Evaluation of Antibiotic Resistance Pattern of Gram-positive Bacilli Isolated From Ready-to-Eat Vegetables Sold in Ota Metropolis, Nigeria

By

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Abstract: In most Nigerian cities, ready-to-eat (RTE) vegetables are purchased directly from street vendors and consumed immediately without necessarily having to cut, peel or rinse them as they have already been presumed to be processed by the vendors. However, the microbiological safety of these vegetables is of special concern due to the possible microbial contamination from incoming raw produce from farms, workers hygiene and handling practices, and the condition of the environment and equipment used to process the vegetables for distribution, marketing and sales. The aim of this study was to determine the incidence and antibiotic susceptibility patterns of Gram-positive *Bacilli* on RTE vegetables; Cabbage, Carrot, Cucumber and Lettuce from two local produce markets within Ota Metropolis. Pure cultures obtained by repeated streaking and identified based on cultural, morphological and biochemical characteristics were subjected to antibiotic susceptibility tests. The total aerobic bacteria present in the RTE vegetable samples Cabbage, Cucumber, Lettuce and Carrot ranged, respectively, from 1.84×10^6 - 2.24×10^6 cfu/g, 1.72×10^6 - 2.48×10^6 cfu/g, 1.51×10^6 – 1.97×10^6 cfu/g and 1.69×10^6 – 2.42×10^6 cfu/g. A total of sixteen bacterial isolates from the RTE vegetables were tentatively identified as *Bacillus brevis (30%), Nocardia spp. (18%), Bacillus spp. (12%), B. subtilis (12%), B. megaterium (6%), B. circulans (6%), B. sphaericus (6%) and B. pumilus (6%).* Although, these bacteria are mostly causative agents of food spoilage and sometimes secondary infections, it is alarming that more than 80% of the bacterial isolates were resistant to at least two antibiotics including erythromycin, cloxacillin, cotrimoxazole, augmentin and streptomycin. The

results of this study raise the spectre of antibiotic-resistance in normal soil microbes derived from RTE vegetables with potential impact on humans through the food chain and environmental exposure.

Introduction

Vegetables, edible plants or parts of a plant which usually exclude seeds and most sweet fruit, are known to have nutrient, micro-nutrients, vitamins and fibers, and are thus vital for human health and well-being (Adebayo-Tayo *et al*., 2012). The health benefits associated with consumption of fresh produce combined with the trend toward consuming street-vended or retailed ready-to-eat (RTE) foods in Nigeria have contributed to an increase in the popularity of RTE vegetables including lettuce, cabbage, carrots and cucumber which do not require further preparation before consumption (Abdullahi and Abdulkareem, 2010; Eni *et al*., 2010). Although the presence of agricultural chemical residues or the presence of metals is of concern (Oluwatosin *et al*., 2010; Wilberforce and Nwabue, 2013); the hazards specific to RTE vegetables reside mainly with microbial contaminants. The possible sources of microbial contamination include resident microflora in the soil (Heyndrickx, 2011), the incoming raw vegetables from farms that may have been exposed to non-resident microflora via animal manures, sewage or irrigation water (Halablab *et al*., 2011), workers' hygiene and handling practices, and the condition of the environment and equipment used to process the vegetables for distribution, marketing and sales (Bruno *et al*., 2005; Muinde and Kuria, 2005).

Some of the microbial pathogens associated with fresh fruit and vegetables include *Listeria monocytogenes* (Berrang *et al*., 1989; Carlin and Nguyen, 1994), *Salmonella* spp. (Abdullahi and Abdulkareem, 2010; Eni *et al*., 2010; Ali *et al*., 2011, Aparecida de Oliveira *et al*., 2011), *Shigella* spp. (Frost, 1995; Odu and Okomuda, 2013), enteropathogenic strains of *Escherichia coli* (Ali *et al*., 2011; Ackers *et al*., 1998; Odu and Okomuda, 2013), Hepatitis A virus (Dentinger *et al*., 2001), and the protozoans *Cryptosporidium*, *Cyclospora* and *Giardia* (Doaa, 2012). *Bacillus*, a heterogeneous genus of closely related species that include, among others, *Bacillus cereus*, frequently isolated from fresh vegetables and RTE vegetable-based foods, an important species involved in food poisoning (Rosenquist *et al*., 2005; Elhariry, 2011), and *B. licheniformis* (Kramer *et al*, 1989; Salkinoda-Salonen *et al*., 1997), *B. subtilis* (Adesetan *et al*., 2013) and *B. pumilus (*Gilbert *et al., 1981*, Meldrum *et al., 2008)*, which are causative agents of food spoilage have also been described as responsible for foodborne disease outbreaks.

