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Sales environment, microbiological and biochemical quality of beef skins intended for human consumption

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The quality and composition of meat and its derivatives are influenced by many factors namely physicochemical, organoleptic factors and microbial contamination. The aim of this study was to evaluate *kpakouma* consumption risk through its chemical and microbiological contaminants. The methodological approach was composed of (i) observation of selling environment, (ii) pesticides and antibiotics residues quantification by HPLC, and (iii) microbiological analysis using selective media and biochemical tests. The data shows that aminoglycosides, penicillin and nitrofuran were not determined both in the black and the white *kpakouma*. Macrolides (0.094±0.004) and beta lactams (0.016±0.0036) are noted only with white *kpakouma*. Lindane (0.215±0.003) and HCH (1.0003±0.003) were only detected among some samples whereas chlorpyrifos, malathion and parathion were not detected in all the tested samples. Concerning the microbial contaminants, according to the European Regulation, all *kpakouma* samples were highly contaminated with *Staphylococcus* species, *Escherichia coli* and *Salmonella*. The isolated *Staphylococcus* spp. were mostly (90%) resistant to vancomycin, no *Staphylococcus* spp. resistance was recorded for ciprofloxacin. *E. coli* and *Salmonella* were all resistant to oxytetracycline, no resistant isolate of *E. coli* was recorded for ciprofloxacin but *Salmonella* strains were at 25% resistant to ciprofloxacin. These results show the non-compliance with the hygiene rules during the sale of *kpakouma* and reaffirm the potentially critical role that can be played by commensals in public health.

Key words: Environment, bacteria, pesticide and antibiotics residues, *kpakouma*, Cotonou.

INTRODUCTION

Given the importance of livestock, the valuation of the by-products is still modest in Africa. The fifth quarter of carcasses is a large group representing 20 to 55% of the living animal weight and is divided into two parts, namely

an edible part that groups the offal and an inedible part that includes the exits (Fiems, 2012). Thus, they include edible parts such as tongue, offal, fat and skins; which can be used for human consumption (Jayathilakan et al., 2012).

Except few countries, export marketing is often in the form of pre-tanned or rawhide (Strasser, 2015). Thus, the global trade in hides and leather goods has undergone a profound change over the past twenty years. Raw hides and skins from developed countries are imported to prepare, process and re-export them as products with added value (Bereda et al., 2016). However, industry professionals and industrialists with good development potential in Africa may, benefit from this historic reversal.

Meat products have been traditionally reported to be the vehicle for many foodborne diseases in humans (Heredia and García, 2018). Its hygienic quality depends on the contamination occurring during transformation process and during cooling, storage and distribution (El Okki et al., 2005; Rani et al., 2017). The bad hygienic quality can be the cause of food poisoning. In addition, in many tropical countries, foods are sold at public places and roadside shops. The main critical points of meat products hygiene are in slaughterhouses where microbial contamination may be occurred (Soepranianondo and Wardhana, 2019). It is reported that 80 to 90% of the microflora of meat products reaching the consumers resulted from contamination occurring at the slaughterhouse (Odeyemi et al., 2020). The presence of microorganisms that can cause food poisoning are viruses and bacteria (Hernández-Cortez et al., 2017). Organisms mostly reported to be involved in food poisoning are *Salmonella*, *Escherichia coli* and *Staphylococcus aureus* (Sina et al., 2011; Attien et al., 2017). The presence of microorganism causing food poisoning is related to several factors such as pathogenicity and resistance to antibiotics (Fisher et al., 2018). Thus, the damage caused by bacteria can be due to their capacity to not only colonize their host but also production of toxins (Kim et al., 2017).

The transformed beef skin ready to be eaten is commonly called *kpakouma* in Benin local language *barriba*. The *kpakouma* is a very important part of cooking habits. This importance is explained by its availability, its accessibility to any type of portfolio and the satisfaction it provides to the consumers. Once considered food for the poor, beef skin is now a "star" in Beninese cuisine. Indeed, it is found more and more in dishes cooked in important events like vegetable sauce. However, it can be cook it in different ways. Due to its importance, it will be interesting to investigate the major critical points of *kpakouma* hygiene. Despite its appreciation by the population, information on the microbiological quality and importance of this commodity is lacking in Benin. Thus, the aim of this study was to appreciate the *kpakouma* selling environment and to evaluate its chemical and microbiological contaminants qualities.

