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RESEARCH ARTICLE

GRAM POSITIVE BACTERIAL ISOLATES FROM SELECTED FRESH VEGETABLES IN ONDO STATE, NIGERIA

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ABSTRACT

Three vegetables, *Brassica oleracea* (cabbage), *Lactuca sativa* (lettuce), and *Cucumis sativus* (Cucumber) were purchased from various markets in Ondo State (Akungba, Ikare, Ore, Akure and Owo), Nigeria and examined to determine their level of bacterial contamination and safety for human consumption. In this research, gram positive bacteria were isolated from these vegetables and the bacterial isolates were tested for antibiotic susceptibility. The Gram positive bacterial isolates from the vegetables include *Brevibacillus laterosporus* and *Lactobacillus spp* from cabbage, *Enterococcus faecalis*, *Bacillus licheniformis*, *Staphylococcus condimenti*, and *Bacillus lentus* from lettuce, and *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus faecalis* from cucumber. Out of the three selected vegetables, cabbage had the highest bacterial levels with total viable count of about 155 x 10^{-4} while cucumber had the lowest with about 74 x 10^{-4}. All the isolates were sensitive to gentamycin while all the isolates were resistant to augmentin except *Bacillus cereus*. Since some of these bacteria are antibiotic-resistant and may likely transfer the resistance gene in natural habitat, this constitute a food safety concern. However, with increasing discoveries of microbial contamination of fresh vegetables, consumers need to be aware of the potential risk involved with their consumption specifically in immune compromised individuals.

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INTRODUCTION

Vegetables are plants consumed in small amount and considered as side dish or relish with solid food in south western part of Nigeria. Their popularity grow with the belief that they give hundred percent nutrient in the body. As a result, fresh vegetables are common in Nigerian markets and sellers work hard to keep the freshness optimum for buyers. One method commonly used in preserving these produce is sprinkling with water. Although sprinkling vegetables with water is found ideal by sellers, there are high possibilities of contamination by microorganism. Contamination may be direct or indirect. Direct contamination relates with the handlers (buyers and sellers). Touching of the produce with dirty hands or exposing of produce to flies are typical examples of direct contamination. The unhealthy surroundings of the market may lead to indirect contamination. Very dirty environment may create huge microbial loads on produce. Consequently, fresh vegetables with huge loads of microbial contaminants may begin to spoil so soon if not sold. However, adequate cleaning processes can reduce the microbial load (Schwab *et al*., 2008). Apparently, various studies have shown presence of spoilage microorganisms in fruits and vegetables. This is as a result of poor pre-harvest and post-harvest practices (Sperber *et al*., 2009). One major pre-harvest source of contamination is irrigating with poor-quality water. This allows foodborne pathogens on the vegetables. Likewise, using raw animal manure as fertilizer can increase the possibilities of the vegetables being contaminated. There are reports indicating the presence of foodborne pathogens in raw vegetables. The common isolates includes *Listeria monocytogenes, Salmonella*, and *Escherichia coli*. Gastrointestinal organisms mostly enteric pathogens have also been found on various produce including lettuce, cabbage and tomatoes (Obieze *et al*., 2011). With the introduction of safety concept- hazard analysis critical control point (HACCP), microbial contamination in vegetables may be reduced however, cases of food poison-ing, intoxication, and spoilage are continually increasing (Meng *et al*., 2002). Also, continuous improvement in preservation will limit the tendencies of contamination as vegetables are consumed without heat treatment. Generally, washing procedures result in a one or two logs reduction in microbial counts. The efficacy of various disinfectants and sanitizing methods varies greatly. This depends on the fruit and vegetable surface, type of microorganism, physiological stage and environmental

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stress conditions (Beuchat, 1998). In Nigeria, information on microbial food safety is limited. There are very few reports explaining the microbial quality of fresh produce. In Calabar, a report indicated the prevalence of foodborne pathogens on these produce (Obieze et al., 2011). However, as the scope of this study is focus on fresh vegetables in Ondo State, this finding is bound to enlighten consumers of salad vegetables (cucumber, lettuce, and cabbage) that may have developed running stomach or dysentery after consuming these produce, to understand that it is as a result of microbial contamination due to poor food hygiene practices either at the markets where these fresh vegetables were bought, or even at home before they are been consumed.

