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Screening of Fruit and Vegetable Salads retailed in Ago-Iwoye, Ogun State for Extended-Spectrum Beta-Lactamase Producing Gram-Negative Bacteria

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Abstract

Extended-Spectrum Beta-Lactamase (ESBL) producing bacteria are of great concern to healthcare because of their resistance to β-lactam antibiotics. This study aims to screen Gram-negative bacteria recovered from fruit and vegetable salads for ESBL production. The bacteria in thirty samples of fruit and vegetable salads purchased from food vendors were isolated and identified with standard microbiological methods. The Gram-negative bacteria recovered were screened for ESBL production by the double disk synergy test (DDST) and brilliance ESBL agar (BEA). The total coliform counts in the fruit and vegetable salads were in the ranges 2.3 - 19.1 x 10^4 cfu/g and 2.8 - 19.4 x 10^4 cfu/g respectively. Ninety-eight (98) Gram-negative bacteria were recovered from the salad samples. They were *Escherichia coli* (34.7%), *Citrobacter freundii* (21.4%), *Enterobacter cloacae* (10.2%), *Enterobacter aerogenes* (9.2%), *Pseudomonas aeruginosa* (9.2%). *Klebsiella oxytoca* (8.2%), *Klebsiella pneumoniae* (4.1%) and *Proteus mirabilis* (3.1%). For ESBL production using DDST, 43. 9% (43) of the isolates were positive for the test. On the brilliance agar, 68.4% (67) showed the expected colour change as outlined by the manufacturer. However, five strains of *K. oxytoca* showed blue growth while sixteen of *C. freundii* had brown growth. ESBL-producing *E. coli* strains that were not detected with DDST grew on BEA. This finding showed that ESBL-producing bacteria are present in fruit and vegetable salads retail in Ago-Iwoye, hence, there is need to take the necessary precautions during the preparation and storage of these food products to prevent contamination by these pathogens and subsequent production of ESBL.

Keywords: Antibiotics, Brilliance ESBL agar, Double Disk Synergy Test (DDST), ESBL bacteria, Fruits, Salads, Vegetables

1. Introduction

Fruits and vegetables provide the human body with the necessary nutrients in the precise amount for growth and development [1]. The importance of raw vegetables in diets has been emphasized, and consumption of fruits and vegetables are recommended daily [2]. Some benefits of fruits and vegetables intake are reduced risk of death from cardiovascular disease and some cancer [3,4], lower risk of type 2 diabetes [5,6] and weight loss [7]. Because of these health benefits, there was global promotion of consumption of fruits and vegetables [8,9], and five servings per day was recommended to American consumers [10]. The consumption of fruits and vegetables has increased worldwide in recent years [11].

There is an increase in demand for minimally processed, pre-packed ready-to-eat fruits and vegetables [12]. Increase in demand for vegetables has resulted in a wide and uncontrolled vending of vegetables especially in the form of salad [13]. Salad is a combination of different chopped, sliced or diced fresh fruits or vegetables which rarely undergo any heat processing. Fruit salads contain a combination of any of these fruits: watermelon, cucumber, apple, pawpaw, pineapple and banana. The vegetables mostly found in vegetable salads are cabbage, tomato, onions, spring onions, lettuce, and parsley. Occasionally, pasta like macaroni and boiled eggs are added and are eaten with toppings like salad cream or mayonnaise. Vegetable salads are usually served along with other foods including rice [14] while both types of salad can be taken as dessert and appetizer [15].

The presence of pathogenic bacteria, parasites and viruses on fruits and vegetables with their possible route of contamination have been documented [16]. Vegetables have been implicated as a vehicle of transmission of foodborne bacteria that may cause illness [17].

Extended-Spectrum Beta-Lactamases (ESBLs) are enzymes capable of conferring resistance to penicillins, broad-spectrum cephalosporins and monobactams. About 10 - 40% of strains of *E. coli* and *K. pneumoniae* express ESBLs in many parts of the world [18] and pose a challenge to clinical microbiologists. These bacteria pose a risk to humans particularly in hospital environments but also by contaminating foods such as ready-to-eat (RTE) vegetables [18]. Kim *et al*. [19] reported that ready-to-eat vegetables, especially sprouted seeds may play a role in spreading antimicrobial resistant bacteria and ESBL genes to humans. The use of polluted irrigation water for crops can aid in the transfer of multidrug-resistant (MDR) *Enterobacteriaceae* onto

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fresh crops [20]. It was reported that most developing countries use untreated water for irrigation of around 10% of crops [21]. Wastewater irrigated crops showed an increased incidence of Enteropathogens [22].

Though studies have been conducted on vegetable salads in some parts of the country, there is still need to further investigate the safety of both fruits and vegetables. Therefore, the aim of this study was to isolate Gram-negative bacteria from fruit and vegetable salads retailed in Ago-Iwoye, Ogun State and screen the isolates for ESBL production using double disc synergy test and brilliance ESBL agar.

