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Occurrence and Molecular Characterisation of *Listeria* Species in Some Fresh-cut Vegetables and Environmental Samples

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MAO and AM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors OMD and OF supervised and managed the analyses of the study and proofread the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Listeriosis remains a foodborne disease to be reckoned with courtesy of its high mortality rate among high-risk persons. This study, therefore, examined the occurrence and the phylogenetic relationship of *Listeria monocytogenes* isolates from different samples. A total of 175 samples (soil, vegetables and water) were analysed using the FDA-BAM for the isolation of *Listeria* species. Molecular identification of *L. monocytogenes* was done by coupling PCR to DNA sequencing analysis of *Listeria* 16S rRNA genes, and the antibiotic susceptibility profile was studied. Analysis yielded 386 bacteria species and *Listeria* species was recorded at 78.24%. Of these, the pathogen and agent of listeriosis in humans, *L. monocytogenes*, was recorded at 16.58% and was isolated from all samples analyzed. Other *Listeria* species identified are *L. innocua, L. rocourtiae, L. grayi, L. fleischmannii,* and *L.* species. Significant difference was observed among the population of isolates from vegetables and water samples, and a significant difference was seen among isolates from soil

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samples at $p < .05$. *Listeria monocytogenes* was significantly higher among isolates from water samples at $p < .05$. Phylogenetic analysis revealed common ancestry among isolates from the different samples analysed. Isolates are suspected to be of lineage I, commonly associated with human clinical cases. All isolates were resistant to ampicillin/cloxacillin and amoxicillin. However, most of the isolates were sensitive to ciprofloxacin at 92.4%. Though not significantly different, resistance among isolates from environmental samples was higher than isolates from fresh cut vegetables. This study established the presence of *L. monocytogenes* in all samples and a high level of resistance to commonly used antibiotics. Hence, good agricultural practices and a high level of hygiene are required to protect against infection by the pathogen via fresh-cut RTE foods.

Keywords: Listeria monocytogenes; listeriosis; ready-to-eat; resistance; phylogeny.

1. INTRODUCTION

Vegetables are plants consumed by humans as food and can be eaten either in a raw form or cooked. They are known to contain high concentrations of vitamins, fibre and minerals, and low in fats and carbohydrates [1]. The inclusion of vegetables in diet is known to reduce the risks of cancer, stroke, cardiovascular diseases and other chronic ailments known to man [2], and Nigeria has been ranked among the top vegetable-producing countries in the world [2]. However, the eating habits of the modern world, especially the increasing demands for ready-to-eat (RTE) foods like minimally processed fruits and vegetables have increased the risk of infection by bacterial contaminants [3]. *Listeria monocytogenes* has been identified as one bacterium widely distributed in vegetation and can survive on plant materials for many years [4,5]. The organism has been linked to several vegetable recalls in the United States within 2015 and 2016 (www.whatcomcounty.us/888/Food-Recalls). The climatic conditions in Nigeria support the growth of *Listeria* species, thus it can thrive and contaminate foods sold in the open especially RTE foods [6]. The tropical weather is warm and humid all year round and many rural places are not very hygienic and have poor water sanitation [6]. Environmental studies of *Listeria* in Nigeria have shown that the organism occurs in known *Listeria* sources such as soil and lakes, and faecal droppings of domesticated ruminants [7; 8]. Other uncommon sources of contamination to man are veterinary surgical material [9] and Naira currency notes [10]. A study of two anthropogenic lakes in Abia State in South East Nigeria by Nwachukwu et al. [11] showed a prevalence rate of 91.67% and 79.17% for 24 samples analyzed from each lake. The authors pointed out the ubiquity of the organism in nature and the organism's reputation as water and food-borne bacterial pathogen as reasons for the high prevalence. Another study by Mawak et al. [12] used the 2-step enrichment method to analyse natural water bodies including rivers, streams and ponds used for irrigation in Jos, Plateau State in the middle belt region of Nigeria. They found that four *Listeria* species namely *L. monocytogenes*, *L. innocua, L. ivanovii* and *L. grayii* were present in 10 out of 30 samples analyzed. Their report suggested that dry season farmers should be educated on measures that would reduce the hazards associated with *Listeria* in their farm produce.

This study investigated the occurrence of *Listeria* species in environmental (terrestrial and aquatic) samples as well as fresh cut vegetables in Ado Ekiti, South Western Nigeria.

