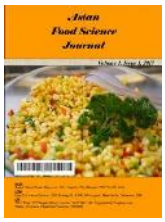

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Molecular Characterisation of Bacteria Isolated from Various Part of Chicken (*Gallus gallus domestica*) Meat

S. A. Wemedo[1*], S. I. Douglas[1] and L. K. Nima[1]

[1]Department of Microbiology, Rivers State University, Port Harcourt, Nigeria.

Authors' contributions

This work was carried out in collaboration between the authors. Authors SAW and SID designed and supervised the study. Author LKN performed the work, statistical analysis, wrote the protocol, managed the analyses and literature searches of the study and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the types of bacteria found in the different parts of Chicken meat using biochemical and molecular techniques.
Study Design: Poultry birds were bought and processed in the respective markets by the butchers. Four parts of the poultry meat were minced and placed in sterile bottles which were taken to the lab for analysis. The control was bought and processed in the laboratory.
Place and Duration of Study: The Mile three and Mile one markets which are amongst the major markets in Port Harcourt metropolis, Rivers State, Nigeria were the area under study. The study duration was for three months.
Methodology: Ten gram of four parts (intestine, gizzard, muscle and skin) each was weighed and transferred into test tubes containing 90ml sterile normal saline. Subsequent 10-fold serial dilution was carried out and aliquots of preferred dilutions were inoculated unto Nutrient and MacConkey agar (TM media, India) plates. Incubation for 24 hours at 37°C followed. Resulting colonies were enumerated and distinct colonies were subcultured to get pure isolates followed by biochemical tests to identify the isolates. The boiling method of extraction was used in extracting the DNA of the

**Corresponding author: Email: samwems@yahoo.com;*

various isolates. rRNA genes of the isolates were amplified using the 27F and 1492R universal primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 µl for 35 cycles.
Results: The total heterotrophic load of the skin ranged from 0.2×108 to 5.5×108 Cfu/g. the bacterial load of the muscle, intestine and gizzard ranged from 0.0×108 to 1.0×108 Cfu/g; 1.2×108 to 2.9×108 Cfu/g and 1.7×108 to 2.0×108 Cfu/g. The coliform load of the skin ranged from 0.2×105 to 2.2×105 Cfu/g, while the total coliform of the muscle, intestine and gizzard ranged from 0.1×105 to 1.9×105 Cfu/g, 1.1×105 to 1.5×105 Cfu/g and 0.2×105 to 2.0×105 Cfu/g respectively. Nine bacteria genera which include *Salmonella, Shigella, Vibrio, Enterococcus, Staphylococcus, Chryseobacterium, Aeromonas, Acinetobacter* and *Escherichia* species were isolated and identified.
Conclusion: The bacteria identified in this study could be pathogenic if foods are not properly prepared. Thus, chicken meats should be properly processed to avoid cross contamination.

Keywords: Parts of the chicken meat; molecular characterisation; bacteria contaminants.