MATERIALS AND METHODS

Experimental design and *kpakouma* sales environment investigation

For this study, the sample size is determined using the work published by Kadam and Bhalerao (2010). Thus, 300 samples of vendors' goods on show were selected using the "convenience" sampling technique (Etikan et al., 2016) for the investigation. The survey was carried out in 10 markets (Dantokpa, St Michel, Aïdjèdo, Midonbo, Wologuèdè, Gbégamey, Vèdoko, Zogbo, Minnontin and Degakon) of Cotonou to collect information on the *kpakouma* selling environment. For the microbial analysis in the laboratory, nine samples were collected from each market.

Sample's collection for chemical and microbial analysis

Three samples of *kpakouma* were randomly collected from three different sellers per market in ten markets. Thus, 90 samples were collected. Once collected, samples were collected in sterile Stomacher papers then carried to laboratory in icebox (4-8°C) for their microbial analysis.

Chemical contaminant detection

Preparation of standards solutions

Stock standards solutions (1000 µg.ml^{-1}) were prepared by dissolving 10 mg of each analyte in 10 ml HPLC grade water. Further dilution was obtained using acetonitrile to obtain working solutions (40, 20, 10, 5, 2.5 and 1.25 µg/ml). All standards were protected from light with aluminium foil; diluted solutions were stored at -20°C and used after a week.

Determination of antibiotics and pesticides residues

The antibiotics and pesticide residues were extracted from *kpakouma* samples using the method previously described by Lehotay et al. (2005). Briefly, 15 g of *kpakouma* samples were mixed with 15 ml of acetonitrile + 1% acetic acid. Extraction salts (6 g of magnesium sulphate + 1.5 g of sodium acetate) were added and mixed before centrifuged (5000 rpm for 1 min). The organic phase (1 ml) was mixed to purified salt (150 mg PSA+ 50 mg $MgSO_4$ + 50 mg C_{18}) and then centrifuged (5000 rpm for 1 min). One hundred microliters of the supernatant were filtered and then transferred to the HPLC (Agilent Technologies 1260 infinity, GmbH & Co. KG, Waldbronn, Germany) vials for antibiotics (sulfamide, tetracycline, macrolides, b-lactams, aminoside, penicillin and nitrofuran) and pesticides residues (lindanes, HCH, DDT,

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Figure 1. Picture showing *kpakoumas*' sales environment.

chlorpyrifos malathion and parathion) quantification. Each solution was analysed on three replications according to HPLC quantification protocol. About 10 µl was injected in the HPLC analytical column at room temperature and the chromatographic peaks for each sample were identified and compared to the determined retention time of the standard solution of each antibiotic and pesticide residues. The limits of detection (LOD) and of quantification (LOQ) of each antibiotic and pesticides residues were determined using the linear regression method as previously described (Shrivastava and Gupta, 2011).

Microbial analysis

Once in the laboratory, 10 g of each *kpakouma* sample was added to 90 ml of sterile bacteriological peptone (Oxoid, Hampshire, England) and then incubated at 37°C for 1 to 3 h for the enrichment (Akoachere et al., 2009). In this study, microbiological analyses focus on staphylococci, *E. coli*, *Salmonella*, and Mesophilic Aerobic Flora (MAF) were enumerated. From the incubated suspension, a decimal dilution was made with peptone water (BioRad, Paris, France) and used for bacterial identification and enumerations. Each of the dilution (0.1 ml) was spread on Baird Parker agar (Biokar Diagnostics, France) with egg yolk (Baird-Parker, 1990; Dennaï et al., 2001) before its incubation at 37°C for 48 h for Gram positive cocci's. EMB agar (Biokar Diagnostics, France) incubated at 37°C for 24 h was used to isolate *E. coli*. The research of *E. coli* was completed by indole production test (Riegel et al., 2006).