Furthermore, this study aims at educating both the sellers and consumers of fresh vegetables to be cautious of the storage process that these vegetables are subjected to, as well as the environment where they are being sold should be strictly cleaned and sanitized. Particularly, much attention is focused on antibiotics that may be used whenever microbial contaminated vegetables are being consumed, as ignorance to the type of antibiotics used may cause greater havoc to the body. In essence, this study of bacteriological quality aims at examining the potential of bacterial contamination of fresh vegetables mostly sold in various markets in Ondo State and to determine their safety for human consumption.

MATERIALS AND METHODS

Sample Collection

The vegetable samples numbering three (each sample from three markets was mixed together) were bought from three different markets namely: Owo, Ikare, and Akungba (Cucumber and cabbage), Akure, Ore, and Owo (Lettuce) markets respectively. The samples were collected inside a sterile polythene bags and were transported to the Microbiology laboratory of Adekunle Ajasin University, Akungba Akoko for Microbiological analysis.

Microbiological Analysis of the Samples

Gram positive bacteria were isolated from the fresh vegetables according to the method of Obieze et al., 2011. The vegetables were aseptically cuts into bits using a sterile knife. Ten grams (10g) of each vegetable were dispensed into 90ml of sterile distilled water inside a beaker and shaken at room temperature. This stock solution was serially diluted in sterile distilled water to 10^{-5}. Inoculum (1ml) was taken from 10^{-2} and 10^{-4} dilutions respectively to seed sterile plates of nutrient agar, all in replicates and labeled. An uninoculated plate was included as a control for the nutrient agar (NA). The nutrient agar plates were then incubated at 37^0C for 48hrs. Combinations of cultural characteristics, cellular morphology, and biochemical characterization were employed in identifying the isolates (Barrow *et al*., 1993). After 48hours of incubation, the NA plates were carefully examined for visible growth. The resultant colonies were counted and the cultural characteristics were carefully studied and recorded. Preparation of pure culture was done by preparing a fresh medium of tryptic soy agar (TSA). For each of the distinct colonies formed, sterile inoculation loop was used to streak the colonies on the sterile plates of the fresh medium and incubation was done at 37^0C for 24hours. The colonies were selected in a way that separate colonies, were picked up to avoid picking more than one colonies at a time. Inoculating loop was flamed red hot to become sterilized and swung for 20s to allow for cooling before use. The purified isolates were then sub-cultured into sterile TSA slants for further studies. The cellular morphology of the pure isolates were determined by gram staining and recorded. All isolates were preserved and maintained on NA for further characterization and full identification.

RESULTS

All isolates have diverse cellular characteristics and cellular morphologies as shown in Table 1. The microscopic view shows that most isolates have irregular shape. Cultural characteristics such as edge, surface and motility are evenly distributed among the isolates. The biochemical characterization results in Table 2 show that all the isolates metabolized glucose except CuE. Similarly, all the isolates metabolized citrate except LeC and all isolates metabolized lactose except LeA and LeC. The predominant bacteria from the samples were *Bacillus species* and *Staphylococcus species*. Antibiotics such as GEN (Gentamycin), COT (Cotrimoxazole), CHL (Chloramphenicol), AUG (Augmentin), AMX (Amoxillin), ERY (Erythromycin), TETRA (Tetracycline) and CXC (Cloxacillin) were tested against all the isolates for susceptibility. All isolates were sensitive to GEN and all isolates were sensitive to CHL except CuD1. Also, all isolates were resistant to AUG except CuA,and all isolates were resistant to AMX except CuA and CuD1, while, CuA is sensitive to all the antibiotics except CXC (Table 3). The number of isolate susceptible or resistant to each antibiotics can be seen graphically in Figure 6.

