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Isolation and Antimicrobial Susceptability of Listeria *Monocytogenes* from Raw Milk and Milk Products in Northern Kaduna State, Nigeria

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Abstract *Listeria (L.) monocytogenes* is an 'emerging food borne pathogen' that poses threat to global food safety. Its occurrence in milk and milk products constitute a negative impact to the dairy industry. Despite its veterinary public health significance, only few studies are available on the prevalence and characteristics of the organism from milk and milk products in Nigeria. The main objectives of this study were to isolate, identify and determine the antibiotic susceptibility patterns of the *Listeria monocytogenes* isolated from raw milk and milk product in Kaduna state Nigeria. A total of 550 samples of raw milk and milk products (fermented milk "Kindrimo" and milk butter "Manshanu") were collected during a cross-sectional study. Of these 550 samples, 193 (35.10%) samples were positive for *Listeria* species- like growth when plated on chromogenic *Listeria* agar, 91 (47.15%) from raw milk, 65 (33.67%) from 'Manshanu' and 37 (19.15%) 'Kindrimo'. A total of 36 (6.55%) were found to be *L. monocytogenes* based on conventional biochemical tests. However, when further subjected to Microbact™ *Listeria* 12L detection Kit (Oxoid, MB1128A) only 3 (8.3%) of the 36 isolates were identified as *L. monocytogenes*, while the others were *L. grayi* 4 (11.1%), *L. ivanovii* 27 (75.0%) and *L. seeligeri* 2 (5.6%). The multiplex PCR assay identified 9 *Listeria (L.) monocytogenes* isolates harbouring the *hly* A gene. The susceptibility of 36 *L. monocytogenes* isolates to 10 antibiotics was evaluated using the disk diffusion method. Isolates from milk samples had an overall resistance of 64.09% to the antibiotics, followed by isolates from 'Kindrimo' (61.67%), and least resistance was observed in isolates from 'Manshanu" (58.33%). Overall, *L. monocytogenes* isolates showed the highest frequency of resistance to ampicillin (100%), followed by penicillin (95%) and cloxacillin (90%). In conclusion, the isolation of *L. monocytogenes* and other *Listeria* species in this study calls for an improved hygienic practices in the milk and milk products production channels and for the enlightment of the Fulani herds men and women by agricultural extension workers. Also, the resistance pattern shown by the isolates is an indication that the use of antibiotics should be regulated to minimize the incidence of antibiotic resistance.

Keywords: *listeria monocytogenes, isolation, antibiotic resistance*

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1. Introduction

Milk is composed of water, carbohydrate, fats, proteins, minerals and vitamins. Milk plays a leading role in improving the nutritional status of human populace, most especially in areas of high prevalence of protein deficiency [1]. However, it often serves as a medium for milk borne diseases [2]. As a practice and source of food, pastoralist usually process fresh milk into various traditional products such as 'Nono' (Sour milk), 'Kindrimo' (sour yoghourt), 'Manshanu' (local butter) and 'Cuku' (Fulani cheese) [3]. Dairy products such as milk, cheese, ice cream and butter have all been implicated as a cause of milk borne diseases [4]. Traditionally fermented milk products are commonly consumed in Asia, Africa, the Middle East and Northern and Eastern Europe. However, preparation and composition of milk product varies from one region to the other [5].

The genus Listeria consists of 10 species; L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi, L. marthii [6], L. rocourtiae [7], L. fleischmannii [8], and L. weihenstephanensis [9]. Recently, five new Listeria species (L. floridensis sp. Nov., L. aquatic sp. Nov., L. cornellensis sp. Nov., L. riparia sp. Nov., and L. grandensis sp were identified from agricultural & natural environments in the United States [10]. No haemolytic activity or PI-PLC activity, suggesting these species lack the genes (hly A and plc A) associated with virulence in L. monocytogenes and L. ivanovii. The absence of virulence genes found in the prf A cluster [11] or LIPI-2 [12] from the draft genome,

further support that none of the new species described here are pathogenic.

L. ivanovii and *L. monocytogenes* are pathogenic for mice and other animals. However only *L. monocytogenes* is commonly associated with human listeriosis. Listeriosis associated infection by *L. ivanovii*, and even *L. seeligeri* is extremely rare in humans. The major mode of transmission of *L. monocytogenes* is via the ingestion of contaminated food [13]. Human listeriosis is a sporadic disease, which is associated with consumption of contaminated milk, soft cheese, under-cooked meat, and unwashed raw vegetables and cabbage [14,15,16]. In human, the illness may range from mild flu-like sickness to severe manifestations (encephalitis, meningitis, septicaemia, abortion, premature birth, stillbirth, and abscesses).

