pH of the samples was recorded using digital pH meter. The chemical analyses of nutrients (nitrate and phosphate) were determined for all water samples following the standard procedures outlined in WHO, (2008). Accordingly, the nitrate (NO_3) content of water samples was determined using sodium salicylate method and phosphate (PO_4) content of water samples was determined using ascorbic acid method using the Palin test system of water analysis. Temp. was determined onsite and the samples were packed and kept refrigerated prior to the analysis of pH, dissolved oxygen (DO), electrical conductivity (EC) and nutrients.

2.5. Fish samples

The live fish samples were clinically examined to determine any abnormalities externally and were grouped into apparently healthy and diseased, and were separately packed into sterile plastic bags and transported to Batu Fisheries and Other Aquatic Life Research Center, Batu, Oromia, south-central Ethiopia, on the sampling day. The samples were dissected using a sterile dissecting scissor by making a transverse incision anterior to the anus towards the ventral part of the head up to the gill covers. Anal incision was made cutting craniodorsally toward the lateral line following the dorsal margin of the peritoneal cavity up to the gill arches and finally a third incision connecting the ends of the two cuts (Leal et al., 2009). The sidewall was then removed to expose the internal organs. Examination of organs was done by observing for any abnormalities including their position, size, color and other signs of internal disease. The digestive tract, gonads and visceral organs were removed by cutting the esophagus disconnecting them from kidneys. Tissue samples of kidney, intestine and liver were taken aseptically using sterile scalpel blade (forceps) and kept in sterile universal bottles of 100 ml capacities separately and homogenized in physiological saline solution. Water samples from each site of the lakes were collected into sterile glass bottles of 100 ml capacities separately (APHA, 1999).

Similarly, about 10gm of meat was cut from processed fish surface from Batu fish processing center with a sterile knife and kept in sterile universal bottles of 100 ml capacities in which it was homogenized with about 10 ml physiological saline solution. From the homogenized samples of both live and processed fish, 1ml aliquot was drawn and further homogenized in a clean, dry sterile beaker containing 9ml of distilled water giving a 1:10 dilution (Society of American Bacteriologists (SAB) procedure (SAB, 1957). All samples were transported on ice to Health Biotechnology Laboratory of Institute of Biotechnology, College of Natural and Computational Science, Addis Ababa University, where they were analysis was done.

2.6. Bacterial isolation and identification

The three types of samples were processed, according to the standard microbiological methods under complete aseptic conditions. Inoculums were taken from each sample of physiological saline solution homogenized intestine, liver, kidney, water and processed fish using sterile inoculating loop and inoculated on Nutrient Agar (Oxoid, England) and incubated at 37°C for 24 hours under aerobic condition. Each type culture colony was picked up and sub cultured on selective and differential media EMB agar medium and XLD agar media (HIMEDIA, India) and incubated at 37°C for 24 hours. Suspected bacterial colonies were picked up and inoculated into Tryptone Soy Broth (HIMEDIA, India) and incubated at 37°C for another 24 hours.

2.7. Morphological observations of isolates

For the identification of selected isolates, colony morphology such as colony form, elevation, margin, surface and pigmentation were studied. Colony color was noted by visual inspection and bacterial cell suspension using fresh culture was used for microscopic examination of isolates with simple staining and differential staining (Gram- staining) following Society of American Bacteriologists (SAB) procedure (SAB, 1957).The morphologically identified isolates were stored at -20°C in 50% glycerol (Fine Chemical, Ethiopia) using cryovial tubes of 1.8ml (IMEC, China) for further biochemical identification.

2.8. Biochemical studies of the isolates

All isolates were identified biochemically by streaking bacterial colonies over TSB and incubated at 37°C for 24 hours and, then identified by using a range of biochemical tests performed according to respective manufacturer's instructions. Following Society of American Bacteriologists (SAB) (SAB, 1957) and Bergey's manual of determinative bacteriology (Holt et al., 1994) procedure, the following biochemical tests of the isolated bacteria were carried out. Indole production was tested by inoculating 10mL of Dev tryptophan broth (HIMEDIA, India) with a pure colony, incubating at 37°C for 24 hours, and adding 2-3 drops of indole reagent. Methyl red (MR) test was conducted by inoculating 10mL of Methyl red Voges-Proskauer (MR-VP) medium (HIMEDIA, India) with a pure colony, incubating at 37°C for 24 hours, and adding 2-3 drops of 0.05% Methyl Red. Voges-Proskauer (VP) test was conducted by inoculating 10mL of Methyl red Voges-Proskauer (MR-VP) medium (HIMEDIA, India) with a pure colony, incubating at 37°C for 24 hours, and adding 2-3 drops of 5% α-nephtol followed by2-3 drops of 40% KOH and shake it and leave open the test tubes for an hour.