Table 1. Cultural Characteristics and Cellular Morphology of Gram Positive Bacterial Isolates from Cabbage, Lettuce and Cucumber

	Cultural characteristics							Cellular morphology			
Isolates id	Pigment	Shape	Edge	Elevat-ion	Surface	Consistency	Motility	Size	Morphology	Arrang-ement	Gram reaction
Ca1	White	Irregular	Rough	Flat	Dull	Transparent	Positive	Short	Rods	Chains	Positive
Ca2	Milky	Irregular	Smooth	Flat	Shinning	Transparent	Negative	Short	Rods with spores	Clustered	Positive
LeA	Milky	Irregular	Rough	Flat	Dull	Transparent	Negative	Small	Cocci	Clustered	Positive
LeB	Creamy	Irregular	Rough	Flat	Dull	Transparent	Positive	Long	Rods	Scattered	Positive
LeC	Creamy	Irregular	Smooth	Raised	Shinning	Transparent	Positive	Short	Rods with spores	Clustered	Positive
LeD	Milky	Irregular	Rough	Flat	Dull	Transparent	Negative	Small	Cocci	Clustered	Positive
CuA	Creamy	Circular	Smooth	Raised	Dull	Opaque	Positive	Short	Rods	Clustered	Positive
CuD_1	White	Irregular	Rough	Flat	Shinning	Transparent	Negative	Small	Cocci	Scattered	Positive
CuE	Cream	Irregular	Smooth	Raised	Shinning	Opaque	Negative	Small	Cocci	Clustered	Positive

Table 2. Chemical characterization and probable identities of gram positive bacterial isolates from cabbage, lettuce and cucumber

Isolates id	Catalase	Coagulase	Methyl red	Voges proskauer	Indole	Citrate	H_2s	Glucose	Lactose	Oxidase	Urease	Probable identity
Ca1	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	*Brevibacillus laterosporus*
Ca2	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	*Lactobacillus sp*
LeA	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	*Enterococcus faecalis*
LeB	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	*Bacillus licheniformis*
LeC	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	*Bacillus lentus*
LeD	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	*Staphylococcus condimenti*
CuA	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	*Bacillus cereus*
CuD_1	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	*Staphylococcus aureus*
CuE	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	*Streptococcus faecalis*

Table 3. Antibiotic susceptibility profile of the gram positive bacterial isolates

Zones Of Inhibition By The Antibiotics (Mm)								
ISOLATES	GEN	COT	CHL	AUG	AMX	ERY	TETRA	CXC
Brevibacillus laterosporus	17	R	23	R	R	19	18	R
Lactobacillus sp	10	20	15	R	R	R	10	10
Enterococcus faecalis	13	R	5	R	R	R	R	R
Bacillus licheniformis	19	R	9	R	R	19	19	R
Bacillus lentus	13	R	12	R	R	10	15	R
Staphylococcus condimenti	15	R	18	R	R	12	18	R
Bacillus cereus	9	13	10	7	7	7	5	R
Staphylococcus aureus	18	R	R	R	20	R	5	5
Streptococcus faecalis	6	9	10	R	R	4	2	R