1. INTRODUCTION

Due to the advancement in the food processing technology, the production and consumption of poultry meat has experienced outstanding increase in recent years [1]. Poultry, especially the meat (chicken meat) which is known to be a very good source of protein with low fat content having little or no religious restriction is presently the best source of animal protein for the low income populations since it is inexpensive and within reach [2,3]. Because of these advantages, large scale consumption of poultry meat is greater than that of other meats [4,5,6]. Furthermore, the increase in the poultry industry especially in the developing nations has provided employment opportunities and also improved the country's economy [7]. In the assessment of the Nigerian poultry sector, it was revealed that the Nigerian poultry industry was estimated at ₦80 billion ($600 million) and was comprised of approximately 165 million birds, which produced 650,000 MT of eggs and 290,000 MT of poultry meat in 2013 [8]. The consumption of poultry meat globally has been reported to surpass the consumption of pork come 2020 and also to record a 27% increase in 2023 [2]. In 2015, the consumption of poultry meat in the EU was reported to be 22.5 kg [2], while in the USA the consumption of poultry meat was reported to be 40.5 kg per capita in the same year (United States Department of Agriculture [9]. The consumption of poultry meat in developed countries has increased at a slower rate than that in developing countries. This is owing to rapid population growth, urbanisation, and lifestyle factors that contribute to a higher protein intake and demand for poultry meat in the developing countries [4]. In sub-Saharan Africa, the largest producer of chicken meat is South Africa with a consumption of 37.47 kg per capita per year in 2014 [10]. In other to produce good quality poultry meat for consumption in a world where the consumption of poultry meat has increased, an efficient and effective food processing is required [11,12]. Furthermore, enhancement of poultry meat to improve its tenderness, juiciness, flavour and shelf life for consumers is being carried out by the poultry industry [13,14]. Each of the ingredients in the enhancement solution provides specific functions. For example, phosphates aid in water retention and maintain the juiciness in meat; salt is added to increase water binding ability; and flavour additives provide desirable flavours for consumers [7]. Also, refrigeration of chicken meat is gradually enhancing the preservation of chicken meat despite the insufficient supply of electricity. According to Grashon [15], consumers are interested in good tasting and healthy food with relevance to nutritional physiology and at the same time, they are afraid of potentially harmful ingredients such as drug residues, intoxicants, allergenic components, and microbial contamination, which may contribute to health problems. Microorganisms such as *Salmonella, Campylobacter, Enterobacter, Escherichia coli*, which are associated with poultry could cause severe health related diseases such as gastroenteritis [16]. According to Baeumler et al. [17], the major source of Salmonella infections in human is via the ingestion of poultry birds. Thus, ensuring the microbial safety of poultry meat products is highly important so as to assure a healthy production and consumption of the meat. Furthermore, contamination of the poultry meat arises during and after slaughtering either from the animal microbiota, the slaughter house environment and the equipment used during the processing processes, and some of these bacterial contaminants can grow or survive during food processing and storage [18,12].

Studies on the molecular characterisation of bacterial isolates from poultry meat are scarce. Thus, this study is aimed at investigating the types of bacteria found in the different parts of poultry meat using molecular techniques.

2. MATERIALS AND METHODS

2.1 Study Area

The Mile three and Mile one markets which are amongst the major markets in Port Harcourt metropolis, Rivers State, Nigeria. The GPS coordinates of the markets are 79258N, 6.998541E and 4.804341N, 6.993728E for Mile three and Mile one markets respectively.

2.2 Sample Collection

A total of six chicken (*Gallus gallus* domestica) were bought from two markets (Mile I and Mile III) which are the major markets in Port Harcourt metropolis. The birds were slaughtered in the markets before they were sent in sterile containers to the microbiology laboratory of the Rivers State University for analysis. Also, the control was slaughtered in the laboratory and the skin, gizzard, muscle and the intestinal content were extracted and placed in sterile labeled containers which were also used for analysis.

2.3 Microbiological Analysis

The method described by Amadi et al. [19] was adopted with slight modification. Approximately 10 g of the various parts was minced and placed in different labeled 150 ml conical flasks containing 90 mL of sterile physiological saline. This was properly shaken to dislodge the microorganisms after which a 10-fold serial dilution was carried out. Aliquots from 10^{-3} dilutions were inoculated into MacConkey agar (TM media, India), while aliquots of the 10^{-6} dilutions was inoculated on freshly prepared nutrient agar (TM media, India) plates. The plates were incubated at 37ºC for 24 hours. Colonies from various plates after incubation were counted and were used to enumerate the CFU/g.

2.4 Purification and Storage of Isolates

Discrete colonies from the various plates for the gizzard, intestine, muscles and skin were purified by continuous streaking on freshly prepared sterile nutrient agar plates. Pure isolates were preserved in 10% glycerol in a frozen state. The isolates were tentatively identified based on morphological characteristics like; size, shape and Gram's reaction. Other biochemical tests which include motility, indole, catalase, Citrate, Coagulase, Oxidase, Vogues Proskauer, sugar fermentation and methyl red test according to [20] were adopted. Bacterial isolates were finally identified using Bergey's Manual of Determinative Bacteriology [21].