2. MATERIALS AND METHODS

2.1 Sample Collection and Laboratory Analysis

A total of 175 samples were collected consisting of 50 soil samples (25 samples from abattoir environments and 25 samples from cultivated lands), 50 water samples (25 samples from rivers and 25 from livestock farm houses), and 75 vegetable samples sold in the market (25 samples each of cabbage, carrot, and lettuce). One gram (1g) of freshly collected soil samples from abattoirs and farmlands was homogenized in 9ml Tryptone Soy broth. A volume of 1ml of this homogenate was then inoculated into 9ml of University of Vermont Medium *Listeria* enrichment Broth (Alpha Biosciences, USA), with a selective agent (Oxoid, UK) after 4 hours of incubation at 30ºC, and then subsequent incubation at 37ºC for 44 hours. The enrichment culture was streaked at 24 and 48 hours on Brilliance *Listeria* agar with differential and selective supplements (Oxoid, UK). After incubation at 37ºC, blue-green colonies on media which expressed Gram positive coccobacilli on

Gram staining were presumed to be *Listeria* species. Vegetable samples (25g of sliced cabbage, carrot, and lettuce) were prepared by the rinsing method of Rovrik et al. [13]. The University of Vermont Medium Modified *Listeria* Enrichment Broth (Alpha Biosciences, USA) used in rinsing the samples was incubated for enrichment. Enrichment was carried out by initial incubation at 30ºC for 4 hours in 500ml of Erlenmeyer flask, after which *Listeria* selective agent (Oxoid, UK) was added before further incubation for 24 - 48 hours at the same temperature. Subculture of the broth was done on Brilliance *Listeria* agar plates and incubated for 24-48 hours at 37ºC. Similarly, blue-green colonies on media which expressed Gram positive coccobacilli on Gram staining were presumed to be *Listeria* species. Twenty-five milliliters (25 ml) portion of each water sample was enriched for *listeria* in 225ml of University of Vermont Medium Modified *Listeria* Enrichment Broth (Alpha Biosciences, USA), incubated for 4 hours at 30ºC, and then supplemented with *Listeria* selective agent (Oxoid, UK) with further incubation at 37ºC for 24-48 hours. Subculture was made from the enrichment culture onto Brilliance *Listeria* agar, containing both selective and differential agents (Oxoid, UK). Blue-green colonies on media at 37ºC for 24-48 hours, which expressed Gram positive coccobacilli on Gram staining, were also presumed to be *Listeria* species.

2.2 Biochemical Differentiation

This was done in reference to Collins et al. [14]. More presumptive identification was done using the Oxoid Biochemical Identification System, OBIS mono kit (ID0600) (Oxoid, UK), which tests for the production of D-alanyl aminopeptidase (DALAase), an enzyme produced by other *Listeria* species except *L. monocytogenes*. Isolates showing no colouration (purple color) in reaction with the reagents is presumed *L. monocytogenes*.

2.3 Molecular Studies

The following procedure was carried out in the Biosciences Center, International Institute of Tropical Agriculture (IITA). This was done by coupling PCR to the DNA sequencing analysis of *Listeria* 16S rRNA genes [15,16]. Genomic DNA was extracted using QIAamp DNA mini kit (250) cat no 51306. Polymerase Chain Reaction (PCR) was performed using 27F (5'-AGAGTTTGATCMTGGCTCAG – 3') and 1525R (5'- AAGGAGGTGWTCCARCCGCA -3') universal primers and PCR protocols were performed as described by Bubert et al. [17]. PCR procedures were as follows: 36cycles, each at 94ºC for 30 seconds, 56ºC for 30 seconds, and 72ºC for 7 minutes. The amplicons were subjected to gel electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and were visualised under UV light. The amplicons were subjected to sequencing reactions using BigDye Terminator v3.1 Cycle Sequencing Kit. The products were loaded unto 3130xl Genetic Analyzer (Applied Biosystems), and molecular sequences were identified by a combination of BLAST and FASTA [18]. Isolates were identified by a > 95% identity value.

2.4 Phenotypic Expression of Pathogenic Traits

The production of lecithinase (phosphotidylcholine phospholipase C, PCPLC) activity was demonstrated on the medium (Brilliance *Listeria* agar with Brilliance *Listeria* differential supplement, Oxoid UK). Production of an opaque white halo around the colony confirms the presence of the enzyme. The production of listeriolysin O was demonstrated on 5% sheep blood agar.