To conduct catalase enzyme production test, a small amount of a colony was transferred directly to a clean glass slide using a sterile loop and add one drop of hydrogen peroxide and look for bubbles.To conduct citrate utilization test, Simmons citrate agar (HIMEDIA, India) slant was inoculated with 24 hours grown colony of the isolates by streaking the slant using straight inoculating wire and the tubes were loosely capped and incubated at 37°C for 24 hours and observe the slant for a color change at 24 hours. Hydrogen sulfide (H_2S) (Triple sugar iron (TSI)) production test was conducted by inoculating 24 hours grown colony

of the isolates with TSI agar (HIMEDIA, India) slant by stabbing the butt and streaking the slant using straight inoculating wire and loosely cape and incubate for 24 hours at 37°C and observe the slant for a color change at 24 hours. Urea production was tested by inoculating 24 hours grown colony of the isolates with Christensen's Urea Agar (HIMEDIA, India) slant by streaking the slant using straight inoculating wire and the tubes were loosely capped and incubated at 37°C for 24 hours and observe the slant for a color change at 24 hours. Sugar or carbohydrate (glucose, sucrose and lactose) fermentation tests was conducted using sugar (HIMEDIA, India) broth medium prepared from different components having peptone (1gm), meat extract (0.3gm), NaCl (0.5gm), sugar (0.5gm) and phenol indictor (0.008gm) per 100ml distilled water. Three tubes having the three different sugars broth medium were inoculated with a pure colony separately, incubating at 37°C for 24 hours and observe the culture for a color change at 24 hours.

2.9. Data analysis

Statistical analysis was performed using IBM SPSS software version 20 (IBM, Chicago, USA). Absence or presence of bacteria of interest in the different sample types was determined. Bacterial species diversity in live and processed fish, and water samples were compared using one way analysis of variance (ANOVA). A significance level of $p<0.05$ was applied at all statistical tests. Descriptive statistics such as proportion and frequency was employed in summarizing the data and in order to characterize and quantify the differences between each lake, organs of fish, sampling sites and fish species bacteria flora populations. Chi-square test of independence was employed in comparing the prevalence of bacterial isolates with respect to place of isolation.

3. Results

A total of 674 samples (630 live fish tissue samples, 24 water samples and 20 processed fish samples) were collected.

3.1. Physicochemical Parameters

The physicochemical parameters of water samples (n=12) analyzed are shown in (Table 1). Accordingly, pH range 5.74 to 9.12 (Hawassa), 6.02 to 9.4 (Langanoo), 8.6 to 8.84 (Ziway) and the electrical conductivity varied from 741.1 to 2156 µS/cm (Hawassa), 902 to 1717 µS/cm (Langanoo) and 236 to 362 µS/cm (Ziway). Similarly, the temperature values of samples were between 24.1°C to 28.7°C (Hawassa),19°C to 26°C (Langanoo), 22°C to 23°C (Ziway) and of DO from 5.66 mg/L to 6.84 mg/L (Lake Hawassa), 4.79 mg/L to 5.47 mg/L (Lake Langanoo) and 2.8 mg/L to 4.5 mg/L (Lake Ziway). The concentration of nitrate range from 3.12±0.2 mg/L to 4.37±0.5 mg/L (lake Hawassa), 2.31±0.26 mg/L to 2.83±0.31 mg/L (lake langanoo) and 0.17±0.12 mg/L to 0.96±0.24 mg/L (lake Ziway) and the overall mean was 3.885 mg/L, 2.51 mg/L and 0.465 mg/L respectively. The concentration of phosphate range from 0.42±0.06 mg/L to 1.96±0.03 mg/L (lake Hawassa), 2.34±0.31 mg/L to 4.48±0.27 mg/L (lake Langanoo) and 0.21±0.04 mg/L to 0.97±0.08 mg/L (lake Ziway) and the overall mean was 1.28±0.9 mg/L, 3.65±0.44 mg/L and 0.64±0.81 mg/L respectively. The mean dissolved oxygen was (6.275 mg/L (lake Hawassa), 5.10 mg/L (lake Langanoo) and 3.93 mg/L (lake Ziway)).