2.5 Molecular Characterisation of the Isolates

The 16S rRNA gene sequencing was used in the molecular characterisation of bacterial isolates. Extraction of DNA was carried out by sub-culturing pure bacteria isolate into Lauria-Bertani (LB) broth and incubated at 37ºC for 24 hours. After incubation, about 2ml of the isolates in LB broth was transferred into 2ml Eppendorf tubes and was spun at 14000 rpm for 3 minutes. The supernatant was discarded and 1000 µl of 0.5 normal saline was added to the sediment and mixed on eLtech XHB vortex machine. The tube was later subjected to heating at 95ºC for 20 minutes on a heating block. After which it was cooled before it was spun again at 14000 rpm for 3 minutes. About 500 µl of the resulting supernatant was transferred into a 1.5ml Eppendorf tubes and was cooled in the freezer at -20ºC. The extracted DNA was quantified using the Nanodrop 1000 spectrophotometer. The 16S rRNA gene of DNA sample was PCR amplified using 16S rRNA universal primers: 27F and 1492R on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 µl for 35 cycles at an annealing temperature of 52ºC for 30 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualised on a UV transilluminator and was sequenced in an ABI 3510 genetic analyser using Big Dye Terminator kit. similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. Downloaded sequences were aligned using ClustalX and the evolutionary history was inferred using the Neighbor-Joining method in MEGA 7.0 [22].

3. RESULTS AND DISCUSSION

The result of the total heterotrophic bacteria load in Table 1 revealed that the bacterial load on the skin ranged from 0.2×10^{8} to 5.5×10^{8} Cfu/g. the bacterial load of the muscle, intestine and

gizzard ranged from 0.0×10^8 to 1.0×10^8 Cfu/g; 1.2×10^8 to 2.9×10^8 Cfu/g and 1.7×10^8 to 2.0×10^8 Cfu/g. The total heterotrophic bacteria on the skin revealed that chicken meat which were bought and slaughtered in the markets had higher microbial loads than those slaughtered in the laboratory. Counts were not recorded for the muscle parts of the control and chicken meat from the mile one market while those from the mile three markets had bacterial counts in the muscles which were perceived to be high. Also, the counts of the total heterotrophic bacterial load of the intestines and the gizzard were higher in the chicken meat slaughtered in the market than those slaughtered in the laboratory.

The result for the total coliform bacteria load is presented in Table 2. The result revealed the presence of coliform bacteria in all the meat parts for the different locations with the exception of the muscle part of the chicken meat from the mile one market. The coliform load of the skin ranged from 0.2×10^5 to 2.2×10^5 Cfu/g, while the total coliform of the muscle, intestine and gizzard ranged from 0.1×10^5 to 1.9×10^5 Cfu/g, 1.1×10^5 to 1.5×10^5 Cfu/g and 0.2×10^5 to 2.0×10^5 Cfu/g respectively. The coliform loads of the intestines were higher in the control than those slaughtered in the respective markets. Similarly, the coliform counts on the skin of the chicken meat from the markets were more contaminated than those of the control. The muscle part of the chicken meat from the mile three market were more contaminated where as those slaughtered in the mile one market were not contaminated and no counts were recorded.

