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Full Length Research Paper

Biopreservative application of bacteriocins obtained from samples *Ictalurus punctatus* and fermented *Zea mays*

Oyinlade C. Ogundare[1*], Simeon K. Odetunde[1], Mutiat A. Omotayo[1], Oluremilekun O. Sokefun[1], Rasheed O. Akindiya[1] and Adetayo Akinboro[2]

[1]Department of Chemical Science, School of Pure and Applied Science, Lagos State Polytechnic, Ikorodu, PMB 21606, Ikeja, Lagos State, Nigeria.
[2]Department of Biochemistry, Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.

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This study evaluated the preservative ability of protein-like cell free supernatants produced by lactic acid bacteria (LAB) isolates from samples of *Ictalurus punctatus* (Cat fish) and slurry of fermented *Zea mays* (Ogi). The LAB strains were separately isolated from understudied samples using De Man, Rogosa and Sharpe (MRS) media at 37°C for 48 h. The isolated strains were characterized with Gram staining, oxidase and catalase tests, microscopy study, carbohydrate fermentation, acid production and NaCl tolerance. Thereafter, the protein concentrations of crude bacteriocin supernatants from the Gram positive, rod shaped, oxidase and catalase negative strains were studied. Also, the growth inhibition of *Bacillus subtilis, Staphyloccocus aureus* and *Escherichia coli*, heat stability, pH tolerance, effect of proteolytic enzyme and biopreservation efficiency of protein-like cell free supernatants (crude bacteriocins) were determined. Biopreservative efficiency of the crude bacteriocin samples was also determined in orange (*Citrus sinenses*) and Titus fish (*Scomber scombrus*). The isolates from intestine of *I. punctatus* and fermented *Z. mays* fermented carbohydrate, and grew optimally at 3% NaCl, and 10 and 37°C, respectively. They inhibited the multiplication of *E. coli* at various extents, but more effective on different strains. The bacteriocins from slurry of fermented *Z. mays* on the other hand, were more potent in *E. coli* (22.7 ± 0.8 mm) than *S. aureus* (7.9 ± 0.1 mm). The biopreservative efficiency of crude bacteriocin from *I. punctatus* was greater than that of Z. *mays.* The LAB obtained from the selected samples produced protein-like substances in form of bacteriocins with potent antibacterial and biopreservative proficiencies through the growth inhibition of tested pathogens and low colony counts on tested food samples, respectively. Bacterial isolates obtained from samples of *I. punctatus* and *Z. mays* can be successfully used in the preservation of food and vegetables.

Key words: *Ictalurus punctatus*, *Zea mays*, bacteriocin, protein-like substances, biopreservative ability.

INTRODUCTION

Ictalurus punctatus and fermented *Zea mays* are parts of the many functional foods that are consumed in West African countries, and are produced through the use of lactic acid bacteria (LAB) during metabolism or production processes. For instance, several LAB strains have been isolated and established from grain products, dairy products, meat and fish products, beer and wine, fruit and its fruit juices, pickled vegetables and mash foods, as

well as during fermentation of plant materials (Liu et al., 2014)

I. punctatus (Channel Catfish) is a fresh water fish and commonly used as one of the protein sources in African diets. It is widely known as 'Eja aro' in western part of Nigeria. The demand for *I. punctatus* has grown significantly in the recent years (Eun et al., 1994). Like any other aquatic animals, the GIT or gut of *I. punctatus* contains series of bacteria which include LAB or compounds obtained from LAB (bacteriocins, organic acid and many more), and these candidates are known for probiotic activity against both Gram-positive or -negative pathogens (Ringø and Gatesoupe, 1998). Moreover, the presence of probiotic LAB or their products in aquatic animals converses immunity to the animals (Behnsenet al., 2013; Shahid et al., 2017).

Fermented *Z. mays* is commonly called Ogi, Pap, Koko and Akamu in different parts of Nigeria. It is taken by both children and adults, and can be processed to give different products. Fermented *Z. mays* is obtained by fermentation of maize in the presence of LAB leading to improvement of nutritional and sensory properties, and shelf life of the fermented *Z. mays* (Adesokan et al., 2010; Ejigui et al., 2005; Ijarotimi and Keshinro, 2011).

In the fermentation of *Z. mays*, two fermentation procedures are applied; natural fermentation in which raw clean *Z. mays* are allowed to ferment naturally by steeping in water at room temperature for a period of 12 to 72 h, and artificial fermentation in which *Z. mays* are exposed to LAB and anti-fungi agents in the presence of water for a period of 12 to 48 h (Alka et al., 2012; Ogodo et al., 2017).

LAB, which are naturally part of the microbial flora that are present in foods such as *Z. mays* or during steeping in the present inoculum during artificial fermentation encourages fermentation via rapid acidification of the food matrix and enhances food safety or production of antimicrobial metabolites, which create a physicochemical environment that prevents the growth of potential spoilage and pathogenic organism, improves food texture, nutritional value, and aroma (Smid and Kleerebezem, 2014).

The benefits of LAB cannot be overemphasized, LAB being part of the component of daily food materials such as poultry, fish, dairy and meat products, may enhance appropriate equilibrium in the intestinal flora, improved digestion of lactose, control serum cholesterol and certain types of cancer (Ali, 2010; Udhayashree et al., 2012). The LAB strains are used as starter culture for important biological processes including fermentation, aroma production, as well as microbiological stability (De Vuyst and Leroy, 2007; du Toit et al., 2011; Smid and Kleerebezem, 2014; Trząskowska et al., 2014). Microbiological stability of food samples in the presence of LAB is achieved by liberation of antimicrobial substances (organic acids, diacetyl, hydrogen peroxide and bacteriocins), and has been reportedly responsible for food preservation (Vignolo et al., 2012; Yang et al., 2014). Reports showed that the addition of antimicrobial substances (bacteriocins) to foods may not pose risks to the consumer's health or affect the nutritional and sensory quality of the food (Vignolo et al., 2012; Woraprayote et al., 2016).

Perez et al. (2014) described bacteriocins as heat stable antimicrobial peptides or proteinaceous compounds that are synthesized in the ribosomes by LAB strains which are naturally found in foods, and are effective in inhibiting the growth of similar or closely related bacterial strains from fermented foods without affecting the producing strain (Ramu et al., 2015). A recent report showed that the peptide compounds are effective on Gram-positive bacteria, and numerous food-borne and pathogenic microorganisms (Barbosa et al., 2017). Although bacteriocins may be sensitive to certain proteolytic enzymes, temperature and pH, their application in food preservation is generally regarded as safe and known to enhance the sensory qualities of the food samples and extend their shelf life (Chang and Chang, 2010; Reis et al., 2012). Therefore, bacteriocins are exploited in food preservation (Del Nobile et al., 2012; Silva et al., 2018). The LAB bacteriocins function by different mechanisms in order to exercise their antimicrobial activity (Deegan et al., 2006). It involves the leakage of proteins, alteration of cell membrane integrity, DNA and RNA (Gould, 2012; Lee and Kim, 2011). Recently, as a result of the safe potency of origins of bacteriocins and extensive scope of efficacy of the peptide substance on pathogenic organisms, attention of researchers has been placed on the use bacteriocins in inhibition of pathogenic organisms in foods, and then application in industrial food preservation (Ghanbari et al., 2013).

The use of preservatives in food safety has been one of the major ways by which foods are made available at all seasons, as their shelf lives are extended via protection of foods from chemical, physical and microbiological alterations that cause food spoilage (Yousef and Balasubramaniam, 2013). Methods involving physical and chemical processes using natural (preservatives obtained from plants, animals or microorganisms) or artificial (synthetic compounds) preservatives are employed in preservation to destroy, remove or inhibit the growth of unwanted microorganisms (Farkas, 2007; Gould, 2012; Lück, 1985). Natural process like drying or roasting is used to kill or reduce the levels of food poisoning causing microorganisms in food products.

*Corresponding author. E-mail: ogundareoyinlade@yahoo.com.

These methods alter the colour of foods, while many of the chemical preservation methods are limited due their side effects. Nitrates, benzoic acid or its salts, formaldehyde, sorbates, parabens, butylated hydroxyl toluene (BHA), and butylated hydroxy anisole (BHA) are responsible for serious health perils such as hypersensitivity, asthma, allergy, cancer, hyperactivity and neurological damage of consumers (Shahidi, 2015; Sharma, 2015). Of all these preservatives, the most commonly used artificial preservative is benzoic acid. Aside from drying and roasting, antioxidant and antimicrobial agents are exploited in the prevention of food spoilage, and increase shelf life of foods and vegetables. These compounds include antioxidant such as vitamins C and E, and antimicrobial: bacteriocin (Davidson et al., 2012). The antioxidant forms of preservative are known to generate free radicals especially when used at a relatively high dosage (Piper et al., 2001).

Summarily, the currently applied methods of food preservative (physical and chemical methods) are limited as a result of the consumer needs for safe and minimally processed foods. The associated limitations have led to recent researches in the production bio-preservatives such bacteriocins. Although, the use of bacteriocins from LAB strains have been previously reported by scientists, but to the best of our knowledge there has been paucity of data as regard the production of proteinaceous bacteriocin produced by LAB isolates obtained from samples of *I. punctatus* and the slurry of fermented *Z. mays*. Therefore, this study attempts to produce and characterize and investigate antibacterial potential of proteinaceous substances from the understudied food sample. Moreover, the bio-preservative activity of the suspected bacteriocins was established against pathogens associated with samples of Titus fish and orange juice.

MATERIALS AND METHODS

Collection of samples for analysis

A total of six (6) samples of life *I*. *punctatus* were randomly collected from nearby Fish-farm in Ikorodu, Lagos State and taken to the laboratory in a cellophane bag containing small quantity of clean water. In the laboratory, the samples of fish were sacrificed, and the obtained intestine was stored at 4°C for about 2 h in readiness for analysis. The slurries of fermented *Z*. *mays* samples were also collected from nearby local producers, stored in ice bath and taken to the laboratory for instant use.

Isolation and identification of bacteriocin producing organisms

The collected samples of *I*. *punctatus* were cut and their intestines rinsed in normal saline. The intestines (1.0 g) were taken from each *I*. *punctatus* and pulverized to paste in normal saline (10 mL) by use of mortar and pestle to give stock solution of 0.1 g sample/mL. Similarly, slurry sample (1.0 g) of fermented *Z*. *mays* was also taken into clean mortal and pulverized to paste in normal saline (10 mL) by use of mortar and pestle to give stock solution of 0.1 g sample/mL. Homogenate of the intestine of *I*. *punctatus* or fermented *Z*. *mays* was centrifuged at 5000 rpm for 10 min to obtain the supernatant. The supernatants obtained from samples of intestine of *I*. *punctatus* or fermented *Z*. *mays* were combined to give homogenates of intestine of *I*. *punctatus* or fermented *Z*. *mays,* respectively. A measure (10 mL) of each supernatant was taken into a conical flask and carefully inoculated into freshly prepared de Man, Rogosa (MRS) broth (40 mL) in order to isolate the possible LAB isolates. The culture was in turn distributed into 10 mL sterilized test tubes and incubated at 37°C for 2 days with persistent shaking on a shaker under anaerobic situations. Every tube exhibiting turbidity was chosen, and further inoculated onto MRS agar plates and incubated for 2 days at 37°C under anaerobic conditions.

Possible LAB plates (plates showing creamy or white colonies) were selected, and further purified for two successful times by aseptically streaking the organisms on MRS agar plates so as to increase the number of pure bacteria. The resulting creamy or white cultures that were established by Gram staining using crystal violet dye, oxidase test trips, cell morphology by examination on microscope and catalase test were branded as LAB. The plates containing pure LAB colonies were stored in the refrigerator for further studies. Additionally, the LAB isolates were further identified by the following assays.

Fermentation of carbohydrates by LAB isolates

Ability to ferments carbohydrate by use of protocol of Tserovska et al. (2002) was adopted with slight modifications. MRS broth (medium containing 1 g beef extract, 10 g protease peptone No. 3, 5 g yeast extract, 2 g K_2HPO_4, 5 g $CH_3COONa{\cdot}3H_2O$, 5 g sodium chloride, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$, 0.17 g phenol red and 1 mL of tween 80) was prepared in distilled water. The aforementioned solution was filtered and used as solvent for preparation of 1% sugar solution (carbon source), this is an orange coloured carbohydrate broth, pH 7.4. The carbohydrate broth (5 mL) was poured into 10 mL test tube and Durham tube was inserted into it so as to detect gas production. The tube was then autoclaved at 121°C for 15 min for glucose, and 121°C for 3 min for lactose, maltose or sucrose. The LAB isolates were aseptically inoculated by use of inoculating loop into different test tubes, and incubated for 37°C. A pronounced air bubble in the Durham tube after 48 h indicates fermentation of sugar with gas production, and lack of gas bubble indicates that fermentation did not occur.

Acid production by LAB isolates

The reaction tubes that have been subjected to fermentation were further studied for acid production. Acid production by the isolates was characterized by the change in the orange colour of the solution in the test tube to yellow colouration as a result of production of acid by the lactic acid bacteria.

Heat tolerance test

The ability of the isolates to grow at various temperatures was investigated by use of Kozaki et al. (1992) method. Pure colonies of LAB isolates were aseptically obtained from MRS agar plates, and inoculated into tubes containing MRS broth. Tubes were incubated in anaerobic jars at temperatures of 10, 27, 37 and 50°C for 48 h. Positive results were determined as formation of turbid or cloud solution. Heat tolerance was monitored following the streaking of 1 mL of broth on sterile MRS agar plates. This was incubated at 37°C for a period of 48 h.

NaCl tolerance

MRS broth (10 mL) containing 3, 5, 7 and 9% (w/w) NaCl was prepared into different test tubes and sterilized (Zou et al., 2013). LAB isolates were inoculated into the MRS broth and incubated at 37°C for 48 h. Test tubes were visualized in order to monitor the growth based on turbidity of the resulting broth. NaCl tolerance was evaluated following the streaking of 1 mL of broth on sterile MRS agar plates. This was incubated at 37°C for a period of 48 h. Tubes containing LAB cultures without NaCl served as positive control.

Production of crude bacteriocins

Gram positive, cocci-shaped organisms, which are found to be oxidase and catalase negative isolates (purified LAB), were inoculated into MRS broth at 37°C for 2 days to obtain bacteriocin as more LAB isolates are produced. At the expiration of fermentation, cells were harvested by centrifugation at 30000 rpm for 15 min. Denaturation was prevented by maintaining temperature range of 2 to 4°C in an ice bath. The resulting cell free supernatant were tested for protein which was quantified by use of Lowry's method (Lowry, 1951). These were reserved as the crude bacteriocin samples.

Determination of protein concentration

The concentrations of protein in crude bacteriocins obtained from LAB isolates from intestines of *I*. *punctatus* and slurries of fermented *Z*. *mays* were determined according to Lowry's method (Lowry, 1951). Briefly, a set of nine test tubes containing 0.5 mL of standard bovine serum albumin (BSA) solutions of concentration ranging from 0 to 2 mg/mL were prepared as standard from a stock BSA (4 mg/mL) solution, and used to prepare a standard curve. The bacteriocins were also dispensed into different test tubes. The standard or sample of bacteriocins (0.1 mL) was separately mixed with 0.1 mL of 2 N NaOH. These were hydrolyzed at 100°C for 10 min in a boiling water bath and cooled to room temperature. Additionally, 1 mL of freshly prepared complex-forming reagent prepared from a mixture of solutions of 2% (w/v) Na_2C0_3, 1% (w/v) $CuS0_4.5H_20$ and 2% (w/v) sodium potassium tartrate in ratio 100:1:1 was added. The reaction mixtures were incubated at room temperature for 40 min and their absorbance values were read at 550 nm. The analysis was done in triplicates and the protein concentration of the bacteriocins obtained from standard curve.

Antimicrobial activity of crude bacteriocins

Antimicrobial activities of bacteriocins against three common pathogenic microorganisms (*Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*) were determined by well diffusion method under anaerobic condition. The activity was considered according to the extent of growth of the test organisms as bactericidal (where there is no growth of the organism in the presence of the bacteriocin) or bacteriostatic (inhibitory activity). Summarily, inoculum of test organisms (1×10^5 CFU/mL) was introduced into freshly prepared nutrient agar plates. This was spread over the plates using swab sticks and four wells (8 mm) were bored into each plate before 20 µl of crude bacteriocin (cell free supernatant) was introduced into each well. The plates were incubated at the 37°C [optimum temperature for indicator microorganisms as documented in previous reports (Noor et al., 3013; Stewart, 2003; Hanim, 2017)] for 24 h. The antimicrobial activity of crude bacteriocins was determined by measuring diameter of clear zone around each well. Values were expressed as mean of triplicate readings.

Heat stability of crude bacteriocins

Protocol of Udhayashree et al. (2012) was adopted with slight modification. A measure of 5 mL of crude bacteriocins in different test tubes was heated at 10, 37, 50, 80 and 90°C for a period of 2 h under pressure. The heat treated bacteriocin samples were then studied for antimicrobial activity on the indicator organisms for which the bacteriocin was bactericidal by use of well diffusion method.

Effect of pH on crude bacteriocins

Aliquot of crude bacteriocins (5 mL) was taken in test tubes and the pH of the contents was separately regulated at pH 2, 4, 6, 7 and 9, using either 1 M solution of HCl or NaOH. The tubes and their contents were left at room temperature for 2 h and assessed for antimicrobial activity by use of well diffusion method (Udhayashree et al., 2012).

Effect of trypsin on crude bacteriocins

Indicator organism that was selected here was *E. coli*. Aliquot of crude bacteriocins (5 mL) was taken into test tubes and treated with trypsin (1 mg/mL) at optimum pH for the bacteriocin substance (pH 7). The control contained no enzyme, but 5 mL of phosphate buffer and bacteriocin. Test tubes and their contents were incubated at 37°C for 2 h and heated at 100°C for 3 min to denature the enzyme. Both the control and samples were studied for antimicrobial activity using well diffusion method according to protocol of Udhayashree et al. (2012).

Biopreservative efficiency of bacteriocins

Healthy ripe oranges (*Citrus sinenses*) obtained from a nearby market were washed, peeled, cut into pieces and pressed on juice extractor. The extract obtained was filtered using filter paper to separate the juice from the orange insoluble fiber. The orange juice was stored in a clean sample bottles at 4°C for further use.

Fresh Titus fish (*Scomber scombrus*) were obtained from nearby market, the flesh was removed and ground in mortal in a measure of 100 g Titus fish to 1 L of 3% NaCl solution so as to obtain a 10% fish homogenate. The homogenate was then stored at 4°C in the refrigerator until analysis. The selected sample solutions were sterilized in an autoclave at 72°C for 2 min. In other to compare the biopreservative ability of the bacteriocins with a chemical preservative, benzoic acid was used as a standard. The assessment was done according to the protocol of Pratush et al. (2012). Briefly, inoculum of *E. coli* (8.5×10^5 CFU/ mL) was introduced to three sets of sterilized glass bottles labelled as Control, Standard and Sample that contain 100 mL of either orange juice or fish homogenate. This was followed by addition of sodium benzoate at a concentration of 600 mg/mL to the Standard, while the Sample was treated with only crude bacteriocin at 600 mg/mL. The test samples were incubated at 37°C for seven days and their microbial counts were monitored daily. The experiment was done in triplicates.

Statistical analysis

Statistical analysis of bacterial growth was achieved by use of comparison at $P<0.05$ value through Turkey test with the aid of GraphPad Prism (version 5.01). Standard deviations for all the analyzed data are indicated by error bars.

Table 1. Characteristics of isolates from intestine of *I*. *punctatus* and slurry of fermented *Z*. *mays*.

Test	Intestine of *I. punctatus*	Fermented *Z. mays*
Growth in MRS broth	Consistent turbidity	Consistent turbidity
Number of colonies on MRS agar	8 smooth round colonies	17 smooth round colonies
Colony morphology	Cream or white coloured rod organisms	Bright white coloured rod organisms
Gram staining	Gram positive non-spore forming	Gram positive non-spore forming
Catalase test	Negative	Negative
Oxidase test	Negative	Negative
Acid production during glucose fermentation	Yes	Yes
Glucose fermentation	Gas production	Gas production
Fructose fermentation	Gas production	Gas production
Maltose fermentation	Gas production	Gas production
Lactose fermentation	Gas production	Gas production
Heat tolerance		
Growth at 10 ºC	Yes	Yes
Growth at 27 ºC	Yes	Yes
Growth at 37 ºC	Yes	Yes
Growth at 50 ºC	No	No
NaCl tolerance		
Growth in 3% NaCl	Yes	Yes
Growth in 5% NaCl	Yes	No
Growth in 7% NaCl	No	No
Growth in 9% NaCl	No	No

Table 2. Protein concentrations of bacteriocin like substance from intestine of *I*. *punctatus* and slurry of fermented *Z*. *mays*.

Test	Intestine of *I. punctatus*	Fermented *Z. mays*
Protein concentrations	108.4 ± 3.9 mg/mL	102.7 ± 3.0 mg/mL

RESULTS

Selection of potential probiotic requires proper identification of the selected organism through morphological, biochemical and most times genotypic characterization (Pham et al., 2014). In the present study, morphological and biochemical properties of LAB isolates from intestine of *I. punctatus* and slurry of fermented *Z. mays* (Table 1) revealed the presence of eight (8) white colour rod shaped micro-organisms in intestine of *I. punctatus* compared to the seventeen (17) that were found in slurry of fermented *Z. mays.* These organisms appeared white in colour. Furthermore, biochemical characterization of the isolated microorganisms showed that there was no liberation of O_2 in the presence of H_2O_2, neither was there a change in the colour of the strip of paper (purple) during oxidase test by use of Kovács oxidase reagent. The isolated organisms liberated acid and gas from glucose during fermentation, and produced gas in the fermentation of other carbohydrates (fructose, maltose and lactose). Table 1 also illustrates the heat and salt (sodium chloride) tolerance capacity of the isolates. The strains were able to grow between 10 and 37°C and tolerated at least 3% NaCl concentration.

Table 2 reveals that the cell free supernatant obtained from cultures of LAB isolates from intestine of *I*. *punctatus* and slurry of fermented *Z*. *mays* contained 108.4±3.91 and 102.7 ± 3.0 mg/mL crude protein, respectively. The proteinaceous supernatants inhibited growth of *E*. *coli*, *S*. *aureus* and *B*. *subtilis* at varied capacity (Table 3) as shown by the diameter of the circle that is formed around the diameter of the cork borer (was used for the well) as a result of the inhibitory activity of proteinaceous supernatants (crude bacteriocins) against indicator organisms. The crude bacteriocin from the isolates from intestine of *I*. *punctatus* was more potent on *B*. *subtilis* (26.0 ± 0.9 mm) than *E. coli* (8.1±0.31 mm), but did not inhibit the growth of *S*. *aureus* at all. The bacteriocin from slurry of fermented *Z*. *mays* on the other hand, was more potent on *E. coli* (22.7 ± 0.8 mm) unlike

Table 3. Antimicrobial activity of crude bacteriocins from intestine of *I*. *punctatus* and slurry of fermented *Z*. *mays*.

Indicator organism	Zones of inhibition of bacteriocin (mm)	
	Intestine of *I. punctatus*	**Fermented *Z. mays***
E. *coli*	8.1 ± 0.3	22.7 ± 0.8
S. *aureus*	No inhibition	7.9 ± 0.1
B. *subtilis*	26.0 ± 0.9	No inhibition

Table 4. Effect of temperature on the inhibitory activities of crude bacteriocins from intestine of *I*. *punctatus* and slurry of fermented *Z*. *mays*.

Indicator organisms	Temperature (°C)	Zones of inhibition of bacteriocin (mm)	
		Intestine of *I. punctatus*	**Fermented *Z. mays***
E. *coli*	10	7.60 ± 0.3	17.10 ± 0.2
	37	7.50 ± 1.0	22.00 ± 0.2
	50	10.10 ± 0.2	19.50 ± 1.8
	80	2.30 ± 0.1	5.30 ± 0.2
	90	No inhibition	No inhibition
S. *aureus*	10	No inhibition	2.40 ± 0.01
	37	No inhibition	7.10 ± 0.21
	50	No inhibition	6.00 ± 0.05
	80	No inhibition	No inhibition
	90	No inhibition	No inhibition
B. *subtilis*	10	16.0 ± 0.3	No inhibition
	37	26.1 ± 0.1	No inhibition
	50	No inhibition	No inhibition
	80	No inhibition	No inhibition
	90	No inhibition	No inhibition

the inhibition of *S*. *aureus* (7.9 ± 0.1 mm).