Table 1
Mean ± SD values of physicochemical parameters of four different sampling sites (S1-S4) of lakes Hawassa, Langanoo and Ziway where from water samples were drawn

Lake Hawassa						
Water Samples	pH	Temp., °C	DO, mg/L	EC, µS/cm	Nitrate, mg/L	Phosphate, mg/L
S1	8.86±0.04	24.6±0.1	5.66±0.05	1961±1.9	4.33±0.4	1.31±0.1
S2	9.12±0.05	24.1±0.1	6.84±0.2	2156±1.4	4.37±0.5	1.44±0.04
S3	5.74±0.04	25.8±0.8	6.78±0.4	741±3.1	3.12±0.2	1.96±0.03
S4	7.92±0.1	28.7±0.1	5.82±0.9	1836±2.6	3.72±0.4	0.42±0.06
Average	7.91±0.1	25.8±0.8	6.275±0.9	1673.5±487	3.885±0.6	1.28±0.9
Lake Langanoo						
Samples	pH	Temp.	DO	EC	Nitrate	Phosphate
S1	9.14±0.04	24±0.51	4.99±0.18	1522±3.2	2.46±0.27	2.92±0.41
S2	9.4±0.05	26±0.48	5.47±0.21	1717±4.9	2.83±0.31	4.48±0.27
S3	6.02±0.04	19±0.39	4.79±0.35	902±1.8	2.44±0.28	2.46±0.29
S4	8.2±0.10	22±0.54	5.16±0.59	1647±7.4	2.31±0.26	2.34±0.31
Average	8.19±0.09	22.75±0.43	5.10±0.54	1447±5.7	2.51±0.29	3.65±0.44
Lake Ziway						
Samples	pH	Temp.	DO	EC	Nitrate	Phosphate
S1	8.61±0.12	23±0.49	4.32±0.31	281±11	0.17±0.12	0.63±0.86
S2	8.84±0.58	22±0.56	4.10±0.22	362±92	0.48±0.04	0.76±0.06
S3	8.6±0.09	22±0.28	2.8±0.30	236±43	0.96±0.24	0.97±0.08
S4	8.67±0.10	23±0.11	4.5±0.28	279±56	0.25±0.11	0.21±0.04
Average	8.68±0.11	22.5±0.36	3.93±0.18	289.5±14	0.465±0.14	0.64±0.81
WHO	6.5-8.5	<40^{o}C	5.0-7.0	750	45	0.1
DO: dissolved oxygen, EC: electric conductivity						

3.2. Live fish

A total of 210 live fish (168 (80%) apparently healthy and 42 (20%) clinically diseased) samples of the three species tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*) and catfish (*Clarias gariepinus*) were obtained from the three lakes. Out of the 210 live fish samples, 90, 50 and 70 fish were collected from lake Ziway, lake Langanoo and lake Hawassa respectively with 105 (tilapia), 60 (common carps) and 45 (catfish).

3.3. Morphology of the isolates

The morphological characteristics of the bacterial strains were determined including Gram staining, bacterial colony's and bacterial cell's morphology. On the basis of colony morphology, all strains were short rod and non- spore former. Colonies of the isolated isolates were found to be different in their form, elevation, margin, surface, colour and optical characteristics.

3.4. Biochemical examination of bacteria

To identify the isolated bacteria, a range of biochemical tests (Indole, methyl red, Voges-Proskauer (VP), Citrate, H_2S gas, Urease, sugar fermentation and catalase test) were carried out. The results showed that different groups of isolates that belonged to 15 species were identified. These were *A. sobria*, *C. koseri*, *C. freundii*, *E. tarda*, *E. aerogenes*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *P. shigelloides*, *P. aeruginosa*, *S. paratyphi*, *S. typhi*, *S. dysenteriae*, *S. flexneri* and *V. parahemolyticus*. Among the tested isolates only *K.pneumonia* showed positive results in Voges-Proskauer (VP) test. In Methyl red (MR) test *E. aerogenes*, *E. cloacae*, *K. pneumoniae* and *P. aeruginosa* isolates showed positive results. All of the isolates except *V. parahemolyticus* were negative to degrade urea. *Edwardsiella tarda* and *S. paratyphi* produced hydrogen sulfide gas.

3.5. Species of isolated bacteria

Different bacterial species isolated from the water, live fish and processed fish samples during the study periods is presented in Table 2. Pathogenic and non-pathogenic bacteria were isolated from the live fish tissue, processed fish and water samples. The most prevalent bacterial isolates were *E. coli* (14.6%) followed by *E. tarda* (9.7%) and *S. paratyphi* (11.7%). The most commonly detected bacterial isolates from processed fish samples were *Salmonella* spp. and common fecal coliforms (*E. coli, K. pneumoniae* and *E. aerogenes*), while, majority of *A. sobria, E. tarda*, *P. shigelloides, P. aeruginosa, S. flexneri* and *V.*

parahemolyticus were isolated from diseased fish (72% of tilapia and carps).The bacterial species isolated from processed fish (*E. coli*, *K. pneumoniae* and *S. paratyphi*) were also recovered from the water samples.