Listeriosis is ranked third of the major pathogens transmitted by food [17]. Studies on the microbiological characteristics of several fermented milk products have been carried out in many countries, including Nigeria [18]. Thus, outbreaks are reported on a regular basis, even though most cases occur unexpectedly [19]. High risk groups include the old, young, pregnant women and immunocompromised individuals [20]. The disease poses a very serious public health problem, due its high case fatality rate of 30%, which might rise to 75% in high risk individuals [21]. It has a high hospitalization rate compared to other food borne infections [22]. With the exception of cephalosporins, fosfomycin, and early quinolones to which innate resistance has been reported [23,24]), *L. monocytogenes* is generally considered sensitive to most clinically relevant antibiotics [23,25]. Even though antibiotics are the drug of choice, multidrug resistant strains have been identified [26]. At present, there is limited data on the prevalence and antimicrobial susceptibility patterns of *L. monocytogenes* in milk and milk-product in Nigeria. Hence the present study was undertaken.

2. Materials and Methods

The study was designed as a cross-sectional study and sampling was done for seven months between the periods of August, 2012 to February, 2013. Samples of raw milk 173(31.5%) and milk products 377(68.5%) making a total of 550, were collected using 50 ml sterile plastic containers (for raw milk and fermented milk 'Kindrimo') and 50 ml polythene bags (for milk butter 'Manshanu') respectively, from four Local government areas (Sabon gari, Lere, Makarfi and Zaria). They were labelled appropriately and transported on ice to the Bacterial Zoonosis Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria where the conventional biochemical analysis and antibiotic susceptibility testing was undertaken. The reference strain, *Listeria monocytogenes* ATCC 19155 used as positive control in this study was kindly provided by Prof. Son Radu of the Centre of Excellence for Food Safety Research, Faculty of Food Science and Technology, University Putra Malaysia, where the molecular studies aspect was carried out.

2.1. Isolation and Identification of *L. monocytogenes*

The isolation of *Listeria monocytogenes* was adapted according to the procedure of Roberts *et al*. [27]. Ten ml of the incubated homogenate were added to 90 ml *Listeria* enrichment broth (Oxoid, CM 0862), which contains selective *Listeria* enrichment supplements (Oxoid, SR 0141E) and incubated at 30^{o}C for 48hrs. A loop full of the 48hrs broth was cultured onto Chromogenic *Listeria* agar (Oxoid, CM 1080) plates, which contains brilliance ^{TM} *Listeria* differential supplement (Oxoid, SR 0228E) and brilliance ^{TM} *Listeria* selective supplement (Oxoid, SR 00227) and the plates was incubated at 37°C for 24-48hrs. Presumptive colonies of *Listeria monocytogenes* having a greenish-blue coloration were picked and streaked on Nutrient agar (Oxoid, CM 0003) slants and incubated at 37°C for 24hrs, before storage at 4-5°C. The suspected isolates were further subjected to conventional biochemical test [Gram staining, oxidase, catalase, β-hemolysis, bile asculine and carbohydrate fermentation test (xylose, Mannitol, Rhamnose, Maltose, Inositol and Sucrose)] as described by [28]. Isolates found to ferment mannitol and xylose, and positive to rhamnose were considered *L. monocytogenes*. Further characterization using Microbact ^{TM} 12L *Listeria* identification kit (Oxoid, MB 1128) was carried out according to the manufactures instructions.

2.2. DNA Extraction and Multiplex Polymerase Chain Reaction

Bacterial genomic DNA was extracted from the *L. monocytogenes* isolates using the Quick-g DNA^{TM} miniprep kit, (Zymo Research, S.A.) following the manufacturer's instructions. Multiplex PCR was used to detect *L. monocytogenes* isolates harbouring *hly* A gene. The primer pairs designated as LM1 & LM2 (LM1 5′–CCT AAG ACG CCA ATC GAA-3′and LM2 –5'-AAG CGC TTG CAA CTG CTC-3′) were used for the detection of *L. monocytogenes* harbouring *hly* A gene. On the other hand, primer pairs designated as U1 and LI1 (LI1 5'–CTC CAT AAA GGT GAC CCT-3′ and U1 5'– CAG CMG CCG CGG TAATWC-3′), were used to amplify a 938bp region in the 16S rRNA gene for the detection of *Listeria* genus [29]. The primers were synthesized by Invitrogen USA, and were 18-20bp in length. *L. monocytogenes* ATCC 19155 was used as a positive control. The PCR amplification was carried out in a 25µL reaction mixture, that consisted of 5µL of 5× PCR buffer, dNTPs (0.5µL), $MgCl_2$ (2µL), Taq DNA polymerase (0.5µL), 0.5µL of each 20pM primer [LM1, LM2, U1 & LI1], 13µL of distilled water and 2µL of DNA template. A 96- well thermal cycler (Applied biosystems, Foster city, CA) was used for the amplification. PCR amplification was carried out in a programmed thermocyler with following conditions; hot start PCR at 95°C for 3mins; followed by 35cycles each, of 30 sec denaturation at 94°C, 15 sec annealing at 53°C, 90 sec extension for 72°C, and final extension at 72°C for 7 mins.