Effects of temperature (Table 4) and pH (Table 5) revealed that the crude isolated bacteriocins were optimally stable at 37 and 50°C for bacteriocins from fermented *Z. mays* and intestine of *I*. *punctatus,* respectively, and pH 6 to 7, respectively against selected indicator organisms. The inhibition of growth of *E. coli* by the trypsin treated bacteriocin that was obtained from LAB isolates was investigated by agar well diffusion method (Table 6). The zone of inhibition (mm) in the presence of the trypsin treated bacteriocin from LAB isolates from intestine of *I*. *punctatus* was reduced, while the one from slurry of fermented *Z. mays* was totally eliminated.

Table 7 describes the biopreservative potential of crude bacteriocins from intestine of *I*. *punctatus* (BI) and slurry of fermented *Z*. *mays* (BZ) on juice of ripe orange and Titus fish. The tested samples were initially sterilized in order to eliminate possible contamination before the assessment. There was a reduction in growth of inoculated organism (*E. coli*) in the orange juice, Titus juice and standard (benzoic acid) compared to control group as the treatment progressed. This was revealed by the reduced values of colony forming units of the indicator (Log CFU/mL) pathogen (Table 7) in all the treatment groups in relation to the control group. The growth inhibition of the pathogen by BZ (9.96 ± 0.09 Log CFU/mL) during the six day of the preservation of orange juice was significantly ($p<0.05$) lower than that of BI (10.96±0.09 Log CFU/mL) or standard preservative (11.70±0.10 Log CFU/mL) in the treated orange juice (Figure 1a). The preservation of Titus fish was a reversal as there was a significant ($p<0.05$) decrease in inhibition of the indicator organism as a result of application of BI (10.00±0.10 Log CFU/mL) to sample of Titus fish (Figure 1b) as at the last (6th) day of treatment in relation to other groups.

Using agar well diffusion method to access the production of antimicrobial agents by the selected bacterial isolates from the *I*. *punctatus* intestine and fermented *Z*. *mays* against three pathogens, the susceptibility of various Gram positive (*S. aureus* and *B. subtilis*) and Gram negative (*E. coli*) bacteria to grow in presence of crude extract of bacteriocin revealed

Table 5. Effect of alteration of pH on the inhibitory activity of crude bacteriocins from intestine of *I. punctatus* and slurry of fermented *Z. mays*.

Indicator organisms	pH	Zones of inhibition of bacteriocin (mm)	
		Intestine of *I. punctatus*	Fermented *Z. mays*
E. coli	2	4.30 ± 0.17	10.10 ± 0.33
	4	6.80 ± 0.19	14.20 ± 0.12
	6	14.10 ± 0.92	19.50 ± 0.61
	7	8.50 ± 0.21	10.30 ± 0.26
	9	5.97 ± 0.19	7.00 ± 0.09
S. aureus	2	No inhibition	4.30 ± 0.41
	4	No inhibition	8.30 ± 0.39
	6	2.01 ± 0.08	10.10 ± 0.17
	7	3.45 ± 0.11	12.00 ± 1.96
	9	No inhibition	11.40 ± 0.99
B. subtilis	2	16.00 ± 1.05	1.40 ± 0.07
	4	26.10 ± 2.01	3.60 ± 0.17
	6	29.60 ±0.98	7.30 ± 0.11
	7	29.40 ± 1.01	5.30 ± 0.18
	9	20.90 ± 1.06	2.90 ± 0.21

Table 6. Effect of trypsin in antimicrobial property of bacteriocins from intestine of *I. punctatus* and slurry of fermented *Z. mays.*

Indicator organisms	Zones of inhibition of the bacteriocins (mm)			
	Intestine of *I. punctatus*		Fermented *Z. mays*	
	Control	Sample	Control	Sample
E. coli	7.90 ± 0.43	4.70 ± 0.05	20.80 ± 2.01	Nil

inhibition against *E. coli*, *S. aureus* and *B. subtilis* at varied degrees (Figure 1). There was an evident reduction in the microbial count of pathogenic organisms on application of bacteriocin with little or no effect on the growth of *E. coli*.

DISCUSSION

LAB comprise a group of diverse microorganisms that generates lactic acid as the major product during the fermentation process, and are also categorized as Gram-positive bacteria that have a number of biotechnological abilities in food industry (Alvarez-Sieiro et al., 2016). LAB produce assorted types of substances that include the metabolic end products, bactericidal or antibiotic-like proteneceous substances that are termed bacteriocins (Klaenhammer, 1988). LAB that associate with food substances are obtained from plant as well as animal origins. The LAB strains are found in milk products, fermented foods, animal intestines or freshwater fishes, soil samples, sugar cane plants, and poultry farms (Barakat et al., 2011). Various types of bacteriocin have been isolated from LAB, for instance: nisin, lacticin and lactosin which are obtained *Lactococcus lactis* and *Lactobacillus sakei* (De Vuyst and Vandamme, 1994; Mørtvedt et al., 1991; Piard et al., 1992). Bacteriocins are relevant in different facet of life, especially in maintenance of food safety in order to extend the shelf life of such food through the formation of fermentation products (Sarika et al., 2010).

In this study, MRS medium were used under anaerobic conditions in order to allow the identification of possible LAB isolates from the selected animal and plant tissues. This was in accordance with the recommendation of Ouali et al. (2014) where the MRS medium were recommended for isolation of different micro-organisms. Reports from Pham et al. (2014), Al Kassaa et al. (2014) and Fontana et al. (2013) established that selection of potential probiotic bacteria (LAB strains) requires proper identification of the selected organism through morphological and biochemical tests as the organism

Table 7. Biopreservative potential of bacteriocins from intestine of *I. punctatus* and slurry of fermented *Z. mays*.

Test food samples	Microbial counts (Log CFU/mL)			
	Control	Sample (BI)	Sample (BZ)	Standard
Ripe oranges				
Day 0	5.93 ± 0.01	5.93 ± 0.07	5.93 ± 0.01	5.93 ± 0.03
Day 1	5.99 ± 0.04	5.98 ± 0.03	6.13 ± 0.02	5.98 ± 0.03
Day 2	6.13 ± 0.11	6.10 ± 0.04	6.16 ± 0.02	6.17 ± 0.05
Day 3	7.54 ± 0.06	7.11 ± 0.04	7.22 ± 0.04	7.48 ± 0.08
Day 4	9.85 ± 0.09	7.90 ± 0.08	8.03 ± 0.06	8.20 ± 0.07
Day 5	10.17 ± 0.08	8.93 ± 0.06	9.95 ± 0.08	9.78 ± 0.09
Day 6	14.99 ± 0.11	9.86 ± 0.10	10.96 ± 0.09	11.70 ± 0.10
Titus fish				
Day 0	5.93±0.03	5.93 ± 0.02	5.93 ± 0.01	5.93 ± 0.03
Day 1	6.29±0.03	5.98 ± 0.03	6.02 ± 0.01	6.19 ± 0.03
Day 2	7.49±0.01	6.10 ± 0.07	6.39 ± 0.03	7.22 ± 0.03
Day 3	8.00±0.10	7.65 ± 0.03	7.65 ± 0.06	7.55 ± 0.04
Day 4	10.30±0.10	7.94 ± 0.07	8.03 ± 0.07	8.11 ± 0.09
Day 5	12.70±0.11	9.99 ± 0.10	10.03 ± 0.09	10.04 ± 0.09
Day 6	15.04±0.13	10.00 ± 0.10	11.01 ± 0.09	11.01 ± 0.08

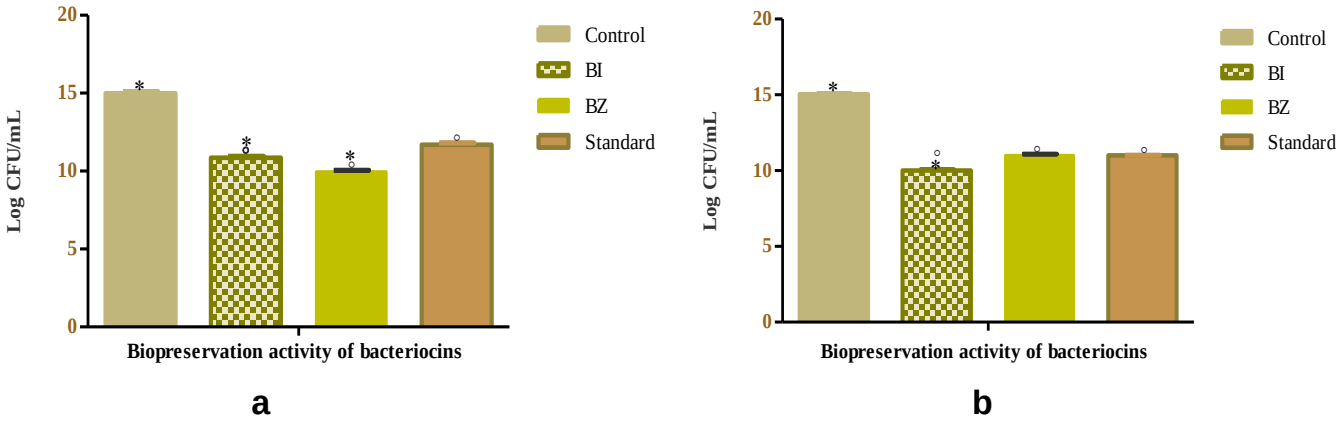

Figure 1. Biopreservative activities of bacteriocins from intestine of *I. punctatus* (BI) and slurry of fermented *Z. mays* (BZ) on orange juice (a) and Titus fish (b) as at day 7. All values are presented as Mean ± Standard Error of Mean of triplicate readings. Comparisons were made between the treatment groups. (*) $p<0.05$ versus Standard (benzoic acid); (◦) $p<0.05$ versus Control.

shows a bacilli shape, and without catalase activity. A total of the 8 isolates obtained from *I. punctatus* intestine and 7 isolates from slurry of fermented Z. *mays* were confirmed to be Gram positive, catalase negative, oxidase negative, non-spore, and white or cream coloured rod micro-organisms. Previous reports also described LAB as genetically and physiologically distinct set of rod-shaped, Gram-positive and catalase negative bacteria (Ashmaig et al., 2009; Dallal et al., 2017; Guetouache and Guessas, 2015).

Furthermore, the isolates which were able to grow in anaerobic condition displayed an ability to ferment carbohydrates such as glucose, fructose, maltose and lactose as they liberate gas in the culture media. Fermentation of carbohydrates by LAB strains has been reported by Rattanachaikunsopon and Phumkhachorn (2010), Zou et al. (2013) and Jose et al. (2015). Also, there was a production of acid from glucose by these isolates, thereby, suggesting the properties of *Lactobacillus* species as described by Wang et al. (2010) and Ni et al. (2015). Previously, LAB have been isolated from both animal and plant sources in a bid to determine their probiotic ability or tendency to liberate antimicrobial substances that can be used in food preservation (Barakat et al., 2011; Tufail et al., 2011). Some of these sources include intestine or gut of fish (Balcázar et al.,

2008; Rao et al., 2015; Ringø et al., 2018; Sica et al., 2012). Fermented food samples including fermented Z. *mays* have also been reported to possess probiotic LAB strains (Onwuakor et al., 2014; Oyedeji et al., 2013; Rao et al., 2015; Zou et al., 2013). The isolated LAB strains produced acid in fermentation broth as an attribute of heterofermenter. Homofermenters are known for production of lactic acid from glucose. Two classes of fermentation strains of LAB (homofermentative and heterofermentative) were previously mentioned by researchers (Akalu et al., 2017; Nigatu et al., 2015). Some of the considerations made in the selection of potential probiotic LAB include optimum growth temperature and effect of salt concentration on their fermentation activities. Table 1 shows that the LAB isolates were stable at relatively high temperature range (10 to 37°C), and can be said to be heat tolerant, therefore the basis for the production of acid in the fermentation broth by the LAB isolates from the increased glycolytic activity. This is an added advantage over thermolabile pathogenic organisms, as the liberated acid reduces the contamination by other microorganisms. The report of this study is in agreement with Qiuju et al. (2013) and Zorriehzahra et al. (2016). The LAB isolates from the two tested samples were osmotolerance at 3% NaCl, while only the LAB isolates from intestine of *I*. *punctatus* could grow in 5% NaCl (Table 1). This indicates that the LAB strains from intestine of *I*. *punctatus* may be more tolerance to osmotic concentrations of NaCl than the strains from slurry of fermented *Z*. *mays*. Van Sinderen and Crowley (2013) and Adnan and Tan (2007) described tolerance of LAB strains to osmotic concentrations of salt like NaCl as an added advantage to commercial applications. Other scientists have previously reported the ability of LAB strains to withstand osmotic concentration resulting from addition of salts (Subramanyam, 2020; Van Sinderen and Crowley, 2013).

Despite the abundant information on production of bacteriocins from terrestrial origins or LAB that are capable of producing bacteriocins, there have been paucity of information on application of LAB especially in bacteriocins production in *I*. *punctatus*. Production of bacteriocin by LAB strains is essential factor in the choice of probiotic bacterial strains (Dobson et al., 2012). The bacteriocins which are proteinaceous substances are used to inhibit the growth of related microorganisms, and are recently applied in food preservation. Table 2 shows that the cell free supernatants obtained from culture of LAB strains from intestine of *I*. *punctatus* and fermented *Z. mays* samples contained proteinaceous substance (suspected to be bacteriocins) with protein concentrations 108.4 ± 3.9 and 102.7 ± 3.0 mg/mL, respectively. This is known as bacteriocins. This is similar to the reports of Udhayashree et al. (2012) and Abbasiliasi et al. (2012).

In a bid to characterize the proteinaceous substance, the antimicrobial activity of the substance was investigated in cultures of *E*. *coli*, *S*. *aureus* and *B*. *subtilis* (Table 3). The indicator organisms were vulnerable to the activity of the crude bacteriocins at varied degrees. Gram-positive bacteria (*S. aureus* and *B. subtilis*) responded positively to inhibition of growth by the crude bacteriocins obtained from intestine of *I*. *punctatus* and *Z. mays*. This is an indication of antibacterial activity of bacteriocins produced by the isolated LAB against the selected pathogens. In the company of these are Gram-negative bacteria (*E. coli*) whose cell membrane is surrounded by lipid rich cell wall as in the case of any Gram-negative bacteria, but still proved sensitive to antibacterial actions of the extracted bacteriocins. Reports from Tufail et al. (2011) and Sankar et al. (2012) revealed the antibacterial activity of bacteriocin against some pathogenic organisms like *E. coli* and *S. aureus*. Yang et al. (2012), Djadouni and Kihal (2012) and Gaamouche et al. (2014) reported the antimicrobial activity of LAB bacteriocins in some Gram-positive bacteria. For instance, Afolayan et al. (2017) and Rather et al. (2017) recounted the antimicrobial activity of substance obtained from LAB isolates from fermented *Z. mays* and gut of fishes, respectively. This work supported the tendency of bacteriocins to affect the growth of both Gram-positive and Gram-negative organisms (Abriouel et al., 2011).

The effects of alteration of temperature and pH on activity of crude bacteriocins from the LAB isolates were determined using *E. coli*, *S. aureus* and *B. subtilis* as indicator organism. The crude bacteriocins were found to be heat stable especially at 37 and 50°C for bacteriocins from fermented *Z. mays* and intestine of *I*. *punctatus*, respectively (Table 4). These results indicate that bacteriocin produced by LAB from intestine of *I*. *punctatus* is more heat stable than the fermented *Z. mays*, as its activity was sustained after the heat treatment at the aforementioned temperature. Bacteriocins that are used as food preservative are usually heat stable since preparation of many food requires heat in one way or the other (Ogunbanwo et al., 2003). Previous reports have also corroborated the present finding that the bacteriocins from the LAB isolates are heat stable (Gómez-Sala et al., 2015; Udhayashree et al., 2012).

Effect of pH on activity of crude bacteriocins from fermented *Z. mays* and intestine of *I*. *punctatus,* respectively (Table 5) was carried out. It was observed that bacteriocin produced by LAB in intestine of *I*. *punctatus* and fermented *Z. mays* were optimally stable at pH 6. This further confirmed the tolerance of bacteriocins from the LAB to acidic rather than the alkaline pH values and that they can be applied in acidic foods (Adesina et al., 2016; Ayed et al., 2015; Li et al., 2015).

Exposure of E. coli to the trypsin treated bacteriocin that were obtained from LAB isolates showed that zone of inhibition (mm) in the presence of the trypsin treated bacteriocin from LAB isolates from intestine of *I. punctatus* was reduced, while the one from slurry of

fermented *Z. mays* was totally eliminated (Table 6). This indicates that crude bacteriocins were inactivated by treatment with trypsin as a result of reduction or elimination of antimicrobial activity when it relates to controls, and further established the antimicrobial substances obtained from the isolated LAB cultures to be bacteriocin; a proteinaceous substance (Sankar et al., 2012).

Biopreservation is a potent natural method of extension of shelf life and safety of foods by using naturally occurring microorganisms, their innate antibacterial agents of specified quality and quantity (Ghanbari et al., 2013). Biopreservative activity of bacteriocin from LAB has been of utmost interest in the recent time. The reduction of microbial population in Titus fish and orange juice after addition of the crude protein-like substances produced from intestine of *I*. *punctatus* and *Z. mays* (Table 7) shows that the bacteriocins can be applied in preservation of food from plant and animal origins. The result also revealed that bacteriocin obtained from LAB in intestine of *I*. *punctatus* is more efficient in Titus fish than bacteriocin from *Z. mays.* Reduction of bacterial counts in food samples after treatment with crude bacteriocins as a measure of preservation has been documented. Gómez-Sala et al. (2016), Ghanbari et al. (2013) and Sarika et al. (2019) observed the extension of shelf life of fish after treatment with bacteriocins. Similarly, Udhayashree et al. (2012) and Ageni et al. (2017) reported a decrease in microbial loads in edible milk and button mushrooms, and in fermented maize (Ogi) and cassava (Fufu), respectively, In addition, bacteriocins from LAB obtained from these food items are efficient in the preservation of the selected test food samples, the crude bacteriocin from fish intestine (BI) was more efficient in Titus fish than in orange juice than the chemical preservative.

Conclusion

The present study revealed that the protein-like antibacterial substances from LAB isolates obtained in the samples of *I. punctatus* (Cat fish) and slurry fermented *Z. mays* (Ogi) possess an extensive spectrum of inhibitory activity against *S. aureus* and *B. subtilis*. The reduction in the microbial load in Titus fish and Orange juice exhibited by these proteinaceous substances (crude bacteriocins) also justify their tendency to preserve sea foods and fruits.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Aflatoxigenic potential of *Aspergillus* section *Flavi* isolated from maize seeds, in Burkina Faso

Hamidou COMPAORE[1*], Serge SAMANDOULOUGOU[1], Fidèle W. TAPSOBA[1], Alima BAMBARA[2], Hissein RATONGUE[3], Ignace SAWADOGO[1], Donatien KABORE[1], Pane B. OUATTARA-SOURABIE[4] and Hagrétou SAWADOGO-LINGANI[1]

[1]DTA/IRSAT/CNRST, 03 BP 7047 Ouagadougou 03, Burkina Faso.
[2]Département de Biochimie-Microbiologie, Laboratoire de Biochimie et d'Immunologie Appliquée (LaBIA), Université Joseph KI-ZERBO, 03 BP 7021 Ouagadougou 03, Burkina Faso.
[3]Université Catholique de l'Afrique de l'Ouest, Unité Universitaire à Bobo-Dioulasso (UCAO/UUB), 1052 Bobo-Dioulasso, Burkina Faso.
[4]Laboratoire National de Santé Publique (LNSP), 09 BP 24 Ouagadougou 09, Burkina Faso (Kadiogo / Centre).

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The frequency of occurrence and four principal kinds of aflatoxin concentration in maize seeds grown in Burkina Faso was investigated. Ten (10) samples collected, were analyzed by high performance liquid chromatography (HPLC) with post-column derivatisation after immunoaffinity column cleanup. Eight strains of *Aspergillus* section *Flavi* were previously isolated from these samples and cultivated on "*Aspergillus flavus* and *parasiticus* agar (AFPA)" to ascertain if they belong to *A. flavus* or *A. parasiticus* species. The qualitative ability of aflatoxin production was also previously performed by fluorescence emission under ultra violet light at 365 nm after four (4) days of incubation at 30 °C on Coconut Agar Medium (CAM). Results showed that 70% of samples were contaminated by aflatoxins. The levels ranged from 0.93 to 58.94 µg/kg. Samples M1 and M10 had high concentrations, 58.94 µg/kg and 70.73 µg/kg; whereas M4 and M5 had low concentrations from 1.68 to 0.93 µg/kg, respectively. In these samples, four were contaminated with aflatoxin B1 (AFB1) and aflatoxin G1 (AFG1), two with AFB1 and aflatoxin B2 (AFB2) and one (01) with AFB1 only. We notice that AFB1 was the most prevalent member of aflatoxins, and AFG2 was absent in all samples.

Key words: Maize, *Aspergillus*, aflatoxins, HPLC, Burkina Faso.

INTRODUCTION

Cereals are a staple food for humans and animals. In Burkina Faso, their annual consumption is estimated at 62% of the food consumed by households (Waongo et al., 2013). Among food crops, maize is the most used product by over 98% of rural households (Bambara, 2021). In Burkina Faso, maize ranks second among

*Corresponding author. E-mail: hamidoucom@yahoo.fr. Tel: +226 25 37 31 31.

cultivated cereals, both in area, production and consumption (Somda, 2016). It also makes a significant contribution to the country's economies. The area devoted to maize cultivation in the country increased from 790,321 ha in 2010 to 1,019,181 ha in 2018 (GDSSS/MAHAD, 2020). National maize production reached 1,700,127 T in 2018 against 1,133,480 T in 2010 (GDSSS/MAHAD, 2020). Maize is grown widely around the tropical world owing to its good adaptation to climate and its popularity. Besides being distributed widely, maize can be used for many purposes, such as animal feed, industrial uses, and is even the staple food in many developing countries. It also makes a large contribution to the economies of developed and developing countries. Nevertheless, the aflatoxin phenomenon is undermining the sector in Africa (Chauhan et al., 2016). Thus, maize is food crop that is easily contaminated with mycotoxins such as aflatoxins that are cancer-causing, immuno-suppressive mycotoxins (Makhlouf, 2019). According to Bambara (2021), 40% of maize production is affected by aflatoxins in developing countries. Contamination of crops with aflatoxin is a global food safety issue (Compaoré et al., 2021). The most important members of AFs are AFB1, AFB2, AFG1 and AFG2. The International Agency for Research on Cancer (IARC) has classified AFB1, AFB2, AFG1 and AFG2 as Group I human carcinogens (IARC, 2002). Among the many known toxins in the world, aflatoxins are highly toxic and carcinogenic compounds that can cause diseases in livestock and humans (Ouattara-Sourabié, 2018). AFs are a group of mycotoxins produced as secondary metabolites by species in *Aspergillus* section *Flavi*. The species most notorious for aflatoxin production are *Aspergillus flavus* (produces only aflatoxins B) and *Aspergillus parasiticus* (produces both B and G aflatoxins) (Kachapulula et al., 2017).