Table2. Occurrence, distribution and prevalence of bacterial species isolated from different sample types from the three study lakes ((number (%))

Sample type	N	+ for ≥1 isolate, n(%)	As, n(%)	Cf, n(%)	Ck, n(%)	Et, n(%)	Ea, n(%)	Ec^{+}, n(%)	Ec*, n(%)	Kp, n(%)	Ps, n(%)	Pa, n(%)	Sp, n(%)	St, n(%)	Sd, n(%
Water	24	12(50.0)	1(8.3)	1(8)	1(8.3)	2(17)	0(0)	0(0)	1(8)	1(8)	0(0)	1(8)	1(8)	0(0)	1(8
Live fish	210	83(39.1)	3(3.6)	3(4)	3(3.6)	10(12)	4(5)	5(6)	12(14)	4(5)	3(3.6)	4(5)	7(8)	7(8)	6(7
Processed fish	20	9(45.0)	0(0)	0(0)	0(0)	0(0)	1(11)	0(0)	2(22)	2(22)	0(0)	0(0)	2(22)	2(22)	0(0
Total	254	103(40.5)	4(3.9)	4(3.9)	4(3.9)	12(11.7)	5(4.9)	5(4.9)	15(14.6)	7(6.8)	3(2.9)	5(4.9)	10(9.7)	9(8.7)	7(6

As: Aeromonas sobria, Cf: Citrobacter freundii, Ck: Citrobacter koseri, Ct: Edwardsiella tarda, Ea: Enterobacter aerogenes, Ec^{+}: Enterobacter cloacae, Ec: Escherichia coli, Kp: Klebsiella pneumonia, Ps: Plesiomonas shigelloides, Pa: Pseudomonas aeruginosa, Sp: Salmonella Paratyphi, St: Salmonella Typhi, Sd: Shigella dysenteriae, Sf: Shigella flexneri, Vp: Vibrio parahemolyticus*

3.6. Bacteria isolated from the live fish tissue samples

Table 3 shows the distribution and frequency of occurrence of bacteria from the tissue samples kidney, liver and intestine of live fish. The majority of bacterial isolates isolated from live fish were from the intestine (Table 3). The most frequently isolated bacteria from live fish tissue samples were *E. coli* (2.4%) in the intestine and (3.6%) in the liver. *Enterobacter aerogenes* is the most frequently isolated isolate from the kidney (2.1%). The frequencies of *E. coli, P. aeruginosa*, *A. sobria*, *Citrobacter* spp., *Shigella* spp., *P. shigelloides* and *V. parahemolyticus* between the three tissue samples were statistically significant.

Table3. Distribution and frequency of occurrence of bacteria from the kidney, liver and intestine of fish (n = 210)

Isolated bacteria	Intestine n(%)	Kidney n (%)	Liver n(%)	Total n(%)	P-value
Aeromonas sobria	1(0.5)	-	2(1.4)	3(1.4)	0.001*
Citrobacter freundii	2(0.9)	-	1(0.5)	3(1.4)	0.001*
Citrobacter koseri	2(0.9)	1(0.5)	-	3(1.4)	0.001*
Edwardsiella tarda	4(1.9)	2(0.9)	4(1.9)	10(4.8)	0.623
Enterobacter aerogenes	1(0.5)	3(2.1)	1(0.5)	5(2.4)	0.372
Enterobacter cloacae	2(0.9)	2(0.9)	1(0.5)	5(2.4)	0.221
Escherichia coli	5(2.4)	2(0.9)	5(3.6)	12(5.7)	0.010*
Klebsiella pneumoniae	1(0.5)	2(0.9)	1(0.5)	4(1.9)	0.061
Plesiomonas shigelloides	1(0.5)	2(0.9)	-	3(1.4)	0.001*
Pseudomonas aeruginosa	2(0.9)	1(0.5)	1(0.5)	4(1.9)	0.015*
Salmonella paratyphi	3(1.4)	2(0.9)	2(0.9)	7(3.3)	0.151
Salmonella typhi	4(1.9)	1(0.5)	2(0.9)	7(3.3)	0.311
Shigella dysenteriae	3(1.4)	-	3(1.4)	6(2.9)	0.009*
Shigella flexneri	3(1.4)	-	3(1.4)	6(2.9)	0.009*
Vibrio parahemolyticus	3(1.4)	1(0.5)	-	4(1.9)	0.004*
Total	37(17.6)	19(9.1)	26(12.4)	82(39)	
* means significant at 5% level					

3.7. Bacteria isolated among different fish species

Table 4 shows the number and percentage n (%) of bacterial species isolated from different fish species.