Five (5) micro litres of the PCR products was electrophoresed in 1.0% Agarose gel, stained with

Ethidium bromide, at 100V for 45min and visualized under UV illuminator (SYNGENE, Biosystems UK, 2000). A 100bp DNA molecular ladder (Promega, Promega Corporation, USA) was included to determine the size of the amplified products.

2.3. Antibiotic Sensitivity

The disc diffusion method was used to determine the antibiotic susceptibility of the *L. monocytogenes* isolates on Mueller Hinton agar [30]. Three to five pure colonies of *L. monocytogenes* isolates were inoculated into 10 ml of brain heart infusion broth (Oxoid, CM 1135) and the test tube was diluted with normal saline to a turbidity equivalent to 0.5 McFarland standards (approximately 10^8 cfu/ml), and incubated at 37°C for 18-24hrs.The entire surface of a dried Mueller Hinton agar (Oxoid, CM0337) plate was swabbed with the 24hrs inoculum using a sterile cotton swab and allowed to dry for 10 minutes at room temperature under a biological hood. Standard antibiotic discs obtained from Oxoid (Hampshire, United Kingdom), Gentamicin (30µg), Ciprofloxacin (5µg), Ampicillin (10µg), Chloramphenicol (30µg), Streptomycin (10µg), Penicillin (10µg), Oxacillin (5µg), Sulfamethoxazole-Trimethoprim (25µg), Tetracycline (30µg) and Erythromycin (15µg) were applied to the Mueller Hinton agar plates using a disc dispenser (Oxoid, ST8090) and the plates were incubated at 37°C for 18 hrs. The zone of inhibition that were observed were measured to the nearest millimetre and used to determine the susceptibility of the isolates according to the Clinical and Laboratory Standards Institute standard guidelines [31]. *Staphylococcus aureus* ATCC 25923 was used as a control strain [32]. Strains were evaluated as susceptible or resistant. Multiple antibiotic resistance (MAR) index for each resistance pattern was calculated by the formula given by [33]

$$\text{MAR} = \frac{\left(\begin{array}{l}\text{Number of antibiotics to which}\\ \text{the isolate was resistance to } (a)\end{array}\right)}{\text{Total number of antibiotics tested } (b)}.$$

2.4. Statistical Analysis

Data obtained was analyzed using the Statistical Package for Social Science (SPSS) version 20.0 (SPSS Inc, Chicago, USA) and the results presented in tables and charts. Frequency distribution and chi square test were calculated for the prevalence as well as antibiotic resistance of *L. monocytogenes* isolates to commonly used antibiotics. P value < 0.05 was considered statistically significant.

3. Result

3.1. Sample Type Distribution

The sample type distribution showed that, of the total 193 (35.10%) samples positive for *Listeria* species when platted on chromogenic *Listeria* agar, 91(52.60%) were raw milk, 65(42.21%) 'Manshanu' and 37 (16.60%) 'Kindrimo'. When the association between the isolation rate for *Listeria* species and the sample type was tested, there was significant association between the two (P<0.0001) (Table 1).

Table 1. Distribution of presumptive *Listeria* species based on sample type

Sample Type	No. sampled	No. (%) of *Listeria* spp	X^2	P-Value
Raw milk	173	91(52.60)		
Manshanu	154	65(42.21)	60.22	0.0001
Kindrimo	223	37(16.60)		
Total	550	193(35.10)		

3.2. Distribution of *Listeria monocytogenes* Based on Sample Type

The distribution of 36 *L. monocytogenes* isolates (Table 2) based on sample type showed that 14(8.09%) of the *L. monocytogenes* were from raw milk, 14(9.09%) 'Manshanu' and 8(3.59%) 'Kindrimo', giving an overall prevalence of 36 (6.55%) for *L.monocytogenes* in this study**.** When the association between the isolation rate for *L. monocytogenes* and the sample type was tested, there was no significant association between the two (P<0.064) (Table 3).

Table 2. *Listeria monocytogenes* isolates identified using conventional biochemical test

