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ABSTRACT

Ensuring safe aquaculture products has been one of the major challenges and concerns for many fish farmers, traders and consumers in developing countries. This is attributed to contamination of fish and fish products by pathogenic and spoilage micro-organisms that are resistant to multiple antibiotics. Additionally, the use of antibiotics in fish farming for prophylaxis has resulted in the transfer of antibiotic-resistant genes thus establishing a reservoir for resistant microorganisms.

***Objectives*: This study was carried out to determine the level of microbial contamination of fish products and antimicrobial resistance patterns of the isolated bacteria to commonly used antibiotics.**

***Design and setting*: Fish products were sampled from fish traders in six markets from Kirinyaga County. The bacteria cultures were enumerated and sub-cultured to obtain pure cultures used to describe their morphological and biochemical characteristics. The Kirby-Bauer agar diffusion technique was used to carry out the antibiotic susceptibility testing.**

***Results*: Based on morphological characteristics, a total of 59 morpho-groups were obtained and were confirmed by biochemical characterization. There was a significant difference ($p = 0.001$) and interaction ($p = 0.037$) of average bacteria count obtained from different fish products. Processed fish resulted to a significant difference ($p = 0.001$) on mean for CFU. Ciprofloxacin was the most effective antibiotic while about 80 % of the isolates showed multidrug resistance.**

***Conclusion*: The study suggests that fish and fish products harbour multi-resistant bacterial isolates that can aid in the dissemination of resistance genes.**

INTRODUCTION

Fish and fishery products are well known for their high nutritional value and quality. They have varied fat content, from low-fat content, saturated fat and cholesterol and yet high in polyunsaturated fatty acids, protein and minerals such as calcium, phosphorus, sodium, potassium, and magnesium [3]. These nutritional benefits provide better alternatives to consumers. Under normal circumstances, quality and freshness of fish and fish products are degraded and deteriorated through microbial attack and biochemical mechanisms. According to Gram and Huss [3], bacterial activity leads to production of unpleasant odor due to conversion of amino acids into biogenic amines, sulfides and organic acids. Fish could be contaminated after harvesting, during handling or on transportation to markets. Highly contaminated fish and fish products are unsuitable for human consumption since they can be a powerhouse for pathogenic bacteria.

The use of antibiotics in fish farms is of importance to human health as resistant bacteria in these farms could be transferred to other bacteria or directly to human pathogens, such as *Staphylococcus* sp., *Salmonella* sp., *Shigella* sp., *Escherichia coli* and *Pseudomonas aeruginosa* have been isolated from fish ponds with some of these isolates exhibiting high levels of resistance to commonly used antibiotics in humans [4].

Studies have also shown that there is a continuous increase in resistance of bacteria to antibiotics due to their widespread use and misuse in fish farming in the treatment of specific and non-specific infections and as growth promoters resulting in the emergence of resistant bacteria strains. According to Gousia *et al.* [8], this makes farm animals become reservoirs of antibiotic-resistant bacterial strains. The apparent occurrence and increase of bacterial strains resistant to routinely used antibiotics in fish hatcheries and their possible human health implications is calling for intensified surveillance. There is limited information on these resistant bacterial strains and the health challenges from fish-borne diseases among fish handlers in the target region. In this study, we hypothesized that the bacterial contaminants in the fish and fish products have varied resistance patterns to commonly used antibiotics in Kirinyaga County. The objectives were to determine the level of microbial contamination of fish and fish products retailed at various Kirinyaga County markets and to determine antimicrobial resistance patterns of bacteria isolates using commonly used antibiotics.

MATERIAL AND METHODS

Study area: The study was carried out in Kirinyaga County located in Central Kenya. It covers an area of 1,478.1 square kilometers and lying at the foothills of Mt. Kenya. It lies at 0°34'23.43"S latitude and at a longitude of 3 7°19'31.7"E. The region is a mild altitude agro climatic zone ranging from 1500 to 2000 meters above sea level with an average annual temperature of 20° C. The main economic activity is farming.

