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Prevalence and antibiogram of *Listeria monocytogenes* isolated from ready-to-eat vegetables and fermented milk in Yola, Nigeria

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ABSTRACT

Aim: *Listeria monocytogenes* (*L. monocytogenes*) has been reported as the major pathogen that contributes to foodborne illness. This study was conducted to determine the prevalence and to characterize *Listeria monocytogenes* isolated from ready to eat vegetables and local fermented milk (Nono) in Yola, Nigeria. **Methods:** A total of 162 samples of cabbage, lettuce, ready to eat salad and fermented milk were examined on PALCAM and Brilliance Listeria Agar. **Results:** Distribution of *L. monocytogenes* from the study showed that cabbage had the highest prevalence of 42.3% followed by lettuce with 26.9%. The least occurrence (11.5%) was obtained from fermented milk. Furthermore, *L. monocytogenes* occurrence was from vegetable obtained from shinko farm (53.9%) while least (19.2%). The total bacterial counts of the ready to vegetables and fermented milk from the study ranged from 6×10^3 cfu/ml to 1.86×10^5 cfu/g. The result also showed that cabbage had the highest contamination from all the study locations and fermented milk from all the study locations had the least total bacterial counts. Antimicrobial susceptibility screening of the isolates revealed that more than 60% of the isolates were susceptible to commonly used antibiotics. However, about 65% of the isolates demonstrated resistance to erythromycin and cotrimoxazole. Also, none of the isolates harbored any plasmids. **Conclusion:** Findings from this study are of public health significance as all the samples studied do not require cooking before they are consumed. Thus, there is need to exercise caution during purchase, processing and consumptions of these vegetables and milk.

Introduction

Listeria monocytogenes (*L. monocytogenes*) is a small, facultatively anaerobic, gram-positive, non-sporulating motile bacterium

with peritrichous flagellation. The organism has been tagged foodborne pathogen in the early 1980s but the infection primarily affects children, the elderly and immunocompromised individuals

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causing serious diseases conditions like septicaemia, encephalitis and meningitis [1]. In pregnant women, *L. monocytogenes* infections have been linked to abortion and stillbirth however. In healthy people, it causes fever, vomiting and diarrhoea. Worldwide; this foodborne disease is relatively rare but serious. About 1,500 cases per year are recorded in Europe and 2,500 per year in US. Because of its high fatality rate (between 20 and 30 %), listeriosis ranks among the most frequent cause of human death due to foodborne illnesses [2]. The World Health Organization (WHO) estimates the global burden of listeriosis to be 172,823 disability adjusted life years (DALYs) from 23,150 illnesses [3]. Listeriosis infections are exceptionally dangerous to high-risk individuals, causing an unprecedented average mortality rate of approximately 30%

Generally ready to eat foods such as vegetables, raw meat, fish, milk, milk related products have been linked with listeriosis. Therefore, possible contamination of foods represents a significant health hazard when the contaminated foods are stored or distributed, even in a refrigerator. Human infections primarily result from eating contaminated food and may lead to serious and potentially life-threatening listeriosis depending on the individual. Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illness [4]. The presence of this bacterium in the processing of foods and its natural distribution within the environment coupled with its inherent resistance and ability to grow in some foods, makes it difficult to control and regulate [5]. The proliferation of vendors of ready to eat vegetables in Yola, Nigeria and other parts of the country often without any regulation puts consumers of such ready to eat vegetables and milk at risk of contracting infection by *L. monocytogenes* and other agent of food infection. The recommended count of *L. monocytogenes* in ready to eat food is reported to be less than 100cfu/g at the time of consumption as specified by the European Commission and the International Commission on Microbiological Specification for foods [6]. However, USDA, [7] recommended zero tolerance for *L. monocytogenes* in ready-to eat foods in US. There are reports of the recovery of *L. monocytogenes* from ready to eat vegetables in different parts of Nigeria [8- 10] but none has been reported for Yola, Nigeria. Besides the problem of contamination of foods with *L.*

monocytogenes, there have been reports on evidences on the emergence of antibiotic resistance among *L. monocytogenes* strains isolated from food products. The resistance of *L. monocytogenes* isolates from food to antibiotics currently used in the treatment of human listeriosis such as penicillin, ampicillin, tetracycline, and gentamicin, has been documented [11]. The report shows that acquisition of movable genetic elements is considered the major mechanism of antibiotic resistance development in *L. monocytogenes*.