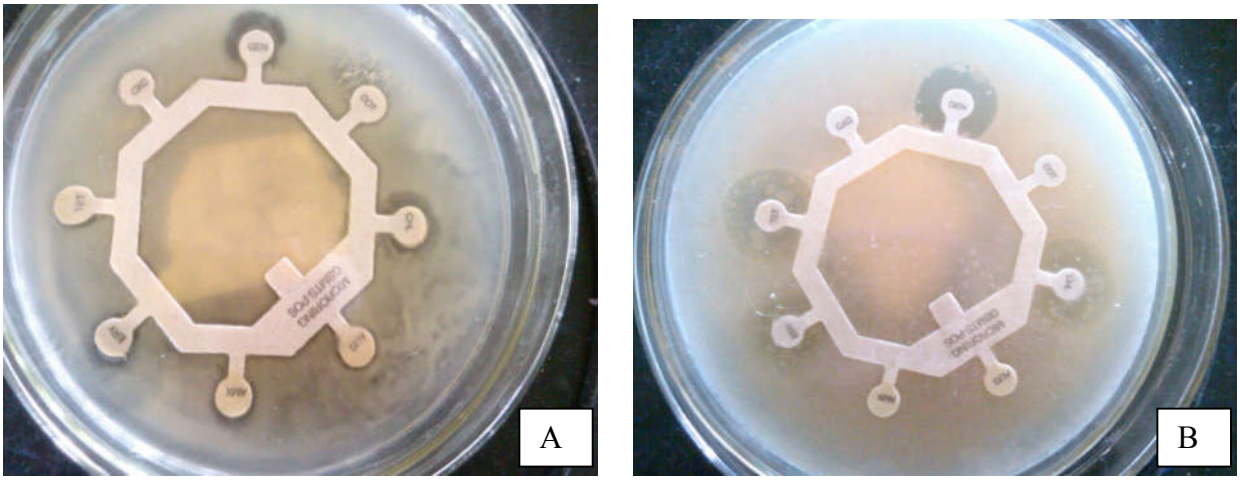

Figure 1. (A) Antibiotic susceptibility result of *Bacillus cereus* (B) Antibiotic susceptibility result of *Bacillus lentus*

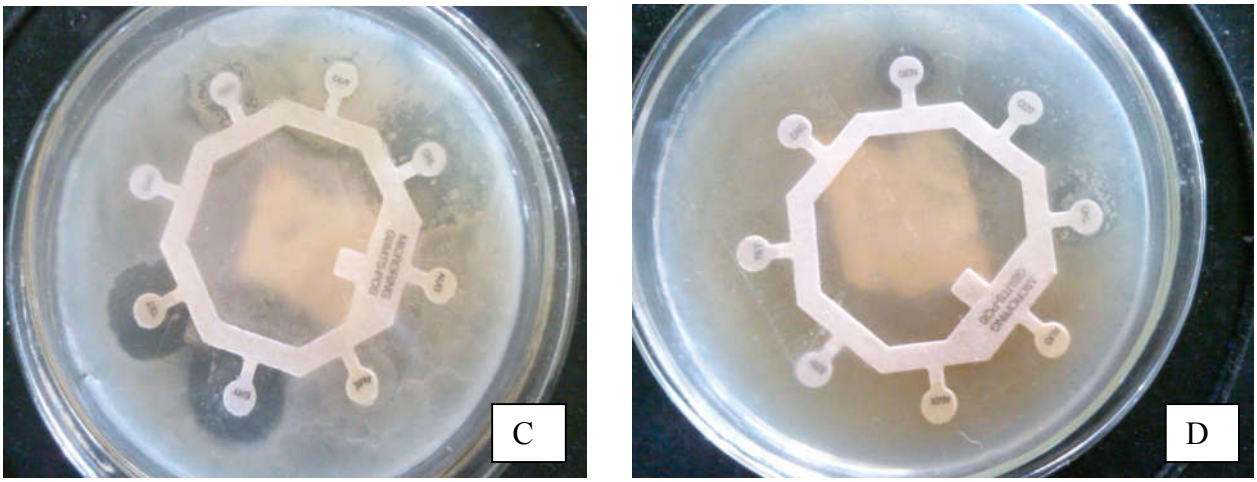

Figure 2. (C) Antibiotic susceptibility result of *Brevibacillus laterosporus* (D) Antibiotic susceptibility result of *Lactobacillus sp*