Table 4
Number (%) of bacterial species isolated from different fish species

Isolated bacteria	Carps*(Cc)* n=60	Tilapia (*On*)n=105	Catfish (*Cg*)n=45	Total n=210	P-value
A.sobria	2(3.3)	-	1(2.2)	3(1.4)	0.001*
C. freundii	-	2(1.9)	1(2.2)	3(1.4)	0.001*
C. koseri	-	1(1.4)	2(4.4)	3(1.4)	0.001*
E. tarda	3(5)	4(3.8)	3(6.7)	10(4.8)	0.098
E. aerogenes	1(1.7)	1(1.4)	3(6.7)	5(2.4)	0.407
E. cloacae	1(1.7)	1(1.4)	3(6.7)	5(2.4)	0.193
E. coli	3(5)	6(5.7)	3(6.7)	12(5.7)	0.007*
K. pneumoniae	1(1.7)	2(1.9)	1(2.2)	4(1.9)	0.670
P. shigelloides	2(3.3)	1(1.4)	-	3(1.4)	0.001*
P. aeruginosa	1(1.7)	2(1.9)	1(2.2)	4(1.9)	0.501
S. paratyphi	2(3.3)	3(2.9)	2(4.4)	7(3.3)	0.184
S. typhi	2(3.3)	3(2.9)	2(4.4)	7(3.3)	0.457
S. dysenteriae	2(3.3)	3(2.9)	1(2.2)	6(2.9)	0.307
S. *flexneri*	3(5)	1(1.4)	2(4.4)	6(2.9)	0.008*
V. parahemolyticus	-	2(1.9)	2(4.4)	4(1.9)	0.014*
Total	23(38.3)	32(30.5)	27(60.0)	82(39.1)	

On: O. niloticus, Cg: C. gariepinus, Cc: *C. carpio* *means significant at 5% level

3.8. Bacteria isolated from the fish based on different study lakes

Escherichia coli were the most frequently isolated bacteria in fish sampled from the three lakes (Lake Hawassa, Lake Ziway and Lake Langanoo) (7.1%, 6.3% and 3.3%) respectively. *Edwardsiella tarda* were the second most frequently isolated bacteria in fish sampled from the three (lake Hawassa, lake Ziway and lake Langanoo) lakes (7.1%, 3.8% and 3.3%) respectively. The percentages of *V. parahemolyticus, C. koseri, C. freundii, A.sobria, E. cloacae*, *E. coli* and *P. aeruginosa* varied significantly (P<0.05) among the fish sampling lakes (Table 5).

Table 5
Number (%) of bacteria isolated from the fish based on study lakes

Isolated bacteria	Lake Hawassa n=70	Lake Langanoo n=60	Lake Ziway n=80	Total n=210	P-value
A.sobria	2(2.9)	-	1(1.3)	3(1.4)	0.001*
C. freundii	2(2.9)	1(1.7)	-	3(1.4)	0.001*
C. koseri	2(2.9)	-	1(1.3)	3(1.4)	0.001*
E. tarda	5(7.1)	2(3.3)	3(3.8)	10(4.8)	0.096
E. aerogenes	1(1.4)	1(1.7)	3(3.8)	5(2.4)	0.206
E. cloacae	2(2.9)	-	3(3.8)	5(2.4)	0.003*
E. coli	5(7.1)	2(3.3)	5(6.3)	12(5.7)	0.016*
K. pneumoniae	2(2.9)	-	2(2.5)	4(1.9)	0.153
P. shigelloides	1(1.4)	1(1.7)	2(2.5)	4(1.9)	0.287
P. aeruginosa	1(1.4)	-	2(2.5)	3(1.4)	0.001*
S. paratyphi	3 (4.3)	1 (1.7)	3 (3.8)	7 (3.3)	0.364
S. typhi	2 (2.9)	3 (5)	2 (2.5)	7 (3.3)	0.068
S. dysenteriae	2 (2.9)	1 (1.7)	3 (3.8)	6 (2.9)	0.413
S. flexneri	3 (4.3)	1 (1.7)	2 (2.5)	6 (2.9)	0.158
V. parahemolyticus	1 (1.4)	-	3 (3.8)	4 (1.9)	0.007*
Total	34 (48.6)	13 (21.7)	35 (43.8)	82 (39.1)	
* means significant at 5% level					