Previous studies in Africa have found that the occurrence of aflatoxins in food products is mainly influenced by favorable conditions such as high moisture content and temperature (Waré et al., 2017). In fact, in the Sahelian zone dry, post-harvest conservation is the only means of ensuring the link between the harvest occurring once in the year and consumption that is permanent and obligatory. The harvests, kept in general under inappropriate conditions, are attacked by insects, rodents and molds (Waongo et al., 2013). Also, in Burkina Faso the improper storage or preservation methods used such as maize bad drying would also lead to attacks by micro-organisms including molds of the genus *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* (Sanou, 2000). Aflatoxins contamination of maize has always remained a topic of debate in terms of international market as well as economic development of country which are part of trade market (Chauhan et al., 2016). In view of the huge economic losses and health problems caused by mycotoxins; a great deal of interest is currently accorded to them throughout the world. Thus to guarantee the health of consumers, each country is obliged to adopt specific legislation for the main mycotoxins in foods liable to harbor toxigenic molds (Waré et al., 2017). Several countries in the world have established or proposed regulatory limits for mycotoxins in foods. European Union countries edited regulations that have been revised periodically to limit their presence in the foods in Europe (EU Regulation 1881, 2006). In Africa certain countries also have regulations on mycotoxins and produce significant research, especially on aflatoxins and fumonisins that affect health (Ezekiel et al., 2014). Unfortunately, Burkina Faso, a country of West Africa has not set a mycotoxin regulation and uses those of *Codex alimentarius* which fixes the maximum levels of AFB1 and total aflatoxins respectively at 2 µg/Kg and 5 µg/Kg (EU Regulation 1881, 2006). Indeed, there are few studies and surveys about the contamination of mycotoxins in maize in Burkina Faso and this does not really reflect the problem in our country (Sanou, 2000). Our farmers therefore often find it difficult to export their products to countries whose mycotoxin regulations are in force. Indeed, because crops in tropical and subtropical regions are more susceptible to contamination due to favorable climatic and their inadequate storage conditions which facilitate the proliferation of molds and their secondary metabolites (Waré et al., 2017). Therefore, the aim of the proposed work is to determine the fungal load of maize samples from Ouagadougou City in Burkina Faso and to quantify the concentration of all kinds of aflatoxins using high performance liquid chromatography. This will confirm the results of the qualitative demonstration of aflatoxins production capacity of molds previously isolated from these maize samples, such as fluorescence emission under ultra violet light at 365 nm and those in "*A. flavus* and *parasiticus* agar (AFPA)".

MATERIALS AND METHODS

Physicochemical analyses

The maize samples were first subjected to physicochemical analyses such as humidity and pH using standards methods. The tests were repeated three times.

Sampling and fungi isolation from maize seeds

A total of ten (10) maize samples in commercially available were collected during the period from December to February 2021 at Ouagadougou markets. Fungi were isolated and purified on Potato Dextrose Agar (PDA) and subculture in "*Aspergillus flavus* and *parasiticus* agar (AFPA)" to identify *A. flavus* and *A. parasiticus* according to Pitt et al. (1983) and Cotty (1994) protocols. Systematic determination and the strains identification were made on Potato Dextrose Agar (PDA) at 25 and 37 °C depending on the methods used by Christensen (1981), Hocking (1982) and Cooney and Emerson (1964). Inoculation was done in three points equidistant.

Reference strains

For comparison of cultural and microscopic characters between strains isolated from maize seeds and those of reference strains, three references belonging to *Aspergillus* section *Flavi* were used. They were UBOCC-A-106031 (*A. flavus* aflatoxinogenic) of French origin, UBOCC-A-111042 (*A. parasiticus var. globosus* aflatoxinogenic) of Japanese origin and S_2 (*A. flavus* aflatoxinogenic) previously isolated from groundnuts and identified in Burkina Faso using molecular method by two (2) PCR based on 28S ribosomal sub unit (D1-D2 region) and the hyper variable ITS1-5.8S-ITS2 region, (Compaoré et al., 2016). This comparison was performed on Potato Dextrose Agar (PDA) medium at 30 °C for seven days.

Aflatoxins quantification

Sample extraction (AOAC, 2005)

Aflatoxins production is confirmed by HPLC using aflatoxins B and G Standard, Romer Biopure, and a blank consisting of the extraction solution. HPLC analysis was performed at the Toxicological Department of the National Public Health Laboratory (LNSP) in Ouagadougou. Ten (10) maize samples were subjected to this analysis.

The principle was used to extract aflatoxin from the samples using suitable organic solvents, to purify this aflatoxin on an immunoaffinity column and then to identify and quantify it. To do this, 25 ± 0.2 g of mix ground maize was weighed and added approximately 3 g of sodium chloride and placed in blender cup. 125 ml of extraction solution methanol-distilled water (70:30; v/v) was added to the sample and the whole was stirred for 20 min. The solution was then filtered through Whatman No.4 filter paper and 15 ml of the filtrate was transferred into a beaker and diluted with 30 ml of distilled water. The diluted homogenized sample solution was filtered through glass microfiber membrane.

Sample cleanup

The immunoaffinity columns were previously conditioned by passing 10 ml phosphate buffered saline (PBS) through the column by gravity and placed on the variant cuvette; the silica gel was allowed to flow. 15 ml of the diluted filtrate (1g sample equivalent) was taken and poured into the immunoaffinity column where it retained the desired molecules. The molecules were then washed with 10 ml of distilled water which was poured twice into the immunoaffinity column. Under gravity, the bound aflatoxins were eluted with 1 ml of pure methanol and air was pushed through the column to collect the last drops of eluate. 1 ml of distilled water was added to the eluate. The eluate was filtered through 0.45 µm Methanol-compatible membrane filter (PTFE or Nylon) and collected into a micro sample vial. Then this solution containing the aflatoxin molecules was sent to the HPLC for the detection and quantification of these aflatoxin molecules. Each experiment was conducted in triplicate and aflatoxins contents were determined according to their corresponding standard curves.

HPLC analyses

Spiked samples were prepared on relevant matrix for each batch of sample to obtain the % recovery. 0.5 ml of the working solution was added (100 µg/kg B1, G1 and 25 µg/kg B2, G2) per 25 g of matrix, mixed well and proceeded to sample extraction. The chromatographic system consisted of an automatic Agilent 1200, with Immunoaffinity column cleanup and post-column derivatisation manufactured by Shim-pack VP-ODS, 4.6 mm (ID) x 150 mm (L). The post-column derivatisation was achieved using a Kobra Cell to obtain electrochemically generated bromine (ISO 16050 CEN/TC-34, 2006); Romer Biopure). It is equipped with an auto-sampler (10 µl, injector vol), a Shim-pack VP-ODS column with a Reverse-Phase C18 (4.6 (ID) x 150 mm (L)) and a fluorescence detector. The detector was set at EX= 350 nm, EM = 450 nm. The mobile phase was isocratic and composed of methanol: water (45:55)-KBr-HNO_3 mixture with 450 ml of methanol, 119 mg of potassium bromide and 87.5 µl of 16 M nitric acid per liter of mobile phase. The flow rate was set at 1 ml/min.

The quality control standard (40 µg/kg calibration solution) was injected 3 times. The % CV of the peak area corresponding to the consecutive injections shall be within ± 10%. 10 µl of each of the Calibration Standards Solutions (2.5 µg/kg, 5 µg/kg, 10 µg/kg, 20 µg/kg, 40 µg/kg and 80 µg/kg) was injected into the HPLC system, followed by sample.

The concentration of Aflatoxins in the sample is calculated using the following formula:

$$\text{C spl } (\mu g/kg) = \frac{\text{A spl}}{\text{Gradient of calibration curve}} \times \frac{\text{Inj Vol std}}{\text{Inj Vol spl}} \times \text{F}$$

Where

$$\text{A spl}: \quad \text{Peak area of sample}$$

$$\text{Gradient of calibration curve}: \quad \frac{\text{Peak area of standard}}{\text{Concentration of standard}}$$

$$\text{F}: \quad \text{Dilution Factor per gram } (\times 200)$$

$$\text{F}: \frac{\text{Total sample extract (ml)} \times \text{Vol of final sample extract } (\mu l)}{\text{Volume for column (ml)} \times \text{Vol for injection into HPLC } (\mu l) \times \text{Weight of sample(g)}}$$

Statistical analysis (XLSTAT. 2016)

The differences in aflatoxins concentration in maize samples between the Ouagadougou zones Burkina Faso and those of physicochemical analyzes were compared by Analysis of variance (ANOVA) using XLSTAT-Pro 7.5.2 software. Interpretation of values was performed using Newman-Keuls test at probability level p = 5%. The results were expressed as mean ± SD and the measures were repeated three times (n=3).

RESULTS AND DISCUSSION

Physicochemical analyses of maize sample

Humidity rate analysis carried out on the ten (10) samples of maize seeds revealed that our samples are not very humid with an average value between 3.91 and 4.66% (Table 1).

These values are lower than the average moisture content of mold growth. In fact, the minimum humidity for certain molds to start growing is 10% (Compaoré, 2017). Our data could be explained by contamination of samples in the field or during storage. Mold strains are able to survive unfavorable conditions for their growth by producing large numbers of spores (Tabuc, 2007). Thanks to its great adaptability to environmental conditions,

Table 1. Humidity rate of maize samples

Sample	Humidity (%)
M1	4.10 ± 0.07^{cd}
M2	4.45 ± 0.07^{ab}
M3	4.07 ± 0.07^{cd}
M4	4.71 ± 0.07^{a}
M5	3.99 ± 0.07^{cd}
M6	4.22 ± 0.07^{bc}
M7	4.48 ± 0.07^{ab}
M8	3.91 ± 0.07^{d}
M9	4.66 ± 0.07^{a}
M10	4.26 ± 0.07^{bc}
Significance level	< 0.0001

For each column and for each maize sample, the means which are in common the same letter are not significantly different according to the Newman-keuls test at the 5% probability level.

Table 2. pH values of maize samples.

Sample	pH
M1	5.93 ± 0.04^{a}
M2	5.83 ± 0.04^{a}
M3	5.89 ± 0.04^{a}
M4	5.95 ± 0.04^{a}
M5	5.98 ± 0.04^{a}
M6	5.94 ± 0.04^{a}
M7	5.96 ± 0.04^{a}
M8	5.99 ± 0.04^{a}
M9	5.93 ± 0.04^{a}
M10	5.96 ± 0.04^{a}
Signifiance level	< 0.0001

For each column and for each maize sample, the means which are in common the same letter are not significantly different according to the Newman-keuls test at the 5% probability level.

A. flavus can grow both on crops in the field, during harvest as well as later, during storage (Makhlouf, 2019). For the different samples, statistical analyses showed significant differences between the different humidity values. From this analysis it appears that the humidity is very variable from one sample to another. The pH results of the various maize samples analyzed indicate that all of the samples are slightly acidic, with pH values ranging from 5.83 to 5.99 (Table 2).

The pH values are very favorable to the growth of molds because according to Gauthier (2016), molds can grow in a pH range from 3 to 8, with optimal growth being rather between 5 and 6. The production of mycotoxins takes place for pH close to optimal growth pH (Makhlouf, 2019). Due to their acidity, many foods are much more prone to fungal than bacterial spoilage (Tabuc, 2007). Statistical analyses showed no significant difference between the different pH values.

Fungi isolation from maize seeds

In our study, ten (10) maize samples were used as a matrix for the collection of strains of *Aspergillus* section *Flavi*. From the consortium of fungi grown on maize, twenty-three (23) isolates were collected. Macroscopic observation of the Petri dishes made it possible to retain only the isolates forming colonies of yellowish and greenish color and have powder aspect. In optic microscopy, we were interested in those with non-septate and hyaline conidiophores. Eight (08) local *Aspergillus* section *Flavi* strains were therefore isolated from the

Table 3. Number of *Aspergillus* section *Flavi* isolates from collected maize samples.

Sample	Number of *Aspergillus* section *Flavi* isolates	Isolates code
M1	1	A_2
M2	1	A_5
M3	0	-
M4	0	-
M5	3	A_4, A_6 et A_8
M6	0	-
M7	0	-
M8	0	-
M9	1	A_3
M10	2	A_1 and A_7
Total	8	-

consortium of fungi grown on maize seeds (Table 3).

Aflatoxins level in maize samples

Results of the quantitative aflatoxin analysis are reported in Table 4. 70% of the samples were found to be contaminated with total aflatoxins. These were M1, M2, M4, M5, M6, M9 and M10. The total aflatoxin contents in the maize samples ranged from 0.93 to 70.78 µg/kg. M1 and M10 had high concentrations (58.86 and 70.78 µg/kg respectively) and M4 and M5 had low concentrations with respective values of 1.67 and 0.93 µg/kg. In all the contaminated samples, aflatoxin B1 was present and the most concentrated with however the absence of aflatoxin G2 as it can be seen from the chromatograms in Figure 1. Out of seven (7) samples, four (04) were contaminated with aflatoxin B1 (AFB1) and aflatoxin G1 (AFG1), two (02) with AFB1 and aflatoxin B2 (AFB2) and one (01) with AFB1 only.

The contamination of our samples with aflatoxins could be explained by the presence of toxigenic strains *Aspergillus* section *Flavi*. Indeed, the latter are the fungal contaminants most frequently encountered in cereals and which secrete aflatoxins there when environmental conditions (temperature, humidity, pH, etc.) are met, as is the case with the pH of our maize samples which are slightly acidic with a pH around 5.83 to 5.99 (Table 2). Maize is considered among the fragile crops at high risk of contamination by toxigenic molds unlike other cereals, particularly barley and wheat (Brahmi and Zahi, 2016).

According to the *Codex alimentarius*, the maximum levels of AFB1 and total aflatoxins (B1, B2, G1 and G2) are respectively 2 and 5 µg/Kg (EU Regulation 1881, 2006). Our results revealed that the contamination rate of the different samples varied from sample to sample. Four (04) samples had total aflatoxin levels between 13 and 71 µg/Kg and therefore well above the *Codex alimentarius* standard. Only the M2, M3, M4, M5, M7 and M8 samples complied with the standard. The high concentration of aflatoxins in our samples could be explained by climatic conditions in Africa, poor agricultural practices and poor crop storage conditions. According to Zinedine (2004), crops grown in hot, humid climates and exposed to toxigenic molds provide optimal conditions for mold growth and can be contaminated with mycotoxins. According to Makhlouf (2019), the infestation of maize, by *Aspergillus flavus* (producer of AFB) before harvest, is often linked to the aggression of the plant by insects and rodents in the field. Cereals can be contaminated either by the spores that are initially found in the cereals or later during storage mainly if it is bad (Waré et al., 2017). Indeed, overripe crops and grain damaged during threshing are postharvest conditions that promote fungal growth in crops (Bhat et al., 2015). In addition, crops, usually stored in inadequate conditions, are attacked by insects, rodents and molds (Waongo et al., 2013). In Burkina Faso, the ears of maize are kept either in bundles or sheaves and hung from branches or above the hearths, or in straw or mud granaries. These storage systems do not protect the food from direct attack by insects and many other pests such as birds and rodents which carry microorganisms (Somda, 2016).

Aflatoxin B1 (AFB1) was predominantly present in all seven (7) contaminated samples with concentrations ranging from 0.54 ± 0.08 to 63.24 ± 0.07 µg/Kg. The maximum values of AFB1 that we obtained are much lower than the results obtained by Vargas et al. (2001) in Brazil, where the level of contamination of maize by aflatoxins reached 129 µg/kg. Nevertheless, our results are superior to those obtained in Ethiopia by Chauhan et al. (2016) which was 53 µg/Kg.

Aflatoxins are produced mainly by *Aspergillus* section *Flavi* species, mainly *A. flavus* and *A. parasiticus*. These two species are both producers of type B aflatoxins, but *A. parasiticus* also produces type G aflatoxins (Vargas et al., 2001). Out of seven (07) samples, three (03) were only contaminated with AFB. *Aspergillus spp*. isolated from these

Table 4. Aflatoxin level in maize samples.

Aflatoxin (µg/kg)	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	*Codex* limit
B1	55.70± 0.21^{b}	1.81± 0.04^{e}	nd	1.67± 0.12^{e}	0.54± 0.08^{f}	9.58± 0.05^{c}	nd	nd	6.73± 0.06^{d}	63.24± 0.07^{a}	2
B2	3.16± 0.07^{b}	nd	nd	nd	nd	nd	nd	nd	nd	7.54± 0.11^{a}	-
G1	nd	0.62± 0.04^{c}	nd	nd	0.39± 0.01^{d}	5.73± 0.06^{b}	nd	nd	6.39± 0.03^{a}	nd	-
G2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-
Significance level					< 0.0001						
AFs	58.86	2.43	nd	1.67	0.93	15.31	nd	nd	13.12	70.78	5

nd= not detected. For each line and for each maize sample, the means which are in common the same letter are not significantly different according to the Newman-keuls test at the 5% probability level.

maize samples would therefore belong either to *A. flavus* or to *A. parasiticus*. Four (04) on the other hand were contaminated with AFB and AFG, indicating the presence in these samples of strains of mold belonging to the species *A. parasiticus*. Samples M1, M4 and M10 would therefore be contaminated by *Aspergillus flavus* strains while M2, M5, M6 and M9 would be contaminated by *A. parasiticus*.

The presence of the different types of aflatoxins in our maize samples would confirm the morphological characterization of our different isolates carried out previously. Thus, the A_1, A_2 and A_7 isolates from sample M10 would be strains of *A. flavus*. A_5 isolated from M2; A_4, A_6, A_8 isolated from M5 and A_3 isolated from M9, would all be strains of *A. parasiticus*. Indeed, in a previous study by Compaoré et al. (2021), we determined by qualitative methods the ability of A_2 and A_3 isolates to produce aflatoxins through blue fluorescence emission when cultured on Coconut Agar Medium (CAM). Both isolates were also subcultured on "*Aspergillus flavus* and *parasiticus* Agar" (AFPA) medium a four days incubation at 30 °C to study their ability to produce aspergillic acid. The results showed that both isolates are capable of producing aflatoxin. Reference strains UBOCC-A-111042 and S_2 which are aflatoxinogenic were also tested in order to compare the obtained results with those of our two maize isolates. The results showed that both isolates are capable of producing aflatoxin. In the present study, the quantitative analysis of the different types of aflatoxins came to confirm the morphological identification carried out previously.

Aflatoxins production and their determination by fluorescence HPLC

Results of the qualitative aflatoxin analysis are reported in Figure 2. Aflatoxin production abilities tested previously by fluorescence under UV light of strains by cultivating them on Coconut Agar Medium (CAM) and AFPA medium were in concordance with those obtained by HPLC determination (Table 5). We have found that both A_2 and A_3 isolates showing fluorescence under UV light produced aflatoxins in CAM.

Conclusion

The present study included ten (10) samples of maize seeds grown in Burkina Faso. The results of the aflatoxin analysis showed that the majority of these are of unsatisfactory sanitary quality and have a fairly high average mold load predominantly dominated by genus *Aspergillus*. A total of eight (8) fungal strains belonging to *Aspergillus* section *Flavi* were isolated and characterized. The quantitative aflatoxin analysis method such as HPLC performed in the present study attests to the results of aflatoxin-producing ability previously performed and confirms that isolates A_2 and A_3 belong respectively to *A. flavus* and *A. parasiticus*. Nevertheless, this identification should be confirmed by Biology molecular methods. In view of the high levels of aflatoxins in cereals in Burkina Faso and the danger represented by the ingestion of contaminated food. The players in the maize sector must observe the Good Practice pre and post-harvest as well as decontamination methods such as biopreservation by lactic acid bacteria and *Bacillus* in order to preserve the health of consumers.

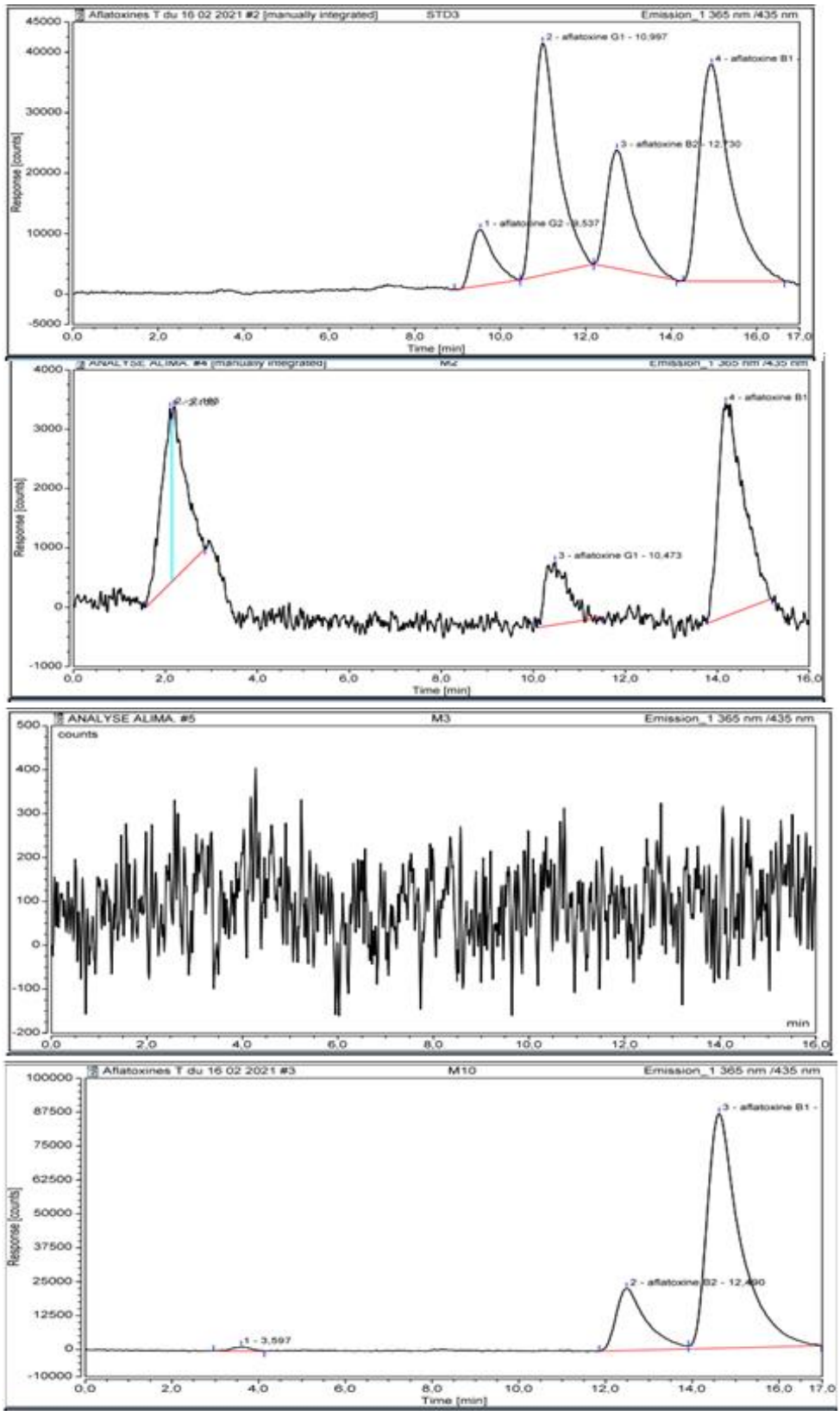

Figure 1. HPLC chromatograms of aflatoxins standards (AFB1, AFB2, AFG1 and AFG2); AFB1 and AFG1 in sample M2, lake of Aflatoxin in sample M3, AFB1 and AFB2 in sample M10.

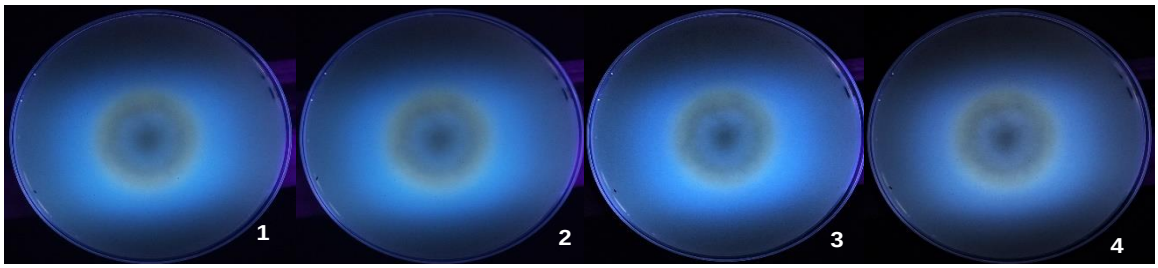

Figure 2. Showed the detection of aflatoxigenic strains by Coconut Agar Medium under UV light at 365 nm on the fourth (4^{th}) day of incubation at 30 °C. (1) control of aflatoxigenic strain UBOCC-A-111042, (2) S_2 aflatoxigenic, (3) A_2 isolate and (4) A_3 isolate showing a blue-green fluorescent ring around the colony.

Table 5. Comparison of aflatoxigenic strains responses for three methods of identification (response on AFPA, fluorescence under UV light on CAM and HPLC response of maize samples source of A_2 and A_3 *Aspergillus spp.* isolates).