Sample size: The ICMSF sampling standard was used to determine the sample size. The sample size was calculated using the following formula:

$$ME = Z\frac{\sqrt{p\,(1-p\,)}}{n}$$

Where:

ME is the desired margin of error = 5%

Z is the z score set at 95% confidence interval

P is the prior judgment correct value of p set at 0.04%

n is the sample size (to be found)

$$0.05 = \frac{1.96\sqrt{0.04x0.96}}{n}$$

$(0.05/1.96)^2 = 0.0291/n$
$0.0025/3.8416 = 0.0291/n$
$n = 60$
Therefore, the sample size was 60 samples per every fish product while in every market, 6 samples we collected per product.

Sampling: Raw fish and fish products (samosas, sausages, cake, skewers, hot dogs, fried fish, balls, fingers and burgers) were randomly sampled from fish vendors in every market, separately packaged in zip locked bags and transported in cool boxes to Kenyatta University for further analysis. The sampled raw fish were of the species, tilapia and catfish.

Isolation and characterization of bacterial isolates: In the microbiology laboratory, the fish samples were skinned aseptically to avoid contamination. Ten grams of fish (only flesh and skin was used) was homogenized with 90 ml sterile distilled water for 3 minutes using a Kenwood blender (De'Longhi Group, United Kingdom). Each fish sample was a composite sample obtained from 3 fishes of the same type.

Tenfold serial dilution was prepared in physiological saline (0.85 % NaCl). After blending, a mix of 1 ml was diluted at 1:10, 1:100, 1:1000 and 1:10000 using sterile peptone water. Spread plate of 0.1 ml of each dilution was done on Nutrient Agar (NA) media (Himedia, India) and incubated at 37^{o} C for 24 hours. The bacteria cultures were enumerated and sub-cultured to obtain pure cultures. The pure bacterial cultures were used to describe their morphological and biochemical characteristics.

Bacterial counts in raw and processed fish and fish products: Ten grams of the meat sample was aseptically homogenized in 90 ml of 0.85 % sterile saline, this effected a 10^{-1} from which a tenfold serial dilution was done up to the fourth diluent. From these dilutions, 0.1 ml was spread plated on nutrient agar plates in triplicates and incubated at 37 ^{o}C overnight. The colony forming units (CFU) were then counted from each plate using a EI colony counter (Bio Technics, India). The most dominant colonies were picked, purified and biochemically tested.

Antibiotic susceptibility testing: Commonly used antibiotics; Tetracycline 30mcg, Streptomycin 10mcg, Ciprofloxacin 30mcg, Ampicillin 10mcg, Gentamycin 10mcg and Penicillin 10mcg (Tan Biotech Ltd, India) were used in this study. This was geared at finding out which antibiotics were best suited at both the elimination and control of these bacterial isolates. Based on identities from morphological, biochemical and Gram staining characteristics, 20 bacterial representative isolates were selected for antibiotic susceptibility testing. The Kirby-Bauer agar diffusion technique was used to carry out the antibiotic susceptibility testing, on Mueller-Hinton agar medium (Himedia, India). McFarland's standard bacterial suspensions were prepared for all of the bacterial isolates to be tested. Sterile forceps were used to place the six antibiotics discs aseptically onto the surface of the agar followed by light pressing of the discs. The agar plates were incubated at 37^{o}C for 24 hours. Diameters of the inhibition zones were measured using well calibrated scale to the nearest millimeter. Minimum inhibitory concentration (MIC) breakpoints for resistance were based on the Clinical and Laboratory Standards Institute (CLSI) criteria and isolates grouped as resistant, intermediate or susceptible [17].

Data analyses: The data on viable counts and antibiotic resistance patterns were analyzed using analysis of variance (ANOVA). Tukey's Honest Significant Difference (HSD) test was used to separate the means at $p< 0.05$. The data analyses was carried out on SAS software Version 9.4.