The bacteria genera identified biochemically in this study were *Escherichia, Acinetobacter* species, *Vibrio, Salmonella, Shigella, Staphylococcus, Chryseobacterium, Aeromonas* and *Acinetobacter* species. The biochemical characteristics as well as their morphology are represented in Table 3. The result presented in Table 4 revealed the presence of eight bacterial isolates which were not evenly distributed in the various part of the poultry meat. *Salmonella, Shigella, Vibrio, Enterococcus, Staphylococcus, Aeromonas, Acinetobacter and E.coli* were the bacterial isolates isolated from the control. *Salmonella* species were only isolated in the intestines. *Shigella* and *Vibrio* species were only isolated from the intestines and gizzard. *Enterococcus* species was isolated from the skin, intestines and the gizzard. *Staphylococcus* was isolated from the skin and intestines of the chicken meat. In the muscle, only *Aeromonas* and *E. coli* species were isolated. *Acinetobacter* was isolated from the gizzard while *E. coli* was isolated from the skin, gizzard, intestine and the muscle (Table 3). The frequency of occurrence of bacterial isolates isolated from the Mile one market is presented in Fig. 2. Seven bacterial isolates were identified in the chicken meats bought from the Mile one market. Three bacterial isolates belonging to *Staphylococcus* (42.9%), *Escherichia* (28.6%) and *Chryseobacterium* (28.6) were isolated from the skin of the chicken meat with *Staphylococcus* having the highest occurrence. *Staphylococcus* (14.3%), *Enterococcus* (35.7%), *Escherichia coli* (28.7%) and *Salmonella* (21.4%) were isolated from the intestines of the chicken meat. While *Escherichia coli* (60%), *Shigella* (20%), and *Aeromonas* (20%) were isolated from the gizzard. Furthermore, the distribution of bacteria genera was not even.

Similarly, seven bacteria belonging to *Staphylococcus, Chryseobacterium, E. coli*, *Enterococcus, Aeromonas, Acinetobacter* and *Shigella* were isolated from chicken meat parts bought from the Mile three market (Fig. 3). The predominant bacterial isolate on the skin was *Chryseobacterium* followed by *Staphylococcus* species. In the muscle, only two bacterial isolates (*Acinetobacte*r and *Enterococcus*) were isolated. *Enterococcus, Escherichia coli, Shigella, Acinetobacter and Aeromonas* were the bacterial isolates isolated from the intestines. *Enterococcus* and *Shigella* species were the most occurring bacterial isolates in the intestines. While only *E. coli* and *Shigella* species were the two bacterial isolates isolated from the gizzard.

The Phylogenetic tree showing the evolutionary distance between the bacterial isolates is presented in Fig. 1. The obtained 16S rDNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database 16S rDNA of B4 which was biochemically identified as *Acinetobacter* species has a high similarity with *Acinetobacter junii* strain 44.2. Isolate B3 was found to have a 99.9% match with *Enterococcus faecalis strain 2*. Also, isolate B6 has a high similarity with *Chryseobacterium* sp (KLBC52), while *Aeromonas caviae* has a close match with isolate B5. Isolates B1 and B2 has a very high percentage similarity with *Escherichia coli strain SAMA_EC.*

Table 1. Total heterotrophic bacterial counts (×10^8 CFU /g) of the various chicken parts from the different locations

Replicate sample	Chicken parts											
	Skin			Muscle			Intestine			Gizzard		
	Control	Mile one	Mile three	Control	Mile one	Mile three	Control	Mile one	Mile three	Control	Mile one	Mile three
1	0.2	9.2	2.9	0.0	0.0	1.4	1.1	2.4	3.1	1.2	1.5	1.2
2	0.2	1.7	8.0	0.0	0.0	0.6	1.2	2.2	2.7	2.2	2.5	2.4
Total	0.4	10.9	10.9	0	0	2	2.3	4.6	5.8	3.4	4	3.6
MEAN ± SD	0.2±0.0	5.5±5.3	5.5±3.6	0.0±0.0	0±0.0	1.0±0.6	1.2±0.1	2.3±0.1	2.9±0.3	1.7±0.7	2.0±0.7	1.8±0.9

Table 2. Total coliform bacterial counts (×10^5CFU /g) of the various chicken parts from the different locations