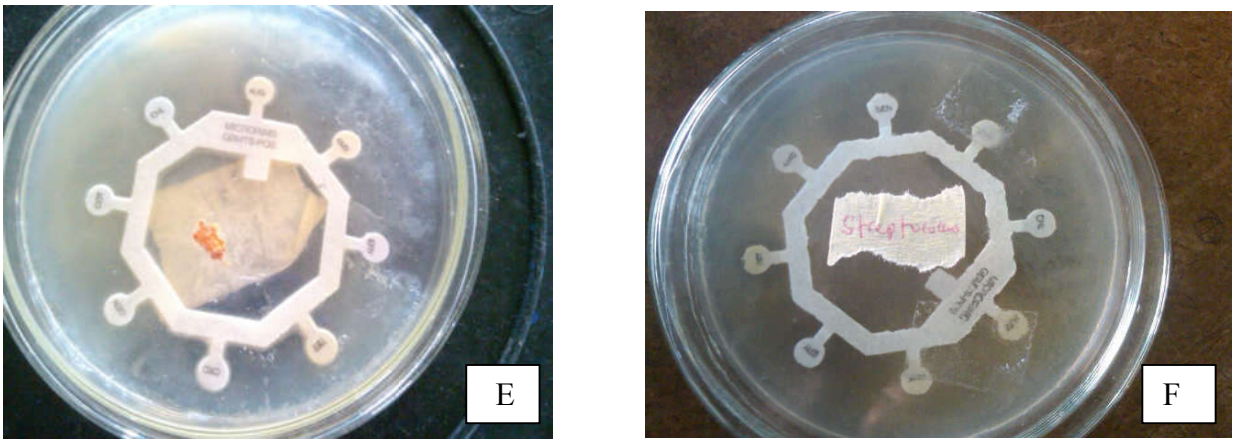

Figure 3 (E) Antibiotic susceptibility result of *Staphylococcus aureus* (F) Antibiotic susceptibility result of *Streptococcus faecalis*

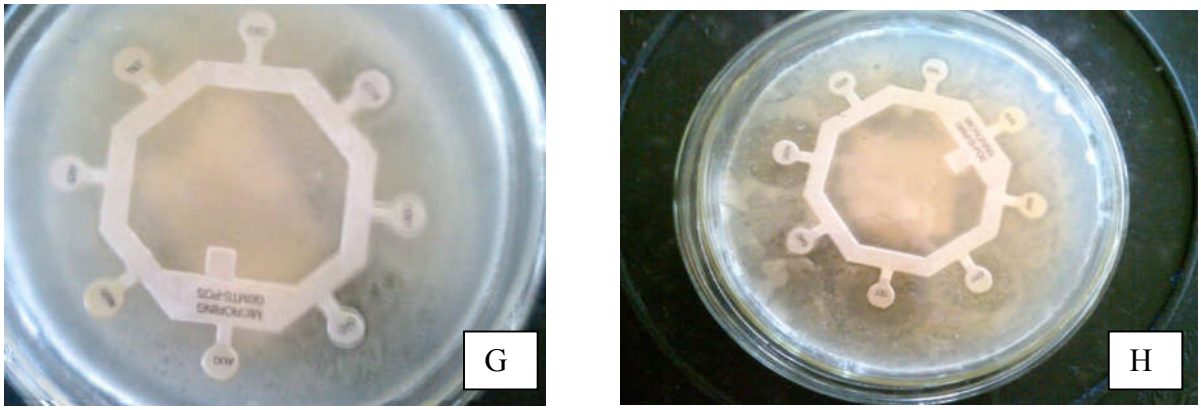

Figure 4 (G) Antibiotic susceptibility result of *Enterococcus faecalis* (H) Antibiotic susceptibility result of *Bacillus licheniformis*