RESULTS

Bacterial isolates from fish products

The results showed that there was a significant difference (p = 0.001) of average bacteria count obtained from different fish products (Table 1). Fish samosas recorded the highest bacteria count of 1.67 x 10^2 cfu/g. On the other hand, the lowest bacterial counts were recorded from fish burger (7.93 x 10^1 cfu/g) and fish cake (7.28 x 10^2 cfu/g) (Table 1). The average bacteria count obtained from fish products in different markets varied significantly (p = 0.001). Fish products from Ndia had the highest mean count of 1.93 x 10^2 cfu/g followed by Kerugoya and Tebere markets with a mean count of 1.63 x 10^2 cfu/g and 1.31 x 10^2 cfu/g respectively. There was also a significant (p = 0.037) interaction of markets versus fish products (Figure 1).

Table 1

Total bacterial counts of fish products from markets in Kirinyaga County

Fish products	**Mean cfu/g±SD**
Markets	
Kerugoya	163.11±16.25b
Kianyaga	54.22±9.18de
Mwea	50.74±6.12e
Ndia	193.48±20.71a
Sagana	79.22±10.08d
Tebere	131.26±15.02c
Fish products	
Burger	79.28±22.78c
Cake	72.78±11.46c
Fingers	120.94±21.13b
Fish balls	109.67±22.51bc
Fried	135.50±18.12ab
Hot dog	99.07±23.58bc
Samosa	167.44±23.39a
Sausage	100.06±19.35bc
Skewer	123.33±19.42b
P values	
Markets	0.001
Fish products	0.001
Markets*fish products	0.037

Values (Means ±SE) followed by dissimilar letters along the columns are significantly different at P≤0.05 using Tukey's HSD test