For the identification of *Salmonella* spp. 10 g of each sample was cut into small pieces in sterile blender jar containing 90 ml of peptone water as a pre-enrichment broth and incubated at 37°C for 24 h. After incubation, 0.1 ml of pre-enrichment culture was transferred into sterile tubes containing 10 ml of Rappaport Vassiliadis broth, and incubated at 42°C for 24 h. After incubation, a loopful of each tube was cultured on *Salmonella Shigella* agar and incubated for 24 h at 37°C. Typical colony of *Salmonella* appears as transparent colonies with or without black centers (depending on the species isolated).

The contamination frequency was calculated from the ratio of contaminated products on all the products whereas the prevalence was obtained by the ratio of the strains isolated on all the biological products tested. Once isolated, the microorganisms were identified using classical morphological (gram staining, shape of bacteria, and Ziehl-Neelsen staining) and biochemical characters (sugar fermentation, Oxidase, Methyl red-Voges-Proskauer, indole and Catalase test) related to the genus identification techniques (Janda and Abbott, 2002).

Susceptibility to antibiotics

Antimicrobial susceptibility of isolated bacteria was determined by the disc diffusion method of Kirby-Bauer on agar Mueller-Hinton (bioMérieux, Marcy l'Etoile, France) using EUCAST recommendations and interpretation (CA-SFM/EUCAST, 2019). After 24 h of incubation, inhibition zone was measured.

The tested antibiotics (Bio Mérieux, France) against *Staphylococcus* spp. were ampicillin (A 10 µg), chloramphenicol (C 30 µg), ciprofloxacin (CIP 5 µg), gentamicin (GM 10 µg), oxacillin (OX 5 µg), oxytetracycline (OT 30 µg), penicillin G (P 10 µg), and vancomycin (VA 30 µg). For *Salmonella* and *E. coli* stains, the tested antibiotics were ampicillin (A 10 µg), cephalothin (KF 30 µg), chloramphenicol (C 30 µg), ciprofloxacin (CIP 5 µg), nalidixic acid (NA 30 µg), oxacillin (OX 5 µg) and oxytetracycline (OT 30 µg).

Data proceeding and statistical analysis

Field investigations have identified the manufacturing methods and sources of microbiological contamination of *kpakouma* samples. The data obtained from the survey and the laboratory analyses were coded and entered with the Excel 2016 spreadsheet. Descriptive statistics such as prevalence, mean and standard deviation were calculated for the quantitative variables. A significant difference between the mean was determined. The Graph Pad Prism 7.00 software was used for statistical analysis and graphs. The test is considered statistically significant if $p<0.05$.

RESULTS

kpakouma sales environment in the markets of Cotonou

In the markets covered by our investigation, it is found that the *kpakouma* is exposed unprotected (Figure 1). Thus, exposition was observed either on a plate (Figure 1a), in the uncovered basin (Figure 1b) or directly on tables (Figure 1c). In addition, considering the color of the products, two kinds were observed: white (Figure 1c) and

Table 1. Average values of antibiotics residues detected in *kpakouma* samples (µg/kg).

Antibiotics	Black (µg/kg)	White (µg/kg)
Sulfamides	0.0615±0.0026	0.0893±0.0006
Tetracyclines	0.0652±0.0024	0.4823±0.0025
Macrolides	ND	0.094±0.004
B-lactams	ND	0.016±0.0036
Aminoglycosides	ND	ND
Penicillin's	ND	ND
Nitrofuran	ND	ND

ND: Not detected.

Table 2. Average values of pesticides residues detected in *kpakouma* samples (µg/kg).

Pesticide family	Pesticides residues	Black	White
Organochlorines	Lindane	ND	0.215±0.003
	HCH	ND	1.0003±0.003
	DDT	ND	ND
Organophosphorus	Chlorpyrifos	ND	ND
	Malathion	ND	ND
	Parathion	ND	ND

brown (Figure 1a and b).

Chemical residues contaminant detected

Two residues of antibiotics (sulfamides and tetracyclines) were founded in both white and black *kpakouma* at variable concentration (Table 1). Macrolides (0.094±0.004) and beta lactams (0.016±0.0036) are noted only with white *kpakouma*. Three antibiotics residues (aminoglycosides, penicillin and nitrofuran) were not determined both in the black and the white *kpakouma*.