Figure 5 (I) Antibiotic susceptibility result of *Staphylococcus condiment*

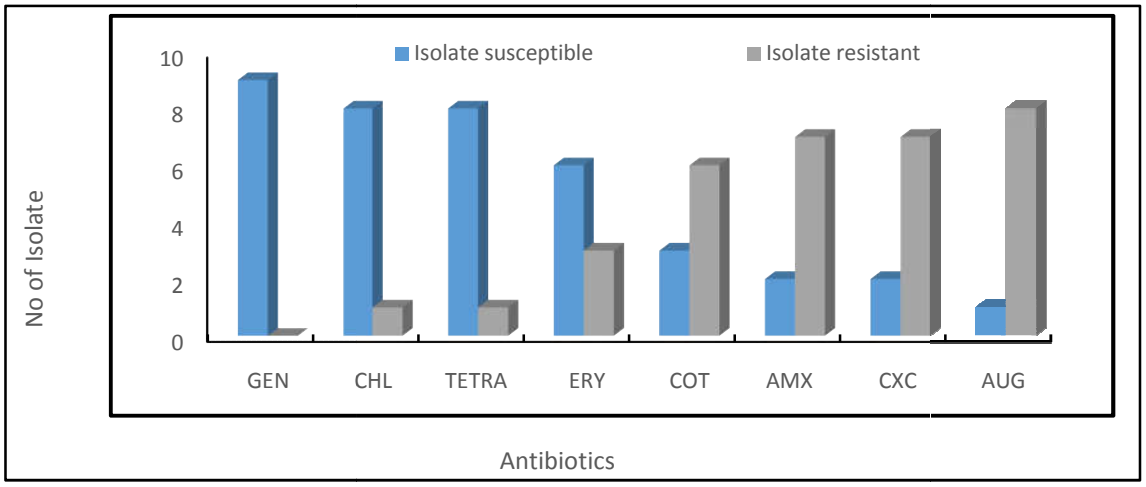

Figure 6. Isolate reaction to selected antibiotics

DISCUSSION

This study shows various gram positive bacterial isolates from selected fresh vegetables; Cabbage, Lettuce, and Cucumber. All samples from each of the selected markets have one or more bacterial isolates. Previous report on microbial contaminant of salad vegetables in Abuja Municipal Area Council have shown that fresh vegetables mostly those used in salad mixtures, have been linked to food poisoning and have been proven to be detrimental to consumer's health (Aboh *et al*., 2010). This is because they have loads of pathogenic microorganisms that produce poisonous toxins. When consumed, these toxins gradually affect the immune system and sometimes may cause inflammation of the human body. The poor practices these produce are subjected to, are fact as they are mostly consumed without proper washing or thermal process (Lund, 1992). Table 1 shows the cultural characteristics and cellular morphology of the bacterial isolates from the selected vegetables. Most of the organisms were creamy on plate; some were milky while others were whitish. Also, most of the isolates were non-motile on the culture plate and have flat elevation with few being motile and raised. The surface of some of the isolates were shinning while some were dull. In addition, most of the isolates have transparency consistency with many having rough edges while few were opaque with smooth edges. In the microscopic view of the isolates, many have rod--shaped with spores, and some are cocci while few are ovoid-shaped. All isolates were positive to gram reaction. Table 2 shows the biochemical characterization and the probable identities of the isolates. The bacterial isolates from cabbage obtained from the markets include; *Brevibacillus laterosporus*, and *Lactobacillus sp.* Isolates from lettuce include; *Enterococcus faecalis*, *Bacillus licheniformis*, *Bacillus lentus*, and *Staphylococcus condimenti*, While those isolates from cucumber include; *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus faecalis*. Generally, this study corroborates the discoveries of Obieze *et al.* (2011) who reported a number of potential food borne pathogens in a varieties of raw vegetables including tomato, lettuce, and cabbage. Enteric pathogens such as *Listeria monocytogenes*, *Salmonella* and *Escherichia coli* were isolated which explains that majority of these salad condiments are contaminated with deadly pathogenic microorganisms. Furthermore, Lynch *et al.* (2009) stressed that fresh salad vegetables have emerged as new carriers of infectious disease- this study have proved it right beyond measure that majority of the isolates have been associated with bacterial contamination, and have been linked with many gastrointestinal diseases. Moreso, this study correlates the work of Tambekar *et al.* (2006) who reported that salad vegetables are consumed raw without thermal treatment as a result linked with bacterial contamination. In this report, *Staphylococcus condiment* that is isolated may not be heat-labile. Further study is recommended.

Sicknesses due to *Staphylococcus aureus* are caused mainly by pre-formed enterotoxins in food (Hennekinne *et al*., 2012). Enterotoxins genes are present in some *Staphylococcus aureus* therefore their ability to cause poisonings in food. Most cases of infections are as a result of *Staphylococcus aureus* in vegetables and soup. Recently, *Staphylococcus condimenti* have been reportedly isolated in soy sauce mash (Misawa *et al*., 2015) however, isolation from fresh vegetables (cucumber, cabbage and lettuce) have not been reported. This study have proved its presence in fresh vegetables. Staphylococcal enterotoxins are heat-resistant and can withstand some normal cooking processes including boiling which makes it difficult to eradicate in fresh vegetables that are minimally processed or not processed at all- therefore in the absence of metabolic active organisms, active toxin can be present (Shimelis *et al.*, 2015). In this study, *staphylococcus aureus* tested positive against coagulase and most coagulase-positive staphylococci do not grow at refrigeration temperatures, they optimally grow at temperatures between 7°C and 48°C. Larger percentage of people carry *Staphylococcus aureus* as contaminants of fresh produce from either farm, markets, or during processing by food handlers or consumers (Hennekinne *et al*., 2012). Also, *Bacillus cereus* isolated in this study is an opportunistic pathogen occasionally associated with infections in humans (Hoffmaster *et al*., 2006). It has a potential of causing systemic infections which of public health concerns. To understand the pathogenicity for treatment or for developing antimicrobial drugs against it, sequencing its genome is very important and crucial (Wijnands *et al*., 2006). In addition, *Enterococcus faecalis* is an isolate from lettuce in this study ar d h ave been found to be universal commensal colonists of human's gastrointestinal tract (Lynette *et al.*, 2006). It is ubiquitous as it spreads and persists in the environment. This is because of its natural strength and vigor. Witte (2000) reported its strain transmission to human intestine with foods and water as possible vectors. Girraffa (2002) further explained this with antibiotic resistant strains discovered in raw foods.