2.5 Phylogenetic Analysis

Representative isolates were selected at random among highly resistant ones to fluoroquinolones and cotrimoxazole antibiotics, as well as strong hemolysis on blood agar. The 16S rRNA sequences were aligned using ClustalW (pairwise and multiple). The evolutionary history among representative isolates across the samples was inferred by using the Maximum Likelihood statistical method, test of phylogeny using bootstrap method (1000 replicates) and Tamura-Nei model [19,20]. Evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA 7) [21].

2.6 Antibiotic Susceptibility Testing

Using the Kirby-Bauer disc diffusion method as recommended by the Clinical Laboratory standards Institute [22]. The commonly used antibiotics against Gram-positive bacteria were employed; pefloxacin (10µg), gentamycin (10µg), ampiclox (Ampicillin/cloxacillin, 30µg), zinnacef (cefuroxime, 20µg), amoxacillin (30µg), rocephin

(ceftriaxone, 25µg), ciprofloxacin (10µg), streptomycin (30µg), septrin (cotrimoxazole, 30µg), and erythromycin (10µg).

2.7 Statistical Analysis

Data generated were analyzed using the Statistical Package for Social Sciences (SPSS 16) and Post Hoc by Duncan test at the significant level of $p < .05$.

3. RESULTS

From all samples analysed, a total of 386 bacteria species were isolated and characterised. Soil samples from the abattoir environment yielded the highest number of bacteria species at 155 (40.16%), while water samples from farm houses yielded the lowest number of bacteria species at 19 (5.00%). In all, the occurrence of *Listeria* species was recorded at 78.24%, and *L. monocytogenes* was recorded at 16.58%. Other *Listeria* species identified are *L. innocua, L. rocourtiae, L. grayi, L. fleischmannii* and *L.* species at 25.13%, 17.88%, 5.44%, 7.00%, and 6.22% respectively (Table 1). *Brochothrix* species was also isolated in the study at 21.76%. *Listeria innocua* and *L. grayi* recorded the highest and lowest occurrence respectively. The pathogenic species, *L. monocytogenes*, was isolated in all samples analysed and was highest in soil samples from the abattoir environment at 100%. All molecularly identified *L. monocytogenes* showed no colouration on reaction to OBIS-mono test kit, produced lecithinase and narrow hemolysis on 5% sheep blood agar (Plate 1). One of the isolates was not amplified, while others showed distinct DNA bands (Plate 2). Frequency of isolates of *L. monocytogenes* and *L. innocua* were significantly higher (p<.05) in water and vegetable samples respectively (Table 2).

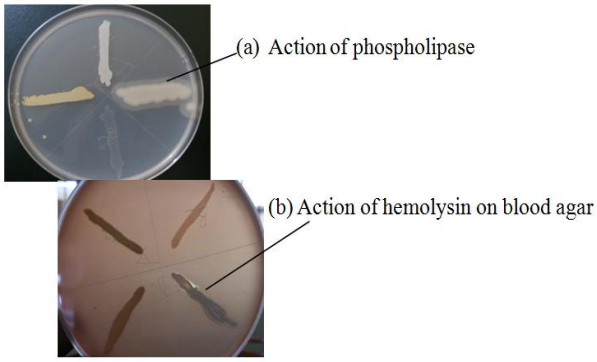


Plate 1. Expression of virulence traits by *L. monocytogenes*

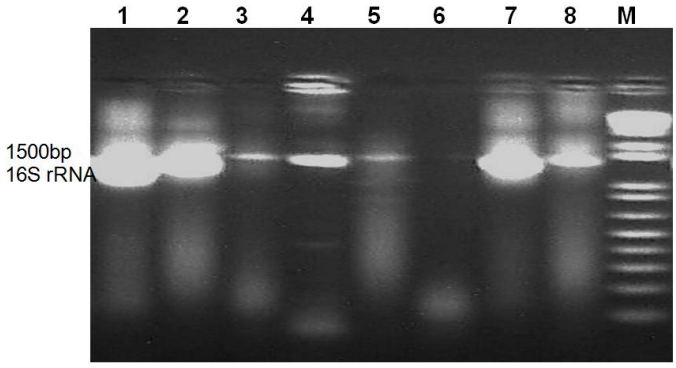


Plate 2. Bands of amplified DNA from representative isolates using PCR

Key: 1- L. monocytogenes from soil (farmland); 2- L. monocytogenes from soil (abattoir); 3- L. monocytogenes from water (farmhouse); 4- L. monocytogenes from water (river1); 5- L. monocytogenes from cabbage; 6- L. monocytogenes from water (river2), 7- L. monocytogenes from carrot; 8- L. monocytogenes from lettuce; M- molecular weight marker