4. Discussion

A total of 15 different Gram-negative bacteria species *A. sobria*, *C. koseri*, *C. freundii*, *E. tarda*, *E. aerogenes*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *P. shigelloides, P. aeruginosa*, *S. paratyphi*, *S. typhi*, *S. dysenteriae, S. flexneri* and *V. parahemolyticus* were isolated from 82 (79.6%) live fish (intestine, kidney and liver), 12 (11.6%) water and 9 (8.7%) processed fish samples. The majority of the isolates isolated from the live fish were originated from the intestines. This result is in line with the findings of Sedlacek et al. (2016) and Kassa and Mitiku, (2021) which shows higher bacterial load at intestine than other organs. In this study the differences in the frequency of each bacteria isolates between groups of the different variables (tissue samples) were more pronounced due to the variation of the frequency of bacteria isolates in the kidney and liver of fish, which were sampled during the study period. The most frequently isolated bacteria from live fish tissue samples were *E. coli* (2.4%) in the intestine and (3.6%) in the liver. And *E. aerogenes* is the most frequently isolated isolate from the kidney (2.1%). The least frequently isolated bacteria from live fish tissue samples were *P. shigelloides* (2.9%). The isolates *E. coli, P. aeruginosa*, *A. sobria*, *Citrobacter* spp, *Shigella* spp, *P. shigelloides* and *V. parahemolyticus* were found to be having statistically significant differences (P<0.05) between tissue samples. Characteristics of the microenvironment at various locations through the alimentary tract of each fish species also influence the taxonomic composition as well as the numerical abundance of bacteria present (Gufe et al., 2019).

Among the water samples examined bacteriologically during the study period, bacteria species *A. sobria, Citrobacter* spp, *E. tarda, E. coli, K. pneumoniae, P. aeruginosa, S. paratyphi, S. dysenteriae* and *S. flexneri* were isolated. (Gisain et al., 2013 and Hiko et al., 2018), studied fresh water (rivers and lakes) to investigate their microbial diversity leading to the identification of different bacterial species such as *Shigella* spp., *E. coli*, *Aeromonas* spp., *Vibrio* spp., *Salmonella* spp and *Pseudomonas* spp. This is similar to the present study findings where these and other different bacterial species were isolated. The presence of these bacteria both in the aquatic environment and in the fish has been often observed elsewhere (Egerton et al., 2018; Wang et al., 2017 and Marinho-Neto et al., 2019). The least frequently isolated bacteria from both live fish and water samples were *A. sobria C. koseri* and *C .freundii* with similar prevalence.

Different Gram-negative bacteria species were isolated from processed fish samples. *Salmonella* spp and fecal coliforms (*E. coli, K. pneumoniae* and *E. aerogenes*) bacteria were detected in 42% of water samples and 64% of processed fish samples. This result is in agreement with several studies which reported the isolation of *E. coli*, *Enterobacter* spp., and *Citrobacter* spp., and *Klebsiella* spp., *P. aeruginosa*, and *Salmonella* from fish products (Shafi et al., 2020 and Khater et al., 2021).This was indicative of post contamination of fish product in the processing system, fecal pollution and poor quality of processed fish which is linked with the practice of inadequate hygienic measure, mishandling, improper storage and use of dirty water during processing and all unhygienic condition of the processes.

Presence of *E. coli* in processed fish samples is attributed to contamination of the fish samples by man through handling and processing (Gufe et al., 2019). (Shafi et al., 2020), studied coliforms such as *E. coli* are usually present in live and fresh fish organs which indicate the faecal contamination from warm blooded animals. This is similar to the present study findings were different coliforms including *E. coli* was isolated. Among the isolated bacteria, *E. coli* was found in all the samples and majority of it was present in live fish followed by processed fish and water, and this might be due to contamination during

handling in the processing center. This result is in agreement with the study of Rani et al. (2016). Even though *Salmonella* spp in fish products mainly originates from the environment rather than from poor standards of hygiene and sanitation, sometimes incidence of this bacterium in fish or similar foods of aquatic habitats may be happened due to external contamination. *Shigella* spp. and *Salmonella* spp. are pathogenic bacteria found in animal or human reservoir and contamination of fish products by these bacteria is almost always due to poor hygiene (poor personal hygiene, poor processing hygiene or poor water quality) (Gufe et al., 2019). This is similar to the findings of (Mzula et al., 2019) who studied the presence of spoilage bacteria (*Salmonella* spp) which make the control of spoilage microorganisms critical for fish product safety. *Citrobacter*, *Enterobacter* and *Klebsiella* are indigenous to general environment and frequently present in fish but most of the isolates are non-pathogenic environmental strains. The bacterial species isolated from processed fish (*E. coli*, *K. pneumoniae* and *S. paratyphi*) were also recovered from the water samples. However, the microbial status of processed fish was distantly related to the microbial conditions of the water.

Except *A.sobria* all the others bacterial isolate were isolated from the tilapia (*Oreochromis niloticus*). Similarly all the others bacterial isolate were isolated from the catfish (*Clarias gariepinus*) with the exception of *P. shigelloides*. However, *C. freundii, C, koseri* and *V. parahemolyticus* were not isolated from carps (*Cyprinus carpio*). Even though differences in frequency and percentage of each particular bacteria species were found among different fish species, stastically significant differences (P<0.05) were shown in *E. coli*, *A. sobria*, *Citrobacter* spp, *P. shigelloides, S. flexneri* and *V. parahemolyticus*. This result is in agreement with the study of Wamala *et al*. (2020) and Feliatra et al. (2020) which indicate the isolation of different pathogenic bacteria; *Aeromonas sobria, Edwardsiella tarda, Plesiomonas shigelloides, Pseudomonas aeruginosa, Citrobacter* spp and *Klebsiella* spp) from tilapia (*Oreochromis niloticus*) and catfish (*Clarias gariepinus*), whereby they could be considered a reservoir or vehicle for food-borne infections and, therefore, a threat to public health.