Organophosphorus residues (chlorpyrifos, malathion and parathion) were not detected in all the tested samples (Table 2). Among the organochlorin, DDT was not detected in both white and black samples whereas lindane (0.215±0.003) and HCH (1.0003±0.003) were only detected among white samples.

Microbial load of collected *kpakouma* samples

The *kpakouma* samples have variable microbial loads depending on the place of collection and the bacterial strain (Table 3). Thus, it is found that all bacterial loads are high, and therefore dangerous for consumption. Globally, the highest loads were recorded with the Mesophilic Aerobic Flora (MAF).

Resistance profile of isolated bacteria from *kpakouma*

Susceptibility of E. coli strains

Figure 2 shows that the susceptibility of *E. coli* strains highly varies with antibiotics ($p<0.0001$). However, it was noted that all strains have been resistant to ampicillin, oxacillin, and oxy-tetracycline while they are sensitive to ciprofloxacin.

Susceptibility of Staphylococcus strains

Figure 3 shows that susceptibility of *Staphylococcus* strains considerably varies with antibiotics ($p<0.0001$). Our data shows that all strains were resistance to ampicillin, oxacillin, oxy-tetracycline and penicillin G while they are sensitive to ciprofloxacin.

Susceptibility of Salmonella subsp. strains

Figure 4 shows that susceptibility of *Salmonella* strains highly varies with antibiotics ($p<0.0001$). The analysis of this figure shows that all *Salmonella* strains were resistant to ampicillin, cephalothin, oxacillin, and oxy-tetracycline while 75% of the isolated strains were sensitive to ciprofloxacin and chloramphenicol.

Table 3. Average number of microbial loads per germ of *kpakouma* samples collected in Cotonou (CFU/g).

Market	*Staphylococcus* spp.	*MAF*	*E. coli*	*Salmonella*
Dantokpa	1.88×10^5	2.50×10^9	6.30×10^4	++
St Michel	2.73×10^5	4.00×10^9	1.33×10^3	++
Gbegamey	4.00×10^5	4.55×10^9	1.70×10^5	++
Wologuèdè	4.00×10^5	5.25×10^9	5.50×10^4	++
Vèdoko	2.15×10^5	3.06×10^9	1.59×10^5	++
Minnontin	2.45×10^5	5.00×10^9	3.00×10^4	++
Midonbo	1.80×10^5	5.00×10^9	3.00×10^5	++
Degakon	2.12×10^5	4.07×10^9	4.10×10^4	++
Aidjèdo	4.50×10^5	5.06×10^9	7.40×10^4	++
Zogbo	3.09×10^5	6.17×10^9	1.70×10^5	++

++: High presence.

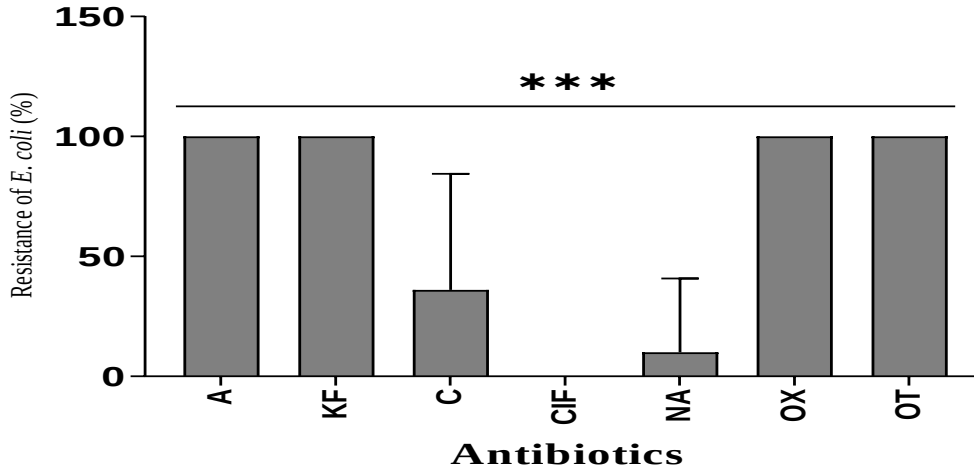

Figure 2. Resistance profile of *E. coli* strains isolated from *kpakouma* to the seven antibiotics. Ampicillin (A), cephalothin (KF), chloramphenicol (C), ciprofloxacin (CIP), nalidixic acid (NA), oxacillin (OX), and oxytetracycline (OT). ***p<0.0001.