Table 1. Frequency distribution of isolates among samples

Bacteria	Soil		Water		Vegetables			Total (%)
	Abattoir (n=25)	Farmland (n = 25)	River (n= 25)	Farm house (n = 25)	Cabbage (n = 25)	Lettuce (n = 25)	Carrot (n = 25)	
Brochothrix	25	09	08	0	15	17	10	**84(21.76)**
L. innocua	23	15	06	05	13	20	15	**97(25.13)**
L. rocourtiaae	24	10	01	03	09	13	09	**69(17.88)**
L. monocytogenes	25	05	08	09	03	07	07	**64(16.58)**
L. grayi	21	0	0	0	0	0	0	**21(5.44)**
L. fleischmannii	15	02	07	02	01	0	0	**27(7.00)**
Listeria spp.	22	0	01	0	01	0	0	**24(6.22)**
Total	**155**	**41**	**31**	**19**	**42**	**57**	**41**	**386**

Table 2. Statistical representation of bacteria population per sample

Bacteria	Soil samples	Water samples	Vegetable samples
Brochothrix	17.00 ± 8.00^{a}	$4.00 \pm 4.00^{a, b}$	$14.00 \pm 2.08^{c, d}$
L. innocua	19.00 ± 4.00^{a}	$5.50 \pm 0.50^{a, b}$	16.00 ± 2.08^{d}
L. rocourtiaae	17.00 ± 7.00^{a}	$2.00 \pm 1.00^{a, b}$	10.33 ± 1.33^{c}
L. monocytogenes	15.00 ± 10.00^{a}	8.50 ± 0.50^{b}	5.67 ± 1.33^{b}
L. grayi	10.50 ± 10.50^{a}	N/A	N/A
L. fleischmannii	8.50 ± 6.50^{a}	$4.50 \pm 2.50^{a, b}$	0.33 ± 0.33^{a}
Listeria spp.	11.00 ± 11.00^{a}	0.50 ± 0.50^{a}	0.33 ± 0.33^{a}

Key: Data are Mean ± SE; values with same superscripts have no significant difference per column at p >.05, while samples with different superscripts have significant difference per column at p <.05. N/A – not applicable

Evolutionary relationship by Maximum Likelihood method revealed three different clusters A, B and C. Sequences in cluster A was supported by a strong bootstrap values 91 and 100%. Isolates from farmhouse water and cabbage shared a 100% common ancestry, and to a lesser extent (91%), both shared common ancestry with an isolate from farmland soil. Sequences of clusters B and C revealed isolates from abattoir soil and river water; and isolates from carrot and lettuce with a bootstrap value of 88% common ancestry respectively (Fig. 1).

