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Listeria Survival and Growth in Fresh Cut Vegetable

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Abstract

Vegetables and fruits are highly nutritious containing minerals and vitamins for sustenance of human health. Contamination is high owing to frequent exposure to soil and water in farms and gardens. Fresh cut vegetables (waterleaf, pumpkin, and cabbage) obtained from three major markets in Owerri, Imo State, Nigeria were subjected to routine microbiological analysis.Characterization and identification of *Listeria monocytogenes* was done with reference to standard identification manuals. Total bacterial counts ranges from 1.0 x 10^7 to 2.94 x 10^8. Water leaf recorded the least counts and pumpkin the highest in all the locations. Fresh cut vegetables obtained from Ekeonuwa market recorded the highest bacterial counts. *Listeria monocytogenes* and *Listeria innocua* were identified as the major contaminants of the vegetables.The percentage distribution showed 61% - 100% for *Listeria monocytogenes* and 72% - 86% for *Listeria innocua* across the sample indicates gross contamination of the vegetables in the markets. The isolates were resistant to Amoxicillin, Rifampicin, Ampicillin and Sulphamethoxazole. Listeriosis, a major foodborne illness associated with foods contaminated with listeria species had been documented. The risk of consuming contaminated fresh vegetable is high due to its patronage. From farm to fork, the fresh leafy produce supply chain (FLPSC) is complex and contains a diverse range of environments where *L. monocytogenes* is sporadically detected during routine sampling of produce and processing. Multi drug resistance recorded by the isolates is worrisome.

Keywords: vegetables, listeria, survival

Introduction

Listeria monocytogenes is an opportunistic bacterial pathogen. It is the causative agent of listeriosis, a disease which predominantly affects immunocompromised people including the elderly, immunosuppressed and pregnant women together with their unborn or new-born babies. *Listeria monocytogenes*, a member of the genus *Listeria,* has been well recognized as a foodborne pathogen and commonly found and isolated from fresh produce (Goldfine and Shen, 2007). Once *L. monocytogenes* enter into the processing facilities, it can survive for long periods due to favourable humidity and temperature (Dalmasso *et al*., 2015); therefore, refrigeration is not the most effective way to control the pathogens growth even when temperature almost reach freezing. Foods such as soft cheeses, spreads, smoked and raw seafood, sprouts, fruit, vegetables, poultry,

soil, water, sheep, cattle, processed foods; dairy foods have all been linked to listeriosis cases (CDC, 2014).Symptoms of Listeriosis are very similar to symptoms such as the flu. These symptoms can be described as fever, nausea, diarrhea, aches, and can even affect the nervous system causing one to be unbalanced. Microbial risk in fresh produce begins at an early stage of production, the pre-harvest stage (Qadri *et al*., 2015; Ranjbar and Halaji, 2018). Pathogens initially attach to the surface of produce, however during slicing pathogens may transfer to the flesh. Produce is rich in nutrients that can support the growth of pathogens (Jordan and McAuliffe, 2018).

Foods which have been previously implicated in *L. monocytogenes* infections include milk, soft cheeses, deli or sandwich meats and fresh produce, which encompass both fresh fruit and vegetables (Jackson *et al*., 2018). Several reports have demonstrated *L. monocytogenes* presence in a wide variety of fresh produce samples (Nightingale *et al*., 2004; Qadri *et al*., 2015; Ranjbar and Halaji, 2018; Kristina, 2022) and other minimally processed foods. Other than a potentially tragic loss of life, the economic consequences of a *L. monocytogenes* outbreak are significant due to a loss of consumer confidence and subsequent drop in product sales and related value (CDC, 2014; Kristina, 2022).*L. monocytogenes* contamination of fresh leafy produce lines, such as cabbage, pumpkin, waterleaf etc are considered 'high-risk' in terms of bacterial contamination because of their leaf structures and proximity to the ground. The fresh leafy produce supply chain (FLPSC), from farm to fork, is complex and contains a diverse range of environments where *L. monocytogenes* can be detected during routine sampling of fresh leafy produce throughout the supply chain. For example, in soil, recently cut vegetable, the processing environment and in the final the product itself, although detection tends to be sporadic (Qadri *et al*., 2015; Choi *et al*., 2018; Ranjbar and Halaji, 2018; Rogolia and Bomar, 2022).