Isolate	Response on AFPA	Fluorescence under UV light on CAM	Aflatoxins production in maize sample
A. flavus UBOCC-A-106031 France	+	+	na
A. parasiticus var. globosus UBOCC-A-111042 Japan	+	+	na
A_2	+	+	+
A_3	+	+	+
A_5	+	+	+
A_4, A_6 and A_8	+	+	+
A_1 and A_7	+	+	+

(-) negative; (+) positive; (na) not analyzed

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Detection of *Legionella pneumophila* as the cause of atypical pneumonia in the water sources of the holy places of Makkah

Sami S. Ashgar and Hamdi M. Al-Said*

Department of Microbiology, College of Medicine, Umm Al-Qura University, Saudi Arabia.

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Legionella pneumophila **is an important pathogen and is involved in more than 95% of cases of severe atypical pneumonia. The current study focused on the ablution water in the grand mosque in Makkah as well as water tanks in hotels surrounding the holy mosque. A total of 100 water samples were collected from ablution water in the Haram and the hotels in the central area around the grand mosque and sent to the research laboratory of microbiology, Faculty of Medicine, University of Umm Al Qura. Samples were filtrated and inoculated onto buffer charcoal-yeast extract agar base and incubated at 37°C in a CO_2 incubator. The plates were examined after seven days of incubation. Isolated organisms were confirmed by using the "Microgen Legionella" (latex agglutination test). Out of the 100 water samples tested, 11 samples were positive for *L. pneumophila*. All positive water samples were from hotels water tanks. This indicates that this type of bacteria existing in the water sources. So requires further research to cover all sources of water to avoid an outbreak of this infection among the pilgrims. To avoid the possibility of this, constant maintenance of hotel water tanks regularly and the use of chlorine in specific proportions according to recommended specifications can help reduce the spread of these microbial infections.**

Key words: Holy Mosque, *Legionella pneumophila*, water tanks, environmental.

INTRODUCTION

Legionnaires disease is considered a major form of travel-associated pneumonia. *Legionella pneumophila* is the main causal agent of this disease (Heuner and Steinert, 2003; Miyashita et al., 2020). This infection is transmitted between people through the air polluted with water droplets loaded with this microbe which may be present in water tanks and warm water systems. Transition occurs during the shower through inhalation of this water, which may be contaminated with this type of pathogenic bacteria. This disease, often in the summer season where the temperature rises which is considered an important environmental factor for the growth and multiplication of this type of bacteria. Symptoms start as high temperature, headache, muscle pain, and coughing. The people with major risk factors for community-acquired Legionnaires are immunocompromised people, chronic diseases, cigarette smokers, and the elderly; they are the most vulnerable to this bacterial infection (Smith

*Corresponding author. E-mail: hamdimustafa1@gmail.com or hmibrahim@uqu.edu.sa. Tel: 966551941735.

et al., 2019). Therefore, *Legionella* is responsible for an uncommon type of human respiratory disease called Legionnaires' disease that could be fatal if not treated (Leoni et al., 2005). This disease was first identified in the seventies of the last century in America and was initially believed to be a flu-like disease when a group of people who attended a conference in America died in 1976 (Terranova et al., 1978; Han et al., 2019). According to some studies issued by the center for disease control and prevention (CDC), more than 20,000 patients every year, in addition to nearly four thousand deaths (Chamberlain et al., 2017; Borges et al., 2016). Interestingly, a published study of formerly collected data, investigated both water samples from different sources include water coolers, sprinkles, in addition to clinical samples collected from patients who were admitted to five specialized hospitals in Makkah during Haj season (Cristovam et al., 2017; Pierre et al., 2017; Ohno et al., 2003; Khodr et al., 2016). Another study revealed that most of the causes of acute pneumonia during the Hajj season are *Haemophilus influenzae* and *Streptococcus pneumoniae*. However, *Legionella* was not mentioned, and this may be due to the lack of tools available for testing for *Legionella*. Another study to identify the etiological agents of severe community-acquired pneumonia during Hajj declared that most of the bacterial infected samples were due to *H. influenzae* and *S. pneumonia*, but no Legionella test was performed (Azhar et al., 2010; Correia et al., 2016; van Heijnsbergen et al., 2015). For a clear understanding of the factors causing the colonization of water pipes and domestic water systems by *L. pneomophilia* in Saudi Arabia, this study was designed to detect the presence of *L. pneumophila* in the water sources in the hotels of the accommodation of pilgrims surrounding the Grand Mosque in Makkah city and to obtain data on *L. pneumophila* to help in controlling this type of infection.

MATERIALS AND METHODS

Collection of samples

The number of samples collected in this study was 100 water samples, 90 of them from the hotel tanks surrounding the campus and 10 samples from the ablution water in the Haram during three months in the middle of the year 2014. The water samples were collected in 1 L sterile bottles containing 1 ml of 0.1 N sodium thiosulfate to neutralize the chlorine in the water samples so that the bacteria to be isolated will not be affected. The source of water for the ablution water in the Grand Mosque in Makkah city is chlorinated municipal water, while the water of hotel tanks is a mixture of chlorinated municipal water and non-chlorinated well water in the city of Makkah. Water is transferred from wells to hotel tanks by vehicles equipped for this. The sample size in the current study was designed according to the international organization for standardization (ISO) 11731. This study was conducted during the year 2014.

Transportation of the specimens

After collecting the samples, they were sent to the microbiology research center in Medicine College, Umm Al-Qura University.

Water sample preparation and analysis

The samples were filtered by using a filtration vacuum pump device. The polycarbonate membrane filter 0.2 um was used for filtering the water samples. The filter was removed and placed in 10 ml of sterile water inside a sterile tube with a capacity of 50 ml. Then the tube was placed in a centrifuge at 3000 rpm for 1 min. The supernatant was removed and the sedimentation was mixed using the vortex to obtain homogenous suspension. Then 0.1 ml of the final concentration was taken and inoculated on a selective medium Buffered Charcoal Yeast Extract (BCYE). The agar plates were incubated under CO_2 conditions at 37°C.

Identification and confirmation

After seven days incubation, the plates were examined by traditional methods, then the suspected bacterial colonies expected to be *Legionella* were re-cultured on blood agar plates and BCYE agar plates media and were re-incubated for three days. Blood agar plates that had no growth indicating that this is *Legionella* spp. Confirmation tests were carried out using, latex agglutination technique "Microgen Legionella" (latex agglutination test).

To differentiate between *Legionella* spp., a serotyping test was performed using the Legionella Latex test (Cat. No. DR0800M oxoid, UK). This test was done by the direct method. All reagents were placed at room temperature before the assay started. The tube was numbered according to the number of samples and then 0.4 mL of 0.85% saline was added to each test tube. A number of growing colonies of each sample were transferred to 0.4 mL of 0.85% saline in the test tube. This was mixed to obtain a homogeneous mixture. One drop of the three reagents and control reagent was distributed in four circles. One drop of the bacterial suspension was added to each of the four circles, then mixed for exactly 1 min. The agglutination in any circle showed *L. pnemophilia* within 1 min. Whereas, other circles that did not show agglutination within 1 min confirmed that it was negative for *L. pnemophilia.*

RESULTS AND DISCUSSION

The time period to collect water samples for the current study was three months, according to the action plan that was designed to conduct this study. Hundreds of water samples were collected, 10 of them taken from the ablution water in Grand Mosque and 90 water samples were collected from water tanks in hotels surrounding the Holy Mosque in Makkah city for detection of *L. pneumophila*. 11 (11%) water samples out of 100 water samples showed positive results of *Legionella* spp. All positive samples were from water samples collected from hotels, while water samples collected from ablution water in Grand Mosque showed negative results. The permits granted to approve the collection of water samples in this study were one-time, so we were unable to repeat the water samples.

These isolates were subjected to confirmatory tests to detection the pathogenic strains that cause atypical pneumonia, and this was done by test, "Microgen Legionella" (latex agglutination test) that confirmed that

Table 1. Distribution of water samples and prevalence of *Legionella pneumophila* according to specimen collection locations.

Specimen collection locations	No. of specimens	No. of positive specimens	Positive Specimens (%)	No. of negative specimens	Negative specimens (%)	No. of *Legionella pneumophila*
Ablution water in Grand Mosque in Makkah	10	0	0	10	100	0
Water tanks in hotels	90	11	12.2	79	87.8	11
Total	100	11	11	89	89	11

11 isolates were related to *L. pneumophila* shown in Table 1. Positive and negative controls were also used during the cultivation of these samples on BCYE and blood agar media to compare them with the water samples results, using a sterile water sample containing a reference strain, the American Type Culture Collection (ATCC) of *Legionella pneumophila* and another sample for sterile water only as a negative control.

Published studies regarding pilgrim's health particularly respiratory tract infections during Hajj showed that pulmonary infections during Hajj are of great burden in Saudi health authorities. The study also showed that 160 admitted patients in Arafat and Mona hospitals during Hajj were diagnosed with respiratory tract infections and were the highest health attributed problem in hospitalized pilgrims during mild weather (Memish et al., 2014; Al-Ghamdi et al., 2003; Madani et al., 2006). Another cross-sectional study in the same year has confirmed that pneumonia was recorded as the highest cause of hospitalization in 808 patients admitted to seven hospitals in Arafat and Mona during the Hajj festival (Khan, 2006). Indeed, these findings were also confirmed by a review article published which studied 689 hospitalized cases belonging to 49 countries in a tertiary hospital in Makah for 5 weeks during Hajj and the findings showed that pneumonia was identified as a major cause of illness and the leading cause of death in 28 patients diagnosed with pneumonia (Alzeer, 2009). Another published review article of respiratory tract infection during Hajj identified these infections as the most common cause of hospital admissions (Mandourah et al., 2012). Interestingly, a published study of formerly collected data, showed both water samples from different sources include water coolers, sprinkles, and storage tanks in Makah areas such as Arafat, Mona, Muzdaliphah, and nearby the Holy Mosque "Haram", and clinical specimens from pneumonia diagnosed patients admitted to five tertiary hospitals in Makkah and the findings showed that pneumonia was the major cause of sickness in pilgrims and recommended with more studies on water sources (Sreenath et al., 2020; Al-Tawfiq et al., 2013, 2014; Spiegelman et al., 2020; Zahran et al., 2018). Absence of documented data on the isolation rates of this type of bacteria cause this disease in Saudi Arabia and in view of the results of previous studies during the Hajj season; the reason for the emergence of this type of bacteria may be due to the fact that these hotels use water from wells which can be the main source of these pathogenic bacterial strains. This requires more research to cover all water sources. As well as the work for the creation of a database on the prevalence rates of this type of bacteria that causes Legionnaires disease and finding mechanisms to resist and control them to avoid the events of outbreaks of this type of disease during the Hajj and Umrah seasons.

Conclusion

Based on the results of the current study, the water samples that showed positive results for *L. pneumophila* were collected from hotel water tanks, while no positive samples were recorded for *L. pneumophila* from ablution water sources in the Holy Haram. It is likely that the reason for the presence of these bacterial strains in the water tanks of hotels is due to the use of well water that is not treated with chlorine often during the congestion in the Hajj and Umrah seasons. To avoid the possibility of this, constant maintenance of hotel water tanks regularly and the use of chlorine in specific proportions according to recommended specifications can help reduce the spread of these microbial infections.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Phenotypic and genotypic characterization of antibiotic resistant gram negative bacteria isolated in Tabuk City, Saudi Arabia

Tarig M. S. Alnour[1,2,3], Elmutuz H. Elssaig[1,3], Faisel M. Abuduhier[1,2], Khalid A. S. Alfifi[4], Mohammad S. Abusuliman[5], Tawfiq Albalawi[6] and Eltayib H. Ahmed-Abakur[1,2,3*]

[1]Department of Medical Laboratory Technologym, Faculty of Applied Medical Science, University of Tabuk, P. O. Box 741, Tabuk -Postal code 71411, Saudi Arabia.
[2]Prince Fahad Research Chair, Department of Medical Laboratory Technology (FAMS), University of Tabuk, P. O. Box 741, Tabuk -Postal code 71411, Saudi Arabia.
[3]Department of Microbiology and Immunology, Faculty of Medical Laboratory Science, Alzaiem Alazhari University, Khartoum north, Sudan, Postal code 11111, Sudan.
[4]Medical laboratory specialist, King Fahad Specialist Hospital, Tabuk, Saudi Arabia.
[5]Laboratory Medicine (Microbiology), King Fahad Specialist Hospital, Tabuk, Saudi Arabia.
[6]Department of laboratory and Blood Bank, King Fahad Specialist Hospital, Tabuk, Saudi Arabia.

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Antimicrobial surveillance and identifying the genetic basis of antimicrobial resistance provide important information to optimize patient care. The present study is an analytical cross sectional study aimed to determine the prevalence of multidrug resistant (MDR), extensively drug resistant (XDR), pan drug resistant (PDR) and extended-spectrum β-lactamases genes among Gram-negative bacteria isolated in Saudi Arabia. A total number of 386 non-duplicate Gram-negative isolates were collected. Identification and susceptibility testing were done using automation system (BD Phoenix™). The extracted DNAs were subjected to multiplex polymerase chain reaction (PCR). The results showed that only 15 (3.9%) of isolates were fully susceptible, the overall prevalence of XDR, MDR, PDR was 129 (33.4%), 113 (29.3%) and 48(12.4%) respectively. High resistant rate was observed against the antibiotic agents of cephalosporins class 79.3% followed by the agents of penicillins class 69.4%. The most dominant resistant gene was bla SHV which was detected in 106/386 (27.5%) isolates, followed by bla CTX-M 90/386 (23.3%). Bla CTX-M showed significant relation with all used antibiotic except ampicillin/clavulanic acid, aztreonam, cefoxtin, and meropene. The isolates which showed frequent resistant genes were: *Klebsiella pneumoniae 90/124 (72.6%), A. baumanni* 37/67 (55.2%)*, and P. mirabilis* 24/44 (54.5%). These findings underscore the need for optimization of current therapies and prevention of the spread of these organisms.

Key word: Multidrug resistant (MDR), extensively drug resistant (XDR), pan drug resistant (PDR), extended spectrum beta lactamase (ESBL), bla SHV, bla TEM, bla CTX-M.

INTRODUCTION

Antimicrobial resistance is described as a condition in which the pathogens escape from the stress of the antibiotic exposure (Alam et al., 2017). The increasing incidence of antimicrobial resistance is a key concern

globally and considered main obstacle in the treatment of patients suffering from bacterial infections (Patil et al., 2019). It has been estimated that about 1.3 to 2 fold rise in mortality is caused by antimicrobial resistant bacteria compared to susceptible infections (Alam et al., 2017). A dramatic evolution has occurred in the significance of infections caused by Gram-negative bacteria (GNB) and associated with considerable mortality (Alam et al., 2017; Patil et al., 2019; Paterson, 2008). The efficiency of the current prophylactic and empiric antibiotic treatment is compromised by the emergence of pan drug resistant (PDR), extensively drug resistant (XDR) and multidrug resistant (MDR) Gram-negative bacteria (GNB) (Patil et al., 2019; Paterson, 2008). The ability to escape from the antimicrobial effects may have contributed to the nature of these organisms, which are heterogeneous, complex group of plasmids-borne and rapidly evolving enzymes which are capable of hydrolyzing cephalosporins, penicillins, aztreonam and monobactams (Fernando et al., 2017; Provenzani et al., 2020).

The American society of infectious diseases identified six top priority dangerous pathogens producing extended spectrum β-lactamases (ESBLs). Three of these six pathogens are antibiotic resistant Gram-negative bacteria: *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and Enterobacteriaceae (Lee et al., 2007). ESBLs have been classified into three major groups: bla SHV, bla CTX-M and bla TEM (Patil et al., 2019). Ting et al. (2013) stated that bla TEM, bla SHV and bla CTX-M genes are super-resistant extended spectrum β-lactamases. In 2017, the WHO published list of global priority pathogens, a catalog of twelve species of bacteria grouped according to their antibiotic resistance under three priority tiers: critical, high, and medium. The critical group involved three pathogens that were Gram-negative bacilli: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* (WHO, 2017). The WHO also notified that the level of resistance to antimicrobial drugs used to treat common infections is reaching a crisis point.

If world administrations do not control infections in order to slow down the growth of drug resistance, entire populations could be wiped out by superbugs (Perez et al., 2014; Ibrahim et al., 2010). There are few novel antibiotic classes targeting gram-negative bacterial infections in the pipeline (Provenzani et al., 2020). However the availability of regional information on the resistance rate is fundamental to implementing efficient treatment protocols against infectious pathogens and may help to prevent infections with multidrug resistance pathogens at the local level (Moolchandani et al., 2017; Ibrahim, 2018). Therefore, the present study is aimed to determine pattern of antimicrobial resistance and to detect ESBL genes among Gram-negative bacteria isolated in Tabuk city, Saudi Arabia.

MATERIALS AND METHODS

The present study is an analytical cross sectional study, conducted in King Fahad Specialist Hospital and prince Fahad Bin Sultan Research Chair (University of Tabuk), Saudi Arabia. A total number of 386 non-duplicate Gram-negative isolates were collected in order to determine the prevalence of MDR, XDR, and PDR and to detect extended-spectrum β-lactamases genes bla SHV, bla CTX-M and bla TEM.

Identification and susceptibility test

Depending on the origin of the samples, each sample was cultured on suitable medium/ media from: MacConkey agar, CLED agar blood agar, and Chocolate agar or Brain Heart infusion broth. Then they were incubated aerobically at 37°C for 24 to 48 h except for the blood culture, which was incubated for 5 to 7 days in broth medium. Growths of corresponding organisms were further sub-cultured for purification purpose. The significant growth was identified to the species level. Identification and susceptibility testing were done using automation system (BD Phoenix™). Identified strains were tested in vitro against several antimicrobial classes including carboxypenicillin (Ticarcillin/Clavulanic acid), Penicillinase resistant penicillin (Ampicillin/Sulbactam and Piperacillin/Tazobactam), Cephalosporins (Ceftazidime and Cefepime), Aztreonam, Carbapenems (Ertapenem, Imipenem and Meropenem), Aminoglycoside (Amikacin, Gentamicin and Tobramycin), Fluoroquinolones (Ciprofloxacin and levofloxacin), Minocycline (Tetracyclin), Glycylcycline (Tigecycline), polymyxin E (Colistin), and sulpha drugs (Trimethoprim/Sulfamethoxazole). Antimicrobial selection for testing depends on types of isolates and site of samples which were done automatically by the program. Based on susceptibility test of the above mentioned antibiotic classes, the isolates were characterized as MDR, XDR and PDR.

Moreover thirteen agents of five antimicrobial classes which represented the most commonly used antibiotics, carboxypenicillin, penicillinase resistant penicillin, cephalosporins, aztreonam and carbapenems, were subjected for further study in order to determine the relation between these antibiotics and ESBL genes. These agents include: ampicillin, ampicillin/clavulanic acid, ticarcillin/clavulanic acid, aztreonam, piperacillin/tazobactam, cefalotin, cefoxitin, ceftazidime, ceftrixone, cefepime, imipenem, meropenem and ertapenem. Quality control and maintenance were achieved according to the manufacturer's guidelines.

The BD Phoenix™ automated identification and susceptibility testing system empowers workflow efficiency, using automated nephelometry, which results in a standardized isolate inoculum and a reduction in potential technologist error along with accurate, reliable and rapid detection of known and emerging antimicrobial resistance (Carroll et al., 2006).

Detection of antimicrobial resistance genes

DNA was extracted from whole 386 GNB isolates, using boiling technique. Few colonies from each isolate were mixed with

*Corresponding author E-mail: eosman@ut.edu.sa. Tel: +966534609127.

Table 1. The primers sequences of ESBLs genes and corresponding size.

Gene	Primer sequences	Size
blaTEM	F-5 -TCGTGTCGCCCTTATTCCCTTTTT-3 R-5-GCGGTTAGCTCCTCC GGTCCTC-3	426
blaSHV	F-5-GTGGATGC CGGTGACGAACAGC-3 R-5 -TGGCGCAAAAA GGCAGTCAATCCT-3	212
blaCTX-M	F-5'TTTGCGATGTGCAGTACCAGTAA3' R-5-CGATATCGTTGGTGGTGCCATA3'	619

molecular biology-grade water (Eppendorf, Hamburg, Germany), the mixture were centrifuged at 15,000 × *g* for 5 min. The supernatant was discharged and the pellet was re-suspended in molecular biology grade water (Eppendorf, Hamburg, Germany) and subjected to boiling at 100°C in a water bath for 20 min, then cooled and centrifuged at 15,000 × *g* for 60s before it was stored at −20°C.

Multiplex polymerase chain reaction (PCR) was done to determine the presence of three ESBLs genes encoding bla TEM, bla SHV, bla CTX-M. Each extracted DNA was tested against the three sets of specific primers in a single test (Table 1). Amplification was performed in a final volume of 25μL containing 5μL of template DNA, 0.5 μL Taq polymerase, 1.0 μL of each primers and 0.2 μL dNTP mixture (10 mM), and finally the volume was completed to 25 μL by molecular biology-grade water to reach volume of 25 μL. The PCR was run according to the following protocol: essential denaturation at 95◦C for 5 min followed by 40 cycle of denaturation at 95°C for 30s, annealing at 60°C for 30 sec and extension at 72°C for 1 min, the final step was extension at 72°C for 5 min. PCR product was run on 2% ethidium bromide agarose gel electrophoresis, and examined with gel imaging system, bands pattern was observed and interpreted according to their size (Table 1).

Analysis

The proportion of resistant for each antibiotic was calculated as the sum of resistant antibiotic relative to the sum of susceptible and resistant. The proportion of resistant class of antimicrobial represent the mean of resistance of all antimicrobial agents belong to that class. Chi-square tests were performed to determine the relation between ESBLs genes and antibiotic resistant using SPSS version 22. *P* value <0.05 was considered significant. The isolates which showed susceptibility to all groups of antimicrobial agents were classified as susceptible, while those that showed resistant to one group were classified as mono-resistant. The isolates resistant to one drug in two groups were classified as resistant to two antimicrobial groups. Multidrug resistant (MDR) denote when the isolates shown resistance to 3 or more antimicrobial group but susceptible to 2 or more. The US Centers for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC) have defined bacteria as pan drug resistant (PDR) when they are non-susceptible to all agents in all antimicrobial categories and as extensively drug-resistant (XDR) when they are non-susceptible to at least one agent in all, but two or fewer antimicrobial categories (Mohapatra et al. 2018). The ethical clearance for this study (UT-86-10-2019) was obtained from the research ethics committee, University of Tabuk (Saudi Arabia).

RESULTS

Table 2 shows the frequency of isolates along with patterns of occurrence of resistant ESBLs genes. The results revealed that the most common isolates were *Klebsiella pneumoniae* 124(32.1%), followed by *A. baumanni* 67(17.4%), *E. coli* 51(13.2%), *P. aeruginosa* 50(13.0%) and *P. mirabilis* 44(11.2%). The isolates showed high resistant rate against the antimicrobial agents of cephalosporins group, 79.3%, followed by the agents of penicillins, 69.4%; while against the agents of carbapenems they exhibited 32.5% resistance rate. In term of individual antimicrobial agent, the isolated Gram-negative bacteria revealed a high resistance rate against ampicillin 262(93.2%), followed by aztreonam 87(90.6%) and cefalotin 157(90.2%). Only 15(3.9%) of isolates were fully susceptible to all used antimicrobials. The overall prevalence of MDR, XDR, PDR was 113 (29.3%), 129 (33.4%) and 48(12.4%) respectively (Table 3).