DISCUSSION

In this study, the presumptive isolation and identification of bacterial isolates indicate the presence of *Staphylococcus* spp., *E. coli* and *Salmonella* spp. The presence of these three genera of bacteria isolated from this food ingredient could be a matter of serious concern. Indeed, those microorganisms were reported to be pathogenic and can be involved in food poisoning (Sina et al., 2011; Musa and Hamza, 2013). This result can also be explained by the existence of multiple sources of contamination, such as the contact of *kpakouma* with the hands of contaminated buyers and sellers. Overall, the present study revealed that 80% of sampled *kpakouma* samples were contaminated with *E. coli* strains. This rate recorded in our study is much higher than that of 10 and 57.92% observed in beef and chicken carcass, respectively in Australia (Phillips et al., 2006) and Benin (Ahouandjinou et al., 2015). It was noticed that the absence of *E. coli* is an indicator of good hygiene practices whereas its presence is an indication of fresh fecal contamination and the potential presence of ecologically similar pathogens. This indicator is a measure of the implementation of good selling process practices, and the prevention of the occurrence and spread of fecal contamination during sales.

Overall, most of the collected samples were contaminated by *Staphylococcus* spp. strains. However, there was a slight variability in the contamination rate depending on the collection site (market). *Staphylococcus* spp. particularly *S. aureus* causes diseases such as folliculitis, furuncles, erysipelas, cellulites, scalded skin syndrome, impetigo, pneumonia, osteoporosis, toxic shock syndrome (Toxemia), and staphylococcal food

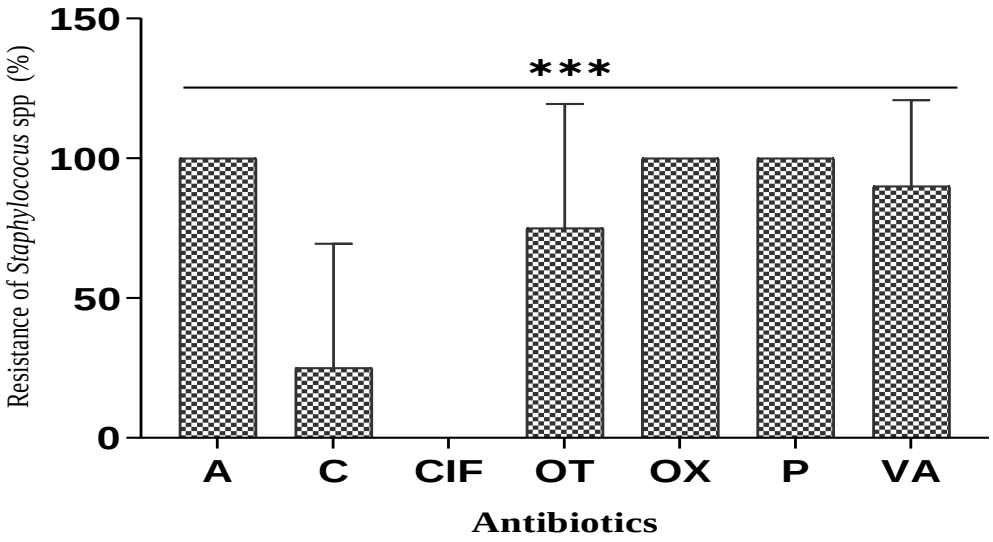

Figure 3. Resistance profile of *Staphylococcus* strains isolated from *kpakouma*. Ampicillin (A), chloramphenicol (C), ciprofloxacin (CIP), oxacillin (OX), oxytetracycline (OT), penicillin G (P) and vancomycin (VA). ***p<0.0001.