In developing countries such as Nigeria, continued use of untreated waste water and manure as fertilizers for the production of raw vegetables is a major contributing factor to microbial contamination that cause numerous food-borne disease outbreaks (Bergogne-Bérézin, 1997; Olayemi, 1997). In addition, the increased consumption of RTE vegetables may increase the risk for transfer of antimicrobial resistance to humans as the eventually present resistant bacteria are not killed (Verraes *et al*., 2013). Food contamination with antibiotic resistant bacteria can be a major threat to public health as the antibiotic resistance determinants can be transferred to other pathogenic bacteria potentially compromising the treatment of severe bacterial infections.

The prevalence of antimicrobial resistance among pathogens from RTE vegetables has increased during the recent decade in developed countries (Duffy *et al*., 2005; Falomir *et al*., 2010; Ruimy *et al*. 2010; Holvoet *et al*., 2013). However, reports of the antibiotic sensitivity of these bacteria are only presently emerging in developing countries (Adesetan *et al*., 2013; Tabashsum *et al*., 2013; Tagoe and Aning, 2011; Adebayo *et al*., 2012; Akinyemi *et al*., 2013). Transmission of antimicrobial resistant bacteria is a potential concern with unhygienic handling of RTE vegetables and fruits. The aim of this research was to isolate bacteria from RTE vegetables sold by street vendors in Ota Metropolis, Nigeria, to characterize the bacterial isolates using conventional methods for Gram positive bacteria and perform antimicrobial sensitivity tests on the bacterial isolates.

Materials and Methods

Sample Collection

Sixteen samples of RTE vegetables were randomly acquired from four street vendors, two each in the local produce markets located at Oju-Ore and Sango areas within Ota metropolis, Ogun State, Southwest region of Nigeria and included: 4 cabbage, 4 carrot, 4 cucumber and 4 lettuce. All the samples were obtained fresh and then wrapped in foil paper and transported in polythene bags to the laboratory and analyzed on the day of purchase.

Total aerobic plate count of bacteria

Bacterial counts were carried out according to standard methods (Swanson *et al*., 2001). A total of ten gram of the RTE vegetable sample was washed in sterile 90ml saline from which 1ml was transferred to the first test tube containing 9mls of sterile distilled water as diluent. This was repeated for the other three sets of tubes to dilute to 10^{-5}. The procedure was repeated for each RTE vegetable sample. From the last two dilutions, 1ml each was dispensed and spread aseptically onto the pre-sterilized Muller-Hinton agar (MHA) plates in duplicate. The plates were packed and incubated at 37^0C for 24hrs. At the end of incubation, the plates were removed and all discrete colonies were counted where possible, multiplied by the dilution factor and expressed as the colony forming units per gram (cfu/g).

Identification and characterization of selected bacterial isolates

Colonies were presumptively identified by colonial morphology on MHA plates and Gram staining characteristics. Pure bacterial cultures were obtained by sub-culturing distinct colonies onto freshly prepared MHA plates followed by incubation at 37^0C for 24 hours. The isolates were confirmed by carrying out biochemical characterization including tests for catalase production, citrate utilization, starch hydrolysis, methyl red, Voges Proskauer (MRVP), spore staining, sugar fermentation and motility (Hemraj *et al*., 2013). The bacterial isolates were further sub-cultured on agar slants and incubated at 37^0C for 24 hours, following which they were stored refrigerated at 4^0C.