S/N	Sample code	Isolation source	Isolation area
1	LMFR151	Raw milk	Lere
2	ZKMM83	Manshanu	Zaria
3	MDGR115	Raw milk	Makarfi
4	MMM103	Manshanu	Makarfi
5	ZKMM84	Manshanu	Zaria
6	LSNK136	Kindrimo	Lere
7	SGMK77	Kindrimo	Sabon Gari
8	MGDR100	Raw milk	Makarfi
9	ZKMR75	Raw milk	Zaria
10	SGHR39	Raw milk	Sabon Gari
11	ZKJR55	Raw milk	Zaria
12	SGHR7	Raw milk	Sabon Gari
13	SGHR12	Raw milk	Sabon Gari
14	SGHR30	Raw milk	Sabon Gari
15	ZKMM87	Manshanu	Zaria
16	ZKMM84	Manshanu	Zaria
17	ZKJR5	Raw milk	Zaria
18	SGMM14	Manshanu	Sabon Gari
19	SGMM34	Manshanu	Sabon Gari
20	SGHR45	Raw milk	Sabon Gari
21	LMFR161	Raw milk	Lere
22	MDGR109	Raw milk	Makarfi
23	SGHR32	Raw milk	Sabon Gari
24	MDGR10	Raw milk	Makarfi
25	ZKJR10	Raw milk	Zaria
26	SGSM9	Manshanu	Sabon Gari
27	LMFR162	Raw milk	Lere
28	SGHR55	Raw milk	Sabon Gari
29	MDGM123	Manshanu	Makarfi
30	SGSK1	Kindrimo	Sabon Gari
31	SGHR6	Raw milk	Sabon Gari
32	SGHR37	Raw milk	Sabon Gari
33	SGSK21	Kindrimo	Sabon Gari
34	LMFK153	Kindrimo	Lere
35	SGMM37	Manshanu	Sabon Gari
36	SGM M11	Manshanu	Sabon Gari

Table 3. Distribution of *L. monocytogenes* based on sample type

Sample Type	Number sampled	No. (%) *L .monocytogenes*	X^2	P-Value
Raw milk	173	14(8.3)		
Manshanu	154	14(9.1)	5.498	0.064
Kindrimo	223	8(3.59)		
Total	550	36(6.55)		

3.3. Distribution of *Listeria monocytogenes* Based on Local Government Areas

The distribution of *L. monocytogenes* based on the local government areas (Sabon gari, Zaria, Makarfi and Lere), showed that Sabon gari had the highest isolation rate of 14(10.9%), followed by Zaria 13(7.9%), Makarfi 5(3.4%) and Lere 4(3.7%). When the association between the isolation rate for *L. monocytogenes* and the local government areas was tested, a statistically significant association was observed (P<0.0413) (Table 4).

Table 4. Distribution of *Listeria monocytogenes* in milk and milk products based on Local Government Areas

L.G.A	No. Sampled	No.(%) Positive	OR	95% CI	X^2	P-Value
Sabo gari	129	141(10.9)	Ref			
Zaria	165	13(7.9)	0.70	0.32-1.55	8.241	0.0413
Makarfi	147	5(3.4)	0.29	0.10-0.83		
Lere	109	4(3.7)	0.31	0.10-0.98		
Total	550	36(25.9)				

3.4. Detection of *hly* A Harbouring *Listeria monocytogenes* Isolates

The PCR results for the detection of *hly* A gene among the 36 *L. monocytogenes* isolates showed that only 9(25.0%) (ZKMM84, SGHR30, SGSK1, ZKJR56, ZKJR39, ZKMR75, SGMM87, SGHR15 & LMFR162) *L. monocytogenes* isolates harboured the *hly* A gene (Figure 1).

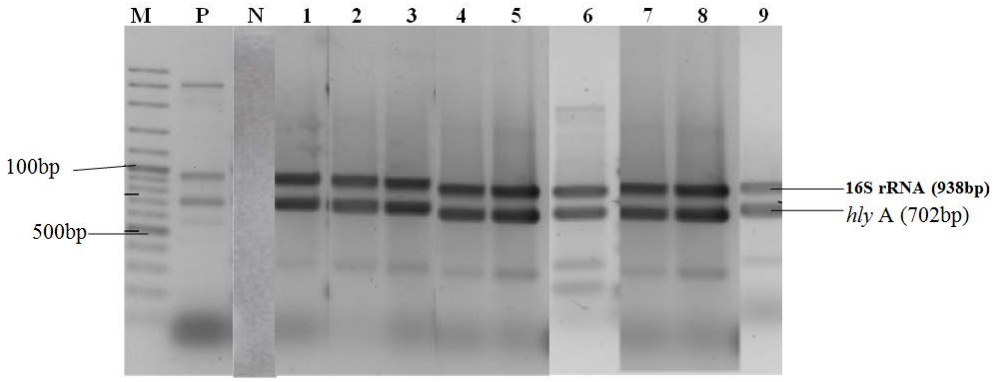

Figure 1. Agarose gel electrophoresis of the PCR products obtained after amplification of the 16S rRNA gene (938bp) and listeriolysin O encoding gene (*hly* A) (702bp) from different *Listeria monocytogenes* isolates from raw milk and milk products. Lane M; 100bp ladder, Lane P: Positive control (*Listeria monocytogenes* ATCC 19155), Lane N: Negative control, Lane 1: *Listeria monocytogenes* (LM) isolate ZKMM84; Lane 2: LM SGHR 30; Lane 3: LM SGSK1; Lane 4: LM ZKJR56; Lane 5: LM SGHR39; Lane 6: LM ZKMR75; Lane 7: LM SGMM87; Lane 8:LM SGHR15; Lane 9: LM LMFR162