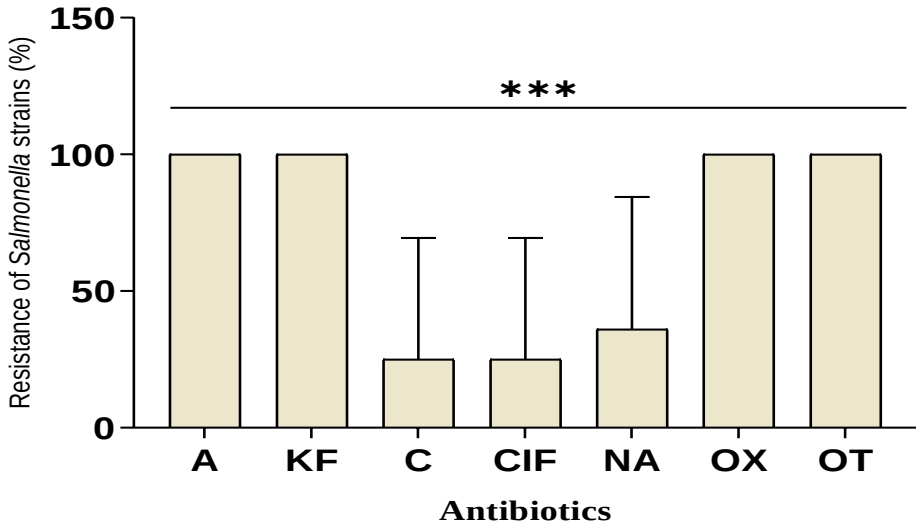

Figure 4. Resistance profile of *Salmonella ssp.* strains isolated from *kpakouma*. Ampicillin (A), cephalothin (KF), chloramphenicol (C), ciprofloxacin (CIP), nalidixic acid (NA), oxacillin (OX), and oxytetracycline (OT). ***p<0.0001

poisoning (Sina et al., 2011; Abdulkadir and Mugadi, 2012). Several authors have found a lower level of contamination than that obtained in this study. This may be due to the sales environment. This result can also be explained by the fact that the mixture water and *kpakouma* would be a broth for the development of aerobic mesophilic germs.

Salmonella prevalence rates recorded in this study are 10 out of 20 samples. *Salmonella* spp. is a strict pathogen and has no habitat other than humans and animals' body; the bacteria is excreted in the faeces or urine and transmitted by food or water which is ingested by another subject (Mølbak et al., 2006). *Salmonella* spp. can cause any of these three types of infections: bacterial food poisoning, enteric fever and systemic fever (Septicemia) (Mølbak et al., 2006).

For most bacteria, there is evidence that increased usage of a particular antimicrobial correlates with

increased levels of bacterial resistance (Granizo et al., 2000); perhaps this explains the high resistance to amoxicillin by the isolates because of its common and prevalent use. Indeed, all isolated strains expressed variable susceptibility to the antibiotics tested. For staphylococci, the highest resistance was observed for vancomycin (90%). The results of the present study have confirmed the frequent resistance to tetracyclines observed previously by some authors (Werckenthin et al., 2001; Aarestrup et al., 2000). It has long been considered the antibiotic of last resort for multi-resistant staphylococcal infections (Schoenfelder et al., 2017). There was no resistance in *Staphylococcus* spp. for ciprofloxacin (quinolone family), which is also the most effective in *Staphylococcus* spp.

All strains of *Salmonella* are resistant to oxacillin, ampicillin, and oxy-tetracycline. This resistance to these molecules, which are old molecules widely used in first intention, is commonly observed in *Salmonella* isolates. This resistance has been reported in several studies on poultry and poultry products (Nayak et al., 2004; Elgroud et al., 2009). *Salmonella* resistance could be transfer from animals to humans, through meat consumption. *Salmonella* strains are 25% resistant to ciprofloxacin. These strains came from the markets of Verdoko, Dégakon, Wologèdè and Dantokpa. Although *Salmonella* strains, highly resistant to quinolones, are still rarely isolated (Schwarz and Chaslus-Dancla, 2001), the decrease in sensitivity should be considered as an alert, since quinolones are antibiotics of last resort against *Salmonella* strains, multi resistant. The evolutionary nature of resistance mechanisms therefore calls for caution.