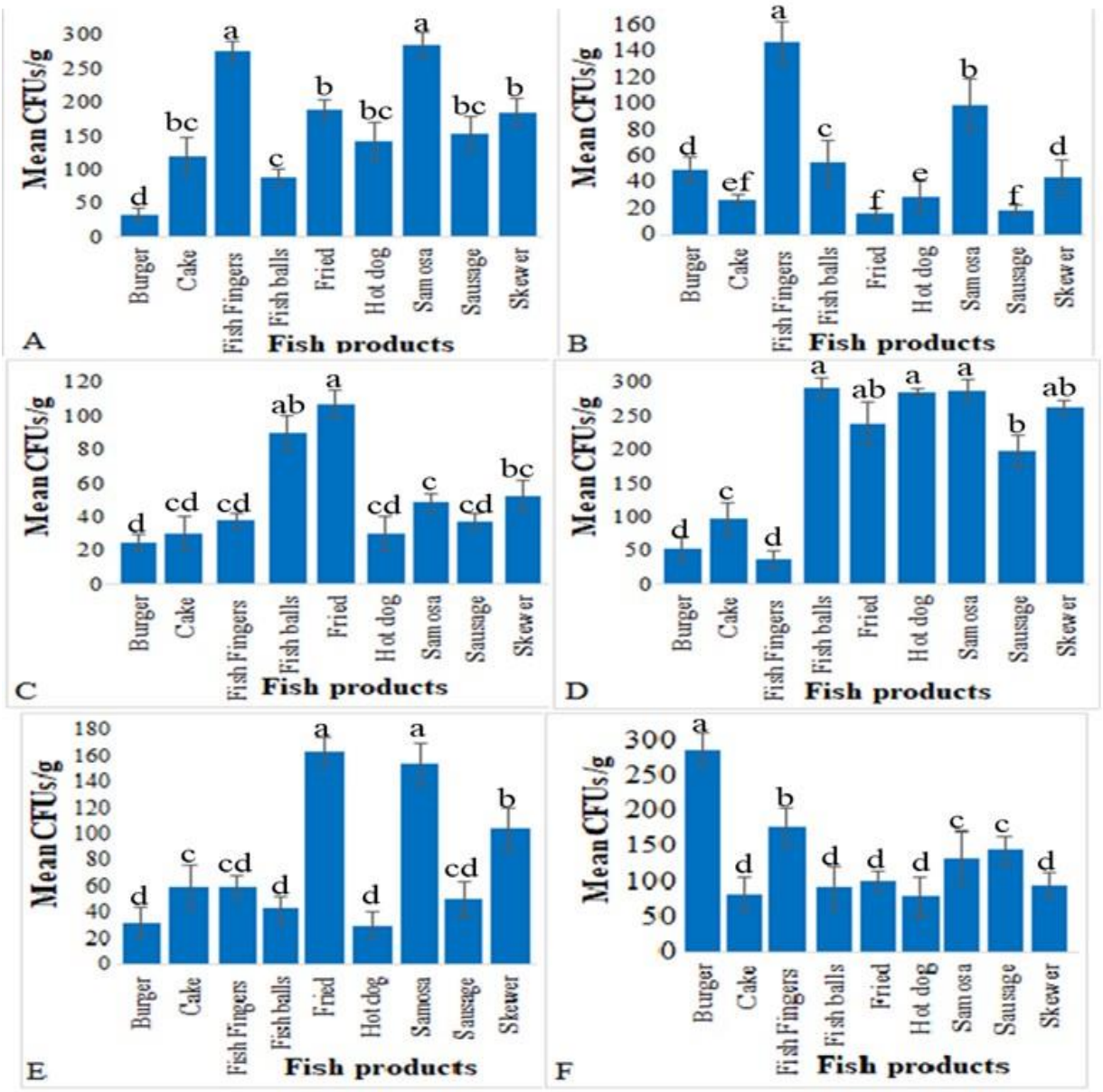

Figure 1: Interactive effects of mean bacterial counts and fish products from different markets in Kirinyaga County. Bars followed by the same letter are not significantly different using Turkey's HSD test at P≤0.05. A, Kerugoya Market; B, Kianyaga Market; C, Mwea Market; D, Ndia Market; E, Sagana Market; F, Tebere.

Processed fish products

Fish processing resulted to a significant difference (p = 0.001) on mean of CFU. Processed or raw fish recorded the highest average CFU while processed fish had significantly low average CFU (Table 2). Average total bacterial viable count from different markets differed significantly (p = 0.013). Samples from Kerugoya market recorded the highest mean total viable count followed by samples from Sagana market (Table 2). The other market with significantly high mean total viable counts was Ndia market with 1.20 x 10^2 Cfu/g (Table 2).

Table 2

Total viable count of bacterial isolates from fresh and processed Catfish and Tilapia fish species

Treatment	Mean
Markets	
Kerugoya	145.83±31.73a
Kianyaga	91.08±14.75b
Mwea	96.00±10.41ab
Ndia	120.91±18.32ab
Sagana	133.75±27.80ab
Tebere	83.91±13.80b
Fish species	
Catfish	108.306±9.31a
Tilapia	115.53±14.03a
Treatment	
Raw fish	161.69±10.63a
processed	62.58±7.78b
P Values	
Markets	0.013
Fish species	0.082
Treatment	0.001

Values (Means ±SE) followed by dissimilar letters along the columns are significantly different at P≤0.05 using Tukey's HSD test.

Morphological and biochemical characteristics

A total of 158 pure bacterial isolates were obtained from the fish and fish product samples from different fish markets in Kirinyaga County. Based on morphological characteristics, a total of 59 morpho-groups were obtained and confirmed by biochemical characteristics. Majority of the isolates groups (27) were from raw fish. The isolated bacteria had varied morphological characteristics. Processed fish products had fewer number of isolates compared to raw fish. Bacterial isolates had morphological characteristics ranging from black centered, round and translucent colonies flat with smooth margins. The bacterial colony size ranged from 0.5 mm to 4.5 mm size. Gram staining reaction had varied results where 24 of the isolates were Gram positive and the rest were Gram negative (Table 3).

Table 3

Biochemical characteristics of representative bacteria isolates from fish and fish products in markets from Kirinyaga County

