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Isolation and antibiotic sensitivity of *Listeria monocytogenes* and *Staphylococcus aureus* in Fruit and Vegetable Salads from some Eateries in Osogbo, Osun state.

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ABSTRACT

The consumption of ready-to-eat (RTE) fruit and vegetable salads have increased owing to the derivable health benefits but these food products can also be vehicles food foodborne pathogens which may subsequently lead to various types of illnesses. This study evaluated the prevalence of *Listeria monocytogenes* and *Staphylococcus aureus* and antibiotic susceptibility pattern in mixed fruit and vegetable salads from six different eateries (A, B, C, D, E, F) within Osogbo metropolis, Osun state. Out of a total of 36 samples (24 vegetable salads and 12 fruit salads), presumptive *Listeria* spp. were identified from A, B and C while *S. aureus* was identified in all the eateries. Besides, only 4.96% were identified as *L. monocytogenes* while 10.44% of *S. aureus* exhibited α-haemolysis. Although the prevalence of *L. monocytogenes* and *S. aureus* are low in each location, all the haemolytic *S. aureus* isolates demonstrated 100% resistance to ampicillin, tetracycline, chloramphenicol and tetracycline while the isolates were generally susceptible to gentamicin. In contrast, *L. monocytogenes* were only 100% resistant to ampicillin. This study calls for awareness among handlers and stakeholders of these RTE food products to pay more attention to the state of raw material being purchased, upload the proper hygiene, avoid abuse of storage temperature and other food safety precautions as the presence of these multi-drug resistant isolates raises public health concerns.

Keywords: Isolation, antibiotic sensitivity, Listeria monocytogenes, *Staphylococcus aureus,* Fruits and Vegetable Salads

Aims Research Journal Reference Format:
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1. INTRODUCTION

Salad is a term broadly applied to many food preparations having a mixture of chopped or sliced ingredients which may be mostly fruits or vegetables (Uzeh et al. 2009). Common vegetables used in a salad include cucumber, cabbage, carrots, and spring onions. Other ingredients such as olives, mushrooms, hard-boiled egg, green beans, cheese, meat or seafood are sometimes added to salads. Fruits and vegetables are well-known sources of useful nutrients in the form of vitamins, minerals, dietary fibers and other phytonutrients including flavonoids, carotenoids and phenolic compounds that may lower the risk of cancer, heart disease and others illnesses (Aune et al. 2017). Although vegetables are a good source of antioxidants and phytonutrients, the salads containing raw vegetables may be unsafe for consumption if not properly processed. It may be due to direct contamination from decaying vegetation, animal faeces, soil surfaces, river and canal waters, effluents from sewage treatment operations, improper harvesting and handling procedures, improper sanitary condition of equipment and transportation practices (Beuchat 1996). These ready to eat (RTE) foods have been identified as vehicles of bacterial agents and generate food safety problems, especially gastroenteritis (Adjrah et al. 2013).

The incidences of some foodborne pathogens in Nigeria have been reported by various researchers. Oni et al. (2010) isolated pathogenic species of *S. aureus, Bacillus* spp*., Proteus* spp. and yeast from different salad samples purchased from food outlets in Ekpoma, Edo state, Eni et al. (2010) reported the incidence of pathogenic *S. aureus, Klebsiella, Salmonella, Actinomycetes* and *Escherichia coli* in fruits and vegetables in Sango Ota, Ogun state while Ajayeoba et al. (2016) also reported the isolation of *Listeria monocytogenes* in RTE vegetables from markets in Southwest Nigeria. Furthermore, more than 90 percent of yearly cases of food poisoning in Nigeria have been caused by *S. aureus, Salmonella* spp., *Clostridium perfringens, Campylobacter* spp., *L. monocytogenes, Vibrio parahaemolyticus, Bacillus cereus, Escherichia coli and Proteus* spp. (Oni et al. 2010). According to Onyeneho and Hedberg (2013), WHO estimates that more than 200,000 people die of food poisoning annually in Nigeria from foodborne pathogens.

Since there are indications that *L. monocytogenes* and *S. aureus* are part of the main pathogens causing foodborne illness in RTE fruit and vegetable salads in Nigeria, literature is depleting on the prevalence of these opportunistic pathogens within some eateries in Osogbo, Osun state. Therefore, this study will evaluate the prevalence and antibiotic-resistant pattern of *S. aureus* and *Listeria monocytogenes* from RTE fruits and vegetable salads in different eateries within Osogbo.