Recently, there are growing concerns about the potential advancement of environmental inhabitant of antibiotic resistance in farmland which relate this study. Specifically, resistance genes are horizontally transferred to natural soil bacteria through irrigating with untreated water or application of manures to farmlands. These genes could be transferred back to animals or humans when crops and produce are harvested and consumed (Johnston *et al*., 2006). While there are documentations of presence and transfer of antibiotic resistance in bacteria linked with animals and foods, there are few or no research as regards resistance profiles of bacteria isolated from raw produce (Van den bogaard *et al*., 2000). This study has been able to prove resistivity in isolated microorganisms from the vegetables. A few studies measuring the predominance of resistance among isolated gram negative microorganisms in raw produce exist however, there are contradictory results as that from Hamilton-miller and Shah (2001) where they characterized the antibiotic susceptibility of enterobacterial flora of salad vegetables. They found high degree of resistance to ampicillin and cephalosporin. Furthermore, a study conducted by some Finnish discovered that members of the Enterobacteriaceae family isolated from vegetables were all susceptible to the antibiotics studied and there were no identification of multidrug-resistant strains (Prazak *et al*., 2002). They studied the resistance pattern among *Listeria monocytogenes* that are isolated from cabbage farms. Their report showed that 98% resistivity of the isolates to at least two drugs and 85% of the isolates were found resistant to penicillin. However, the antibiotic resistivity styles among Enterococcus strains that are cultured from fresh vegetables are not very well understood (Thai *et al*., 1995).

From this study, the antibiotic sensitivity patterns of the bacterial isolates were shown. *Lactobacillus sp* was sensitive to GEN, COT, CHL, ERY, TETRA and CXC while *Brevibacillus laterosporus* was sensitive to GEN, COT, CHL, ERY and TETRA. Similarly, *Enterococcus faecalis* was sensitive to GEN and CHL while *Bacillus licheniformis* was

sensitive to GEN, CHL, ERY, and TETRA. In addition, *Bacillus lentus* was sensitive to GEN, CHL, ERY, and TETRA while *Staphylococcus condimenti* was sensitive to GEN, CHL, ERY, and TETRA. Furthermore, *Bacillus cereus* was sensitive to all the antibiotics except CXC while *Staphylococcus aureus* was sensitive to GEN, AMX, TETRA, and CXC. Lastly, *Streptococcus faecalis* was sensitive to GEN, COT, CHL, ERY and TETRA.

Generally, all the isolates were sensitive to GEN and all the isolates were sensitive to CHL except *Staphylococcus aureus.* In contrast, all the isolates were resistant to AUG except *Bacillus cereus* and all the isolates were resistant to AMX except *Bacillus cereus* and *Staphylococcus aureus*. Also, *Bacillus cereus* is sensitive to all the antibiotics except CXC. Therefore, active drug may be prescribed and be useful for patients that have been infected from microbial contaminated fresh vegetables.

Conclusion

To conclude, lettuce, cucumber and cabbage have been found to be carriers of some gram positive bacteria and present potential sources of the organisms. These organisms were isolated from produce being sold in three different markets from different locations in Ondo State which calls for urgent awareness of the risk involved in consuming these produce. The antibiotics susceptibility results shows the recommended antibiotics that can be used when these contaminated produce are consumed. So far, there is no record in the literature on the isolation of most of the isolates obtained from the fresh vegetables grown in Nigeria. This explains that many of these pathogenic bacteria are emerging from fresh produce in the country therefore, thorough precautions and practices should be followed during processing of these vegetables in order to destroy the microorganisms before they find their way inside the human body.

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