Owing to the potential risk of food-borne illness from this bacterium, source tracking, risk assessment and understanding the ability of *L. monocytogenes* to survive in the FLPSC should be considered key factors in tackling *L. monocytogenes* contamination of fresh leafy produce and reducing risk to the consumer.

In this report *L. monocytogenes* contamination in fresh cut vegetables and the mechanisms behind survival in this environment.

Materials and Methods

Description of sample locations:

Fresh cut vegetables (waterleaf, pumpkin, and cabbage) were purchased from open daily markets (Ekeonunwa market, Relief market and School road market) in Owerri, Imo State, Nigeria. The market is densely populated with beehive of activities.

Collection of Samples:

Samples were obtained randomly, wrapped into a clean polyethylene bags, labeled and taken to the laboratory. Samples collected were fresh and intact.

Preparation of media and diluents

Listeria agar (LA) was prepared and used for the isolation of *Listeria* species, especially *Listeria monocytogenes* and other species. The media was supplemented with antibiotics and antifungal agents. Diluents and media were sterilized according to Beishir(1987) and Cheesbrough(2000).

Preparation of samples and inoculation

One hundred grams (100 g) of each samples (water leaf, pumpkin, cabbage) was blended in 900 ml of sterile physiological saline in a stomacher blender and swirled to mix thoroughly to obtain 10^{-1} dilution. Further dilution was carried out until the desired dilution was obtained. Aliquot portion (0.1 ml) of appropriate dilution was inoculated into the pre-sterilized and surface

dried LA. Inocula were spread evenly to ensure uniform and countable colonies. Plates were incubated at ambient temperature for 24-48 hours for the development of visible colonies (Cheesbrough, 2000).

Determination of microbial population and characterization

Colony counts obtained on the media were counted and expressed as colony forming units per gram (CFU/g) of the total population (Harrigan and McCance, 1990). Microbial isolates were characterized based on cultural (colonial), microscopic and biochemical methods with reference to standard manuals. The identities of the isolates were confirmed with reference to standard manuals (Buchanan and Gibbon, 2000).

Standardization of Inoculum and Determination of Multi-Drug Resistant

Twenty four hour old pure cultures of test isolates (*Listeria* species) were washed three consecutive times in a sterile buffer and standardized using McFarland method with cell turbidity equivalent to 1.5 x 10^8Cfu/ml (Cheesbrough, 2000; Sharma, 2000).

One-tenth milliliter (0.1 ml) of the standardized pure cultures of test isolates were spread evenly on a freshly prepared surface dried Mueller Hinton Agar (MHA) medium and allowed to stand for 30 mins. Five commercial antibiotics (oxoid) of known concentrations were placed at equal distance on the medium previously seeded with the test organisms. The entire set was incubated at 37^0C for 24-72 h. Zone of inhibition was measured in triplicates and the mean recorded (Cheesbrough, 2000; Sharma, 2000).

Results

Table 1 shows the total bacterial counts isolated on fresh cut vegetables cultivated on listeria ager. Samples from the different markets designated as sampling location showed varied bacterial population signifying the level of contamination. Bacterial counts ranges from 1.0 x 10^7 to 2.94 x 10^8. Water leaf recorded the least counts and pumpkin the highest in all the sampling locations.Fresh cut vegetables obtained from Ekeonuwa market recorded the highest bacterial counts. The identities of the listeria species is shown in Table 2. The percentage distribution showed 61% - 100% for *Listeria monocytogenes* and 72% - 86% for *Listeria innocua* across the sample locations (Table 3). Prevalence of listeria species in fresh cut vegetables is shown in Table 4. Both isolates were resistant to amoxicillin, ampicillin and Sulphamethoxazole. *L. monocytogenes* is sensitive to amikacin, cephalexin, erythromycin and streptomycin whereas *L. innocua* is susceptible to amikacin, cephalexin, erythromycin, streptomycin and rifampicin (Table 5). *L. monocygenes* is resistant to rifampicin in addition to the three antibiotics mentioned above (Table 5).