Screening for resistance genes showed that most of Gram-negative isolates harbored with resistance genes 198/386(51.3%), while isolates free from resistant genes were 188 (48.7%) (Table 2). Bla SHV was the most dominant gene, which was detected in 106/386 (27.5%) isolates; followed by bla CTX-M and bla TEM, which was detected in 90/386 (23.3%) and 78/386 (20.2%) isolates, respectively (Figure 1). Single resistant gene was detected in 137/386 (35.5%) isolates, coexistence of two genes were detected in 41/386 (10.6%) isolates, while triple genes were present in 20 (5.2%) isolates. Bla CTX-M showed significant relation with all used antibiotic except ampicillin/clavulanic acid, aztreonam and cefoxtin, while bla SHV exhibited significant statistic relationship with Piperacillin/ Tazobactam, Cefalotin, ceftazidime, cefepime, imipenem and meropenem. Bla TEM displayed significant relation only to ampicillin/clavulanic acid, ceftazidime, cefepime and imipenem. Ceftazidime and cefepime agents of cephalosporins class were least effective agents as they showed significant relation to the three resistant genes (Table 4). The isolates which showed the most frequent resistant genes were *K. pneumonia* 90/124 (72.6%), *A. baumanni* 37/67 (55.2%), *E. coli* 22/51 (43.1%), *P. aeruginosa* 8/42(19.0%) and *P. mirabilis* 24/44 (54.5%) (Table 2).

DISCUSSION

Bacterial resistant to different classes of antimicrobial agents are major threat to humanity and are of high risk, which may return the world to pre-antimicrobial era (Mohapatra et al., 2018; Koulenti et al., 2019). While

Table 2. Frequency of isolates and patterns of occurrence of resistant ESBLs genes.

Isolate	Frequency of isolate (%)	Occurrence of resistant encoding gene				
		None (%)	Patten of occurrence			
			Single gene (%)	Two genes (%)	Three genes (%)	Total (%)
A. baumannii	67/386 (17.4)	30 (44.8)	31(46.3)	4 (6.0)	2(3.0)	37/67(55.2)
C. freundii	3/386 (0.78)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	1/3 (33.3)
C. youngae	1/386 (0.26)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Comamonas testosteroni	1/386 (0.26)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)
E. cloacae	7/386 (1.8)	5 (71.4)	2 (28.6)	0 (0.0)	0 (0.0)	2/7 (28.6)
E. coli	51/386 (13.2)	29 (56.9)	15 (29.4)	6(11.8)	1 (1.2)	22/51(43.1)
K. pneumoniae	124/386(32.1)	34 (27.4)	45 (36.3)	29 (23.4)	16 (12.9)	90/124(72.6)
M. morgani	12/386 (3.1)	9 (75.0)	3 (25.0)	0 (0.0)	0 (0.0)	3/12 (25.2)
P. aeruginosa	50/386 (13.0)	42 (84.0)	7(14.0)	1 (2.0)	0 (0.0)	8/50 (16.0)
P. hauseri	2/386 (0.52)	2 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
P. mirabilis	44v (11.2)	20 (45.5)	22 (50.0)	1 (2.3)	1 (2.3)	24/44 (54.5)
P. stuartii	16/386 (4.1)	9 (56.3)	7 (43.8)	0 (0.0)	0 (0.0)	7/16(43.8)
S. marcescens	8/386 (2.1)	5 (62.5)	3 (37.5)	0 (0.0)	0 (0.0)	3/8(37.5)
Total	386	188	137	41	20	386 (100)

Table 3. Phenotypic characterization of antibiotic resistance among Gram-negative bacteria.

Phenotypic characterization of isolate	Frequency	Percentage
Mono-resistant	28	7.2
Resist to 2 antimicrobial group	53	13.7
MDR	113	29.3
XDR	129	33.4
PDR	48	12.4
Fully Sensitive	15	3.9
Total	386	100%

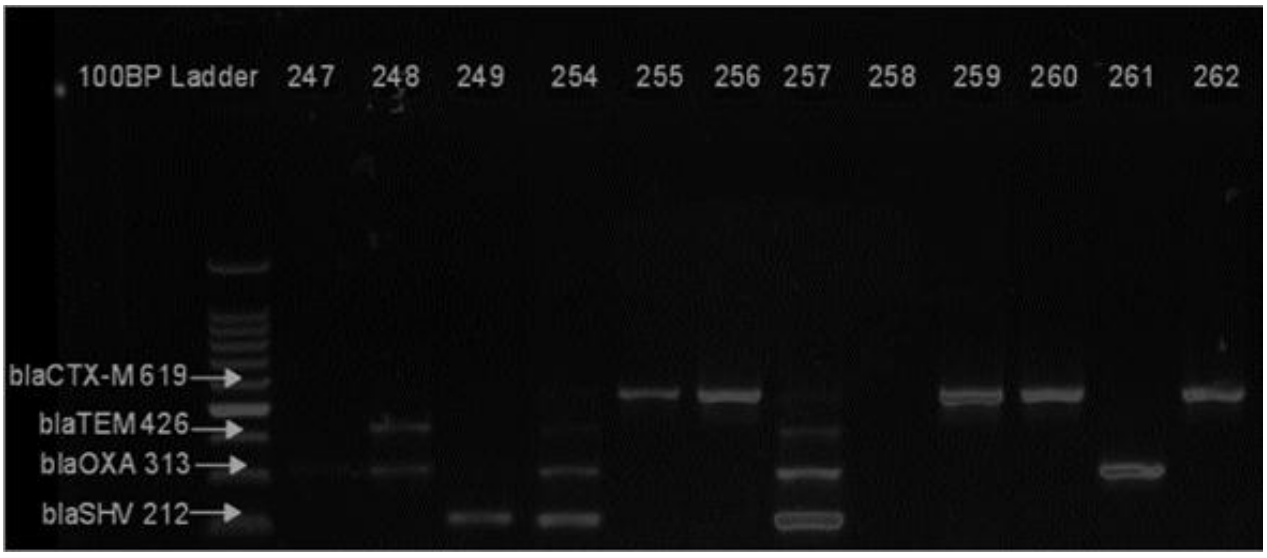

Figure 1. Multiplex polymerase chain reaction (PCR) result of ESBLs genes encoding bla TEM, bla SHV, bla CTX-M.

Table 4. Phenotypic resistance along with frequency of ESBLs genes.

Beta lactam groups	Antimicrobials	Resistance Nr (%)	Genes		
			blaSHV Nr (%)	blaTEM Nr (%)	blaCTX-M Nr (%)
Penicillinase resistant penicillins and carboxypenicillin	Ampicillin	262/282 (93.2)	92/262 (35.1)	60/262 (22.9)	78/262 (29.8) *P* = 0.005
	Ampicillin/ clavulanic acid	78/141 (55.3)	46/78 (58.9)	21/78 (26.9) *P* = 0.022	32/78 (41.0)
	Ticarcillin/Clavulanic acid	96/161 (59.6)	24/96 (25)	18/96 (18.8)	21/96 (21.9) *P* = 0.003
Azetreonam	Aztreonam	87/96 (90.6)	24/87 (27.6)	23/87 (26.4)	11/87 (12.6)
Monobactam / sulbactam	Piperacillin/ Tazobactam	168/249 (67.5)	59/168 (35.1) *P* = 0.006	41/168 (24.4)	58/168 (34.5) *P* = 0.000
Cephalosporins	Cefalotin	157/174 (90.2)	61/157 (38.9) *P* = 0.043	37/157 (23.6)	53/157 (33.8) *P* = 0.017
	Cefoxitin	88/174 (50.6)	42/88 (47.7)	22/88 (25)	29/88 (33)
	Ceftazidime	325/386 (84.2)	98/325 (30.2) *P* = 0.016	75/325 (23.1) *P* = 0.002	87/325 (26.8) *P* = 0.002
	Ceftriaxone	185/210 (88.1)	64/185 (34.6)	46/185 (24.9)	64/185 (34.6) *P* = 0.021
	Cefepime	322/386 (83.4)	99/322 (30.7) *P* = 0.014	75/322 (23.3) *P* = 0.001	88/322 (27.3) *P* = 0.001
Carbapenems	Imipenem	201/375 (53.6)	56/201 (27.9) *P* = 0.003	52/201 (25.9) *P* = 0.016	51/201 (25.4) *P* = 0.020
	Meropenem	158/381 (41.5)	55/158 (34.8) *P* = 0.042	33/158 (20.9)	47/158 (29.7) *P* = 0.032
	Ertapenem	2/80 (2.5)	0/2 (0)	1/2 (50)	0/2 (0)

active surveillance systems are set up in many countries in Europe, USA and Asia, little is reported on antimicrobial resistance status among Gram-negative bacteria in the Middle East, Africa and Saudi Arabia (Zowawi, 2016). The present study determined the prevalence of MDR, XDR, PDR and extended-spectrum- β–lactamases genes (TEM, SHV, CTX-M) among Gram-negative bacteria in Saudi Arabia.

The third generation cephalosporin such as ceftazidime and cefoperazone marked by stability to the common beta-lactamases of Gram-negative bacilli and these compounds are highly active against Enterobacteriaceae (Maina et al., 2012; Arumugham and Cascella, 2021). The isolated Gram-negative bacilli in this study showed high resistant rate to the antibiotic agents of cephalosporins class (79.3%), followed by the agents of penicillinase resistant penicillins which showed 69.4% resistant; while the agents of carbapenems had least resistant rate. The highest resistance rate was reported against ampicillin (93.2%), followed by aztreonam (90.6%) and cefalotin 157(90.2%). Similar trend of resistance was observed by Ruppé et al. (2015) who own the dramatic increase in the rates of resistance to third-generation cephalosporins to spread of plasmid-borne extended spectrum beta-lactamases (ESBLs) in Enterobacteriaceae and to occurrence of sequential chromosomal mutations, which may lead to the overproduction of intrinsic beta-lactamases, hyper-expression of efflux pumps, target modifications and permeability alterations in non-fermenting Gram-negative bacteria. The serious finding in our study was emerging of carbapenems resistant, Carbapenems agents considered the most active and potent agents against multidrug resistant (MDR) (Sheu et al., 2019). This finding is totally contradictory to that reported by Zaman et al. (2015) who determined the susceptibility pattern of Gram-negative bacilli isolated from a teaching hospital in Jeddah, Saudi Arabia and reported 100% sensitivity of enterobacteriaceae to carbapenems. Koulenti et al. (2019) concluded that carbapenem-resistant Enterobacteriaceae are particularly challenging to treat. However, WHO have listed carbapenem-resistant Enterobacteriaceae among the top tier of the antibiotic resistant that pose the greatest threat to human health (WHO, 2017).

The overall prevalence of MDR, XDR and PDR in this study was 29.3, 33.4, and 12.4% respectively. Several studies were conducted in Saudi Arabia and showed high resistance rate among Gram-negative bacteria, most of these studies focused on susceptibility per individual pathogen (Alam et al., 2017; Ibrahim et al., 2010; Ibrahim, 2018; Zowawi, 2016; Al Yousef, 2016; El-Saed et al., 2020). However, Ibrahim (2018), reported higher rate of MDR in Southwest Saudi Arabia (67.9%). The present study showed high PDR and less XDR compared to that reported by Mohapatra et al. (2018). Increasing antimicrobial resistant in Saudi Arabia may also be due to increased cross geographic transmission of drug resistant strains. Saudi Arabia is the capital of the Islamic world and has great number of expatriates, which makes it a potential center for the import and export of multi drug resistant strains (Ahmed‑Abakur and Alnour, 2019), a recent study by Leangapichart et al. (2016) showed that returned travelers from Hajj had acquired MDR A. baumannii and NDM producing E. coli during the Hajj event.

The findings from this study showed that more than half (51.3%) of isolated Gram-negative harbored with resistant genes, while isolates free from resistant genes were 48.7%. Only 3.9% of isolates were fully susceptible to the used antimicrobials. This finding in alignment with Munita and Arias (2016) report and with Patil et al. (2019)

results, who showed increase in the resistant rate among gram-negative pathogen and stated that Gram-negative bacteria are continuously evolving mechanisms to deactivate clinically important antimicrobial drugs by acquisition of resistance elements such as bla SHV, bla TEM and bla CTX-M. However antimicrobial resistant is an outcome of multifaceted microbial interactions such as microbial characteristics to gain resistance genes, selective pressure owing to inappropriate use and widespread of antibiotics, resistance may arise by the acquisition of de-novo mutation during treatment or by acquisition of integrative or replicative mobile genetic elements that have evolved over time in microbes in the natural ecosystem (Munita and Arias, 2016).

The results from this study indicate that bla SHV was the most prevalent resistant gene which was detected in (27.5%) isolates, followed by bla CTX-M (23.3%). Similar results indicated bla CTX-M and bla-SHV as the most prevalent genotypes of ESBLs producing gram-negative pathogens were reported in several countries (Paterson, 2008; Maina et al., 2012; Tian et al., 2010; Bajpai et al., 2017). Abrar et al. (2019) conducted three years study to determine the distribution of bla SHV, bla TEM, bla OXA and bla CTX-M genes in clinical isolates, they reported bla CTX-M as most dominant gene followed by bla OXA, bla TEM and bla SHV. Asokan et al. (2019) stated that the bla CTX-M gene indicates bacterial evolution due to cover prescription or weak enforcement of existing antibiotics policies. Moreover Provenzani et al. (2020) specified that the resistance rate grows rapidly when antibiotics are used inappropriately.

The present study showed that *Klebsiella pneumoniae* (72.6%), *A.baumanni* (55.2%), *P.mirabilis* (54.5%) *E.coli* (43.1%), and *P.aeruginosa* (19.0%) were the highest isolates harbored with of ESBLs genes. These findings are in agreement with Maina et al. (2012), Ibrahim (2018) and Asokan et al. (2019). Enterobacteriaceae such as *K. pneumoniae, E. coli, Proteus spp, P. aeruginosa* are naturally competent and can uptake naked DNA from the environment in suitable conditions (Patil et al., 2019). In our study bla CTX-M showed significant association to all used antibiotic agents of cephalosporin. This finding is in alignment with Maina et al. (2012), who stated that bla CTX-M type extended-spectrum β-lactamases (ESBLs), showing resistance to third and fourth-generation cephalosporins and to aztreonam. Similar result was reported by Nachimuthu et al. (2020).

Conclusion

Infections caused by multi-resistant Gram-negative pathogens negatively influence patient outcomes and costs. This study showed that only 3.9% of isolates had susceptibility to all used antibiotics, high resistant rate was observed against the antimicrobial agents of cephalosporins class and penicillinase resistant penicillin. The most dominant gene was bla SHV.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Effect of bacteriocins from lactic acid bacteria obtained from *Zea mays*-based "*Ogi*" on foodborne bacteria from contaminated cabbage

Orji J. O.[1], Ayogu T. E.[1], Amaobi C. B.[1], Moses I. B.[1*], Elom E. E.[1], Uzoh C. V.[2], Otu J. O.[1], Chukwunwejim C. R.[3], Okeh C. O.[4], Ikegbune C.[5], Peter I. U.[6] and Igwe C. P[1].

[1]Department of Applied Microbiology, Faculty of Sciences, Ebonyi State University, Abakaliki, Nigeria.
[2]Department of Biology/Microbiology/Biotechnology, Alex Ekwueme Federal University, Ndufu-Alike, Ikwo, Ebonyi State, Nigeria.
[3]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.
[4]Department of Microbiology, Alex Ekwueme Federal University, Ndufu-Alike, Ikwo, Ebonyi State, Nigeria.
[5]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Science, Chukwuemeka Odumegwu Ojukwu University, Igbariam, Anambra State, Nigeria.
[6]Department of Public Health, Faculty of Health Technology and Engineering Federal College of Dental technology and Therapy, Trans-Ekulu, Enugu, Nigeria.

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The objective of this work was to evaluate the effect of bacteriocins from lactic acid bacteria (LAB) in *Zea mays*-based *Ogi* on some foodborne bacteria contaminating cabbage in Abakaliki, Nigeria. Ten (10) samples (5 samples of *Z. mays*-based *Ogi* and 5 samples of suspected contaminated cabbage heads) were aseptically collected and analyzed using standard microbiological methods. Five different *Lactobacillus* isolates (A, B, C, D, and E) were isolated from the *Z. mays*-based *Ogi* while 5 different species of bacterial pathogens; *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp., and *Shigella* spp were isolated from cabbage heads. Results showed that *Lactobacillus* isolates exhibited high inhibitory effect against foodborne bacteria (*S. aureus*, *E. coli*, and *Shigella* spp) isolated from cabbage with inhibition zone diameter (IZD) ranging from 14 to 20 mm. A very high antimicrobial activity against foodborne bacteria isolated from cabbage was also observed for the crude bacteriocin at pH of 2. The stability of the antimicrobial affinity of the bacteriocin decreased as pH rises from 6 to 7. This study has shown that bacteriocin has antimicrobial activity against foodborne bacteria contaminating cabbage and could be used as bio-preservatives instead of hazardous chemical preservatives with adverse effects on the human body.

Key words: Lactic acid bacteria, bacteriocin, *Zea mays*, cabbage, fermented food, *Ogi.*

INTRODUCTION

Traditional fermented foods prepared from cereals such as maize (*Zea mays*) are consumed especially by the middle and low income earners in many West African countries. "*Ogi*", an acid fermented cereal gruel, made from maize serves as weaning foods for infants, food for the convalescent and the elderly, as well as breakfast meal by different age groups in Nigeria (Chilaka et al., 2016). *Ogi* has been known to exhibit health promoting

properties such as in the control of gastroenteritis in animals and man. *In vitro* and *in vivo* data have been obtained on the probiotic effects (hypolipidemic, hepatoprotective, and antibacterial) of some lactic acid bacteria (LAB) isolated from *Ogi* (Oyetayo and Osho, 2004; Aderiye et al., 2007). LAB are known for their potential of producing antimicrobial compound and other value-added products. They have the ability to inhibit growth of pathogenic microorganisms, degrade mycotoxins, and with probiotic capabilities (Mokoena, 2017). Bacteriocins are small ribosomally synthesized antimicrobial peptides against which the producer species is immune and act against other bacteria in a bactericidal or bacteriostatic manner (Hill et al., 2017). Most of the bacteriocins are bactericidal with some exceptions that are bacteriostatic (Jeeravatnam et al., 2005). Bacteriocin-producing LAB strains protect themselves from their own toxins by the expression of a specific immunity protein, encoded in the bacteriocin operon (Mokoena, 2017). The application of bacteriocin in food is effective against food spoilage/pathogenic bacteria, and it is assumed to be degraded by protease in the gastrointestinal tract which makes it safe for human consumption (Cleveland et al., 2001; Pal et al., 2015). The increasing interest in bacteriocins as alternatives to antibiotics and chemical food preservatives guided this study interest to evaluate the effect of bacteriocins from LAB in *Z. mays*-based *Ogi* on some bacterial pathogens contaminating cabbage in Abakaliki, Nigeria with a view to understanding its application to avoid food spoilage.

MATERIALS AND METHODS

Collection of *Zea mays*-based *Ogi* samples

Five (5) samples of *Z. mays*-based *Ogi* were randomly purchased at Abakpa Market, Abakaliki metropolis, Ebonyi State between June and October, 2019. Samples were aseptically collected and transported within two hours to the Department of Applied Microbiology Laboratory, Ebonyi State University, for bacteriological analysis.

Isolation and identification of LAB from *Zea mays*-based *Ogi* samples

Pour plate technique was used for the isolation of LAB. One gram of each '*Ogi*' samples were dissolved in 10 ml of water and swirled to mix properly. A tenfold serial dilution was performed and an aliquot for the 10^{-7} and 10^{-8} dilution factors were inoculated on Mann Rogosa and Sharp (MRS) agar with 50 µg/ml of nystatin to suppress the growth of fungi. The inoculated plates were incubated at 37°C for 72 h in anaerobic jar, and suspected LAB colonies were then sub-cultured on MRS agar to obtain pure culture (Harley and Prescott, 2002; Sharma, 2009). All isolates were identified by standard techniques such as Gram staining, and biochemical tests such as citrate utilization, oxidase, indole, methyl red, Voges Proskauer (VP), and sugar (lactose, glucose, sucrose, fructose, maltose and mannitol) fermentation (Holt et al., 1994; Harley and Prescott, 2002; Sharma, 2009).

Isolation of foodborne bacteria from contaminated cabbage samples

Cabbage samples collected from Abakpa Main Market, Abakaliki, Ebonyi State were processed by removing their outer leaves and 20 g of each of the samples were weighed, washed with distilled water and blended in an electric blender. The samples were put in a clean beaker containing 20 ml of sterile water and sieved. The resulting filtrate was serially diluted, and 1 ml of 10^{-7} and 10^{-8} dilutions were plated on Cysteine Lactose Electrolyte Deficient (CLED) agar, Mannitol salt agar (MSA), *Salmonella-Shigella* (SS) agar. The plates were incubated at 37°C for 24 h. The discrete colonies of each of the bacterial isolates were identified by standard biochemical tests (Holt et al., 1994; Harley and Prescott, 2002; Sharma, 2009) and sub-cultured on nutrient agar to obtain pure cultures.

Determination of antagonistic activity of LAB

The antagonistic activity of LAB isolates was tested by the agar well diffusion technique with the cell-free supernatant of each isolate. A standardized suspension of each isolated bacterium (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Shigella* spp., *Salmonella* sp.) from the cabbage samples was prepared and inoculated on the surfaces of prepared MHA plates using sterile cotton swab. The plates were dried and a sterile cork borer with a diameter of 4 mm was used to cut uniform wells in the agar plates. Each of the wells was filled with 0.1 ml aliquot of the test LAB. The plates were incubated at 37°C for 72 h. Isolates exhibiting highest zone of growth inhibition were selected and screened for bacteriocin production.

Screening and selection of LAB with antagonistic activity

The isolates were screened and selected using agar well diffusion technique using the cell free supernatant. A sterilized molten Mueller-Hinton (MHA) was prepared and dispensed into the petri dishes and allowed to solidify. A lawn of indicator organisms such as *E. coli*, *S. aureus*, *P. aeruginosa*, *Shigella* spp. and *Salmonella* sp. were made by spreading their suspension over the surfaces of prepared MHA plates with aid of sterile cotton swab. The plates were allowed to dry and a sterile cork borer with a diameter of 4 mm was used to cut uniform well in the agar plates. Each of the wells in the MHA plate was filled with 0.1 ml aliquot of the test LAB isolates. The plates were then incubated at 37°C for 72 h. Isolates exhibiting maximum zone of growth inhibition were selected and used for bacteriocin production.

Purification of crude bacteriocin production

Bacteriocin was extracted from LAB that had highest growth inhibition by growth in 1 L of MRS broth for 72 h at 30°C under anaerobic conditions (Ogunbanwo et al., 2003). Extract was

*Corresponding author. E-mail: ben_iyke70@yahoo.com. Tel: +2348134136233.

obtained by centrifuging the culture at 12,000 rpm for 15 min to separate the cells. The pH of the cell-free culture supernatant was adjusted to 6.5 with 1 M NaOH. Catalase (1 mg/ml) was added to remove hydrogen peroxide from the supernatant (Daba et al., 1991). The supernatant was filtered through a 0.45-µm pore size membrane and the protein was precipitated using 80% (w/v) saturated ammonium sulphate. The mixture was stirred for 1 h and stored at 4°C. After precipitation, the mixture was centrifuged at 16,000 rpm at 4°C for 30 min, and pellet was stored at 42°C. The pellet was separated from impurities by dissolving 1 ml in distilled water in an Eppendorf tube and centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet containing bacteriocin was washed with deionized water. The mixture was dispensed into another Eppendorf tube and centrifuged at 10,000 rpm for 15 min at 4°C to spin down the proteins, and the supernatant discarded. The pellet was dissolved in 500 µl of 0.1 M sodium phosphate buffer (pH 7.0) and the total volume was made up to 2 ml. The sample was loaded into a pre-treated dialysis tubing cellulose membrane (18-20 mm length) and dialyzed in a 3-L 0.1 M sodium phosphate buffer (pH 7.0) for 2 h; the buffer was changed and the sample further dialyzed overnight at 4°C. After 24 h dialysis, the sample was added to an Eppendorf tube, centrifuged at 10,000 rpm at 4°C for 30 min and stored at -20°C.

Purification of proteins from crude bacteriocin samples

The crude bacteriocin samples were dispensed into Eppendorf tubes and centrifuged at 12,000 rpm for 15 min at 4°C to pellet down the cells. The supernatant was poured into a 50-ml capacity beaker in an ice pack at a temperature of 4°C and 15 g of ammonium sulphate $(NH_4)_2SO_4$ in dry form was measured and dissolved in the supernatant. The mixture was stirred for 1 h and stored at 4°C for 24 h. The stirring was carefully done to prevent foaming that may lead to protein denaturation (Hensyl et al., 1994).