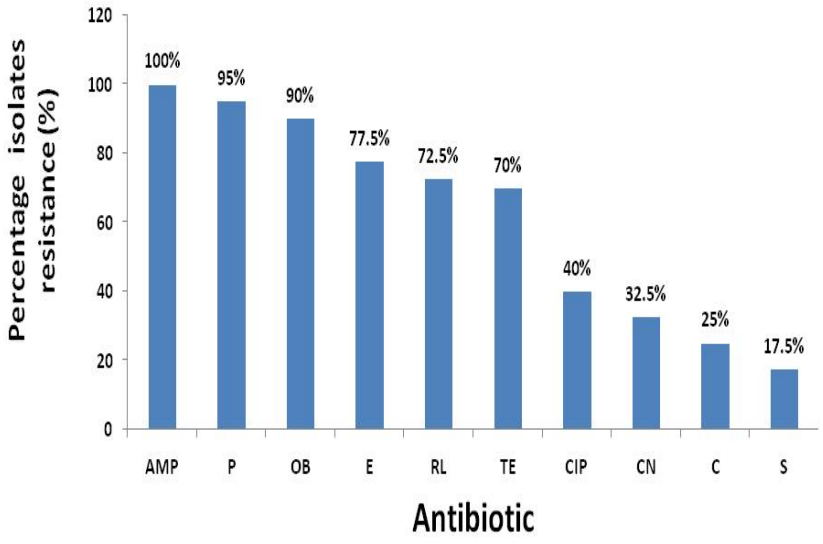

Figure 2. Percentage resistance of *Listeria monocytogenes* to a panel of 10 antibiotic agents

Keys: AMP=ampicillin; P=penicillin; OB=oxacillin; E=erythromycin; RL=sulfamethoxazole; TE=tetracycline; CIP=ciprofloxacillin; CN=gentamicin; C=chloramphenicol; S=streptomycin

3.5. Antibiotic Resistance Pattern

All the isolates tested were resistant to ampicillin (100%), they also resisted penicillin (95%), followed by cloxacillin (90%) and resistance to streptomycin was the least (17.5%), followed by resistance to chloramphenicol with (25%) (Figure 2).

Table 5. Antibacterial susceptibility of 36 *Listeria monocytogenes* isolates from milk and milk products

	No. of *Listeria*	*monocytogenes* (n=36)
Type of antibiotics	**Susceptible (%)**	**Resistant (%)**
Chloramphenicol	26 (72.2)	10 (27.8)
Ampicillin	0 (0)	36 (100)
Ciprofloxacillin	20 (55.6)	16 (44.4)
Gentamicin	23 (63.9)	13 (36.1)
Streptomycin	29 (80.6)	7 (19.4)
Sulfamethoxazole	11 (30.6)	25 (69.4)
Tetracycline	9 (25.0)	27 (75.0)
Erythromycin	9 (25.0)	27 (75.0)
Oxacillin	4 (11.0)	32 (88.9)
Penicillin	2 (5.6)	34 (94.4)

The antimicrobial resistance profiles of the isolates showed that all the isolates were resistant to at least one or more antimicrobial agents and majority (75%) of the isolates the isolates were resistant to more than 3 antimicrobial agents**.** The susceptibility testing of the 36 *L. monocytogenes* isolates gave 30 different antimicrobial resistance patterns (Appendix 1).

3.6. Multiple Antibiotic Resistance

The antibiotic resistances profile of the *L. monocytogenes* isolates are shown in (Table 6). The results revealed that all the isolates were resistant to at least one antibiotic. However, only 3 (8.3%) of the isolates were resistant to less than 3 antibiotics, while 33 (77.5 %) of the isolates were resistant to 3 or more antibiotics (Table 6).

Table 6. Multiple drug resistance of *Listeria monocytogenes* isolates

Antibiotics	**Frequency**	**Percentage (%)**
Resistant to less than 3	3	8.3
Resistant to 3 or more	33	91.7
Total	36	100

3.7. Multiple Antibiotic Resistance Index

The results of this study revealed that majority of the isolates 32(88.9%) had MAR index greater than 0.2, with 14(38.8%) of the isolates having considerably higher MAR index (0.6-0.9). Only 4(11.11%) of the *L. monocytogenes* isolates had MAR index less than 0.2 (Table 7 & Appendix 2). Hence, it was not surprising to find 53.3% of the isolates with high MAR index originated from raw milk samples.