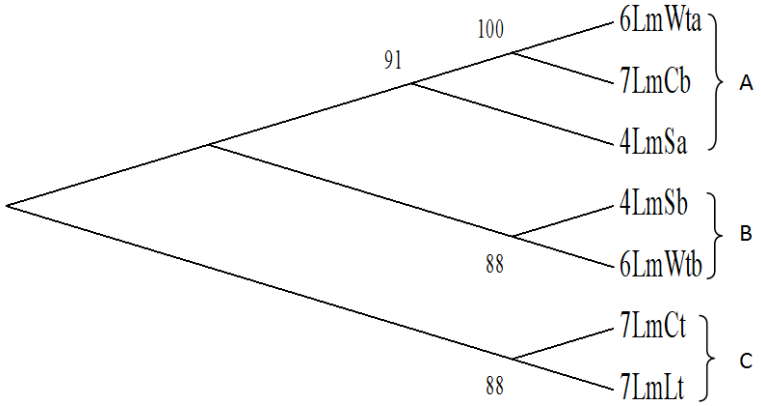
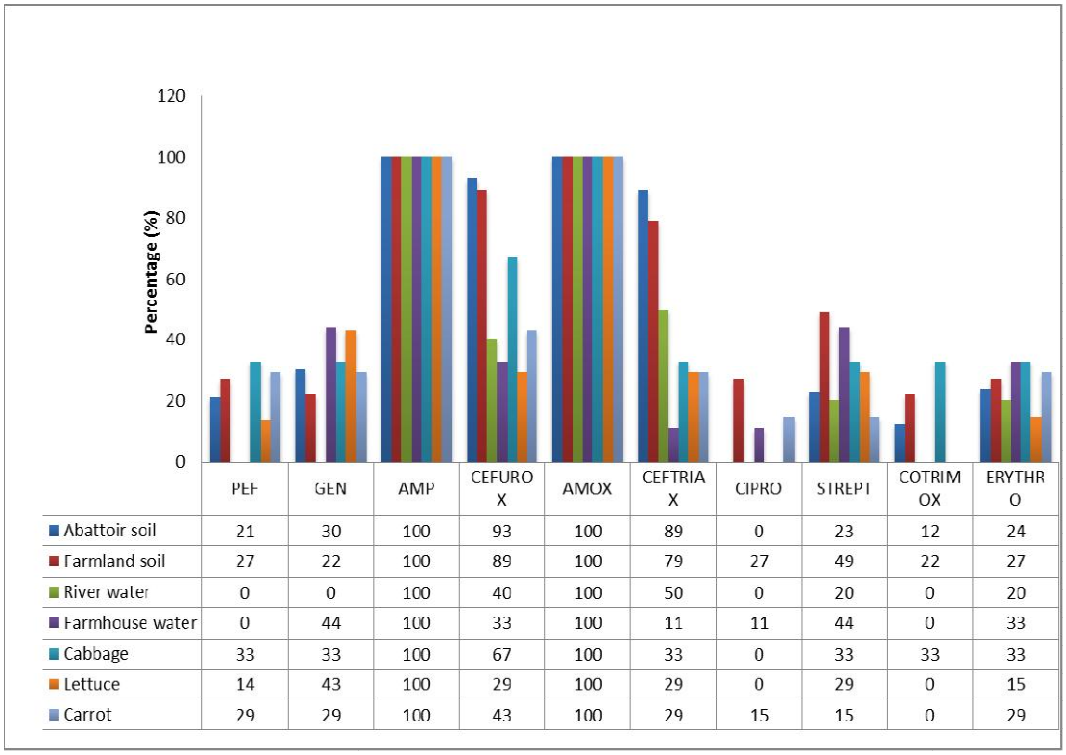


Fig. 1. Molecular phylogenetic analysis by maximum Likelihood method

Key: 4LmSa- L. monocytogenes from soil (farmland); 4LmSb- L. monocytogenes from soil (abattoir); 6LmWta- L. monocytogenes from water (farmhouse); 6LmWtb- L. monocytogenes from water (river); 7LmCb- L. monocytogenes from cabbage; 7LmCt- L. monocytogenes from carrot; 7LmLt- L. monocytogenes from lettuce



	PEF	GEN	AMP	CEFUROX	AMOX	CEFTRIAX	CIPRO	STREPT	COTRIMOX	ERYTHRO
Abattoir soil	21	30	100	93	100	89	0	23	12	24
Farmland soil	27	22	100	89	100	79	27	49	22	27
River water	0	0	100	40	100	50	0	20	0	20
Farmhouse water	0	44	100	33	100	11	11	44	0	33
Cabbage	33	33	100	67	100	33	0	33	33	33
Lettuce	14	43	100	29	100	29	0	29	0	15
Carrot	29	29	100	43	100	29	15	15	0	29

Fig. 2. Percentage resistance of Antibiotics against *L. monocytogenes* per sample

Key: PEF – pefloxacin, GEN – gentamycin, AMP – ampicillin/cloxacillin, CEFUROX – cefuroxime, AMOX – amoxicillin, CEFTRIAX – ceftriaxone, CIPRO – ciprofloxacin, STREPT – streptomycin, COTRIMOX – cotrimoxazole, ERYTHRO – erythromycin.