Tests		TSI				SIM			MIU				MR-VP		Cell characterization		POSSIBLE IDENTITY
Isolates	Origin	S	B	H_2S	G	H_2S	I	M	M	I	U	C	MR	VP	Cell shape	G. staining	
A4	WTRM5	Y	Y	+	+	+	-	+	+	-	+	+	+	-	Rd	-	*Proteus* sp.
A6	WTRM6	P	Y	+	+	+	-	+	+	-	+	+	+	-	Rd	-	*Citrobacter* sp.
A7	FtNRM1	Y	Y	+	-	+	-	+	+	-	+	-	+	-	Rd	-	*Proteus* sp.
A9	WCTM6	Y	Y	+	-	+	+	+	+	+	+	+	+	-	Rd	-	*Aeromonas* sp.
A11	WCRM6	P	Y	+	+	+	+	+	+	+	+	-	-	+	Rd	-	*Citrobacter* sp.
B1	SaCTM1	P	Y	+	+	+	-	+	+	-	-	-	+	-	Rd	-	*Citrobacter* sp.
B2	SmCTM1	Y	Y	-	-	-	+	-	-	+	+	+	-	+	Rd	-	*Klebsiella* sp.
C1	FCTM1	P	Y	+	+	+	-	+	+	-	-	-	+	-	Rd	-	*Salmonella* sp.
C2	FCTM5	P	Y	+	+	+	+	+	+	+	+	+	+	-	Rd	-	*Citrobacter* sp.
C3	SmCTM1	P	Y	+	+	+	-	+	+	-	+	-	+	-	Rd	-	*Citrobacter* sp.
D1	SmCTM1	Y	Y	-	+	-	-	+	+	-	-	-	-	+	Rd	-	*Citrobacter* sp.
D2	SmCTM2	Y	Y	-	+	-	-	+	+	-	-	-	-	+	Rd	-	*Enterobacter* sp.
D3B	WTRM2	Y	Y	-	+	-	-	+	+	-	-	-	-	+	Rd	-	*Enterobacter* sp.
E1	WCTM1	Y	Y	+	+	+	-	+	+	-	-	+	-	+	Rd	+	*Bacillus* sp.
E2	WCTM1	P	Y	-	+	+	-	+	+	-	-	-	+	-	Rd	-	*Enterobacter* sp.
F1	FfNRM1	P	Y	-	-	-	+	-	-	+	-	-	-	-	Rd	-	*Citrobacter* sp.
F2	SkCTM4	P	Y	-	+	-	+	-	-	+	-	-	+	-	Rd	-	*Citrobacter* sp.
F3	WTRM5	Y	Y	-	+	-	-	-	-	-	-	-	+	-	Rd	-	*Escherichia* sp.
G7	SmCRM5	Y	Y	+	+	+	-	+	+	+	+	-	+	-	Rd	-	*Raoultella* sp.
G11	FfNRM1	Y	Y	+	+	+	-	+	+	-	+	-	+	-	Rd	-	*Proetus* sp.
H2	BuCTM4	Y	Y	+	+	+	-	+	+	-	-	+	-	+	Rd	+	*Bacillus* sp.
H3	FcCTM4	Y	Y	+	+	+	-	+	+	-	-	+	-	+	Rd	+	*Bacillus* sp.

Key: P-Pink; Y-Yellow; TSI-Triple Sugar Iron; S-Slant; B-Butt; H_2S-Hydrogen sulfide; G-Gas; SIM-Sulfide Indole Motility; I-Indole; M-Motility; MIU-Motility Indole Urease; U-Urease; C-Citrate; MR-Methyl Red; VP-Vogues Proskauer; + Positive; - Negative. (The identity of the isolates have expounded in appendix 1)

Antibiotic resistance patterns

Twenty (20) bacterial isolates were selected based on morphological and biochemical characterization where every bacterial identity was represented and were subjected to the six antibiotics. Of the six antibiotics, Penicillin recorded the highest antibiotic resistance at 90%. All the isolates were susceptible to Ciprofloxacin (100%). The bacteria were also resistant to for Ampicillin (65 %), Gentamycin (20 %), Tetracycline (15 %) and Streptomycin (10 %). There was a significant difference in the action of antibiotics against the bacterial isolates (P=0.001) (Table 4). Tetracycline was highly effective against isolate H18-SaCTM1 followed by isolate M10-SmCTM1. Among the bacterial isolates, D3B- WTRM2 was most resistance (67 %) against antibiotics used followed by I1-FcNTM1 (56 %) and F3-WTRM5 (51 %). Other bacterial isolates that showed multidrug resistance include A4-WTRM5 (50 %), J12-FfCRM6 (45 %), D1-SkCTM4 (39 %), H12-BCTM5 (37 %), A6-WTRM6 (35 %), C1- FCTM1 (35 %), H10-WTRM5 (34 %), K6- BCTM4 (33 %), B2-SmCTM1 (30 %), and H18- SaCTM1 (30 %). However, isolates M10-SmCTM1 and K1-SkCTM4 had no multidrug resistance.