Determination of bacteriocin activity

Agar well diffusion assay was used to determine the bacteriocin activity of the LAB isolates (Khalid, 2011). Ten ml of partially purified bacteriocin was serially diluted up to 10^{-2} using saline diluent. An overnight culture of bacteria isolated from cabbage grown in tryptic soy broth (TSB) at 37°C was diluted in saline to a 0.5 McFarland standards. The suspension was inoculated on the surface of MHA plates using a sterile cotton swab. The plates were dried and wells were done as described above. Each of the wells was filled with 0.1 ml aliquot. The plates were kept at 4°C for 2 h to ensure diffusion of the supernatant fluid into the agar, and then incubated at 37°C for 24 h. The antimicrobial activity was determined by measuring the diameter of zones of inhibition around the wells.

Determination of effect of pH on antibacterial activity of crude bacteriocin

Sodium hydroxide (NaOH) and hydrogen chloride (HCl) was added to separate tubes to obtain pH values of 2, 3, 4, 5, 6, 7, and 8 confirmed with Jenway pH meter. The solutions were kept at room temperature for 2 h. Aliquots of 50 µl from each test tube were placed in wells of MHA plates inoculated with overnight broth cultures of the isolated bacteria (*S. aureus*, *E. coli*, *P. aeruginosa, Salmonella* sp., and *Shigella* spp.) from cabbage. The plates were incubated at 30°C for 24 h and the zones of inhibition around the wells were measured.

RESULTS

Isolation and identification of LAB from *Zea mays*-based *Ogi* samples and foodborne bacteria from cabbage samples

Results showed that five different lactic acid bacteria species; *Lactobacillus* isolates A, *Lactobacillus* isolates B, *Lactobacillus* isolates C, *Lactobacillus* isolates D, and *Lactobacillus* isolates E were obtained from *Z. mays*-based *Ogi* samples collected from Abakpa Main Market, Abakaliki, Ebonyi State (Table 1). Five micororganisms (*S. aureus*, *E. coli*, *P. aeruginosa*, *Salmonella* sp., and *Shigella* spp.) were isolated from cabbage samples randomly collected from Abakpa Main Market, Abakaliki (Table 2).

Antagonistic activity of lactic acid bacteria isolated from *Zea mays*-based *Ogi* against food-borne bacterial isolates from cabbage

The inhibition zone diameters (IZDs) of *Lactobacillus* isolates (A, B, C, D, and E) against the foodborne bacteria (*S. aureus, E. coli, P. aeruginosa, Salmonella* sp., and *Shigella* spp.) are shown in Figure 1. *Lactobacillus* isolates A, B, C, D, and E had inhibition zone diameters (IZDs) which ranged from 10 - 15 mm, 10 - 16 mm, 0 - 12 mm, 0 - 10 mm and 10 - 20 mm, respectively against the isolated foodborne bacteria (*S. aureus, E. coli, P. aeruginosa, Salmonella* sp., and *Shigella* spp.) (Figure 1).

Determination of the antimicrobial activity of the diluted crude bacteriocin from *Lactobacillus* isolates A, B, C, D, and E obtained from *Zea mays*-based *Ogi* against foodborne bacteria from cabbage

Results showed that 0.1 aliquot of the crude bacteriocin from *Lactobacillus* isolate A exhibited IZDs of 12, 10, 14, 10, and 12 mm against *S. aureus, E. coli, P. aeruginosa, Shigella* spp., and *Salmonella* sp. respectively while at 0.01 aliquot, IZDs of 7, 4, 9, 6, and 7 mm were recorded against *S. aureus, E. coli, P. aeruginosa, Shigella* spp., and *Salmonella* sp. respectively (Figure 2).

At 0.1 aliquot of the crude bacteriocin from *Lactobacillus* isolate B, inhibition zone diameters (IZDs) of 11, 9, 12, 14, and 15 mm were recorded against *S. aureus, E. coli, P. aeruginosa, Shigella* spp., and *Salmonella* sp. respectively, while at 0.01 aliquot, an inhibition zone diameter of 8, 5, 4, 8, and 6 mm were recorded against *S. aureus, E. coli, P. aeruginosa, Shigella* spp, and *Salmonella* sp. respectively (Figure 2).

At 0.1 aliquot of the crude bacteriocin from *Lactobacillus* isolate C, IZDs of 10, 12, 10, 10, and 9 mm were recorded against *S. aureus, E. coli, P. aeruginosa, Shigella* spp.,

Table 1. Morphological, physiological, and biochemical characteristics of bacteria isolated from *Zea mays*-based *Ogi* samples.

Morphological characteristics		Physiological and biochemical characteristics												Suspected Organisms
		Gram reaction	Motility test	Catalase test	Mannose	Arabinose	Sugar fermentation test					Fructose	Maltose	
Shape	Colour						Lactose	Ribose	Glucose	Mannitol	Sucrose			
Rod	White	+	-	-	+	-	+	+	-	+	+	-	+	*Lactobacillus* isolate A
Rod	Cream with a secreted edge	+	-	-	-	-	+	+	+	-	+	+	+	*Lactobacillus* isolate B
Rod	Dirty white	+	-	-	-	+	+	+	+	+	+	-	-	*Lactobacillus* isolate C
Cocci bacilli	Light Yellow	+	-	-	+	-	+	+	+	-	+	+	+	*Lactobacillus* isolate D
Rod	Dirty white on medium	+	-	-	+	-	-	+	+	-	+	+	+	*Lactobacillus* isolate E

+ = Positive, - = Negative.

Table 2. Morphological, physiological, and biochemical characteristics of bacteria isolated from cabbage sample.

Morphological characteristics		Gram reaction	Motility test	Physiological and Biochemical characteristics											Suspected Organism
				Citrate Test	Oxidase Test	Coagulase Test	Indole Test	Sugar Fermentation Test			Catalase Test	Urease Test	Voges Proskauer	Methyl Red	
Shape	Colour							Lactose	Glucose	Sucrose					
Cocci	Yellow on mannitol salt agar	+	-	-	-	+	-	+	+	-	+	+	+	+	*Staphylococcus aureus*
Rods	Opaque yellow on CLED	-	+	-	-	-	+	+	+	+	+	-	-	+	*E. coli*
Rods	Greenish on CLED	-	+	-	+	-	-	+	+	+	+	-	-	+	*P. aeruginosa*
Rod	Pink on SS agar	-	-	-	-	-	-	-	+	-	+	-	-	+	*Shigella* spp.
Rods	Black on SS agar	-	+	-	-	+	-	-	+	-	+	-	-	+	*Salmonella* sp.

+ = Positive, - = Negative.

and *Salmonella* sp. respectively, while at 0.01 aliquot, an IZD of 5, 8, 7, 5, and 10 mm were recorded against *S. aureus, E. coli, P. aeruginosa, Shigella* spp., and *Salmonella* sp. respectively (Figure 2).

Bacteriocin crude extract were not recovered from *Lactobacillus* isolate D and E.

Effect of pH on stability of bacteriocin

The results of the effects of pH on the stability of bacteriocin produced by LAB isolated from *Z. mays*-based *Ogi* against foodborne bacteria isolated from cabbage samples revealed that the inhibition zone for all bacterial isolates decreased as the pH increased, ranging from IZD of 21 to 0 mm (Table 3).

DISCUSSION

Lactic acid bacteria (LAB) are important organisms

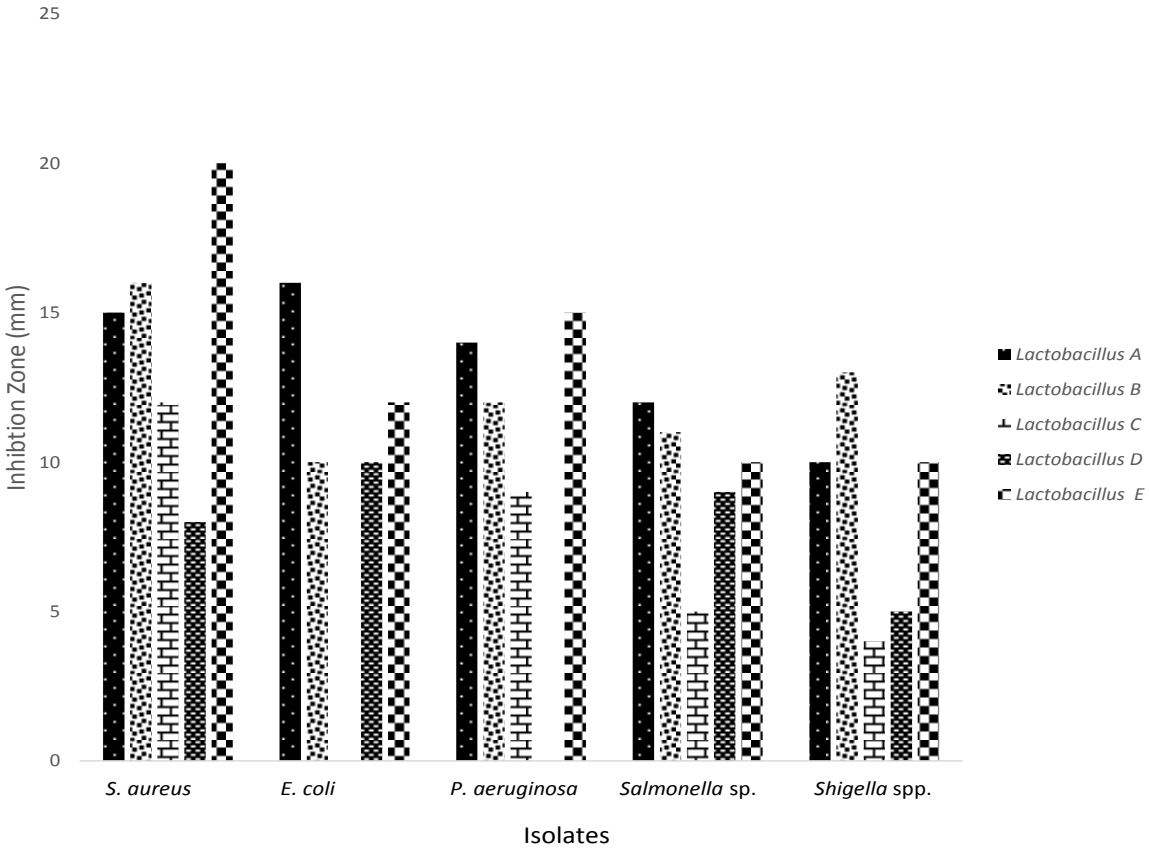

Figure 1. Antagonistic activity of lactic acid bacteria isolated from *Zea mays*-based *Ogi* against foodborne bacteria from cabbage.

recognized for their fermentative ability as well as their health and nutritional benefits. They produce various compounds such as bacteriocins bacteriostatic or bacteriocidal proteins during lactic acid fermentation. A large number of bacteriocins from lactic acid bacteria have been characterized until today, and many different studies have indicated the potential usefulness of bacteriocin as food preservative.

This present study was designed to evaluate the effect of bacteriocins from lactic acid bacteria (LAB) in *Z. mays*-based *Ogi* on some bacterial pathogens contaminating cabbage in Abakaliki, Nigeria. This study has shown that bacteriocin has antimicrobial activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*, *Salmonella* sp., and *Shigella* spp.) foodborne bacteria contaminating cabbage.

In this study, a total of five *Lactobacillus* species were isolated from *Z. mays*-based *Ogi*-based. These results are similar to the work of Ohenhen et al. (2015) who isolated five different species of *Lactobacillus* isolates from fermented *Ogi* samples. Gram-positive (*S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*, *Salmonella* sp., and *Shigella* spp.) bacterial pathogens were also isolated from the contaminated cabbage collected from Abakpa Main Market. This is also in agreement with the report of Sujeet and Vipin (2017), who observed the presence of the same foodborne bacteria in cabbage and other salad vegetables. The bacteria found are mostly enteric, which suggest a possible feacal-oral transmission. They have also been implicated as common foodborne pathogens causing gastrointestinal illnesses such as diarrhoea (Clevland et al., 2001). It is a well-known fact that food is a valuable source of nutrients for microbes to grow; and as these organisms grow on the food, they may cause spoilage such as bad taste, unpleasant smell, and poor appearance (Pal, 2013). The isolated LAB from *Z. mays*-based *Ogi* showed antimicrobial activity against foodborne bacteria isolated from cabbage. The antagonistic activity of LAB isolated from *Z. mays* based *Ogi* against foodborne bacteria revealed that even though *Lactobacillus* isolates A, B, and E had antimicrobial effect on all the indicator microorganisms, the effect on *S. aureus* was best; evident in the larger zones of inhibition. This could be a result of bacteriocin exhibiting bactericidal activity against closely related producers' strains. This is in agreement with the work of Tannock (2004) who stated that the inhibitory action of LAB is mainly due to the accumulation

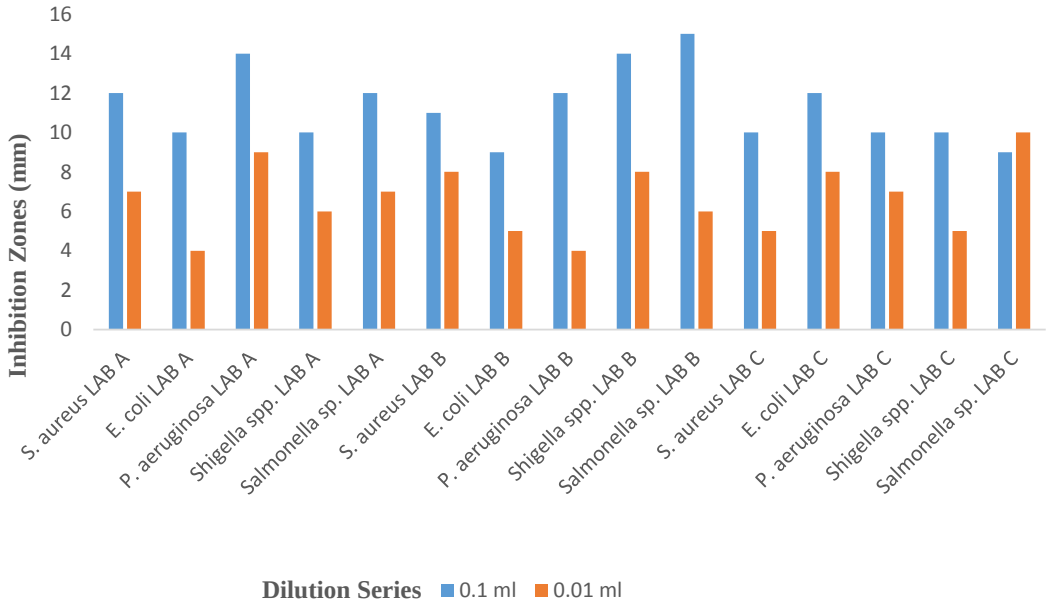

Figure 2. Antimicrobial activity of the diluted crude bacteriocin from *Lactobacillus* (LAB) isolates A, B, C, D, & E from *Zea mays*-based *Ogi* against foodborne bacteria from cabbage samples.

Table 3. Effect of pH on the stability of the antimicrobial activity of bacteriocin against tested foodborne bacterial isolates.

pH	Isolate				
	S. aureus	***E. coli***	***P. aeruginosa***	***Salmonella* sp.**	***Shigella* spp.**
2	21	17	14	19	18
3	14	13	11	12	11
4	13.5	10	9	10	11
5	11	11	10	9	10
6	9	7	4	5	4
7	5	3	5	2	3
8	3	1	4	NI	1

NI - No Inhibition.

of main primary metabolites. He also stated that LAB are capable of producing antimicrobial compounds such as formic acid, benzoic acid, hydrogen peroxide, and bacteriocins. The reason for the high inhibition zone diameter in *S. aureus* could be as a result of the cell wall structure and the physiological characteristics of the organisms (Tannock, 2004). It was reported that Gram-negative bacteria tend to resist antimicrobial compounds as a result of their complex cell wall composition (Pal et al., 2015). This is in contrast with the work of Ohenhen et al. (2015) who observed highest and lowest zone of inhibitions for *E. coli* and *S. aureus* respectively by *Lactobacillus plantarum*. Other *Lactobacillus* isolates; C, and D were less effective against the foodborne bacteria isolated from cabbage. The antimicrobial activity of the diluted bacteriocin from *Lactobacillus* isolate A, *Lactobacillus* isolate B, and *Lactobacillus* isolate C obtained from *Z. mays* showed that all the isolates have antimicrobial effect on the indicator microorganisms at 0.1 ml dilution while at 0.01 ml dilution, it recorded lower inhibition zone. This implies that optimal inhibition zone occurs at 0.1 ml. This result is in agreement with the findings of Kwon et al. (2002) who reported that bacteriocins had broad spectrum of activities against fish pathogens. It has also been reported that certain LAB bacteriocins, especially the class 2 bacteriocin, *pediocin*, can inhibit Gram-negative bacteria such as *Shigella* spp., *Salmonella* sp., and *Pseudomonas* species.

Hwanhlen et al. (2011) also reported that lactic acid was able to cause sub-lethal injury to *E. coli*. Similar properties have also been assigned to acetic acid (Hwanhlen et al., 2011). Because bacteriocin does not act equally against target species, many researchers have examined the activity of bacteriocin on specific

bacteria species and strains (Castro et al., 2011). Indirect evidence suggests that such injury involve disruption of the lipopolysaccharide (LPSP) layer (Castro et al., 2011). Our study also showed that the crude bacteriocin extract partially purified from LAB displayed a very high antimicrobial activity at the pH 2, compared to the antimicrobial activity displayed at pH 6 and 7. No antimicrobial activity was observed in the diluted crude bacteriocin extract at pH 8. *Lactobacillus* isolates have tolerance to low pH. This observation is similar to that reported by Ogunbanwo et al. (2003) who indicated that purified bacteriocin extract recovered from *L. plantarum* was more active at pH 2 and 6, than at pH 10 and 12. The increased sensitivity of *S. aureus* to the pH amended crude bacteriocin extract could be as a result of the cell wall and physiology of the bacterium. However, Gram-positive bacteria only have a thick-like cell wall, made of peptidoglycan which constructs about 90% of the cell wall (Omar et al., 2006). This observation is in agreement with a report by Rammelsberg and Radler (1990) who observed that antimicrobial activity of purified bacteriocin extracted from *L. parecasei* subsp. *tolerans* was more active against *S. aureus* and *Listeria monocytogenes* than *E. coli*. The diluted crude bacteriocin extract at pH 8 exhibited less antimicrobial activity against the indicator organisms. It is possible that a high pH value had a negative effect on the antibacterial activity of the extract. Hence, further studies are needed in the extraction, characterization, and purification of bacteriocin from LAB with a view to utilizing them as alternatives to harmful chemical preservatives and as potential sources of probiotics and antimicrobial agents.

Conclusion

This study has shown that bacteriocin from lactic acid bacteria (LAB) has antimicrobial activities against one Gram-positive (*S. aureus*) and four Gram-negative (*E. coli*, *P. aeruginosa*, *Salmonella* sp., and *Shigella* spp.) foodborne bacteria isolated from cabbage. The bacteriocins produced by the LAB isolates in this study were noted to have maximum activity at pH 2. This study show the possibility that bacteriocins could be used as bio-preservatives in acidic food products like fruits juices instead of chemical preservatives which may have adverse effects on human body after consumption.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Prevalence and characterization of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from poultry in Ouagadougou, Burkina Faso

Souleymane Soré[1,2*], Fatimata B. Josiane Diarra[3], Juste Isidore Bonkoungou[3], Charles Sawadogo[1], Boubié G. Bationo[1], Hervé Ky[1], Patrick Djim-Madjim Madingar[1], Abdoul Salam Ouédraogo[5] and Idrissa Sanou[2,4]

[1]Medical Biology Laboratories, Health Ministry, P. O. Box 7022, Ouagadougou 03, Burkina Faso.
[2]School of Sciences, Health and Technologies, Saint Thomas d´Aquin University, P. O. Box 10212, Ouagadougou 06, Burkina Faso.
[3]Laboratory of Molecular Biology, Epidemiology and Surveillance of Bacteria and Viruses Transmitted by Food (LaBESTA), Center for Research in Biological, Food and Nutritional Sciences (CRSBAN), Graduate School of Science and Technology (EDST), University of Ouaga I Professor Joseph KI-ZERBO, Ouagadougou 03 BP 7021, Burkina Faso.
[4]Tengandogo Teaching Hospital, P. O. Box 104, Ouagadougou CMS 11, Burkina Faso.
[5]Souro Sanou University Hospital, Bobo-Dioulasso, Burkina Faso.

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This study aimed to determine the prevalence of extended spectrum beta-lactamase producing (ESBL) and multidrug resistant *Escherichia coli and Klebsiella pneumoniae.* A cross-sectional study was conducted in Ouagadougou in two poultry farms and two slaughterhouses. 375 cloacal swabs and 46 environment samples were collected and members of Enterobacteriaceae were isolated on EMB agar containing 4 µg/L of cefotaxime. *E. coli* and *K. pneumoniae* were identified using biochemical tests and ESBL production was confirmed by the double-disc synergy test. Antibiotic susceptibility was determined by the disc diffusion method. Prevalence of faecal ESBL producing *E. coli* and *K. pneumoniae* was 12.11% (95% CI = 9.3-15.6). In sampling sites, the prevalence were 5.15% in Farm A, 2.22% in Farm B, 17.50% in slaughterhouse C, 20.59% in slaughterhouse D and 19.57% in environment. *E. coli* (n = 43) and *K. pneumoniae* (n = 13) were frequently identified. ESBL-producing *E. coli* and *K. pneumoniae* MDR was 89.29% (95% CI = 78.5–95.0). Resistance to aminoglycosides was 6.25% in poultry and 10.00% in slaughterhouse, fluoroquinolones 32.5% in slaughterhouse, sulfonamides 100% in poultry and 82.50% slaughterhouse, tetracycline 100% in poultry and 95.0% in slaughterhouse. This study showed that antimicrobial resistance in poultry in Ouagadougou portends a serious problem.

Key words: *Escherichia coli*, *Klebsiella pneumoniae,* extended-spectrum β-lactamase (ESBL), poultry, farms, slaughterhouses, Ouagadougou.

INTRODUCTION

The discovery of antibiotics has been a major breakthrough in human history. However, the emergence of multidrug resistance (MDR) among pathogenic bacteria such as *Escherichia coli* and *Klebsiella*

pneumoniae is an important public health problem in human medicine, animal husbandry, veterinary medicine, and livestock management (Montso et al., 2019). These two species are associated with a wide range of infections such as pneumonia, urinary tract infections, septicemia, and soft tissue infections in humans (Ghosh et al., 2019). They also cause infections in cats, dogs, birds, horses, monkeys, pigs, rats, elephants, and poultry (Mobasseri et al., 2019; Russo et al., 2021). Specifically *E. coli* infections in chicken viz airsacculitis, cellulitis, pericarditis, perihepatitis, and respiratory distress, are critical production-limiting disease for the poultry industry (Ghosh et al., 2019).

By 2050, predictions estimate that over 10 million of deaths and ≈ nearly 100 trillion USD economic loss would result from antibiotic resistance worldwide (Maestre-Carballa et al., 2019). Although this multi-resistance to bacteria is due to the use of antimicrobials on a given chicken farm varies, used by farmers who may have less knowledge of antimicrobials, poorer bio-security practices (including housing other species in close proximity to chickens), and, consequently, a higher burden of disease is even less predictable. Globally, 63,000 tons of antibiotics are being used in livestock, which will further increase to 10,5000 tons in 2030 (Boeckel et al., 2015). These practices contribute to the widespread increase of antimicrobial resistant pathogens in human, livestock, and the environment, which consequently leads to the prolonged hospital stay for patients, financial burden to the society, and even fatal consequences (Klein et al., 2018).

Infections caused by multidrug resistant bacteria are associated with higher mortality, morbidity and healthcare costs (Ndir et al., 2016). Enterobacteriacae producing extended-spectrum-β-lactamases (ESBLs) represent main challenges to antibiotic therapy, with increasing prevalence rates throughout the world (Doi et al., 2017). In Burkina Faso, the number of clinical infections with ESBL-producing organisms is increasingly high (Ouedraogo et al., 2016). Precise information about the spreading of ESBL-organisms influenced by poultry and foodborne is poorly known; however, the proportions of ESBLs carriage in animal vary between countries and farms, but they generally range from 10 to 50% for the faecal carriage in healthy animals, and up to 95% in chicken raw meat (Saidani et al., 2019). Current knowledge concerning the presence of ESBL-producing *E. coli* and *K. pneumoniae* on poultry in Burkina Faso is limited because few studies have been carried out in this aspect. The aim of this study was to determine prevalence and characterize extended-spectrum β-lactamase-producing *E. coli* and *K. pneumoniae* isolated from poultry in Ouagadougou.