Determination of antibiotic susceptibility

The selected bacterial isolates were tested for susceptibility to 8 different antibiotics by the disc diffusion method on MHA plates (Oyetibo *et al.,* 2010). The Gram positive antibiotics (Abtex Biologicals Ltd, Liverpool, UK) tested were: erythromycin 5 µg (ERY), cloxacillin 5 µg (CXC), cotrimoxazole 25 µg (COT), augmentin 30 µg (AUG), tetracycline 30 µg (TET), gentamicin 10 µg (GEN), chloramphenicol 10 µg (CHL) and streptomycin 10 µg (STR). The antibiotics discs were placed on MHA agar plates previously seeded with cell suspension with a turbidity of 0.5 McFarland standards. The plates were incubated at 37^0C for 24 h and observed for zones of inhibition. The zone of inhibition diameter (mm) for the antibiotic sensitivity to be termed susceptible was ≥12 while for resistance to the antibiotic was ≤12.

Results

Samples of RTE vegetables from two different produce markets in Ota, Ogun State were examined for microbial quality using the aerobic plate count. The total aerobic bacteria present in the RTE vegetable samples Cabbage, Cucumber, Lettuce and Carrot ranged, respectively, from 1.84×10^6 - 2.24×10^6 cfu/g, 1.72 x10^6 - 2.48 x 10^6cfu/g, 1.51x10^6 – 1.97 x 10^6 cfu/g and 1.69 x 10^6 – 2.42 x 10^6 cfu/g

(Table 1).

A total of sixteen bacterial species isolated from the total plate count of cultures from the RTE vegetable samples were determined to belong to the genera *Bacillus* (82%) and *Nocardia (18%)* on the basis of Gram stain, cellular and colonial morphology (Table 2). The biochemical tests further confirmed the identity of several isolates from the *Bacillus* as follows: *B. brevis* (30%), *B. subtilis* (12%), *B. megaterium* (6%), *B. circulans* (6%), *B. sphaericus* (6%) and *B. pumilus* (6%).

The results of antibiotic sensitivity tests on the bacterial isolates shown in Table 3 indicate that more than 80% of the selected Gram-positive bacilli bacteria from the RTE vegetable samples exhibited resistance to at least two antibiotics including erythromycin, cloxacillin, cotrimoxazole, augmentin or streptomycin.

Discussion

The standard (aerobic) plate count can provide a general indication of the microbiological quality of a food, although it does not differentiate between the natural microflora, spoilage microorganisms, or pathogenic microorganisms. The presence of aerobic organisms in food products reflects existence of favorable conditions for the multiplication of microorganisms. In this study, all the RTE vegetable samples examined, irrespective of the vendor or produce market from whom or where they were acquired had mean contamination levels of ≥ 1 x 10^5 cfu/g. The New South Wales (NSW) Food Authority (2009) recommends the standard limit for bacterial count of ready-to-eat foods to be <1 x 105 cfu/g. In this regard, our findings suggest that the RTE vegetables examined in this study are unsatisfactory for human consumption without further actions on the part of food handlers and consumers. Similar results documenting microbial contamination of RTE vegetables have been reported in Nigeria (Eni *et al*., 2010; Abdullahi. and Abdulkareem, 2010; Ieren *et al.*, 2013) and elsewhere (Aparecida de Oliveira *et al*., 2011; Ali *et al.*, 2011; Tabashsum *et al*., 2013).

In this study, more than 80% of the bacterial species isolated from the RTE vegetable samples were from the genus *Bacillus* and identified as follows: *B. brevis* (30%), *B. subtilis* (12%), *B. megaterium* (6%), *B. circulans* (6%), *B. sphaericus* (6%) and *B. pumilus* (6%). The higher rate of incidence of *Bacillus* on the vegetables may be a reflection of soil microflora contamination (Rosenquist *et al*., 2005, Contzen *et al*., 2014). Compared to other bacteria that contaminate raw vegetables, *Bacillus* spp. owe their persistence to the ability to produce spores, which can withstand high temperature and ultraviolet sun rays that may kill and reduce other bacteria load in vegetables during exposure and display for sale. This study confirms several reports documenting incidence of *Bacillus* species on raw vegetables (Abdullahi. and Abdulkareem, 2010; Obieze *et al*., 2011; Tabashsum *et al*., 2013; Meldrum *et al*., 2008).