This work was aimed at determining the prevalence and antibiogram of *L. monocytogenes* in ready to eat vegetables and fermented milk sold in Jimeta-Yola.

Materials and methods

Study area

This study was conducted in Yola, North East Nigeria. The town is situated in the semi-arid belt of Northern Nigeria. It lies along the Benue River with 9 o 10' N to 9 o 15' N and 12 o 20' E to 12 o 30' E, Yola is the capital city and administrative centre of Adamawa State, Nigeria. Located on the Benue River, it has a population of 336,648. The study area were three major markets in Yola metropolis notable for trading in ready to eat vegetables and fermented milk labelled markets 1, 2 and 3.

Sample collection

A total of 260 samples comprising Cabbage (62), Lettuce, (46), ready to eat salad (31) and local fermented milk (23) were purchased from three markets in Yola metropolis from vendors. These samples were bought and packaged in sterile plastic containers as they are sold to consumers. They were labelled appropriately and transported to the Microbiology Laboratory, Modibbo Adama University, Yola for processing and analysis.

Sample processing

For cabbage, ready to eat salad and lettuce, 10 g of each of the sample was weighed into 90 ml of peptone water in a blender and homogenized for 3 minutes. [12]. For fermented milk, 10 ml was measured into sterile test tube containing 90 ml of peptone water and vortexed to mix. After homogenizing the samples, each was serially diluted up to 10⁻⁵.

Total bacterial count

After processing of the samples, 1 ml of the 10⁻³ dilution of the homogenized samples was transferred unto the surface of plate count agar and

spread using a sterile bent glass rod. this was allowed to stand for 15 minutes on the bench top before incubating at 37⁰ C for 24 h.

Isolation and identification of *L. monocytogenes*

The method of *L. monocytogenes* isolation reported by Swetha et al. [6] was adopted. Ten (10 ml) millilitres each of homogenized cabbage, lettuce, ready to eat salad was inoculated into 90 ml of Listeria enrichment broth and incubated at 37⁰ C for 24 h. After enrichment, a loopful of the enriched inoculum from the broth was streaked onto PALCAM agar plate and incubated at 37⁰ C for 24 h. Green colonies surrounded by a black zone on PALCAM agar plate were considered presumptive *L. monocytogenes*. The presumptive *L. monocytogenes* isolates were subjected to Gram staining, catalase, oxidase, indole, methyl red, voges proskaeur, citrate utilization, nitrate test, urease test, motility test, CAMP, and sugar fermentation tests (Rhamnose, xylose, glucose). The isolates were then confirmed on brilliance *Listeria* agar. Confirmed isolates were then stored on nutrient agar for further use.

Haemolysis test

The isolates were inoculated aseptically on to 7% sheep blood ager (SBA) and incubated at 37⁰ C for 24 h. The haemolytic zones around the colonies showed a beta zone haemolysis around the colonics indicate a positive test of *L. monocytogenes*

Christie, Atkins, Munch-Petersen (CAMP) test

This was performed according to the procedure described by Guo et al. [13]. A β-haemolytic strain of *Staphylococcus aureus* was grown overnight on 7% SBA at 37⁰ C and a colony was streaked again on freshly prepared 7% SBA in a manner that the streak allows for streaking of *Listeria* colonies. *Listeria* isolates was streaked at 90⁰ angle and 3 mm apart before incubating them at 37⁰ C for 24 h. A positive reaction was indicated by an enhanced zone of haemolysis at the intersection of the test and indicator strain.