Replicate sample	chicken parts											
	Skin			Muscle			Intestine			Gizzard		
	Control	Mile one	Mile three	Control	Mile one	Mile three	Control	Mile one	Mile three	Control	Mile one	Mile three
1	0.2	3.6	0.0	0.1	0	0.0	1.7	2.4	0.8	1.3	3.8	0
2	0.1	0.0	4.3	0.0	0	3.7	1.2	0.4	1.4	1.0	0.1	0.4
Total	0.3	3.6	4.3	0.1	0	3.7	2.9	2.8	2.2	2.3	3.9	0.4
MEAN ± SD	0.2±0.1	1.8±2.6	2.2±3.0	0.1±0.1	0	1.9±2.6	1.5±0.4	1.4±1.4	1.1±0.4	1.2±0.2	2.0±2.6	0.2±0.3

Table 3. Morphology and biochemical characterisation of bacterial isolates

S/N	Cultural	Morphological	Biochemical	Sugar fermentation	Probable organism
B(1&2)	Circular, convex, smooth, pink, opaque	Rod shaped(bacillus) gram negative	Catalase positive, oxidase negative, methyl red positive, voges prokauer negative, indole positive, citrate negative, urease negative, coagulase negative, gas positive, H_2S negative.	Glucose positive, lactose positive, manitol positive sucrose positive	*Esherichia coli*
B4	Circular, convex, smooth, slightly opaque with entire margin, colorless	Gram negative rods	Catalase positive,oxidase negative,indole negative,urease negative,citrate positive,coagulase negative,H_2S negative	Glucose positive,mannitol negative,sucrose negative,lactose positive, galatose positive	*Acinetobacter* sp
B10	Convex yellow because of the acid,from fermented sucrose,which turns the indicator bromothymol blue of the medium into yellow.	Short,curved,coma shaped gram negative bacilli	Catalase negative,oxidase positive,indole positive,methyl red negative, voges prokauer positive,gas negative,H_2S negative	Glucose positive,manitol positive,sucrose positive,lactose negative.	*Vibrio* sp.
B9	Circular,convex,smooth, black center ,translucent,opaque due to production of H_2S.	Rod shaped(bacillus) gram negative	Catalase negative, oxidase negative, indole negative methyl red positive, voges proskaur negative, TSI alkaline/acid, urease negative, gas negative,H_2S positive	Glucose positive, lactose negative, manitol positive, sucrose negative.	*Salmonella* sp.

S/N	Cultural	Morphological	Biochemical	Sugar fermentation	Probable organism
B8	Circular,convvex,smooth,pink colonies without black centre on SSA, transparent on macconkey	Short gram negative bacilli(rod)	Catalase positive, citrate negative, methyl red positive, voges prokaur negative, oxidase negative,urease negative,gas positive,H_2S negative.	Glucose positive, lactose negative, mannitol positive, sucrose negative	*Shigella* sp.
B7	Circular, convex, smooth, golden yellow	Cocci grape-like clusters, gram positive	Catalase positive, citrate positive, coagulase positive, gas negative,H_2S negative, indole negative, methyl red positive, oxidase negative, urease positive, voges prokaurer positive	Glucose positive, lactose positive, fructose positive, galactose positive, mannitol positive, sucrose positive	*Staphylococal* sp.
B6	Colonies are smooth, convex, yellow pigmented	gram negative bacilli (rod shaped).	Catalase positive, oxidase positive, indole positive, urease negative	Glucose negative, mannitol negative, sucrose negative	*Chryseobacterium* sp.
B5	Colonies are smooth, convex, transparent on nutrient agar	Gram negative bacilli (rod)	Catalase positive, oxidase positive, citrate positive,	Glucose positive, manitol positive, sucrose positive	*Aeromonas* sp.
B3	Colonies are smooth, convex.	Gram positive cocci	Catalase negative, oxidase negative, methyl red negative, voges proskaur positive, indole negative, citrate negative, urease negative,H_2S negative, gas negative, coagulase negative.	Glucose positive, frutose positive ,lactose positive, manitol positive, sucrose positive	*Enteroccoccus* sp.