2. MATERIALS AND METHODS

Location of study

This study was carried out in Osogbo, Osun State is an inland state in southwest Nigeria. The state is in the tropical rain forest zone. It covers an area of approximately 14,875sq km and it lies approximately on latitude 7.77ºN of the equator and longitude 4.57ºE of the Greenwich meridian and about 1100m above the sea level.

Source of Materials

All antimicrobial disc and media used for this research work were purchased from Oxoid (UK). 0.5 McFarland standard was prepared in the laboratory according to the specification.

Sampling spot

A total of 36 samples (24 vegetable salads and 12 fruit salads) were randomly purchased from six different eateries (A, B, C, D, E, F) within Osogbo metropolis depending on the availability of the ready-to-eat (RTE) vegetable. The salads were collected in sterile zip-lock bags and stored at 4 ºC before analysis. Microbiological analysis was performed within 3 h of collection.

Methods

All experiments were carried out in duplicates.

Detection and Enumeration of *Listeria monocytogenes* and *Staphylococcus aureus*

The detection of *Listeria monocytogenes* and *Staphylococcus aureus* were carried out according to the method described by Ajayeoba et al. (2016) and Eni et al. (2010) respectively. Suspected colonies were further identified with gram staining and other biochemical tests according to the method of (Cheesbrough 2006).

Determination of α-haemolytic activity in *S. aureus* and β-haemolytic *L. monocytogenes*.

The β - haemolytic activity was carried out according to the modified methods described by Dąbrowski et al. (2003). A distinct colony from a fresh culture was inoculated on blood agar and incubated at 37 ºC for 24h. The colonies were observed for the green colour zone (α-haemolysis) a narrow transparent zone (β-haemolysis) around the colonies.

CAMP test for *L. monocytogenes*

The CAMP test on the test isolates was carried out according to the methods described by Rapeanu et al. (2008). A 24 h old reference isolates of *Staphylococcus aureus* (ATCC 25923) was streaked vertically on blood agar with a distance of about 4 cm between them. The suspected test isolates were carefully streaked horizontally between them without touching the vertical streaks. The plates were incubated for 24 h at 35 ºC and examined for enhanced β- haemolysis at both ends proximal to the reference isolate. Antibiotic susceptibility test. The antibiotic susceptibility of the test isolates was carried out according to the methods standardized by the Clinical and Laboratory Standards Institute (CLSI, 2012). Suspensions of *L. monocytogenes* was prepared by using a sterile swab to pick colonies from a 24 h old culture into test tubes containing 0.85% normal saline to obtain turbidity optically comparable to that of 0.5 McFarland standard. Another swab was dipped into the cell suspension and spread evenly on the surface of Mueller- Hinton agar (CM0337B). The following panel of antimicrobial disks was used for each test isolates: ampicillin (AMP - 10µg), ciprofloxacin (CIP - 5µg), chloramphenicol (C -30µg), tetracycline (TET - 30µg), gentamicin (CN - 10µg) and trimethopim/sulphamethoxazole (SXT - 1.25/23.75µg). The zone of inhibitions was measured and interpreted with the breakpoint standards for the zone of diameter provided by (CLSI 2012) guidelines, to classify the antibiotic sensitivity of each isolates as 'susceptible', 'intermediate' or 'resistant'.

3. RESULT AND DISCUSSION

Isolation and identification of *Listeria monocytogenes* and *Staphylococcus aureus*

Fruits and vegetables are known to be healthy food supplements and are consumed with other food dressings with minimal processing. These RTE foods, if not properly handled and processed, are particularly known to harbour different microorganisms that may result in food-borne illnesses (Ajayeoba et al. 2016, Eni et al. 2010). Therefore, the microbiological safety of these RTE foods is important because its consumption has increased owing to a greater appreciation of their food values. Out of the six different fast foods (A, B, C, D, E, F) within Osogbo metropolis, only three locations (A, B, C) showed visible growth of presumptive *Listeria* spp. on Brilliance *Listeria* agar.