All isolates were 100% resistant to ampicillin/cloxacillin and amoxicillin while resistance to ciprofloxacin in *L. monocytogenes* from all samples were reduced at 7.6%, followed closely by cotrimoxazole and pefloxacin at 9.6% and 17.7% respectively. Resistance to cephalosporins (cefuroxime and ceftriaxone) was recorded at 56.3% and 45.7% respectively (Fig. 2). All isolates in this study are resistant to more than one class of antibiotics.

4. DISCUSSION

Food products of animal and plant origins have been reported to be sources of food borne pathogens vis-à-vis storage, handling, distribution and preparation. *Listeria monocytogenes* has been reported to be pathogenic in humans and animals as the causative agent of listeriosis. It has also been reported as the agent of several food borne disease outbreaks and serious infection among the elderly, new born, pregnant women and anyone in an immunocompromised state; causing fatality rates as high as 20 – 30% and infected persons may show with signs of septicaemia and meningitis [23]. The ubiquity nature of the organism in water, soil and plant materials, its ability to withstand wide adverse environmental conditions, as well as the possession of virulent factors places the organism on priority list among food borne pathogens [24].

In this study, all samples analysed were contaminated by *L. monocytogenes*, most especially in fresh vegetables such as cabbage, lettuce and carrots; common components of salad (a ready-to-eat food). This may have been contaminated either from the farmland during irrigation, storage or wash water. Storage is especially problematic for vegetable products in Nigeria due to shortage and irregular power supply to keep them fresh. Most of these products are sold exposed to natural elements instead of being refrigerated. This report agrees with the record of occurrence of *L. monocytogenes* in vegetables used to make ready-to-eat salads from Turkey [25], as well as reports of Ajayeoba et al. [26] from South Western States of Nigeria. The report had *L. monocytogenes* at 28.28% in cabbage, 9.02% in carrots, 23.36% in cucumber, 19.67% in lettuce and 19.67% in tomatoes. The report also had Lagos State recording the highest occurrence of *L. monocytogenes* in vegetables, followed by Ondo (48.89%), Oyo (48.75%), Ogun (44.09%), Osun (34.38%) and Ekiti (33.33%). Ieren et al. [27] recorded the occurrence of *L. momocytogenes* in coleslaw and conventional salad at 4.4% and 1.7% in Zaria, Nigeria, stating that cabbage and lettuce had the highest occurrence among other vegetables. Other reports on the occurrence of *L. monocytogenes* are at varying percentages from different countries in RTE vegetables and salads from supermarkets and retail outlets have been recorded [28,29,8,30,31,4,5,32].

All rivers from which water samples were analysed were used for different domestic chores ranging from car/motorcycle wash, laundry, irrigation and even to bathe by people of the community, while the farmhouse water samples were associated with poultry houses. The occurrence of *L. monocytogenes* in the river water samples could be due to the continual dumping of wastes products (domestic, agricultural and industrial), and surface run-offs of farmlands contaminated with animal dung. Farmhouse contamination may be due to low level hygiene practice. These results are in agreement with several reports where *L. monocytogenes* is a common contaminant of water and also a source of infection in animals and plants. Its presence in water bodies serves as an indicator of faecal contamination [33,12,11,34]. All soil samples from the abattoir environment were positive to *L. monocytogenes* which show a high level of contamination and may perhaps contribute to infection of animals slaughtered in the environment as well as their products prior to marketing and consumption. The abattoir environ is largely contaminated by cattle faecal droppings which may be identified as the source. Most of the cattle are free-range animals and organism may have been ingested via grazing. The soil environment has been identified as the natural reservoir of *L. monocytogenes*, hence the source of contamination in food [35]. The results from this study re-echoes previous research works in terms of growth and survival of *L. monocytogenes* in soil due to cattle faecal droppings [7], soil available water storage, temperature, proximity to water, roads and urban development, grazing, irrigation, wildlife observation and cultivation [36,37].

Listeria monocytogenes is widely distributed in the environment and possible cross-contamination of different biomes is common. This study showed that isolates from the different biomes shared an evolutionary relationship, thus

a possible common source. This is possible by humans and animals as agents of dispersion via usage of contaminated water for irrigation, surface run-off of top soil into water bodies during erosion, free-range cattle grazing, use of contaminated animal dung as manure, and indiscriminate defecation.