RTE vegetables contaminated with unacceptable levels of *B. cereus* and a range of other species, for example, *B. pumilus*, *B. subtilis*, and *B. licheniformis*, are unsafe and considered to be injurious to health and/or unfit for human consumption (Kramer 1989; Meldrum *et al*., 2008). Antimicrobial resistance genes in soil microflora, food spoilage or opportunistic pathogenic strains contaminating food form an indirect risk to public health, as they increase the gene pool from which pathogenic bacteria can pick up resistance traits (Verraes *et al*., 2013). The observation of resistance to multiple antibiotics by the organisms isolated from RTE vegetables in this study suggests a substantial risk for transfer of antimicrobial resistance to humans because the eventually present resistant bacteria are not killed as they are often consumed without having undergone prior preservation or additional processing. As a consequence, transfer of antimicrobial resistance genes between bacteria after ingestion by humans may occur. Microbiologically safe RTE vegetables are essential to maximize the health benefits promised by adequate consumption of these produce. Proper washing of fruits and vegetables using water supplemented with varying concentrations of acetic or citric acid has been demonstrated to be essential for decontamination (Eni et al., 2010; Tagoe and Aning, 2011) before human consumption.

In conclusion, the results of the present study on RTE vegetables collected from two Ota markets (Oju_Ore and Sango), clearly revealed that soil microflora; *Bacillus* and Nocardia species, closely associated with these produce are resistant to multiple antibiotics, and as such pose substantial risk for transfer of antimicrobial resistance to humans without having undergone prior preservation or additional processing.

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Table 1: Total aerobic bacteria present in the RTE vegetable samples analyzed (cfu/g)

	Total aerobic bacteria (cfu/g)			
	Oju-Ore		Sango	
RTE vegetable	**Vendor O1**	**Vendor O2**	**VendorS1**	**Vendor S2**
Cabbage	1.84×10^6	2.24×10^6	1.98×10^6	1.79×10^6
Cucumber	2.04×10^6	1.72×10^6	1.92×10^6	2.48×10^6
Lettuce	1.97×10^6	1.88×10^6	1.51×10^6	1.81×10^6
Carrot	2.26×10^6	1.69×10^6	1.79×10^6	2.42×10^6

TABLE 2: Identification of selected bacterial isolates from RTE vegetable samples

Bacterial isolates	Colonial morphology	Gram reaction	Cellular morphology	Catalase production	Citrate utilization	Starch hydrolysis	Spore	Glucose	Xylose	MR	VP	Motility	Probable organisms
CUCS2	Large, smooth, convex, entire, opaque, creamy-white	+	Rod	+	+	+	+	+	_	_	_	+	*B. megaterium*
CUCS4	Thin, transparent, spreading	+	Rod	+	_	+	+	+	+	_	_	+	*B. circulans*
CARS1	Small, yellowish-white, irregular, glistening, filamentous	+	Rod	+	+	_	+	_	_	+	+	+	*Norcadia spp.*
CARS4	Flat, smooth, entire, opaque	+	Rod	+	_	_	+	_	_	+	_	+	*B. sphaericus*
LETS1	Circular, raised, smooth, entire	+	Rod	+	+	_	+	_	_	_	_	+	Bacillus spp.
LETS2	Small, yellowish-white, irregular, glistening, filamentous	+	Rod	+	_	_	+	_	_	+	+	+	*Norcadia spp.*
CABS2	Circular, yellow, raised, dull	+	Rod	+	+	_	+	_	+	_	+	+	Bacillus spp.