All the 504 colonies isolated were gram-positive and catalase-positive but only 72 isolates fermented L-rhamnose while other fermented D-xylose. However, 25 of these isolates were positive for β-haemolytic test and CAMP test (Table 1). This shows that there may be other opportunistic microorganisms (probably other isolates of *Listeria* spp.) present in the RTE fruit and vegetable salads. Although the prevalence was higher in vegetable salads, the prevalence of presumptive *Listeria monocytogenes* was generally low within eateries and below the European Union limit of 100 CFU/g (European Union 2007) but the identification of these pathogen poses a significant health risk to consumers. This also corroborates previous reports from Brazil (Porto and Eiroa 2001) and United Kingdom (Little et al. 2007). Generally, the percentage of presumptive *L. monocytogenes* showing β- haemolysis (for both fruit and vegetable salads) was higher in point A (1.98), followed by point C (1.59) and B (1.39) respectively. The distribution of *Listeria* spp. in the RTE food product is shown in Table 1. The differences in the prevalence of this pathogen in different eateries may be due to the level of enlightenment, state of the raw materials used in the preparation, proper storage temperature and implementation of hygienic conditions. In contrast to the prevalence of *Listeria* spp. in the RTE food products, *S. aureus* isolates were identified in all six locations and all tested positive to the standard biochemical test. The level of *S. aureus* in the fruits and vegetable salad was lower than the guideline of National Food Safety Standard-Pathogen Limits for RTE items (GAIN Reports 2012) of 100 CFU/g. A total of 26 isolates exhibited β- haemolysis and the percentage of presumptive *S. aureus* showing β- haemolysis (for both fruit and vegetable salads) was higher in point C (4.02), followed by point A (2.01), B and E (1.61), D (1.20) while there was no haemolytic isolate in point F.

Table 1: Prevalence and distribution of *Listeria* spp. and *Staphylococcus aureus* in RTE fruits and vegetable salads

Sampling spot	Type of RTE food	Number of microorganism identified within location					
		Listeria monocytogenes			*Staphylococcus aureus*		
		Total number of isolates identified (CFU/g)	Total number of isolates positive*	Total number of β-hemolytic isolates	Total number of isolates identified (CFU/g x10^3)	Total number of isolates positive*	Total number of α-hemolytic isolates
Point A	FS	57 (11.31)**	8 (1.59)	2 (0.39)	13 (5.22)	13 (5.22)	4 (1.61)
	VS	92 (18.25)	10 (1.98)	8 (1.59)	17 (6.83)	17 (6.83)	1 (0.40)
Point B	FS	99 (19.64)	9 (1.78)	3 (0.60)	12 (4.82)	12 (4.82)	0 (0.00)
	VS	68 (13.49)	13 (2.58)	4 (0.79)	31 (12.45)	31 (12.45)	4 (1.61)
Point C	FS	79 (15.68)	8 (1.59)	3 (0.60)	14 (5.62)	14 (5.62)	4 (1.61)
	VS	109 (21.63)	24 (4.76)	5 (0.99)	42 (16.87)	42 (16.87)	6 (2.41)
Point D	FS	0 (0.00)	0 (0.00)	0 (0.00)	19 (7.63)	19 (7.63)	3 (1.20)
	VS	0 (0.00)	0 (0.00)	0 (0.00)	29 (11.65)	29 (11.65)	0 (0.00)
Point E	FS	0 (0.00)	0 (0.00)	0 (0.00)	5 (2.01)	5 (2.01)	1 (0.40)
	VS	0 (0.00)	0 (0.00)	0 (0.00)	35 (14.05)	35 (14.05)	3 (1.20)
Point F	FS	0 (0.00)	0 (0.00)	0 (0.00)	4 (1.61)	4 (1.61)	0 (0.00)
	VS	0 (0.00)	0 (0.00)	0 (0.00)	28 (11.24)	28 (11.24)	0 (0.00)
Total		504 (100.0)	72 (14.28)	25 (4.96)	249 (100.0)	249 (100.0)	26 (10.44)

FS= fruit salad; VS= vegetable salad
*Total number of isolates that tested positive to all standard biochemical test
** Percentage of isolate per type of RTE food per location

Haemolysis is one of the important virulent factors for *L. monocytogenes* and *S. aureus*. While β-haemolysis in *L. monocytogenes* could depict the presence of virulent genes that can it aids in disruption of phagocytic vacuole, release of bacteria into cytoplasm and consequently triggers degranulation and lipid mediator generation (Swetha et al. 2012), the cytotoxicity of α-haemolysis in *S. aureus* is mediated through transmembrane pore formation resulting in characteristics DNA fragmentation and cell death through apoptosis (Xiaohong and Yanjun 2011). The presence of these haemolytic isolates calls for more sensitization in handling RTE food in eateries. Opportunistic pathogens can cause life-threatening infections mainly in immunocompromised people and have various effects on the sensory quality of the produce.