Table 7. Multiple antibiotics resistance (MAR) index of *Listeria monocytogenes* isolates

MAR Index	**Frequency**	**Percentage (%)**
Less than or equal to 0.2	4	11.1
Greater than 0.2	32	88.9
Total	36	100

4. Discussion

Among the 550 samples tested, 193(35.1%) were presumptively positive for *Listeria* spp. based on their colour and colonial morphology when plated on Chromogenic *Listeria* agar (Table 1). In a similar study, [34] obtained a prevalence rate of 83 (18.6%) for *Listeria* species from 446 raw milk samples in Tehran, Iran. Based on their ability to ferment sugars (Rhamnose, Xylose and Mannitol) and other conventional biochemical tests, 36(6.55%) of the 196 (35.1%) presumptive *Listeria* species were identified as *L. monocytogenes* (Table 2). The prevalence of 6.55% obtained for *L. monocytogenes* in this study is comparable to other surveys conducted in other countries on raw milk and milk products. Reference [35] reported a prevalence of 5.7% from bovine raw milk produced in the North Eastern Algeria, whereas in China, the prevalence of *L. monocytogenes* was found to be very low (0.23 – 1.2%) [36]. A lower prevalence (16.6%) for *Listeria* species was obtained for 'kindrimo' in this study, as compared to (52.6%) raw milk and (42.2%) 'Manshanu' (Table 1). This lower prevalence obtained could be due to the conversion of lactose during fermentation by the lactic acid bacteria present in the ' Kindrimo' to lactic acid, which lowers the pH of the kindrimo and hence the inhibition of *Listeria* species. Reference [37] reported that fermented milk products are rich in lactic acid bacteria; they also reported that bacteriocin produced by lactic acid bacteria has been found to reduce counts of *Listeria* species in cheese and Yoghurt. The implication of this finding is that raw milk and milk products which are ready to consume products, are mostly consumed without pasteurization, as such could serve as sources of infection to the population especially the high risk individuals.

The distribution of *L. monocytogenes* based on sample type followed the same pattern as the distribution of *Listeria* species, where a lower detection rate of 8(3.6%) for *L. monocytogenes* from 'kindrimo' as compared to 14(18.1%) for raw milk and 14(9.1%) for 'Manshanu'; the only difference was that there was no statistically significant association (P=0.064) between the isolation rate of *L. monocytogenes* and the sample type (Table 2).

The prevalence of 6.6 % for *L. monocytogenes* obtained in this present study (Table 2) is lower than 48.33 % reported by [38] in goat milk, and 22.4 % by [39] in cattle milk in Sokoto. In other regions of the world, similar studies have reported prevalence rates of 0.57 % in Istanbul from foods [40], 5 % in India from bovine raw milk [41], 11.4 % in Iran in ready-to-eat foods [34].

Contamination of food by *L. monocytogenes* may occur at any point from farm to the table [42].The high nutritional value of milk and milk products make them suitable for pathogenic organisms including *L. monocytogenes* to thrive [43].

The low prevalence of *L. monocytogenes* obtained in Makarfi 5 (3.4%) and Lere 4 (3.7%) in this study could be attributed to the large number of samples of 'Kindrimo' sampled from these two local government areas as compared to the large number of samples of raw milk sampled in Sabon gari and Zaria local government areas, which gave a statistically significant association between the isolation rate for *L. monocytogenes* and the local government areas (P<0.0413) (Table 3).

The Microbact 12L *Listeria* identification kit was able to identify 3 (8.3%) out of 36 biochemically identified isolates as *L. monocytogenes*, while the rest were identified as non- *L. monocytogenes* (*Listeria grayi* 4 (11.0%), *Listeria ivanovii* 27 (75.0%) and *Listeria seeligeri* 2 (6.0%)). The identification rate of 3 (8.3%) for *L. monocytogenes* obtained in this study by the Microbact system is lower than 12 (30.0%) obtained by [44] out of the 40 *L. monocytogenes* isolates from raw meat and meat products in Zaria, Nigeria. The applicability of Microbact 12L (Oxoid) for identification of *Listeria monocytogenes* in this environment requires reconsideration and validation. Reference [45] also obtained a prevalence of 39(13.0%) for *L. monocytogenes* out of 400 abattoir chicken isolates from Khartoum, Sudan. In this study *L. ivanovii* had the highest identification rate of 75.0%, which is similar to the study of [44,45], where *L. ivanovii* had the highest identification rate of 47.5% and 48.5% respectively.

The identification of *L. ivanovii* and other supposedly non-pathogenic *Listeria* species (*L. seeligeri* and *L. grayi*) is significant, since these non-pathogenic *Listeria* species like *L. grayi, L. seeligeri and L. innocua* have been found to cause disease in both immunocompetent and immunocompromised individuals [46].

From the perspective of food hygiene, the presence of non-pathogenic species such as *L. innocua* may indicate potential contamination with *L. monocytogenes* [47]. *L. ivanovii* is pathogenic for animals and it has been reported to be associated with gastroenteritis and bacteraemia in a 55 year old man with renal transplant [48].

Listeriolysin O (LLO) produced by *Listeria monocytogenes* and encoded by hly A gene, is one of the most virulent factors associated with the organism. One of the best ways to detect and confirm *Listeria monocytogenes* is through the detection of the virulence factors. The LLO –encoding gene (hly A) is present only in virulent strains of the species and is required for virulence [49,50].