Table 4

Antibiotic resistance patterns of selected bacterial isolates from fish and fish products

Isolates	Tetracycline	Streptomycin	Ciprofloxacin	Ampicillin	Gentamycin	Penicillin
D1-SkCTM4	24.33±0.33 de	19.33±0.67bcde	30.00±0.00 ab	11.33±1.45fghi	20.20±0.41cde	6.67±0.67 c
H12-BCTM5	29.00±1.52 a	23.00±1.00 ab	32.67±0.33 a	14.67±0.68 ef	22.00±0.57b	9.00±0.00 c
A6-WTRM6	24.33±0.33 de	21.66±0.33 abcd	26.00±0.58 d	29.00±0.57a	20.33±0.33bcde	18.00±4.51 b
C1- FCTM1	19.33±0.67 fg	18.67±0.33 bcde	26.68±0.33 cd	14.00±1.15efg	18.00±0.58cde	6.00±0.00 c
H10-WTRM5	25.00±0.58 cde	20.68±0.67abcde	31.00±0.58 ab	13.00±1.00efgh	20.67±0.67bcd	7.33±0.33 c
A4-WTRM5	7.33±0.33 ij	19.00±0.00bcde	32.00±0.00 a	14.67±3.84 ef	20.67±0.33bcd	10.00±1.15 c
K6- BCTM4	24.33±0.33 de	20.33±0.33abcde	30.00±0.00 ab	8.00±0.00 hij	20.00±1.00bcde	6.00±0.00 c
B2-SmCTM1	18.67±0.33 g	16.68±0.33 e	31.32±0.88 ab	18.33±0.67cde	16.67±0.67e	6.00±0.00 c
M7-SmCTM5	25.00±0.58 cde	22.33±1.21 abc	29.23±0.67 bc	8.67±0.88ghij	21.67±1.33bc	6.00±0.00 c
H21-FCTM4	22.00±0.00 ef	18.00±0.57 cde	25.67±0.33 j	7.33±0.33hij	18.67±0.33bcde	6.00±0.00 c
A9-WCTM6	25.67±0.33 bcd	23.00±0.58 ab	30.00±0.67 ab	17.00±0.58def	22.00±0.58b	11.33±0.33 c
J6- WTTM5	25.00±0.58 cde	20.67±0.33abcde	29.33±0.68 bc	27.67±0.33 ab	21.00±0.58bcd	21.00±0.58 b
H23-WCTM6	28.33±0.67 ab	22.00±0.58 abcd	30.67±0.67 ab	16.00±0.58 ef	21.00±0.58bcd	11.00±0.58 c
H18- SaCTM1	30.33±0.33 a	20.00±0.58 abcde	31.67±0.88 ab	11.67±0.33fghi	22.00±0.58b	7.33±0.33 c
D3B- WTRM2	14.67±1.33 h	9.00±3.00 f	24.33±0.88 d	6.00±0.00ij	11.67±0.33 f	6.00±0.00 c
M10-SmCTM1	29.67±0.67 a	6.00±0.00 f	32.33±0.33 ab	27.67±0.88 ab	22.33±1.45b	28.00±1.00 a
K1-SkCTM4	28.00±0.00 abc	24.33±0.33 a	31.33±0.67 ab	23.67±0.33abc	27.67±0.33a	22.67±0.67 ab
I1-FcNTM1	23.67±0.33 de	21.33±0.33abcde	26.67±0.88 cd	6.33± 0.67j	21.33±0.88bcd	6.67±0.33 c
J12-FfCRM6	10.00±0.00 i	20.67±0.67abcde	32.33±0.33 ab	22.00±0.58bcd	20.67±0.67bcd	10.00±0.00 c
F3-WTRM5	6.00±0.00 j	17.33±0.67 de	32.33±0.33 ab	6.00±0.00ij	17.67±0.88de	6.00±0.00 c
P- Value	0.001	0.001	0.001	0.001	0.001	0.001

Key: values shown are mean zones of inhibition (mm) plus the standard error for the means of three independent measurements. Values that are present on the same column lacking common letters differ significantly based on Tukey's HSD p<0.05.