Antibiotic sensitivity

The agar disc diffusion method as described by Clinical and Laboratory Standard Institute (CLSI) [14] was used to determine the antibiotic susceptibility of *L. monocytogenes* isolates on Mueller Hinton agar. A sterile pipette was used to transfer 1 ml of standardized inoculum adjusted to 0.5 McFarland (approximately 10⁸ cfu/ml), onto the surface of Mueller Hinton agar. A sterile bent glass rod was then used to spread the inoculum on the

entire surface of Muller Hinton agar (Oxoid) plate. It was allowed to stand for 10 minutes at room temperature. Standard antibiotic disc obtained from Oxoid (Hampshire, United Kingdom), gentamicin (30 µg), ciprofloxacin (5 µg), penicillin (10 µg) chloramphenicol (30 µg) ceftriaxone (10 µg), azithromycin (10 µg) oxacillin (5 µg), cotrimoxazole (25 µg), tetracycline (30 µg) and erythromycin (15 µg) were applied to the Muller Hinton agar plates using a disc dispenser (Oxoid) and the plates were incubated at 37⁰ C for 18 h. The zone of inhibition observed were measured to the nearest millimetre and used to determine the susceptibility of the isolates according to the CLSI guidelines.

Plasmid profile analysis

Screening for the presence of plasmid *L. monocytogenes* was done using the direct and indirect method. The indirect method of screening for plasmid using 10% sodium dodecyl sulphate for curing of plasmid using antibiotic resistance gene as markers described by Mirmomeni et al. [15]. The direct method of screening for plasmid involving plasmid extraction using the alkaline lysis method and agarose gel electrophoresis described by Peterkin et al. [16]

Results

Result of cultural, morphological, and biochemical characteristics of bacteria isolated from the sample collected showed that the isolates were catalase positive, methyl red positive, beta haemolytic and CAMP positive Gram positive non-sporulating rod (**Table 1**). In all, 26 samples representing 16.0 % yielded growth of *L. monocytogenes in vitro*. The results showed that all the isolates were virulent because of the positivity of the CAMP test.

Results of the distribution of *L. monocytogenes* in relation to the type of sample collected showed that the highest *Listeria* occurrence was from cabbage (17.7%) followed by ready to eat salad with an occurrence of 16.1%. The least *L. monocytogenes* occurrence was from fermented milk (13.0%) (**Table 2**). Chi square analysis of the difference in the distribution of *L. monocytogenes* in relation to type of sample collected is however not statistically significant at $p=0.05$.

The frequency and distribution of *L. monocytogenes* based on sample collection point showed that samples collected from market No. 1

had the highest occurrence of *L. monocytogenes* of 19.4 % followed by market No. 2 (14.6 %). The sample location with the least *L. monocytogenes* occurrence was market No. 3 11.9 % (Table 3). The difference in the occurrence of *Listeria* from the different sampling location is not significant at $p=0.05$.

The total bacterial counts of the ready to vegetables and fermented milk from the study ranged from 6×10^3 cfu/ml to 1.86×10^5 cfu/g. The result also showed that cabbage had the highest contamination from all the study locations (1.56×10^5 cfu/g - 1.86×10^5 cfu/g). Furthermore, fermented milk from all the study locations had the least total bacterial counts.

The result of the antibiotic susceptibility pattern of *L. monocytogenes* isolates from this study is shown in table (4). The isolates predominantly demonstrated highest susceptibility to azithromycin (88.6%), gentamycin (80.8%), ofloxacin (76.9%), ciprofloxacin (73.1%) and ceftriaxone (73%). However, some of the isolates demonstrated highest resistance to tetracycline (92.3%) and penicillin (88.4%).

Result of plasmid curing experiment showed that there was no difference between the pre curing and post curing antibiogram of the isolates. Furthermore, agarose gel electrophoresis showed no plasmid band after plasmid DNA extraction using standard protocol.

Table 1. Biochemical, virulence and fermentation reaction of *Listeria Monocytogenes* isolates.