MATERIALS AND METHODS

Type, period and sampling area of study

This was a cross-sectional study conducted during

the period August 1 to November 30, 2020. Two poultry farms located in rural area of Ouagadougou designated A and B, their proximity environment (chickens drinking water, food and farms space) and two poultry slaughterhouses designated C and D were used in this study. It was:

- Farm A, located in Pabré GPS (Longitude: -1.592197, Latitude: 12.536435) which grows chickens like Isa-brown, Holland blue and Harco.
- Farm B, located in Pabré GPS (Longitude: -1.557643, latitude: 12.518307) which grows local chickens, guinea fowl, coquelet, turkey, Isa-brown.
- Slaughterhouse C, located in Kamboinsin GPS (Longitude: -1.548692, Latitude: 12.439923), which sells chickens like Isa-brown and Holland blue.
- Slaughterhouse D , located in Tanghin GPS (Longitude: -1.5158699, Latitude: 12.3946333) which sells local chickens and guinea fowl.
- Farms environment which group poultry drinking water, food, and their caecal.

Samples and data collection

Written informed consent was obtained from the poultry farms managers before enrollment. Each manager was interviewed using a questionnaire to obtain information on age, sex, poultry breed and use of antibiotics in treatment or supplementation of their poultry. Poultry were selected at random from each farm; sampled poultry were separated from others; and in slaughterhouses, 10 chickens were selected each collection day for 19 days. Cloacal samples were taken by swabbing the cloacae of each poultry, using sterile swab soaked in sterile physiological saline. Environmental samples were taken from chicken's drinking water, food, farms space. All swabs and environment samples were transported in cooler boxes and stored at 4°C.

Isolation and identification of enterobacteriaceae

On August to November 2020, a total of 375 cloacal swabs and 46 environment samples were screened. Swabs were seeded broth-heart-brain (HiMedia, india) and incubated overnight at 37˚C to improve the bacteriological yield. After this enrichment, 10 µl of the broth was transferred to eosine methylen blue (EMB) agar (HiMedia, india) supplemented with 4 µg/L of cefotaxime and incubated at 37°C for 24 h to screen for ESBL/AmpC-producing Enterobacteriaceae. Predominant colonies of different morphotypes were identified at species level using phenotypic characteristics, Gram staining, oxidase, and fermentation tests. Bacteria Gram-negative, oxidase negative and fermentation-positive isolates were biochemically identified using in-house biochemical tests (Triple sugar iron agar, Sulfur-indole-motility test, Simmons´s citrate agar, and urease test). Finally, bacterial isolates were stored at -80°C in brain heart infusion broth supplemented with 20% glycerol

*Corresponding author. Email: soulsore11@yahoo.fr. Tel: (00 226) 72 04 29 15.

Table 1. Distribution of poultry according to age group and gender.

Characteristics		Farms		Slaughterhouse		Total
		A	B	C	D	
Sex	Cocks (n)	35	16	100	12	163
	Hens (n)	62	74	20	56	212
Age group (Month)	1-2	0	0	0	0	0
	2-3	19	61	20	12	112
	3-4	31	10	100	50	191
	4-5	0	13	0	6	19
	5-6	6	6	0	0	12
	≥6	41	0	0	0	41

n: number.

Phenotypic ESBL/AmpC testing

All *E. coli* and *K. pneumoniae* isolates were screened for ESBL production by the double-disk synergy test (DDST) using cefotaxime 30 µg, ceftazidime 30 µg , ceftriaxone 30 µg, cefepime 30 µg and amoxicillin/clavulanic 20/10 µg (HiMedia Laboratories Pvt. Ltd). Phenotypic detection of AmpC production was carried out for *E. coli* and *K. pneumoniae* strains that were either resistant to cefoxitin and/or resistant to ≥3 β-lactam antibiotics (CA-SFM, 2013).

Antimicrobial susceptibility testing

ESBL-producing *E. coli* and *K. pneumoniae* isolates were analysed by Kirby-Bauer disk diffusion technique to determine the resistance patterns of the isolates (CA-SFM, 2013). The strains were tested for antimicrobial susceptibility using the cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), ceftriaxone (CRO 30 µg), Cefepime (CPM 30 µg), cefoxitine (CX 30 µg), amoxicillin/clavulanic (AMC (20/10 µg), imipinem (IPM 10 µg), gentamicin (CN 10 µg), ciprofloxacin (CIP 5 µg), trimethoprim/sulfamethoxazole (SXT,1.25/23.75 µg), and Tetracycline (TE 30 µg) (HiMedia Laboratories Pvt. Ltd). Zones of inhibition were measured with a precision caliper and isolates exhibiting resistance to at least three antimicrobial agents tested were considered as multidrug-resistant strains (CA-SFM, 2013). Zone diameters were compared with the EUCAST criteria to determine if isolates were resistant, intermediate, or susceptible.

Quality control

Standard protocoles have been strictly followed during laboratory analyzes. The expiration dates of culture media, reagents and other consumables have been checked and documented. A verification of the contaminants of the media already prepared was carried out by sterility test. A quality control was carried out in order to test fertility of media. Each new batch was checked before use by testing *E. coli* ATCC 25922 which is one of the standard control strains. During the detection of ESBL *E. coli and K. pneumoniae*, *K. pneumoniae* ATCC 700603 (ESBL positive) and *E. coli* ATCC 25922 were used as controls for this study.

Statistical analysis

Data entry was performed and statistical analysis of the results was done using XLSTAT 2017 version 19.5. The distributions of the variables were compared by the χ^2 independence test. The significance level was set at 5%.

RESULTS

Characteristics of poultry

Distribution of poultry according to age group and gender

The majority of the birds included were hens with a frequency of 212/375 (56.53% CI= 51.5-61.5). The overall sex ratio was 0.76. The minimum age was 2 months with a maximum of 20 months (Table 1).

Distribution of poultry according to breed and notion of antibiotics supplementation

According to the breed of poultry, the Isa-brown breed was the majority (107/187) (57.21% CI=50.1 -64.1) followed by Holland blue (66/187) (35.29% CI=28.8-42.4). Oxytetracycline and polymixin were used in all breeds on both intensive livestock farms (Table 2).

Prevalence of *Escherichia coli* and *Klebsiella pneumoniae*

The 421 samples analysed originated mostly from poultry farms (n=187), slaughterhouse (n=188) and poultry environment (n=46). *E. coli* and *K. pneumoniae* isolates were detected in 51 out of 421 samples analyzed (12.11%) (95% CI = 9.3-15.6). *E. coli* and *K. pneumoniae* were isolated from all four sites (farms and slaughterhouse) but with varying proportions: 5/97 (5.15%) in Farm A, 2/90 (2.22%) in Farm B, 21/120 (17.50%) in Slaughterhouse C, 14/68 (20.59%) in

Table 2. Distribution of poultry according to breed and antibiotics used.

Characteristics		Poultry breed				Total (n)
		Isa-brown (n)	Harco (n)	Holland blue (n)	Turkey (n)	
Farms	A	47	8	42	0	97
	B	60	0	24	6	90
Antibiotics	Oxytetracycline	107	8	66	6	187
	Colistine	107	8	66	6	187
	Quinolones	47	8	42	0	97
	Phenicol	60	0	24	6	90
	Amoxicilline	47	8	42	0	97

n: number.

Table 3. Frequency of ESBL producing *E. coli* and *K. pneumoniae* isolated from farms and slaughterhouse according to sampling area.

	Code	No.	Positive	Prevalence (%)	95% CI
Farms	A	97	5	5.15	2.2 - 11.5
	B	90	2	2.22	0.6 - 7.8
Slaughterhouse	C	120	21	17.50	11.7 - 25.3
	D	68	14	20.59	12.7 - 31.7
Environment	E	46	9	19.57	10.6 - 33.2
Total		421	51	12.11	9.3 - 15.6

No.: Number, CI: Confidence internal.

Slaughterhouse D, and 9/46 (19.57%) in Environment farm (Table 3). *E. coli* (n = 43) and *K. pneumoniae* (n = 13) were frequently identified bacterial species.

Prevalence of conjugative ESBL-AMPc-producing *E. coli and K. pneumoniae*

A total of 421 from poultry and slaughterhouse were further analysed. Combined ESBL- and AmpC-producing phenotypes were observed in 4 of 421 (0.95%) of the isolates.

Distribution of multi-drugs resistance *E. coli* and *K. pneumoniae* among study area

ESBL-producing *E. coli* and *K. pneumoniae* resistant to more than three antimicrobial classes, was 50/56 (89.29%) (95% CI = 78.5–95.0). Resistance to the aminoglycoside, fluroquinolonones, sulfonamide, and tetracycline classes were dominant, observed in 8.93, 23.21, 87.5 and 96.43% of the isolates, respectively. Among site of sampling, frequency of MDR were high to Slaughterhouse C (42.00% CI=29.4-55.8), Slaughterhouse D (26.00% CI= 15.9-39.6 and Farms environment (18.00%, 95% CI = 9.8-30.8) (Figure 1).

Antimicrobial resistance of all *Escherichia coli* and *Klebsiella pneumoniae* strains

All isolates were sensitive to carbapenems. Prevalence was observed in ciprofoxacin (32.5% in slaughterhouse), gentamicin (6.25% in poultry and 10.00% in slaughterhouse), sulfamethoxazole – trimethoprime (100% in poultry and 82.50% slaughterhouse), tetracycline (100% in poultry and 95.0% in slaughterhouse), cefoxitin (2.5% in slaughterhouse), and amoxicillin/clavulanic acid (100% in poultry and 95.0% in slaughterhouse). Absolute resistance was observed in cefotaxime (100%), ceftriaxone (100%), cefepime (100%) and ceftazidime (100%) (Figure 2).

DISCUSSION

During the study, prevalence (12.11%, 95% CI = 9.3-

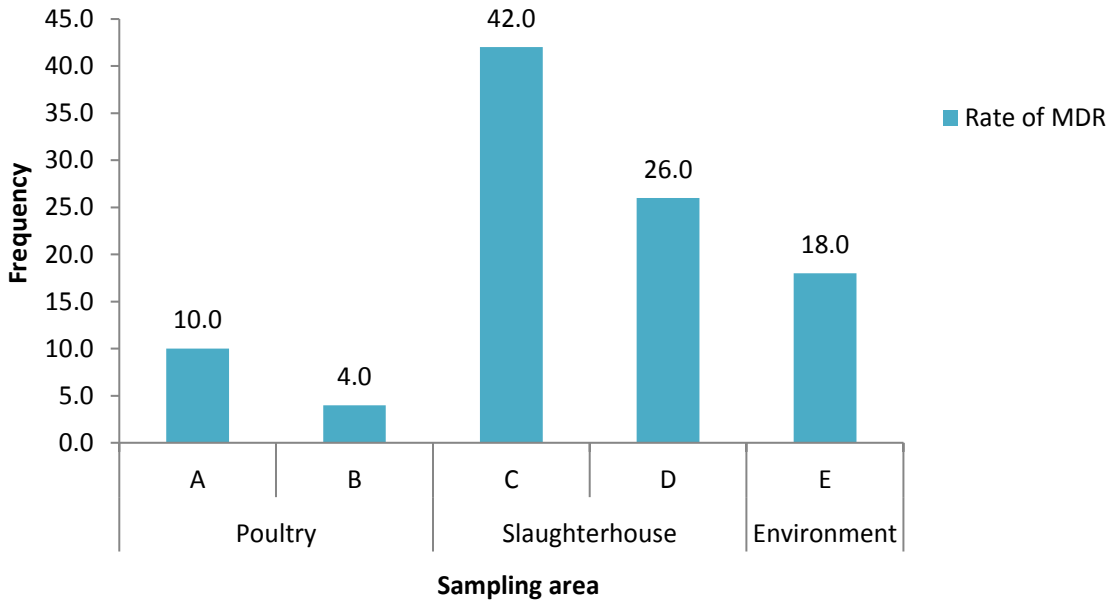

Figure 1. Repartition of MDR isolates from poultry farms, slaughterhouses and farms environment in Ouagadougou.

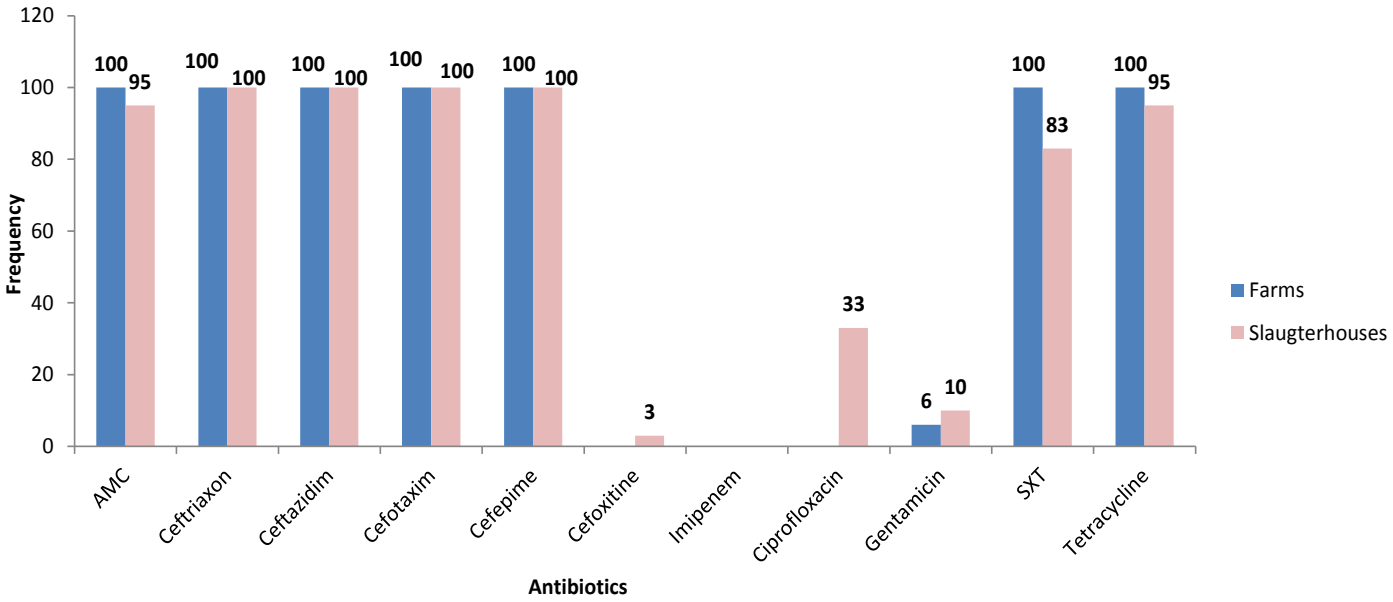

Figure 2. Percentage of antimicrobial resistance of all *Escherichia coli* and *Klebsiella pneumoniae* strains in this study.
AMC: Amoxiccilline/clavulanic acid, SXT: sulfamethoxazole – trimethoprime.

15.6) of ESBL producers was seen in isolates from poultry farms, slaugterhouse and farms environment. This frequency could be explained by the fact that antibiotics had been widely used in healthy animals for growth promotion, a practice now banned in West Africa and in other countries, but still active in others (Maamar et al., 2016). In Burkina Faso, the use of antibiotics is excessive and not controlled in animal health (Samandoulougou et al., 2015). This high prevalence of ESBL producing *E. coli* and *K. pneumoniae* detected in poultry feces could increase human fecal carriage if the microbial flora of the chicken meat is not well inactivated. This frequency of multiresistant enterobacteria isolated in poultry could be concealed in humans and environment by the consumption of contaminated meat. The lack of bio-security in some farms could explain the possible

dissemination of these clonal isolates. ESBL/AmpC producing strains might have been transmitted vertically from breeding flocks to chicks and established in the poultry environment (Vounba et al., 2019). Under the pressure of antibiotic selectivity, drug-resistant bacteria emerged, disseminated in healthy poultry and can spread to humans through consumption of contaminated food, from direct contact with poultry, or by environmental spread (Montso et al., 2019). In the present study, in all two farms, two poultry slaughterhouses and environment poultry were colonized by ESBL producers *E. coli* isolates. The rate of fecal carriage of these multidrug resistant bacteria in healthy chickens varied from one site to another (2.22 to 20.59%). These results confirm that chicken farms constitute a reservoir of ESBL producing *E. coli* and *K. pneumoniae* isolates, which might reflect a high antibiotic pressure for selection of resistant bacteria in this ecosystem (Umair et al., 2019; Saidani et al., 2019; Ghosh et al., 2019; Mobasseri et al., 2019). This prevalence is higher than those observed in Bobo Dioulasso in 2019 where the poultry faecal carriage of ESBL producing *E .coli* and *K. pneumoniae* was 0.8% (Sanou et al., 2019). However, our results are similar to those found in Pakistan where prevalence rate was 13.7% (Umair et al., 2019) and Tunisie where prevalence was between 4 and 67.3% (Saidani et al., 2019). In our study, like in other several countries, cephalosporins are not used for poultry, but high prevalence of ESBL-producing bacteria remains (Sanou et al., 2019). This suggests that there are additional sources for the contamination with ESBL-producing bacteria in livestock. Our situation may be explained by an environmental contamination. Indeed, ESBL-producing bacteria may spread to environment by waste products from human activities and animal production (Soré et al., 2020).

The ESBL *E. coli* and *K. pneumoniae* showed any resistance to imipenem. These two species also showed resistance to ciprofoxacin (33.0% in slaugtherhouse and 0% in farms), gentamicin (10.0% in slaugtherhouse and 6.0% in farms) and sulfamethoxazole – trimethoprime (83.0% in slaugtherhouse and 100% in farms), tetracycline (95.0% in slaugtherhouse and 100% in farms). This resistance to fluoroquinolones, aminoglycosids, sulfamethoxazole – trimethoprime and tetracyclines might have resulted from overuse due to easy access, and lack of control of these antibiotics in the market. In addition to that, most isolates presented multiple-associated resistances, highlighting that ESBL producing *E. coli* can be selected using other veterinary-licensed antibiotics besides broad-spectrum cephalosporins (Diab et al., 2017). Plasmids carrying genes encoding ESBLs is known to also carry other genes conferring resistance to fluoroquinolones, aminoglycosides, tetracycline, and cotrimoxazole (Ouédraogo et al., 2017). The high resistance of cephalosporins explained by Ceftiofur was still systematically used at the hatchery to prevent omphalitis in broiler chicken farms during the period of our sample collection, which could therefore explain the high prevalence of cephalosporin resistance, as demonstrated by a previous study from France (Vounba et al., 2019). However, gentamicin resistance was not highly prevalent although it is not used as aminoglycosides in poultry in the country (Samandoulougou et al., 2015).

Of the 56 ESBL *E. coli* and *K. pneumoniae*, 50 isolates (89.29%) showed multidrug resistance that is resistance to at least three antimicrobials (Figure 1). This prevalence of MDR Enterobacteriacae, was 14.0% in farms, 68.0% slaughterhouses and 18.0% in farms environment. All the strains from farms and farm environment showed resistance to tetracycline, which is used widely in feed supplements (Mobasseri et al., 2019). In general, *E. coli* and *K. pneumoniae* MDR are becoming a serious issue in humans and animals, with an increasing resistance to most available antibiotics (Mobasseri et al., 2019; Stuart, 2002). The presence of these various plasmids, often mediating resistance to several antimicrobials, could also explain the high prevalence of MDR mentioned previously (Vounba et al., 2019).

Conclusion

The results shown in the present investigation emphasize the role of poultry in the spread of ESBL-producing *E coli* and *K. pneumoniae*, and the risk that these microorganisms can reach humans through the food chain. Therefore, close surveillance of antimicrobial resistant bacteria from poultry and food production livestocks should be established as a priority. Almost all of the isolates were MDR, with resistance to major antibiotics used in human medicine such as broad spectrum beta-lactams, fluoroquinolones, aminoglycosides, trimethoprim– sulfamethoxazole and tetracycline. However, in order to avoid the selection of resistant mutants, farmers should refrain from supplementing antibiotics in poultry feed. Also, in order to reduce the dissemination of these multiresistant bacteria in the environment and in humans, a strengthening of hygiene at the level of farms and slaughterhouses is recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Heavy metals and microbial contamination of palm oil produced and sold at some markets in Kogi East Area, Kogi State, Nigeria

Enemuor S. C.*, Adige A. A. and Okechukwu V. C.

Biocatalysis and Environmental Health Research Group, Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria.

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The physicochemical, microbial and heavy metal contamination of palm oil samples randomly selected from three different markets (Ankpa, Anyigba and Idah Markets) in Kogi State, Nigeria, was investigated. The results revealed the presence of moisture (0.5, 0.4, 0.4%); impurity (0.4, 0.4, 0.3%) and free fatty acid (13, 12.8, 12.3%) for Ankpa, Anyigba and Idah Markets respectively. The microbial contaminants isolated were *Enterobacter* sp, *Bacillus* sp, *Proteus* sp, *Micrococcus* sp, *Staphylococcus* sp, *Pseudomonas* sp, *Aspergillus* sp, *Candida* sp, *Mucor* sp, *Rhizopus* sp and *Penicillium* sp. The presence (ppm) of heavy metals in the samples was analyzed using atomic absorption spectroscopy for chromium, cadmium, lead, arsenic, copper and iron. All the tested metals except chromium and lead were detected in samples from Anyigba Market with the mean concentration of arsenic (0.29), iron (4.66), cadmium (0.001) and copper (0.006). The metals detected in samples from Idah Market were cadmium (0.46), arsenic (0.19), iron (7.34), chromium (0.04) and copper (0.03). Only three metals, arsenic (0.04) cadmium (0.01) and iron (1.88) were detected in samples from Ankpa Market. From the findings, the microbial load of the samples fell within the acceptable limits stipulated by World Health Organization limits. However, most of the heavy metals assayed for were above acceptable limits. The present findings indicate the need for refining of locally produced palm oil to eliminate metal contaminants because of the health implication of their accumulation in the body.

Key words: Heavy metals, microbial contamination, palm oil, Kogi East, Nigeria.

INTRODUCTION

Palm oil is an edible vegetable oil obtained from the fruit of the oil palm tree (*Eleais guineensis*). Palm oil is regarded as the most widely produced and consumed vegetable oil in the world (Shahbandeh, 2021) and ranks among the most important oil producing crops in Sub-Saharan Africa (Tagoe et al., 2012). Elaeis guineensis Jacqu original habitat is in West Africa's tropical rain forests (www.fao.org). Oil palm tree is an economic plant that grows abundantly in equatorial West Africa, both in the wild and in plantations. It is cultivated extensively in Southeast Asia where Malaysia and Indonesia are ranked as the largest producers and exporters of oil palm products (Uning et al., 2020). The major countries where oil palm thrives in West and Central Africa include

*Corresponding author. E-mail: simeon.enemuor@unn.edu.ng. Tel: +234 803 7789 633.

Nigeria, Benin, Togo, Ghana, Cote de'Ivoire, Liberia, Sierra Leone, Cameroun, Angola, Zaire, Congo and Angola. In Nigeria the main oil palm growing area is the South, especially the South-Eastern and Mid-Western regions (Raw Materials Research and Development Council, Nigeria, 2004).

Palm oil itself is reddish because it contains high amount of beta-carotene (Akinola et al., 2010). Crude (unrefined) palm oil is an important ingredient in the diet of many people in West Africa (Uning et al., 2020). Chemically, palm oil, like other seeds is a fatty acid ester of glycerol commonly called triglycerides (Akpanabiatu et al., 2001). Palm oil and its products are useful for cooking/frying, in local dishes, soap manufacture, as source of vitamin A, E and K (Oguntibeju et al., 2009). The value of palm oil depends largely on its quality. Okogbenin et al. (2014) isolated a number of food pathogens from freshly prepared palm oil and some of them have potential to secrete toxins in the palm oil. Palm oil becomes prone to contamination by microorganisms found in the production environment, raw materials and equipment used for the processing, as well as those used for storage and distribution. Contamination of palm oil with heavy metals could pose potential risk to humans because of their bioaccumulation in the body (Engwa et al., 2018; Tchounwou et al., 2012).