In this study the multiplex PCR identified 9 *Listeria monocytogenes* isolates harbouring *hly* A gene. The amplified genes for *L. monocytogenes* and *Listeria* genus produced PCR products of 702bp and 938bp in size respectively. The PCR protocol used in this study was based on the amplification of *hly* A gene by using a set of primers, LM1 and LM2. Reference [29] recommended LM1 and LM2 primers as the best primer pair to be used for the detection of *L. monocytogenes*, because they are designed to amplify specific fragments in *L. monocytogenes* strains that are genetically and biochemically assessed as belonging to this specie, on the basis of the presence of the 702bp and 938bp amplicons [29].

Reference [51] was also able to amplify the DNA at their expected amplification site. The result of this study is not in agreement with the result of [52], who reported the carriage of *hly* A gene by all the isolates of *L. monocytogenes* tested. The inability of some of the *L. monocytogenes* isolates in this study to harbour the *hly* A gene, may be due to the non virulent characteristics of some *L. monocytogenes* isolates; because some may be environmental isolates or it may be possible that some *L. monocytogenes* strains may lack one or more virulence determinants because of spontaneous mutations [53].

In this study, the antibiotic susceptibility testing showed that all the isolates were resistant to ampicillin, followed by resistance to penicillin (95%). The least frequency of resistance by the isolates was obtained for streptomycin (17.5%) and chloramphenicol (25%) (Figure 1). A high rate of resistance to ampicillin (100%) is agreement with the result of [39,40] where they reported that all the isolates from milk sold in Sokoto, Nigeria and processed meat sold in Selangor, Malaysia respectively were reported to be resistant to ampicillin. Similarly reference [54] in their study of antibiotic resistance of *L. monocytogenes* isolates from salad vegetables and vegetable salads in Zaria reported the organisms to be resistant to ampicillin (92.9%), erythromycin (64.3%), penicillin (85.0%) and tetracycline (75.0%).

Reference [55] reported that all the isolates from various foods were susceptible to erythromycin; this is at par with the results obtained in this study where 77.5% of the *L. monocytogenes* isolates were resistant to erythromycin. Reference [44,56,57] all observed a 100% susceptibility of *Listeria monocytogenes* isolates to gentamicin, which is not in agreement with the results obtained in this study, where 32.5% of the isolates were resistant to gentamicin. The study of [56], reported a favourable activity of penicillin and ampicillin against *L. monocytogenes*, which is contrary to the findings of the present study, where all the isolates were resistant to ampicillin (100%), 95% to penicillin and 72.5% to sulfamethoxazole. The detection of *L. monocytogenes* resistant to sulfamethoxazole in this study is of particular interest, since the combination of trimethoprim-sulfamethaxozole is the second choice drug of treatment for ampicillin/penicillin resistant strains and also used in patients with allergy to penicillin [58].

It is assumed that the high prevalence of antibiotic resistance observed among the isolates in this study may not be unconnected with the indiscriminate use and abuse of these antibiotics in livestock production in Nigeria as the case in Denmark [59]. When infections are caused by *L. monocytogenes* strains resistant to the first choice drugs, more expensive therapies must be used, also a longer duration of illness and treatment in hospitals, increases health care cost which will result in economic burden on the families and society [60].

A total of 34 antibiograms observed in this study, revealed a high diversity of antimicrobial resistance of the *L. monocytogenes* isolates. Since the first isolation of a multi-resistant strain of *L. monocytogenes* in France in 1988 [61], multi-resistant strains have been extensively isolated. All the 36 *L. monocytogenes* isolates were multidrug resistant. Reference [39] also reported that 20% of the *L. monocytogenes* isolates from dairy products in Sokoto were resistant to more than two antibiotic agents and all the isolates were resistant to at least one antibiotic agent. Reference [54] also reported that 64.3% of the isolates were resistant to more than 4 antibiotic agents and all the isolates (100%) were resistant to more than two antimicrobial agents. The data obtained in this study on antimicrobial resistance suggest the importance of continued surveillance of emerging antibiotic resistance in *L. monocytogenes* in order to control the pathogen and ensure effective treatment of human listeriosis.

The MAR index was determined for the 36 *L. monocytogenes* isolates. The results revealed that 32 (88.9%) of the isolates had MAR index higher than 0.2, an indication that these isolates originated from a high risk

source of contamination e.g., farm animals that are exposed to antibiotics [62, 63].

Multiple antibiotic resistances in *Listeria* spp has been attributed to antimicrobial selective pressure and gene transfer mechanisms between and among *Listeria* species and close relatives, such as *Enterococcus*, *Staphylococcus* and *Streptococcus* species [64]. The high MAR index observed in most of the isolates with the exception of four isolates (MDGM123, SGHR 32, SGHR 39 and MDGR115) is an indication that even though few of the isolates were not multi drug resistant, almost all the isolates would have spread from an environment of high antibiotic use or from high risk sources of contamination. Hence, it was not surprising to find 53.3% of these high MAR index isolates originated from the raw milk samples. Infections caused by resistant microorganisms may result in failure to respond to treatment, result in prolonged illness, higher health care expenditure and greater risk of death [65].