As observed in this study, all the haemolytic isolates demonstrated multiple antibiotic resistance. Multi-drug resistant *S. aureus* have been reported recently been reported in RTE vegetables, meat and meat products (Wu et al. 2018a, Wu et al. 2018b). The spread of antibiotic-resistant isolates of *S. aureus* is of great public and clinical concern in the treatment of staphylococcal infections. Although *L. monocytogenes* are susceptible to various antibiotics (Hof, 2003), the occurrence of antibiotic-resistant isolates of presumptive *L. monocytogenes* in RTE fruits and vegetables reported in this study corroborates the reports of other authors in Nigeria (Ieren et al. 2013, Uzeh et al. 2009), India and New Zealand (Soni et al. 2014, Zhu and Hussain 2014). The occurrence of resistant isolates of the organism thus necessitates the need for surveillance to monitor temporal and geographical shifts in resistance patterns and the associated phenotypes and genotypes. All the haemolytic *S. aureus* exhibited 100% resistance to ampicillin, tetracycline, chloramphenicol and tetracycline, while resistance varied too other types of antibiotics (Table 2) all the isolates were susceptible to gentamicin (except isolate 16). In contrast, *L. monocytogenes* were only 100% resistant to ampicillin will resistance to other antibiotics varied (Table 3).

The high percentage of resistant isolates of *S. aureus* and *L. monocytogenes* in the RTE fruit and vegetable salads towards these antibiotics corroborates previous reports (Ieren et al. 2013, Jamali et al. 2013, Saba et al. 2017, Wu et al. 2018a, Wu et al. 2018b). The prevalence of drug resistant *S. aureus* and *L. monocytogenes* in RTE fruit and vegetable salads in eateries within Osogbo requires awareness among handlers and stakeholders to pay more attention to the state of raw material being purchased, upload the proper hygiene, and other food safety precautions. The results of this study emphasize the prevalence of *S. aureus* and *L. monocytogenes* isolated from different eateries in Osogbo. Although the level of contamination was low, the presence of haemolytic isolates demonstrating multiple antibiotic resistance raises concerns on possible foodborne illnesses. Hence, there is a need to adopt strategies to encourage proper personal hygiene and proper enlightenment on food safety techniques on food and food processing environment to prevent contamination of minimally processed foods like fruit and vegetable salads.

Table 2: Antibiotics Susceptibility Pattern of haemolytic *S. aureus* in fruit and vegetable salads

Isolate identification	Type of antibiotic disc used					
	CN	CIP	SXT	E	AMP	TE	C
EAFS 1	S	S	R	R	R	R	R
EAFS 2	S	S	S	R	R	R	I
EAFS 3	S	S	R	R	R	R	R
EAFS 4	S	S	R	R	R	R	R
EAVS 1	S	I	R	R	R	R	R
EBVS 1	S	I	R	R	R	R	R
EBVS 2	S	S	R	R	R	R	R
EBVS 3	S	S	R	R	R	R	R
EBVS 4	S	I	I	R	R	R	R
ECFS 1	S	S	R	R	R	R	R
ECFS 2	S	R	R	R	R	R	R
ECFS 3	S	S	R	R	R	R	R
ECFS 4	S	S	R	R	R	R	R
ECVS 1	S	I	R	R	R	R	R
ECVS 2	S	S	S	R	R	R	R
ECVS 3	R	I	S	R	R	R	R
ECVS 4	S	I	R	R	R	R	R
ECVS 5	S	S	S	R	R	R	R
ECVS 6	S	S	R	R	R	R	R
EDFS 1	S	S	S	R	R	R	R
EDFS 2	S	S	R	R	R	R	R
EDFS 3	S	S	S	R	R	R	R
EEFS 1	S	S	S	R	R	R	R
EEVS 1	R	S	R	R	R	R	R
EEVS 2	S	S	R	R	R	R	R
EEVS 3	S	I	R	R	R	R	R

R: Resistance, S: Susceptible I: Intermediate
EAFS – Eatery point A Fruit Salad
EAVS - Eatery point A Vegetable Salad
EBVS - Eatery point B Vegetable Salad
ECFS - Eatery point C Fruit Salad
ECVS - Eatery point C Vegetable Salad
EDFS - Eatery point D Fruit Salad
EEFS - Eatery point E Fruit Salad

CN - Gentamicin; CIP – Ciprofloxacin; SXT - trimethopim/sulphamethoxazole; E- Erythromycin; AMP – Ampicillin; TE – Tetracycline; C - Chloramphenicol

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