Table 1 Total Counts of Bacterial isolates on Listeria Agar

Samples and location of sampling	Sample code	Total Counts (Cfu/g) on LA
School Road Market Cabbage (SHMC)	SHMC1	3.9×10^7
	SHMC2	1.69×10^8
	SHMC3	0
	SHMC4	2.94×10^8
Ekeonuwa Market Cabbage (EKMC)	EKMC1	9.4×10^7
	EKMC2	1.1×10^7
	EKMC3	6.0×10^6
	EKMC4	4.2×10^7
Relief Market Cabbage (RFMC)	RFMC1	9.8×10^7
	RFMC2	6.0×10^6
	RFMC3	$1.44\ 10^8$
	RFMC4	1.0×10^7
Relief Market Pumpkin (RFMP)	RFMP1	0
	RFMP2	0
	RFMP3	1.44×10^8
	RFMP4	0
School Road Market Water Leaf (SHMW)	SHMW1	4.3×10^7
	SHMW2	3.6×10^7
	SHMW3	1.5×10^7
	SHMW4	3.8×10^7
Ekeonuwa Market Pumpkin (EKMP)	EKMP1	5.6×10^7
	EKMP2	1.11×10^8
	EKMP3	2.94×10^8
	EKMP4	4.1×10^7
Relief Market Water leaf (RFMW)	RMW1	1.5×10^7
	RFMW2	2.1×10^7
	RFMW3	3.0×10^7
	RFMW4	3.2×10^7
Ekeonuwa Market Water leaf	EFKMW1	2.0×10^6
	EKMW2	1.81×10^8
	EKMW3	1.2×10^7
	EKMW4	1.00×10^7
School Road Market Pumpkin (SHMP)	SHMP1	7.8×10^7
	SHMP2	3.3×10^7
	SHMP3	1.58×10^8
	SHMP4	2.91×10^8

LA, Listeria Agar; RFM, Relief Market; EKM, Ekeonuwa Market; SHM, School Road Market; W, Water leaf ; C, Cabbage; P, Pumpkin leaf

Table 2: Colonial, Microscopic and Biochemical Characteristics of Bacterial isolates on Listeria Agar

Colonial characteristics	Microscopic morphology	Cat	Oxi	MR	VP	NO_3	XYL	Coag	Mann	Rha	Lac	CAMP	HEAM	Identity of isolates
Blue-green colonies with opaque halo	Small Gram negative rods, non-spore forming, motile with peritrichous flagellation	+	-	+	-	-	-	-	-	+	+	+	+	*Listeria monocyto genes*
Gray-green colonies with black halo	Small Gram negative rods, non-spore forming, motile with peritrichous flagellation	+	-	+	-	+	-	-	-	+	+	+	+	*Listeria innocua*

Cat, catalase; MR, Methyl Red Reduction Test; VP, Voges Proskaeur; Mn, mannitol; Rh, Rhafinose; CAMP, Christie Atlkins Munch Peterson; HAEM, Heamolysis Test; Lac, Lactose; XYL, Xylose; OXI, Oxidase; Coag, Coagulase

Table 3 Percentage distribution of Listeria isolates in samples from different markets

Sample location	*Listeria innocua*	*Listeria monocytogenes*
Relief Market	86%	100%
Ekeonuwa Market	72%	61%
School Road Market	77%	100%

Table 4 Prevalence of *Listeria* species in fresh cut vegetables

Types of sample	No. of samples analyzed	*Listeria monocytogenes*	*Listeria innocua*
Cabbage	200	180 (90.0%)	160 (80.0%)
Pumpkin	200	120 (60.0%)	110 (55.0%)
Water leaf	200	200 (100.0%)	180 (90.0%)
Total number	600	500	450

Number in Parenthesis indicate percentage

Table 5 Antibiotic susceptibility Test (*Listeria* spp)