There are reports of heavy metals contamination of palm oil sold in markets from other areas (Olafisoye et al., 2020; Aigbemu et al., 2017; Nnorom et al., 2014; Asemave et al., 2012). Eastern part of Kogi State, Nigeria, is known for production and distribution of palm oil to other states in Nigeria, especially the Federal Capital Territory, Abuja and northern part of the country. However, earlier report in this area evaluated only the fungal contaminants in ready to use palm oil sold at Anyigba Market (Enemuor et al., 2012). There is no report on evaluation of heavy metals contamination of palm oil produced from this area. There is, therefore, the need to investigate the levels of microbial and heavy metals contamination in palm oil produced from this area and sold at the three major markets located in the area.

MATERIALS AND METHODS

Study area

The study was conducted in the Kogi East area of Kogi State, Nigeria, from three different main (local) markets located at Anyigba (located on 7.4934º N and longitude: 7.1736º E), Ankpa (located on 7.4053º N and longitude: 7.6223º E) and Idah (located on latitude 7.1138º N and longitude 6.7440º E). Kogi East area was chosen for the study because of abundance of oil palm plantations in the area and the processing of palm oil is mostly by crude traditional methods. These markets were selected because crude palm oil is distributed in commercial quantity from there to other states in Nigeria especially to the northern part of the country including Abuja, the Federal Capital Territory.

Collection of samples

From each local market ten palm oil samples were randomly selected and bought from ten different sellers (local producers) who employed traditional methods of processing making a total of thirty samples in all. The samples were collected in sterile universal bottles and care was taken not to contaminate the samples during and after collection. The samples from each market were packed in a cooler and transported to the laboratory for microbiological and heavy metals analyses.

Determination of physicochemical properties

Determination of free fatty acids

The free fatty acid content of palm oil samples was determined using the British Standard Method (1976). Five grammes of palm oil sample were measured into Erlenmeyer flask and 50 ml diethyl ether diluted with absolute ethanol in the ratio of 1:1 (v/v) was added. The mixture was heated and swirled at intervals until the oil was completely dissolved. Six drops of phenolphthalein indicator were added and the mixture was titrated against 0.1 M KOH until the end point (orange, red or pink colour which persisted for about 15 s). The titre values were recorded and the free fatty acids (FFA) content was calculated in percentage from the relation:

$$FAA = \frac{Molar\ concentration\ of\ KOH \times titre\ value \times relative\ molar\ mass\ of\ KOH}{Weight\ of\ palm\ oil\ (g)}$$

Determination of moisture content

The moisture content of the palm oil samples was determined using the British Standard Method (1976). Erlenmeyer flask was weighed (W_1) and 5 g of palm oil sample was added and reweighed (W_2). The sample was dried in the oven for 2 h at 105 ºC. It was allowed to cool in a desiccator and its final weight determined (W_3).

This process was carried out in duplicates for all the samples. The moisture content was expressed in percentage from the formula:

$$Percentage\ moisture\ content = \frac{W_2 - W_3 \times 100}{W_2 - W_1}$$

Determination of percentage impurity

The degree of impurity in the oil samples was determined by the method described by Tagoe et al. (2012). A glass funnel was lined with filter paper, washed with hexane and dried at 105 ºC for 30 min. The funnel was allowed to cool and weighed as W_1. Erlenmeyer flasks (100 ml) were weighed (W_2) and 2 g of oil added and reweighed (W_3). Hexane (20 ml) was added to the oil and the flask swirled and heated on a heating mantle to homogenize the mixtures. The mixture was then poured through the funnel and allowed to drain. The flask was rinsed with hexane to recover any remaining particles and poured through the funnel and allowed to drain completely. The funnel thereafter was dried in the oven at 105

Table 1. Some physicochemical properties of palm oil samples from the three areas.

Sample	Ankpa Market			Anyigba Market			Idah Market		
	FFA content (%)	Moisture content (%)	Impurity content (%)	FFA content (%)	Moisture content (%)	Impurity content (%)	FFA content (%)	Moisture content (%)	Impurity content (%)
1	10.2 ±0.1	0.4 ± 0.1	0.0 ±0.0	10.2 ±0.2	0.4 ±0.0	0.0 ±0.0	14.2 ±0.1	0.2 ±0.0	1.0 ±0.0
2	13.0 ±0.0	0.2 ±0.0	0.5 ±0.0	13.0 ±0.1	0.2 ±0.0	0.5 ±0.0	12.5 ±0.0	0.4 ± 0.0	0.0 ±0.0
3	11.3 ±0.1	0.4 ±0.0	0.5 ±0.0	11.3 ±0.1	0.4 ±0.0	0.5 ±0.0	13.3 ±0.0	0.2 ±0.0	0.5 ±0.0
4	12.0 ±0.1	0.2 ±0.0	0.0 ±0.0	12.0 ±0.1	0.2 ±0.0	0.0 ±0.0	11.8 ±0.0	0.6 ±0.0	0.0 ±0.0
5	14.1 ± 0.0	0.6 ±0.0	0.5 ±0.0	14.1 ±0.0	0.6 ±0.0	0.5 ±0.0	10.2 ±0.0	0.4 ±0.0	0.5 ±0.0
6	12.3 ±0.1	0.6 ±0.0	0.0 ±0.0	12.3 ±0.2	0.6 ±0.0	0.0±0.0	12.5 ±0.0	0.2 ±0;0	0.0 ±0.0
7	12.8 ±0.0	0.2 ±0.1	1.0 ±0.0	12.8 ±0.0	0.2 ±0.0	1.0 ±0.0	12.3 ±0.0	0.4 ±0.1	0.0 ±0.0
8	14.4 ±0.5	0.4 ±0.0	1.0 ±0.0	14.4 ±0.1	0.4 ±0.0	1.0 ±0.0	09.8 ±0.0	0.8 ±0.0	0.0 ±0.0
9	13.6 ±0.2	0.4 ±0.1	0.0 ±0.0	13.6 ±0.0	0.4 ±0.0	0.0 ±0.0	11.5 ±0.1	0.6 ±0.0	0.5 ±0.0
10	14.1 ±0.1	0.6 ±0.0	0.0 ±0.0	14.1 ±0.0	0.6 ±0.0	0.0 ±0.0	15.5 ±0.0	0.2 ±0.0	0.0 ±0.0
Mean	12.8	0.4	0.4	12.8	0.4	0.4	12.4	0.4	0.3
SON permissible limit	3-5	0.29	0.2						

SON = Standard Organization of Nigeria.

for 30 min, cooled and reweighed (W_4). This process was carried out in duplicates for all the samples. Impurity in the oil was expressed as a percentage from the formula below:

$$Percentage\ impurity = \frac{W_4 - W_1 \times 100}{W_3 - W_2}$$

Determination of microbial contaminations

A stock solution of each of the samples was made by dissolving 1ml of each palm oil sample in 8 ml of prepared peptone water and thereafter 1 ml of sterile Tween 80 was added using sterile 2 ml syringe. Each solution was prepared in a Bijou bottle, capped and shaken vigorously to obtain a homogenized solution. Three fold serial dilutions were made from each stock solution by measuring 1ml of the stock solution into 9 ml of prepared peptone water in the test tube labelled as 10^{-1} and down to 10^{-3}). Aliquots of each dilution (10^{-1}-10^{-3}) were inoculated on three Nutrient Agar (NA) and Sabouraud Dextrose Agar (SDA) plates using the pour plate method. The NA plates were subsequently incubated at in an incubator at 37ºC while the SDA plates were incubated at room temperature (28 ºC) for 72 to 120 h. All bacterial and fungal isolates were sub-cultured unto freshly prepared NA and SDA plates for proper identification. Standard bacteriological and mycological identification process was strictly followed.

Determination of heavy metals

The concentrations of lead (Pb), cadmium (Cd), iron (Fe), arsenic (Ar) and copper (Cu) in the palm oil samples were determined by Flame Atomic Absorption Spectrometry (AAS). Each sample (5 g) was digested with 20 ml of acid mixture (650 ml Conc. HNO_3; 80 ml perchloric acid; 20 ml Conc. H_2SO_4).

RESULTS AND DISCUSSION

The result of some physical characteristics of the palm oil samples from the different markets are shown in Table 1. The mean moisture contents of samples from the three Markets in this study were slightly above the permissible levels for palm oil by Standard Organization of Nigeria (SON) (SON, 2000). This slight increase could be attributable to the fact that traditional methods employed in processing palm oil in these localities do not subject it to boiling at elevated temperature which could have reduced the moisture content. Orji and Mbata (2008) reported that the traditional methods employed in palm oil processing in Nigeria produced low quality product in terms of free fatty acids, moisture and impurity contents. Other workers reported on the need for improved processing technique to ensure better quality of palm oil sold in the Nigerian markets (Adebayo-Oyetero et al., 2019). However, Japir et al. (2017) reported that the physicochemical properties of high free fatty acid of crude palm oil and low

Table 2. Aerobic viable plate count of palm oil samples from the three areas.

Sample	Ankpa		Anyigba		Idah	
	Bacterial count (cfu/ml)	Fungal count (cfu/ml)	Bacterial count (cfu/ml)	Fungal count (cfu/ml)	Bacterial count (cfu/ml)	Fungal count (cfu/ml)
1	$1.05\times10^{2}\pm1.70$	$1.85\times10^{2}\pm1.50$	$1.00\times10^{2}\pm0.80$	$1.92\times10^{2}\pm1.50$	$1.20\times10^{1}\pm0.00$	$2.00\times10^{2}\pm1.00$
2	$1.31\times10^{2}\pm1.40$	$1.60\times10^{2}\pm1.20$	$2.20\times10^{2}\pm1.10$	$1.68\times10^{2}\pm1.00$	$1.70\times10^{1}\pm0.50$	$1.40\times10^{2}\pm0.90$
3	$1.80\times10^{1}\pm1.50$	$1.33\times10^{2}\pm2.20$	$2.80\times10^{1}\pm0.70$	$1.05\times10^{2}\pm0.70$	$1.50\times10^{1}\pm0.60$	$5.00\times10^{1}\pm0.30$
4	$2.30\times10^{1}\pm0.00$	$1.54\times10^{2}\pm0.80$	$1.20\times10^{2}\pm1.80$	$1.92\times10^{2}\pm1.60$	$2.00\times10^{2}\pm1.10$	$1.30\times10^{1}\pm1.50$
5	$1.73\times10^{2}\pm1.10$	$2.70\times10^{1}\pm0.40$	$2.00\times10^{2}\pm0.10$	$1.38\times10^{2}\pm0.50$	$1.30\times10^{1}\pm1.00$	$1.80\times10^{1}\pm1.20$
6	$1.54\times10^{2}\pm1.00$	$3.00\times10^{1}\pm2.80$	$1.30\times10^{2}\pm1.20$	$1.06\times10^{2}\pm1.60$	$1.50\times10^{1}\pm2.20$	$1.90\times10^{2}\pm1.00$
7	$1.30\times10^{2}\pm1.00$	$2.60\times10^{1}\pm2.40$	$3.00\times10^{1}\pm1.50$	$2.00\times10^{2}\pm1.00$	$1.90\times10^{1}\pm0.20$	$1.20\times10^{1}\pm0.80$
8	$2.07\times10^{2}\pm1.30$	$1.54\times10^{2}\pm1.70$	$1.89\times10^{1}\pm2.00$	$3.00\times10^{2}\pm1.00$	$2.80\times10^{1}\pm1.90$	$1.37\times10^{2}\pm2.40$
9	$2.00\times10^{2}\pm1.20$	$1.60\times10^{2}\pm0.00$	$1.20\times10^{2}\pm1.40$	$2.80\times10^{1}\pm00.0$	$2.00\times10^{1}\pm0.70$	$2.70\times10^{1}\pm1.00$
10	$1.20\times10^{2}\pm2.10$	$1.38\times10^{2}\pm0.00$	$1.00\times10^{1}\pm1.70$	$1.05\times10^{2}\pm2.50$	$1.06\times10^{2}\pm1.60$	$1.30\times10^{1}\pm0.20$
Mean	1.14×10^{2}	1.17×10^{2}	1.15×10^{2}	1.26×10^{2}	4.45×10^{1}	8.00×10^{1}
SON Permissible limit	2×10^{4}					

Legend: SON = Standard Organization of Nigeria.

free fatty acids of crude palm oil were consistent with the Malaysian standard for crude palm oil with the exception of free fatty acid percentage, hydroxyl value and moisture content.

Although the mean bacterial and fungal counts (Table 2) in the oil samples were within acceptable limits the high moisture content could lend to their multiplication especially as the storage time prolongs. This could lead to ease of spoilage and rancidity and shorten the shelf-life. Other workers have isolated several microbes in ready-to-use palm oil sold in different markets in Nigeria (Okechalu et al., 2011, Enemuor et al., 2012, Okogbenin et al., 2014, Seiyaboh et al., 2018, Odoh et al., 2016). Bacillus species which was isolated can cause food poisoning, bacteremia and endocarditis. It has been reported that Bacillus spores are dormant and highly resistant to lethal effects of heat and ultraviolet radiation. Aspergillus and Mucor species also produce heat resistant spores and have been noted for their ability to survive in oil by producing lipase enzyme which can lead to rancidity of the oil. The frequency of the bacterial and fungal isolates is shown in Figures 1 and 2 respectively.

Figure 1, shows the frequency of isolation of bacterial species from palm oil samples from the three markets located at Ankpa, Anyigba and Idah. The bacterial isolates included: Bacillus, Staphylococcus, Enterobacter, Proteus, Micrococcus and Pseudomonas spp. The frequency of isolation of Bacillus sp. is highest in samples from Ankpa Market while Entrobacter sp. was highest in samples from Anyigba Market. Although Staphylococcus sp. had the highest frequency in samples from Idah Market, it should be noted that samples from Idah Market had the lowest bacterial load in comparison with Ankpa and Anyigba Markets. Bacillus, Enterobacter and Proteus spp. were all isolated from samples from the three markets.

Figure 2, shows the frequency of isolation of fungal species in the three markets located at Ankpa, Anyigba and Idah. The fungal isolates included: Candida, Aspergillus, Rhizopus, Mucor and Penicillum spp. All the fungal species were isolated from samples from the three markets. Candida sp. had the highest frequency from the three markets followed by Aspergillus sp. Samples from Anyigba Market had the highest fungal load than that from Ankpa and Idah Markets followed by samples from Ankpa Market. Although the frequency of Candida sp. in samples from Idah Market was high however, they (samples) had the least fungal load comparatively. All the microorganisms isolated in this study have been implicated in similar studies by other workers (Okechalu et al., 2011; Enemuor et al., 2012; Okogbenin et al., 2014; Ohimain and Izah, 2015; Odoh et al., 2016; Seiyaboh et al., 2018;

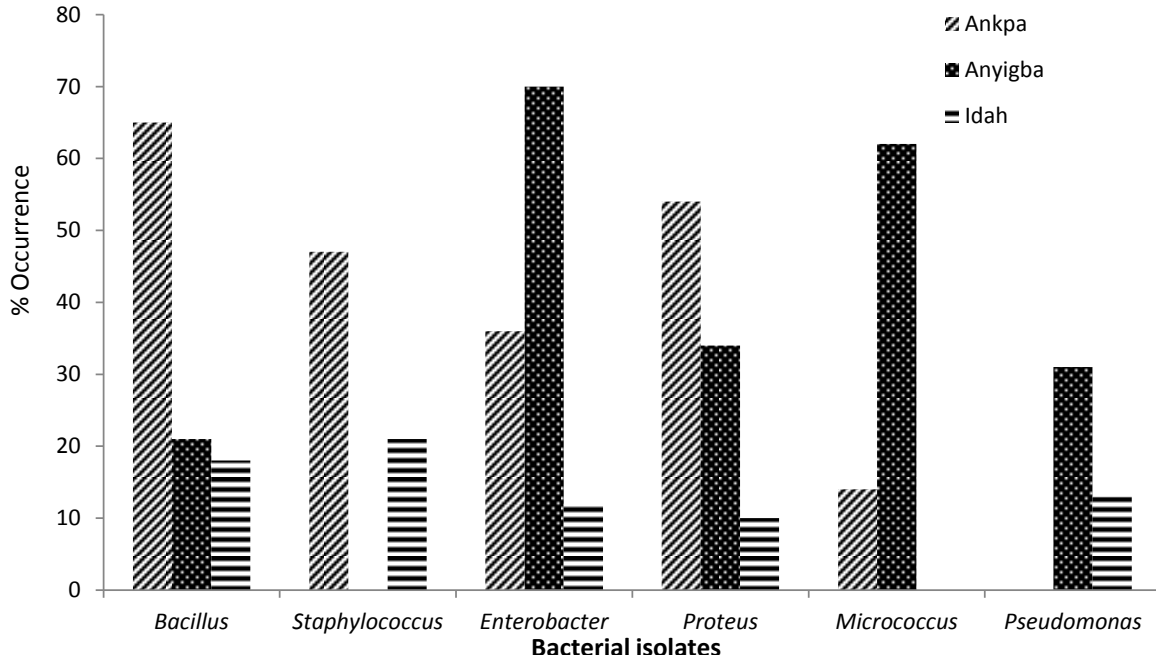

Figure 1. Percentage frequency of occurrence of bacterial isolates in palm oil samples.

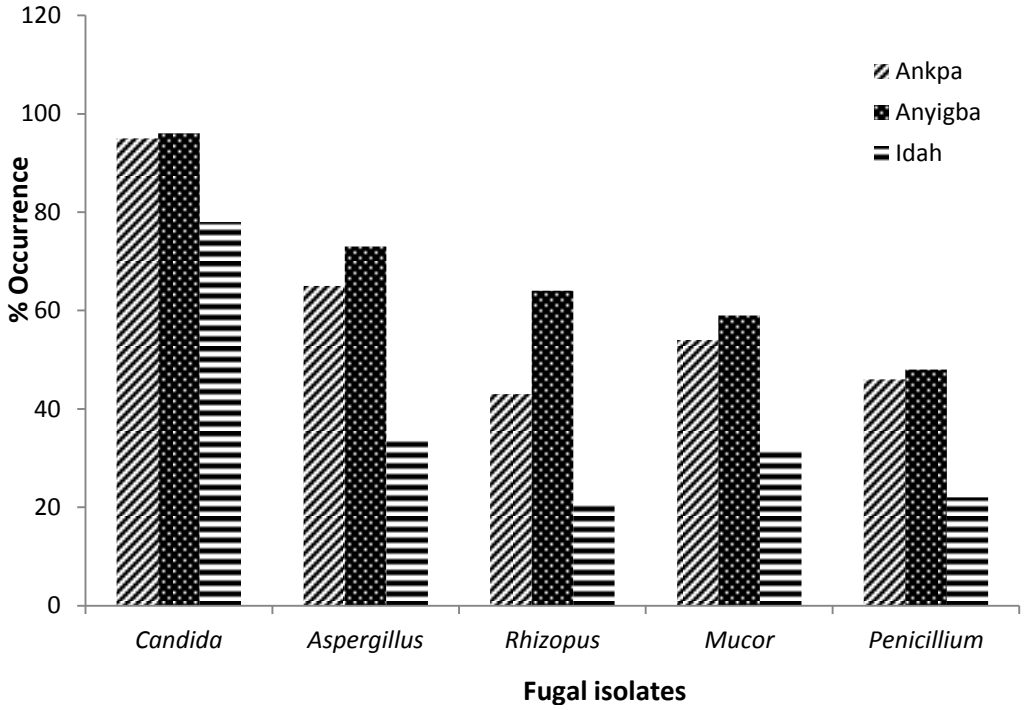

Figure 2. Percentage frequency of occurrence fungal isolates in palm oil samples.

Ngangjoh et al., 2020). It was observed that palm oil samples from Idah area had lowest microbial load and frequency of the isolated microorganisms (Table 2, Figures 1 and 2). The fact that the mean concentrations of Cd and Fe in palm oil samples from Idah (Table 3) were higher than in samples from Ankpa and Anyigba (and above WHO acceptable limit) could offer some explanations for the low microbial load. Several reports

Table 3. Comparison of detectable metals in palm oil samples from Ankpa, Anyigba and Idah areas.

Metal	Mean concentration (ppm)			
	Ankpa Market	Anyigba Market	Idah Market	WHO limits
Cadmium (Cd)	0.010	0.001	0.460	0.050
Chromium (Cr)	<0.001	<0.001	0.040	0.100
Lead (Pb)	<0.001	<0.001	<0.001	0.010
Arsenic (As)	0.040	0.290	0.190	0.010
Copper (Cu)	<0.001	0.006	0.030	1.300
Iron (Fe)	1.880	4.660	7.340	1.000

WHO: World Health Organization; ppm: parts per million.

have elucidated on the toxicity of heavy metals on microbial growth by interfering with the biochemical and physiological properties of microorganisms (Igiri et al., 2018; Oijagbe et al., 2018; Xie et al., 2016; Chen et al., 2014; Das et al., 2012). Table 3 shows the mean concentrations of some heavy metals in the palm oil sample from the three local markets.

The mean concentrations of As and Fe were above acceptable limits in all the samples from the three area markets.

Arsenic can cause a number of human health effects and diet has been reported as the largest source of exposure to arsenic (Tchounwou et al., 2012). Therefore, consumption of the palm oil sold in these markets exposes the consumers to these health risks. The mean concentration of Cd in the samples from Idah Market was also above WHO permissible limits. It has also been reported that human exposure to Cd is possible through eating contaminated food (Tchounwou et al., 2012). Blood vessels are considered to be the organs of Cd toxicity and Cadmium compounds are classified as human carcinogens by several regulatory agencies (Tchounwou et al., 2012).

Several studies conducted in different parts of Nigeria reported detectable amounts of heavy metals including Cd, Cr, Ar and Pb in the ready-to-consume red palm oil samples sold at the local markets (Adepoju-Bello et al., 2012; Aigbemu et al., 2017; Asemave et al., 2012; Tor et al., 2017; Nnorom et al., 2014; Ogabiela et al., 2010). Some of the detected metal concentrations in these studies were reported to fall within permissible limits. They also speculated that source of metals in edible oils could be from soil, environmental or implements during processing.

Obviously, palm oil sold in markets in urban areas and cities in Nigeria could be supplied from different geographical locations within the country that have oil palm plantations. Soils from different locations differ in metallic content. Idah area has oil palm plantations and is located at the bank of River Niger and upstream are located Ajaokuta Steel Company and iron ore mining activities at Itakpe within the same region. These industrial activities tend to confirm the possible metallic content of soil in this area. Again, when the river overflows its banks during the long raining seasons the soil could be contaminated the more with metals coming down from the wash offs from the industries located upstream. The bioavailability of metals in soils on oil palm plantations in Nigeria has been assessed (Olafisoye et al., 2016). They concluded that the concentrations of Pb and Cu were highest in the organic fraction and that metals in the organic phase are more easily released into soil solution comparatively. In Malaysia, other workers determined selected heavy metal concentrations in an oil palm plantation soil and concluded that the soil was uncontaminated but noted that the detectable amounts of metals were from application of chemical fertilizers (Ab-Manan et al., 2018). In another study on the accumulation and risk assessment of metals in palm oil from contaminated oil palm plantation soils, Olafisoye et al. (2020) reported that there is a correlation between the accumulations of metals from soil to palm oil. Apart from oil palm trees assimilating these metals from the soil more contamination of the palm oil by these metals could come from the contaminated water used by locals in the processing because they do have access to treated water supply.

The source of Fe contamination could as well be from wears of metallic implements and equipment used in local processing of palm oil in these three localities as its average concentration in the samples from these zones is above permissible limits. Iron poisoning has always been of interest mainly to pediatricians because children are highly susceptible to iron toxicity (Erber, 2012; Gupta, 2016; Kleiner, 2018).

Conclusion

From the findings, the microbial load of the samples fell within the acceptable limits stipulated by regulatory agencies. However, most of the heavy metals assayed for were above acceptable limits. The present findings indicate the need for refining of our locally produced palm oil to eliminate metal contaminants because of the health implication of their accumulation in the body.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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