CABS4	Small, yellowish-white, irregular, glistening, filamentous	+	Rod	+	–	–	+	–	+	–	–	+	*Norcadia spp.*
CUCO1	Thin, flat, translucent, smooth, spreading	+	Rod	+	+	–	+	+	–	–	+	+	*B. brevis*
CUCO3	Rough, wrinkled, opaque, dull, spreading with brownish tinge	+	Rod	+	+	+	+	+	+	+	+	+	*B. subtilis*
CARO1	Thin, flat, translucent, smooth, spreading	+	Rod	+	+	–	+	+	+	–	–	+	*B. brevis*
CARO4	Smooth, thin, flat, translucent, yellowish white	+	Rod	+	+	–	+	+	+	–	–	+	B. pumilus
LET01	Thin, flat, translucent, smooth, spreading	+	Rod	+	+	–	+	+	–	–	+	+	*B. brevis*
LETO4	Thin, flat, translucent, smooth, spreading	+	Rod	+	+	–	+	+	+	–	+	+	B. brevis
CABO1	Rough, wrinkled, opaque, dull, spreading with brownish tinge	+	Rod	+	+	+	+	+	+	+	+	+	*B. subtilis*
CAB03	Thin, flat, translucent, smooth, spreading	+	Rod	+	+	–	+	+	+	–	+	+	*B. brevis*

TABLE 3: Antibiotic susceptibility pattern of the bacterial isolates from RTE vegetable samples

	BACTERIAL ISOLATES AND ZONES OF INHIBITION (mm)														
ANTIBIOTICS	Bmeg	Bcir	Nspp	Bsph	Bspp	Nspp	Bssp	Nssp	Bbre	Bsub	Bbre	Bpum	Bbre	Bbre	Bsub	Bbre
COT	R(0)	R(0)	R(0)	S(26)	S(18)	S(15)	S(19)	S(18)	S(16)	S(16)	S(16)	R(0)	S(18)	S(14)	R(0)	R(0)
CHL	S(10)	S(14)	S(16)	S(20)	S(16)	S(14)	S(20)	S(14)	S(20)	R(0)	S(18)	S(13)	S(18)	S(18)	S(14)	S(16)
CXC	R(0)	S(13)	R(0)	S(18)	R(0)	S(14)	S(18)	R(0)	S(13)	R(0)	R(0)	R(0)	R(10)	S(18)	R(18)	R(0)
ERY	S(15)	R(8)	R(0)	R(0)	R(0)	R(0)	R(0)	R(10)	R(8)	R(0)	R(10)	S(14)	R(10)	R(8)	R(10)	S(20)
GEN	R(10)	S(22)	S(20)	S(18)	S(18)	S(18)	S(20)	S(20)	S(22)	S(20)	S(22)	S(20)	S(20)	S(20)	S(18)	S(20)
AUG	R(0)	R(8)	R(0)	S(18)	S(14)	S(18)	S(22)	R(10)	S(26)	R(0)	R(0)	R(0)	S(28)	R(10)	R(0)	S(14)
STR	R(8)	S(18)	R(8)	R(0)	S(16)	R(0)	R(0)	S(16)	S(18)	R(0)	S(13)	S(14)	S(20)	S(14)	R(8)	S(18)
TET	R(6)	S(20)	S(14)	R(10)	S(18)	S(13)	S(14)	S(22)	S(18)	S(14)	S(20)	S(18)	S(24)	S(14)	S(18)	S(18)

Key: Bmeg = *Bacillus megaterium*, Bcir = *Bacillus circulans*, Nspp = *Nocardia* spp., Bsph = *Bacillus sphaericus*, Bspp = *Bacillus* spp, Bbre = *Bacillus brevis*, Bpum = *Bacillus pumilus*, Bsub = *Bacillus subtilis*, COT = 25 µg Cotrimoxazole, CHL = 10 µg Chloramphenicol, CXC = 5 ug Cloxacillin, ERY = 5 µg Erythromycin, GEN = 10 µg Gentamicin, AUG = 30 µg Augmentin, STR = 10 µg streptomycin, TET = 30 µg Tetracycline, S = Susceptible to antibiotic effect, R = Resistant to antibiotic effect.