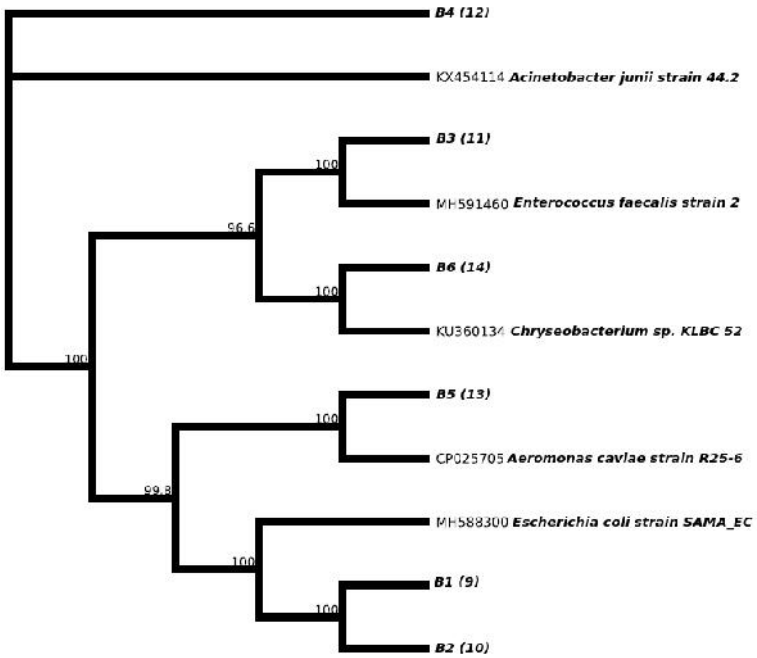

Fig. 1. Phylogenetic tree showing the evolutionary distance between the bacterial isolates

Table 4. Bacterial isolates isolated from the various parts of the chicken meat (control)

Bacterial isolates	Skin	Muscle	Intestine	Gizzard
Salmonella species	-	-	+	-
Shigella species	-	-	+	+
Vibrio species	-	-	+	+
Enterococcus sp	+	-	+	+
Staphylococcus species	+	-	+	-
Chryseobacterium species	-	-	-	-
Aeromonas species	-	+	-	-
Acinetobacter species	-	-	-	+
Escherichia coli	+	+	+	-

Key: +: present, -: absent

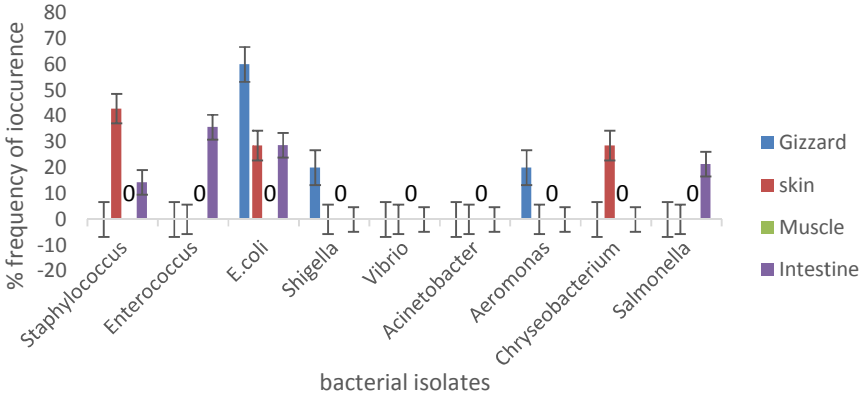

Fig. 2. Frequency of occurrence of the different bacterial isolates in the various part of the poultry meat in Mile 1