In conclusion, the study has demonstrated the presence and distribution of *Listeria monocytogenes* and other Listeria species in raw milk and milk products in the area, this suggest the need for improved milk safety through the implementation of hygienic measures at all level from production to consumption with particular emphasis on ready to eat milk and milk products. Also the isolation of *Listeria monocytogenes* strains that are resistant to ampicillin and penicillin are of great concern, since these two antibiotics are the treatment of choice for listeriosis in combined with an amino glycoside. *L. monocytogenes* presence in RTE foods is of public health concern as these food items are consumed without previous heat treatment

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Appendix 1. Antibacterial resistance patterns (Antibiograms) of 36 *L. monocytogenes* isolates from raw milk and milk products

Sample No.	Type of Sample	Resistance pattern
SGHR6	Raw milk	AMP, C, CIP, CN, RL, TE, E, OB, P
SGHR7	Raw milk	AMP, CIP, CN, S, RL, TE, OB, P
LMFR12	Raw milk	AMP, CN, S, RL, TE, OB, P
ZKMR75	Raw milk	AMP, C, CIP, RL, E, OB, P
SGHR32	Raw milk	AMP, C, CIP, RL, E, OB, P
SGSK21	Kindrimo	AMP, C, CIP, RL, E, OB, P
SGMM14	Manshanu	AMP, C, RL, TE, E, OB, P
SGHR30	Raw milk	AMP, CN, RL, TE, E, P
MDGR109	Raw milk	AMP, CIP, CN, S, TE, P
ZKJR10	Raw milk	AMP, CIP, CN, E, OB, P
LMFR162	Raw milk	AMP, C, CN, TE, O, P
SGHR37	Raw milk	AMP, RL, TE, E, OB, P
SGMK77	Kindrimo	AMP, CIP, CN, S, TE, P
LMFK153	Kindrimo	AMP, CIP, CN, E, OB, P
ZKMM84	Manshanu	AMP, CIP, CN, S, TE, P
ZKMM87	Manshanu	AMP, C, RL, TE, E, P
MGDR100	Raw milk	AMP, RL, TE, E, P
ZKJR55	Raw milk	AMP, RL, E, OB, P
ZKJR5	Raw milk	AMP, CIP, CN, RL, TE
ZKMM83	Manshanu	AMP, TE, E, OB, P
LMFR161	Raw milk	AMP, TE, E, OB, P
LNSK136	Raw milk	AMP, TE, E, OB, P
MMM103	Raw milk	AMP, TE, E, OB, P
SGHR12	Raw milk	AMP, CIP, RL, TE
SGHR15	Raw milk	AMP, RL, E, P
SGMM34	Manshanu	AMP, E, OB, P
SGMM9	Manshanu	AMP, C, CIP, TE
SGMM37	Manshanu	AMP, TE, E, P
SGMM11	Manshanu	AMP, TE, OB, P
ZKMM84	Manshanu	AMP, E, P
SGHR25	Raw milk	AMP, TE, E
MDGR115	Raw milk	AMP, RL
SGHR33	Raw milk	AMP, TE
SGHR12	Raw milk	AMP, CIP
SGSK1	Kindrimo	AMP, CIP
MDGM123	Manshanu	AMP

Appendix 2. MAR Index Analysis of *L. monocytogenes* isolates from raw milk and milk products Samples

S/N	Isolate	No. of antibiotics to which the isolate was resistant (a)	MAR Index (a/b)
1	LMFR151	7	0.7
2	MDGR115	2	0.2
3	MGDR100	5	0.5
4	ZKMR75	7	0.7
5	SGHR 39	2	0.2
6	ZKJR55	5	0.5
7	SGHR7	8	0.8
8	SGHR12	4	0.4
9	SGHR30	6	0.6
10	ZKMR84	3	0.3
11	ZKJR5	5	0.5
12	SGHR25	3	0.3
13	LMFR161	5	0.5
14	MDGR109	6	0.6
15	SGHR32	2	0.2
16	MDGR100	7	0.7
17	ZKJR10	6	0.6
18	LMFR162	6	0.6
19	SGHR15	4	0.4
20	SGHR6	9	0.9
21	SGHR37	6	0.6
22	LSNK136	5	0.5
23	SGMK77	7	0.7
24	SGSK1	5	0.5
25	SGSK21	3	0.3
26	LMFK153	3	0.3
27	MMM103	5	0.5
28	ZKMM84	6	0.6
29	ZKMM87	6	0.6
30	SGMM14	7	0.7
31	SGMM34	4	0.4
32	SGMM9	4	0.4
33	MDGM123	1	0.1
34	SGMM37	5	0.5
35	SGMM11	4	0.4
36	ZKMM83	5	0.5

Legend: Total No. of antibiotics to which the isolates were subjected to (b) =10
NO. Of antibiotics to which the isolate was resistant to (a)
MAR = Multiple antibiotics resistance.