All *L. monocytogenes* isolated in this study produced both listeriolysin and phospholipase, factors known to aid in the pathogenicity of the organism. Both factors aid in the escape of the organism from membrane bound vacuoles, thus releasing the organism into the cytoplasm of the cell [38]. However, research works have it that demonstration of both factors *in-vitro* is not reliable or satisfactory enough to certify the virulence of the organism [39,40]. This is due to the fact that the genes responsible for these proteins may sometimes bear a mutation, thus producing truncated or non-functional variants of the proteins [40,41,42]. However, the occurrence of the organism in foods should raise a concern especially in developing countries where storage and handling of food products are below good manufacturing standard practices.

There are four known lineages of *L. monocytogenes*; I, II, III and IV and are often found overlapping the different ecological niches. Most isolates of lineages I and II are known to cause human clinical cases [43]. However, lineage II is most common in foods, abundant in the natural and farm environments and associated with animals and some human clinical cases [43]. Although the lineages of the isolates in this study were not determined, they are however suspected to be of a subset of lineage I known to carry a listeriolysin S hemolysin, as demonstrated in this study (Plate 1), which is not present in isolates belonging to lineages II, III, or IV [44,43]. Isolates of lineage I are commonly associated with human clinical cases, especially serotypes involved in multistate outbreaks in the USA [43,45]. The occurrence of isolates of lineage I in the environment is of concern as this may aid the spread into different biomes and possibly the food chain network. *L. monocytogenes* from any source is considered a potential pathogen. The strong evolutionary relationship among isolates from different biomes in this study establishes the ubiquity and ecological niche overlap known to the isolate.

Listeriosis is treated with antibiotics especially in persons categorised as high-risk individuals experiencing fever and other non-specific symptoms [35]. It is believed among experts that no tests or treatment be given to persons that does not have any symptoms, even if the person is of the higher-risk category. However, even with prompt treatment, some listeriosis cases result in death, particularly in older adults and in persons with serious medical problems [35]. Research reports of over two decades have it that the pattern of antimicrobial susceptibility of *L. monocytogenes* has been relatively stable for many years; 35 years in the UK [46,47]. In vitro, the organism was susceptible to penicillin, ampicillin, gentamicin, erythromycin, tetracycline, rifampicin, and chloramphenicol, but only moderately susceptible to quinolones [46,48]. Threlfall et al. [47] reported 1-5% tetracycline resistance in *L. monocytogenes* from humans and food in the UK. Morobe et al. [28] also reported 100% potency of erythromycin and ampicillin, while resistance was recorded at 19.30%, 29.82% and 15.79% with streptomycin, cotrimoxazole, and gentamycin respectively. However, results from this study showed a 100% resistance of the organism to the beta-lactam group (ampicillin/cloxacillin and amoxicillin), 26% resistance to erythromycin, 29% resistance to gentamicin, and 30% resistance to streptomycin; which corroborates other research results [49,27,50,51]. There was, however, a marked high resistance percentage to ciprofloxacin at 8% as against 0% in reports of Yakubu et al. [49] but was, however, lower when compared to the report of Ieren et al. [27] at 14.3%. The third generation cephalosporins (cefuroxime and ceftriaxone) showed weak to average potency against *L. monocytogenes*, which may be due to its use as a component in the selective isolation of the organism. Cephalosporins have been reported generally to be ineffective and should not be used therapeutically [52]. Resistance in bacteria has been attributed to abusive use of these agents in veterinary medicine and in humans with subclinical symptoms [53]. Though not significantly different, resistance among isolates from environmental samples was higher than isolates from fresh cut vegetables, 43.5% and 40.4% respectively. Considering that *L. monocytogenes* is slowly becoming antibiotic resistant, a continued surveillance of emerging antimicrobial resistance of this pathogen is important to ensure effective treatment of human listeriosis.

5. CONCLUSION

Listeria monocytogenes was isolated in all samples analysed in this study, most especially

the vegetables used in salads, and thus calls for caution especially in RTE foods consumed. The isolates in this study suggest a possible human clinical case if ingested in high loads or by high risk persons. Cattle contribute to amplification and dispersal of *L. monocytogenes* into the farm environment and the bovine farm system has been linked to subtypes responsible for human listeriosis cases and outbreaks [54]. Food safety, therefore, should entail a high level of hygiene in all processes from 'farm to table'. All strains of *L. monocytogenes* should be considered pathogenic unless otherwise proven.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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