Test	<i>L. monocytogenes</i>
CAMP	+
Catalase	+
Gas production	-
Gram reaction	+
H ₂ S Production	-
Haemolysis	+
Indole	-
Methyl Red	+
Nitrate Reduction	-
Oxidase	-
Voges Proskauer	+
Urease	-
Xylose fermentation	-
Rhamnose fermentation	+
Glucose fermentation	+

Table 2. Average Total Bacterial Count by sample by market (CFU/g).

Sample type	Market No. 1	Market No. 2	Market No. 3
Cabbage	$1.86 \times 10^5 \pm 0.21$	$1.74 \times 10^5 \pm 0.44$	$1.56 \times 10^5 \pm 0.27$
Lettuce	$3.6 \times 10^4 \pm 0.33$	$3.9 \times 10^4 \pm 0.38$	$4.2 \times 10^4 \pm 0.51$
Ready to eat salad	$3.8 \times 10^4 \pm 0.45$	$2.5 \times 10^4 \pm 0.29$	$5.2 \times 10^4 \pm 0.22$
*Fermented milk	$1.6 \times 10^4 \pm 0.6$	$2.6 \times 10^4 \pm 0.9$	$6 \times 10^3 \pm 0.13$

KEY * = Total bacterial count in cfu /ml SEM= Standard error of mean

Table 3. Distribution of *Listeria Monocytogenes* based on sample types.

Sample type	No collected	Number positive (%)	P value
Cabbage	62	11 (17.7)	
Lettuce	46	7 (15.2)	0.9502
Ready to eat salad	31	5 (16.1)	
Fermented milk	23	3 (13.0)	
Total	162	26 (16.0)	

Table 4. Distribution of *Listeria monocytogenes* based on area of collection.

Sample collection area	Number collected	Number positive (%)	P value
Market No. 1	72	14 (19.4)	0.7434
Market No. 2	48	7(14.6)	
Market No. 3	42	5(11.9)	
Total	162	26 (16.0)	

Table 5. Antibacterial susceptibility profile of *Listeria monocytogenes* isolates from study area.

Type of antibiotics	Susceptible (%)	Resistant (%)
Azithromycin	23 (88.6)	3 (11.5)
Gentamycin	21 (80.8)	5 (19.2)
Ciprofloxacin	19 (73.1)	7 (26.9)
Chloramphenicol	16 (61.5)	10 (38.5)
Erythromycin	9 (34.6)	17 (65.3)
Cotrimoxazole	8 (30.8)	18 (69.2)
Ceftriaxone	19 (73.0)	7 (26.9)
Ofloxacin	20 (76.9)	6 (23.1)
Penicillin	3 (11.5)	23 (88.4)
Tetracycline	2 (7.7)	24 (92.3)

Discussion

The consumption of vegetable in Nigeria and Adamawa State in particular is on the increase mostly due to that fact that their proven nutritional and medicinal benefit is being appreciated. This increase in vegetable consumption has brought with it the greater risk of infection from food pathogens especially listeriosis. From this study, *L. monocytogenes* prevalence from ready to eat vegetables and fermented milk was 16.0 %. This is lower than the 44% reported by **Ajayeoba et al.** [8] from vegetables in South West Nigeria and the 22.2% prevalence reported from ready to eat foods in Africa by **Dufailu et al.** [3]. The prevalence from this study is higher than the 3.9% reported from vegetables in Zaria by **Ieren et al.** [17]. Result obtained from this study confirmed earlier reports that vegetables are important vehicles for the transmission of *L. monocytogenes*. This further indicates that *L. monocytogenes* is prevalent in the study area and therefore pose a serious risk to consumers of ready to eat vegetables and fermented milk especially children, the elderly, immunocompromised individuals and pregnant women because they are the most vulnerable.