The observed resistance of *E. coli* to oxy-tetracycline can be attributed to a mutation affecting the structure of porins or decreasing their synthesis. One or more modifications of the porins are at the origin of acquired resistance to beta-lactams, quinolones, chloramphenicol, sulfonamides, trimethoprim and tetracyclines (Liwa and Jaka, 2015). In our study, 10% are resistant to nalidixic acid. These strains came from the markets of Dantokpa and Wologèdè. These results are inferior to those found by Bodering et al. (2017) where they found that *E. coli* strains were resistant to nalidixic acid of 23.08%. Quinolones are currently the largest group of antibiotics. Their interest is related to their low toxicity and especially to the absence of plasmid resistance (Pham et al., 2019). Studies in Nigeria have shown that environmental strains are more resistant to quinolones/fluoroquinolones than clinical strains, and this is explained by the intensive use of these antibiotics in veterinary medicine as growth factors and in the treatment infections (Chigor et al., 2010). Sometimes, the resistances can be of chromosomal origin and this by means of a mutation at the level of the gene, causing a modification of the site of attachment of the antibiotic, or by active efflux; but most often, they are of plasmid origin, therefore transferable horizontally between bacteria of the same species, or even bacteria of distant species (Gassama-Sow et al., 2006).

In this study, strains of *E. coli*, *Salmonella* and *Staphylococcus* spp. showed high resistance prevalence. It is likely that these isolated strains have been subjected to selection pressure by previous use of antibiotic therapy. The consequence is the selection of many resistant strains from the outset to several families of antibiotics that can contaminate consumers of *kpakouma* and make it difficult, if not impossible, all treatments with antibiotics (Amarasiri et al., 2020).

Sulfamides and tetracyclines were founded in all the *kpakoumas'* samples whereas macrolides and beta lactams are noted only with white *kpakouma*. As therapeutic agents, antibiotics have been used in animal production to prevent or control infectious diseases (Dixon, 2001). Meanwhile, growth promoting agents are used to improve the feed conversion efficiency, while antimicrobial agents are added to make more nutrients available to the animal. Those practices have increasingly contributed to the development of bacterial resistance to certain antibiotics (Butaye et al., 2001). Most veterinary drugs used are recommended to be only administered for therapeutic purposes under strict control (van Peteghem and Daeselaire, 2004) with the aim to reduce the development of antimicrobial resistance (Reig and Toldrá, 2009). Comparing the two kinds of *kpakouma*s, the white one shows the high number of residues. This observation can be explained by the fact that the black *kpakouma* is exposed to heat source. Indeed, the black one obtained after grilling is the fresh skin. The heat probably contributes to destroy original molecules.

Concerning the pesticides residues contaminants, some well-known are dioxins, organophosphorus and organochlorine pesticides. In our study, organophosphorus residues are not detected whereas among the organochlorine, DDT was not detected. Pesticide contaminants are quite extensive worldwide, making their control very difficult and can be accumulated in foods and cause damage to consumers. Thought most of the organochlorine pesticides were banned, they are persistent and stable. They can be present in feeds used for farm animals because of the remaining environment for many years, constituting a risk of long-term exposure (Moats, 1994). In our study, the reasons for such contamination can include the use of contaminated ingredients to feeding beefs, lack of control and inadequate processing when using antibiotics (Croubels et al., 2004). However, environmental contaminants are rather difficult to control particularly in our conditions where cows are fed in open air with wild herbs. This habit is favorable for potential toxicity in the meet product (Heggum, 2004). The use of pesticides in farms has no doubt helped developing countries' green revolution, but these are involved in polluting air, water and land. It also

posing serious public health problem by entering in to food chain, thus, it will be interesting to investigate the toxins production capability of the isolated pathogens after their molecular characterization.

Conclusion

The manufacturing techniques of the *kpakouma* and the various apprehensions of the populations consuming this commodity were established. Some collected samples contain antibiotics and pesticides residues. In addition, the microbiological quality of the *kpakouma* samples reveals that all the samples were dissatisfaction for the three criteria, namely, FAM, *E. coli* and *Salmonella* independently to the collection place. Considering the resistance profiles against the antibiotics tested, the results are alarming because of the high resistance rates recorded. Thus, the dangers associated with *Staphylococcus*, *E. coli* and *Salmonella* can be aggravated by the noticeable increases in this antibiotic resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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