DISCUSSION

Bacterial contamination

Total bacterial count is an important general index of assessing fish quality, level of contamination and health concern. The results showed significant difference on the mean bacterial count from one fish product to another. For instance, samosa fish products recorded the highest mean bacterial count (cfu/g) compared to other products across the markets. This high bacterial load may be as a result of poor handling after preparation. According to Gram and Dalgaard [7] , fish products have a unique odor which attracts flies resulting to bacterial contamination if not properly stored. The mean bacterial counts differed significantly across the markets assessed. Ndia, Kerugoya and Tebere recorded the highest. The variation can be attributed to the level of sanitation, environmental and personal hygiene and the level of health awareness. However, other markets such as Mwea had the least mean bacterial count. The findings may be linked to better storage facilities among the fish vendors and good personal hygiene. Additionally, Kasozi *et al*. [9] reported that some markets may record low contamination levels due to close and strict monitoring and supervision from public health authorities.

Bacterial isolation and morphological characteristics

During the study, 158 pure bacteria were isolated from fish and fish products from markets in Kirinyaga County. Fish species have been documented to harbor diverse bacterial isolates [10]. Majority of the isolates were from raw fish unlike other processed fish products. Fish processing methods significantly reduced bacterial population present on the fish skin and other fish surfaces, retard bacterial spoilage and hence less contaminated. Several studies have documented isolation of low bacterial isolates from processed fish products compared to raw fish [11]. In contrast, Pal *et al*. [12] reported possibility of processed fish products being more contaminated especially when processing and post-processing handling is done in unhygienic environment.

The isolates had diverse morphological characteristics and therefore grouped into 59 morpho-groups with similar characteristics. Raw fish mostly harbor bacteria present in their water habitat both pathogenic and non-pathogenic which depicts similar characteristics [3]. The colony elevation of the isolates was raised, convex or flat while for transparency, majority of the isolates were translucent and were opaque. Majority of the bacterial isolates based on Gram staining reaction were Gram-negative. According to Dhanya and Mathew [15], major bacteria genera that have been isolated and identified from different fish species were dominated by Gram-negative bacteria. The common genera comprise of; *Pseudomonas*, *Enterobacteriaceae, Vibrio* sp, *Achromobacter*, *Escherichia coli* and some members of *Flavobacterium*. However, Gram-positive bacteria were also present.

Antibiotic resistance patterns

Majority of the isolates belonged to *Citrobacter* sp (31 %), *Proteus* sp (16 %), *Enterobacter* sp (13 %) and *Bacillus* sp (10 %). Other bacterial genera that were identified include *Aeromonas* sp, *Klebsiella* sp, *Salmonella* sp, *Escherichia* sp, and *Raoultella* sp. *Enterobacter* sp were the most resistance to four antibiotics (67 %). In addition, 80 % of the isolates exhibited multidrug resistance to the antibiotic used. This pose a medical hazard because some isolates such as *Bacillus sp.* have been implicated as a food borne disease causing bacteria based on their resistant endospores. Isolate H12-BCTM5 was susceptible to four of the six antibiotics tested, susceptible to Ampicillin and resistant to Penicillin. This implies that antibiotics Ciprofloxacin, Gentamycin, Streptomycin and Tetracycline can be used as controls to reduce and eliminate these bacterial isolates.

CONCLUSION

Antimicrobial resistance patterns of the selected bacterial isolates against the six commercially available antibiotics showed that Ciprofloxacin was the most effective antibiotic while Penicillin was the least effective. There were also some resistant isolates to a number of antibiotics available in the market. Compliance of fish vendors to good hygienic practices (GHP) is crucial in ensuring food safety. In addition, prudent antimicrobial use in fish handling is required to reduce development of drug resistance pathogens. There is also need for continued research on antimicrobial resistance genes and horizontal gene transfer (HGT) in foodborne pathogens from fish and correlation between antimicrobial resistance and virulence features.

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