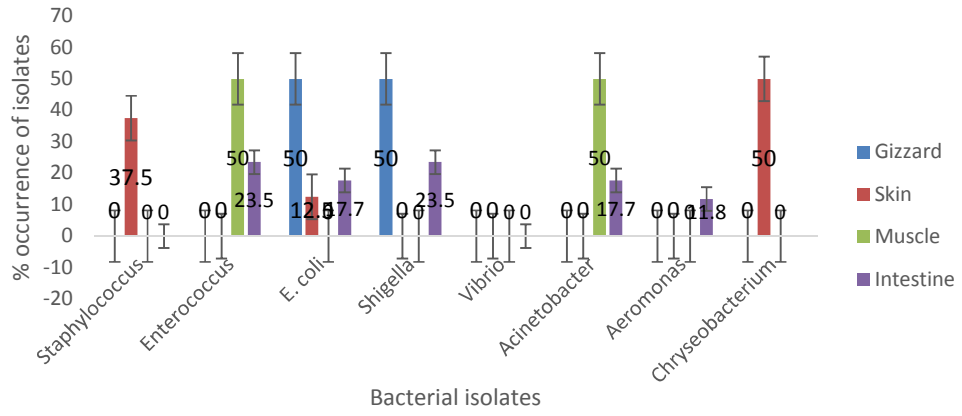

Fig. 3. Frequency of occurrence of the different bacterial isolates in the various part of the poultry meat in Mile 3

The food we eat could enhance or determine the type of microorganisms that lives in us. Thus, there is a relationship between microbes and the food we eat [23]. The presence of micro-organisms which may have been introduced during processing could either be from the normal flora or from the environment and they may cause spoilages and other food borne diseases [24].

In this study, the mean microbial populations of the chicken meat especially those slaughtered in the markets were very high and exceeds the 105 cfu/g recommended limit of bacterial contamination for foods by International microbiological standards for total bacterial plate count [24]. Nine bacteria genera which include *Salmonella, Shigella, Vibrio, Enterococcus, Staphylococcus, Chryseobacterium, Aeromonas, Acinetobacter and Escherichia* species were isolated. *Salmonella, Shigella, Enterococcus* and *Escherichia coli* which are present in this study have been isolated from the gut of poultry birds by previous studies [25,26,10,24,27]. *Enterococcus* has been isolated in poultry meat and pasteurized milk in a previous study [28]. Furthermore, the *E. coli* isolated from the skin could be due to contamination of the skin with faecal matter from the intestinal content which may have been during processing or during feeding (when the birds were still in the poultry farm). Isolation of *Chryseobacterium* from the skin and intestine could be due to contamination of the skin by the microbe which is known to be found in the soil and water. Thus, its presence in the intestine could be that the poultry bird may have picked it up either during feeding or drinking of water. This is in agreement with Calderon et al. [29]. Generally, *Aeromonas* and *Acinetobacter* which are present in this study could be due to contamination of the environment or the feeds or could be normal flora of the poultry birds. Furthermore, these microorganisms are found in both water and soil and contamination of the poultry meat could be attributed to poor hygiene, as well as environmental contaminations since these microbes are also found in the environment. This is in agreement with previous studies [30,31]. The bacterial isolates in this study could be pathogenic especially when they come in contact with humans. Nosocomial infections arising from *Chryseobacterium* species have been reported [29]. Dent et al. [32] have reported infections of the urinary tract caused by *Acinetobacter* species. *Escherichia coli* and *Enterococcus* species which are normal flora of the intestines of animals have been reported to cause gastro intestinal disorders [16]. The water baths used during the process have a washing effect that diminishes the bacterial loads, but can also promote cross-contamination between the slaughtered meat [33,3]. Thus, this could be the reason why the microbial load was very high and could also be the contributing factor of the diversity of microbes and the presence of some microbes in the muscles which is believed to be sterile. This is in agreement with Amélie et al. [25] who had reported the sterility of the muscles of live birds.

4. CONCLUSION

The bacterial populations of the various parts of the poultry meat in this study were very high. Nine bacteria genera which were isolated in this study have been reported to cause serious

diseases ranging from nosocomial infections to gastroenteritis. Thus, during slaughtering and processing of poultry meats, good hygiene should be practiced and utensils should be sterile to avoid contamination or cross contamination from the various parts of the meat. Also, poultry farmers should ensure that their farms meat hygienic standards so as to curb disease transmission.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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