Listeria monocytogenes distribution from the study showed that cabbage had the highest occurrence of *L. monocytogenes* (17.7%) followed by ready to eat salad (16.1%). These findings are in agreement with earlier reports by **Ajayeoba et al.** [8] who reported that cabbage had the highest *L.*

monocytogenes prevalence (28.28%) in South East Nigeria. They further reported that In Lagos and Ondo states, Cabbage had the highest occurrence of *L. monocytogenes* of 7.38% and 4.92% respectively. The presence of glucose in cabbage which can be used by the organism as a source of carbon and energy could be possible reason for the highest prevalence of *L. monocytogenes* in cabbage. The presence of *L. monocytogenes* in cabbage and ready to eat salad from the study area is not surprising since some farmers of vegetables in the area rely heavily on the use of water contaminated with sewage sludge to irrigate the soil where they cultivate these vegetables. This practice has been reported to increase contamination of ready to eat vegetables. Furthermore, storage of vegetables in unhygienic environments, unhygienic handling during harvest, transport and market activities as well as the use of contaminated water to wash or sprinkle the products to keep them fresh during the characteristic hot and dry season temperature of Yola, Nigeria can add to the *L. monocytogenes* contamination [18]. Also, that *L. monocytogenes* was recovered from these ready to eat vegetables implies that these products failed microbiological standards for such foods because the USDA [6] recommended zero tolerance for *L. monocytogenes* in ready-to eat foods.

The frequency and distribution of *L. monocytogenes* based on sample collection point showed that samples collected from ‘Shinko’ farm had the highest occurrence of *L. monocytogenes* of 19.4 % followed by Jimeta main market (14.6%). This difference could be attributed to chance and not sample location. Recovery of this pathogen from these markets is not strange as *L. monocytogenes* has also been isolated from ready to eat products in different markets across the globe. These include the works of **Porto et al.** [19], **Little et al.** [20] and **Meloni et al.** [21] who reported recovery of *L. monocytogenes* in Brazilian markets, United Kingdom and Italy respectively. The differences in the level of contamination of the RTE vegetables and fermented milk in different markets may be due to the handling, transportation or environment in which these products are stored, processed and retailed. The other reason is the fact that there is no standard handling, processing, storage and dispensing, method in Nigeria for these products and even where there are, they are not enforced. In all these markets, these products are not stored and retailed in a safe environment. Reports have shown that ready to eat foods are a major public health hazard in developing countries due to lack of basic infrastructures and facilities to maintain their diversity, mobility, and temporary nature which could initiate microbial contamination from sand, water, dust, and air [22, 23].

The result of the antibiotic susceptibility pattern of *L. monocytogenes* showed that the isolates demonstrated highest susceptibility to azithromycin (88.6%), gentamycin (80.8%) and ciprofloxacin (73.1%). This finding is consistent with previous work reported by **Oyinloye** [24] that *L. monocytogenes* isolates from Ekiti, South-West Nigeria were susceptible to ciprofloxacin. This is good because these drugs can be used to effectively control possible infections with these isolates in the study area. Furthermore, the isolates demonstrated the highest resistance to tetracycline (92.3%), penicillin (88.4%). Also, varying degrees of resistance was observed to norfloxacin (57.2%), levofloxacin (71.4%), and ciprofloxacin (71.4%). This is worrisome because this trend will pose a problem to chemotherapy as the isolates tend to possess several resistance genes that could bring about treatment failure if the choice of antibiotic is made without using laboratory support.

Result of plasmid profile analysis using the direct and indirect method, method from this study

showed that the isolates do not carry plasmids. The absence of plasmids among *L. monocytogenes* isolates from this study brings in a ray of hope in the management and control of *Listeria* infections in the study area although plasmids have been extracted from listeria in other studies.

Conclusion

The results obtained from this study have shown that *Listeria monocytogenes* is present in salad, ready to eat vegetables and local fermented milk sold in Jimeta. These findings are of public health significance because it shows that consumers of ready to eat vegetables and fermented milk stand the risk of listeriosis. This therefore requires that cabbage and lettuce need to be washed more thoroughly to reduce their chances of being involved in the transmission of *L. monocytogenes*. Furthermore, those handling local fermented milk should observe good hygiene practice to minimize the chances of contamination of the milk with *Listeria* and other pathogenic microbes.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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