Characterization of the bacterial isolates from diseased fish of this study revealed that majority of them are *A. sobria*, *E. tarda*, *P. shigelloides, P. aeruginosa*, *S. paratyphi*, *S. typhi*, *S. dysenteriae, S. flexneri* and *V. parahemolyticus* which were considered as a potential pathogenic bacteria. This result is in agreement with several studies which reported the isolation of pathogenic Gram-negative bacteria from tissue samples of different diseased fish showing clinically sign of disease and healthy (Tesfaye et al., 2018 and Wamala *et al*., 2020). Similarly Naim *et al*. (2019) also reported the presence of Gram-negative bacteria particularly *E. coli, K. pneumoniae, E. aerogenes, E. cloacae, C. freundii, C. koseri, P. vulgaris, P. mirabilis, Salmonella* spp., *Shigella* spp., *Serratia* spp., *Pseudomonas* spp., *Yersinia* spp., *Aeromonas* spp. and *Vibrio* spp. in raw fish sampled from Karachi, Pakistan. The presence of the microorganisms in internal fish organs could indicate the breakdown of immunological defense mechanisms (Chandrarathna *et al*., 2019; Hossain et al., 2019 and Mzula et al., 2019).

All the fifteen (15) bacterial isolates were isolated from lake Hawassa fish samples. Except *C. freundii* the others bacterial isolate were also isolated from lake Ziway fish samples. But only 66.7% of the bacterial isolates were isolated from lake Langanoo fish samples. Even though differences in frequency and percentage were recorded at any particular bacterial isolates among the three sampling lakes; statistically significant difference was only recorded in *E. coli*, *A. sobria*, *Citrobacter* spp, *E. cloacae*, *P. aeruginosa* and *V. parahemolyticus*(P<0.05). It is accepted that fish possess a specific intestinal microbiota, but nutritional status or feeding habits, species (attribute to complexity of the fish digestive system) and the environmental conditions (salinity of the habitats and the bacterial load in the water) are the most influential factor which change the fish intestinal microbiota composition (Egerton et al., 2018).

Among the isolated bacteria from lake Hawassa majority of the isolates were obtained from *Amora Gedel* (S1) (22.9%) and *Referal hospital area* (S4) sites (18.6%). In lake Langanoo most isolates were isolated from *O'etu* (S2) site (10%). Similarly, most of the bacteria were isolated from *Cafeteria* (S3) (18.8%) and *Abosa* (S1) (13.8%) sites of lake Ziway. During the study period differences were observed in the bacterial prevalence and frequency across the sampling sites of each lake. This may be generally attributed to the relative distance and degree of exposure to the nearby point source pollution around the study area. Most of *E. coli* (2.4%) was isolated during this study period from the intestine of fish. The occurrence of faecal coliforms in fish intestine reflects the warm-blooded animal pollution level of the water and also indicates that the organism can probably survive and multiply when fish and water temperatures are 28^0C or higher (Gufe et al., 2019).

The physicochemical parameters of water samples indicate that mean values of pH, temperature, dissolved oxygen and nitrate in all sampling sites of the three lakes found to be within the normal range at which most freshwater fish species become non-stressed. The concentrations of nitrate in each lake were within the limit of WHO a standard showing that the selected sites of each lake was less polluted by nitrogenous waste materials. However, highest concentration of phosphate (4.48 mg/L) was recorded at lake Langanoo *O'etu site* (S2) which were higher than the limit of WHO standards (WHO, 2008). These could be due to pollution from different domestic sewages, surface runoff from phosphate containing fertilizers and certain industrial wastes that led to eutrophication and can lead to low productivity of the lake. Unlike pH and temperature of lake Ziway, temperature and EC of lake Hawassa and lake Langanoo indicates to much differences between water samples during the study period. The mean dissolved oxygen were high indicating that there was a good aeration at each lake during the study period, which may be attributed to lower temperature and good flow rate (Garg et al., 2010). Studied temperature oscillated between 19 and 28.7°C among sampling sites of each lake. The effect of stress on fish depends on the severity of the stress, its duration and the physiological state of the fish.