Bacterial isolates	AK 30 µg	CL 30 µg	E 15 µg	AMC 30 µg	RD 5 µg	S 10 µg	AMP 10 µg	SXT 25 µg
Listeria innocua	14.0±0.01	5.0±0.01	16.0±0.02	0	12.0±0.02	7.0±0.01	0	0
Listeria monocytogenes	24±0.02	20.0±0.02	10.0±0.01	0	0	14.0±0.01	0	0

AK, Amikacin; CL, Cephalexin; E, Erythromycin; AMC, Amoxicillin; RD, Rifampicin; S, Streptomycin; AMP, Ampicillin; SXT, Sulphamethoxazole

Discussion

Fresh produce are commonly part of human diets due to their healthy nature. They are a significant source of vitamins, minerals, and fiber (Choi *et al*., 2018; Jordan and McAuliffe, 2018; Kristina, 2022; Rogolia and Bomar, 2022). The regular consumption of these products, along with maintaining a healthy lifestyle, such as frequent exercise has been found to protect from chronic diseases (Qadri *et al*., 2015). These benefit coupled with the promotion of healthy diets has led to a constant increase in the consumption of fresh products over the past decades. Considering this there has also been an increase in outbreaks related to produce (Goldfine and Shen, 2007; Choi *et al*., 2018; Jackson *et al*., 2018).

The growth and survival of*L. monocytogenes* and *L. innocua* on three fresh cut vegetables randomly samples from three popular markets in Owerri Imo State, Nigeria suggest evidence of gross contamination. *Listeria* species are abundant in soil, vegetation, animal and human excreta (Nightingale *et al*., 2004; Ranjbar and Halali, 2018; Kristina, 2022). Food products are contaminated with a foodborne pathogen such as *L. monocytogenes* along the pathway of food production such as planting, harvesting, picking, distribution and serving. Sources of contamination by *L. monocytogenes* canoriginate from the environment, for example: irrigated waters, wash waters, and soil. For example, heavy rains on a crop, especially crops that grows low to the ground such as cantaloupes where there is contamination of soil, the splash of rain could splash bacterium onto the edible surface, thus, contaminating the productNightingale *et al*., (2004).

However, in farm areas, the soils and waters can become contaminated by the use of raw or improperly composted manure (Nightingale *et al*., 2004). Most products used as compost manure for crop consist of chicken and cattle faeces and animal carcasses, which can contain foodborne pathogens and ultimately contaminate crops, and water source (Nightingale *et al*., 2004). Raw manure applied to crops can contaminate water table through leaching, runoff, and by irrigation waters increases the risk of soil and vegetables becoming contaminated by *L. monocytogenes.* (Nightingale *et al*., 2004; Rogolia and Bomar, 2022; Kristina, 2022).Contamination of produce with *L. monocytogenes* is not limited to packing houses or fields. Contamination can also occur in restaurants and consumer kitchens, where the pathogen can grow in areas where foods are being handled and cooked, refrigerated and placed on un-cleaned countertops.Survival of *L. monocytogenes* in these environments is vehicle to its transmission to foodstuffs. For example, *L. monocytogenes* can persist in a food processing facility for months and re-contaminate product passing through that facility (Kristina, 2022).The study reports an increase in *L. monocytogenes* and *L. innocua* in all the vegetables sampled in the markets. This portends high risk of listeriosis in the consumption of vegetables contaminated with *Listeria* species (Choi *et al*., 2018; Jackson *et al*., 1918.

In the global spheres, vegetable are eaten raw or added as spices to garnish already prepared foods that are not further processed before eating. Several reported have incriminated *Listeria monocytogenes* as a major foodborne contaminants and outbreaks of listeriosis (Nightingale *et al*., 2004; CDC, 2014). Multi drug resistance reported in the study is worrisome as reported by Conter *et al*. (20090 and Srinivasan *et al*. (2005)

Listeria monocytogenes is a foodborne pathogen that can have serious consequences in RTE foods. By practicing hygiene and following local, state and government regulation, proper handling, storing and shipping can reduce the possibilities of contamination of *L. monocytogenes*. *L. monocytogenes* is a real foodborne pathogen that spreads, and have word-wide distribution (Ranjbar and Halaji, 2018) affecting a large population because of difficulty in treatment (Temple and Mahata, 2000).

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