5. Conclusions

Numerous bacterial isolates colonizing the freshwater fish in the rift valley lakes of Ethiopia were identified in this study. The identification of *A. sobria*, *C. koseri*, *C. freundii*, *E. tarda*, *E. aerogenes*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *P. shigelloides, P. aeruginosa*, *S. paratyphi*, *S. typhi*, *S. dysenteriae, S. flexneri* and *V. parahemolyticus* is important as these bacteria plays a considerable role as potential pathogenic bacteria for fish as well as human and as an indicator of food quality as spoilage microorganism which affects fisheries industry and causes a huge loss of commercial fish production. In this respects, rapid detection and identification of bacterial fish pathogens is helpful to develop preventive measures and control the spread of pathogens, and contaminations of fish products. The bacteriological analyses of processed fish indicate occurrence of hygiene indicator bacteria at relatively higher level. The mean values of pH, temperature, dissolved oxygen and nitrate in all water samples found to be within the normal range at which most freshwater fish species become non-stressed. From the findings, it can be suggested as fresh processed fish available in fish markets of Batu city may act as the carrier of potentially pathogenic

bacteria which may cause food-borne illness to human. Therefore, necessary hygienic steps should be followed during the processing of fish and in the delivery place as well.

6. Declarations

Biotechnology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

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6.2 Author Contributions

Conceived and designed the experiments, collect samples, performed the laboratory works, analyzed the data and wrote the paper.

6.3 Competing interests

I declare that there is no competing interest regarding the publication of this research article.

6.4 Data Availability and materials

All relevant data and materials are within the paper and it's supporting information files

6.5 Ethics approval

Not applicable in this section.

6.6 Consent for publication

Not applicable in this section.

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Figures

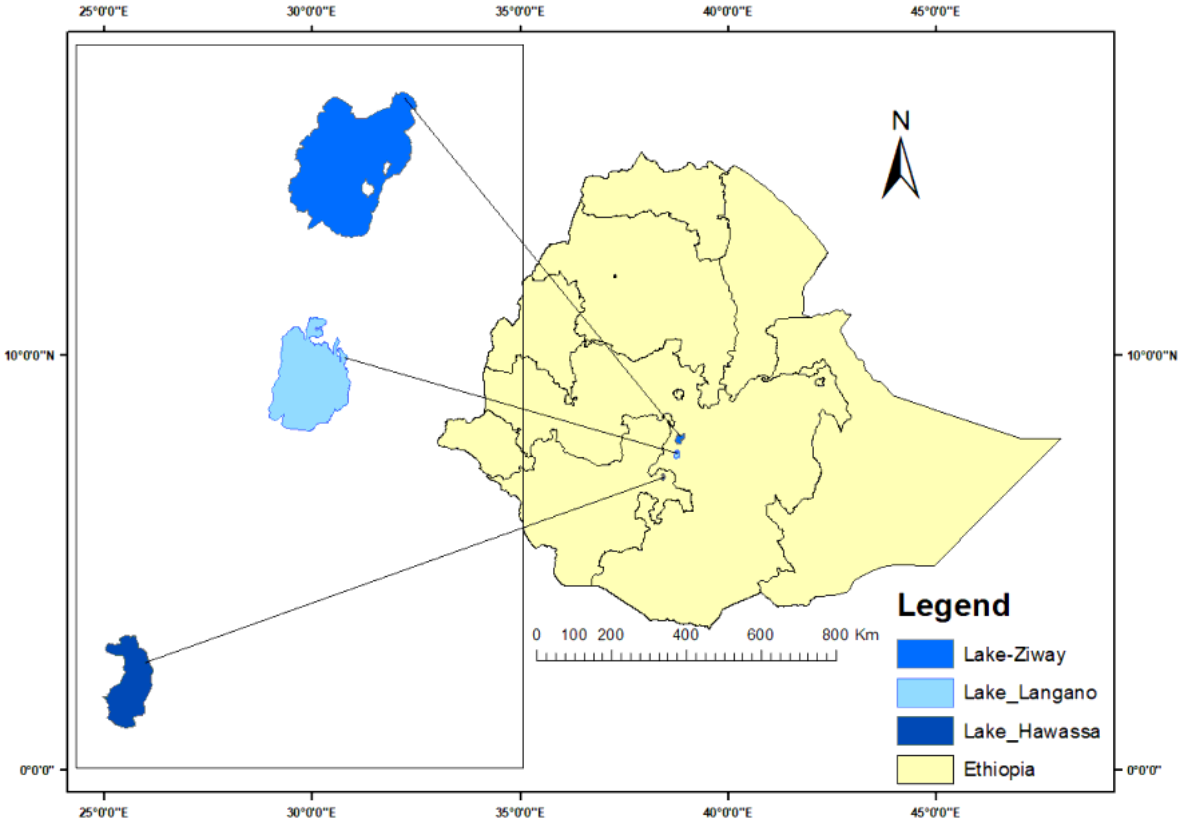

Figure 1

Map of the study area