2. Materials and Methods

2.1 Sample collection

Thirty samples of salads consisting fifteen (15) samples each of vegetable and fruit salads were purchased randomly from canteens in Olabisi Onabanjo University main campus and Ago-Iwoye town inside separate sterile plastics and transported immediately to the Microbiology laboratory for immediate analysis.

2.2 Isolation of Microorganisms

Ten (10) grams of each fruit and vegetable salad sample was aseptically cut into pieces in a conical flask containing 90 ml of distilled water and serial dilution was prepared up to 10^{-6} following the standard plating method. From 10^{-3} dilution, 1 ml of the inoculum was placed in a sterile Petri dish. MacConkey agar that has been allowed to cool was carefully poured in the plate. The plates with the inocula were allowed to solidify and incubated invertedly at 35 ºC for 24-48 h. The colonies obtained were further streaked on nutrient agar plates to obtain a pure colony [23].

2.3 Characterization and Identification of Bacteria Isolates

The bacterial colonies were differentiated first on the basis of their colonial morphology followed by microscopic examination after Gram staining. Pure culture of bacterial isolates was characterized using various biochemical tests namely motility, catalase, oxidase, indole, citrate utilization, urease, methyl red, Kligler Iron Agar and sugar fermentation tests [23,24].

2.4 ESBL Detection Methods

2.4.1 Phenotypic Screening

The gram-negative bacteria were first screened with ceftriaxone. Each isolate was standardized with 0.5 McFarland solution and was spread on Mueller Hinton agar (MHA) plates using a sterile cotton swab. A disc of ceftriaxone (CTR 30µg) was placed at the centre of the plate. The plates were incubated at 37 ºC for 24 h. Zones of inhibition ≤ 25 mm indicate potential ESBL producer [25].

2.4.2 Double Disk Synergy Test (DDST)

The Gram-negative bacteria were standardized with 0.5 McFarland solution and was spread on Mueller Hinton agar (MHA) plates using a sterile cotton swab. A disc of augmentin (amoxycillin/clavulanic acid) (20 /10 µg) was placed on the surface of Mueller Hinton agar; then disc of ceftriaxone (CTR 30 µg) and ceftazidime (CAZ, 30 µg) were kept in such a way that each disc was 20 mm from the Augmentin disc (centre to centre). The plates were incubated at 37 ℃ for 24 h. Extension of the edge of the inhibition zone of ceftriaxone and ceftazidime disc on the side exposed to the disc containing amoxicillin-clavulanic acid was recorded as positive for ESBL [25].

2.4.3 ESBLs Screening on BEA

Culture of bacteria that has been adjusted to 0.5 McFarland turbidity standards was picked using a sterile inoculating loop and was streaked on the Brilliance ESBL Agar plate and incubated for 24 h at 37 ℃. Change in colour from a semi-opaque background indicates a positive result. *Klebsiella, Enterobacter, Serratia* and *Citrobacter* (KESC group) express galactosidase, resulting in green colonies. *E. coli* however, express galactosidase and glucuronidase producing easily distinguished blue colonies (β-galactosidase *E. coli* appeared pink). *Proteus, Morganella* and *Providencia* are able to deaminate tryptophan resulting in tan-coloured colonies with brown halo (as written in the enclosed manual).

3. Results and Discussions

3.1 Results

The total coliform counts in the fruit and vegetable salads were in the ranges of 2.3 - 19.1 x 10^4 cfu/g and 2.8 - 19.4 x 10^4 cfu/g respectively. The highest coliform count was observed on vegetable salad with cream with a count of 25.2 x 10^4 cfu/g (Table 3.1).

The results of the biochemical test performed on the isolate are presented on Table 2. Ninety-eight (98) bacteria isolates were identified belonging to eight (8) bacteria species. All the isolates were catalase positive. The bacteria recovered were *E.cherichia coli*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*. *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Proteus mirabilis*. Table 3 represents the incidence of Gram-negative bacteria in the salad samples, with *E. coli* having the highest occurrence of 34.7% while *P. mirabilis* had 3.1%. Fig. 1 presents the result of the screening with DDST. Out of the ninety-eight (98) Gram-negative bacteria recovered, 43. 9% (43) of the isolates were detected as ESBL producers in the salad samples with the test.

On the Brilliance ESBL agar, 67 (68.4%) isolates were ESBL producing bacteria (Table 4). Thirty-two (92%) *E. coli* and 4 (100%) *K. pneumoniae* isolates showed either

blue/red and green colonies respectively, 1(12.5%) *K. oxytoca* strain had green colony, 1 (4.8%) *C. freundii* isolates showed the expected green colour, 3 (100%) *P. mirabilis* isolates have a tan with halo growth while 5 (55.6%) *P. aeruginosa* isolates showed a reddish-brown colony. All the *E. aerogenes* and *E. cloacae* colonies did not show any change in colour. Also, some strains of *K. oxytoca* and *C. freundii* had blue and brown colonies respectively.

Table 3.1: Total coliform counts of bacteria in fruit and vegetable salad ($x10^4$ cfu/g)

Fruit Salad Code	TCC ($x10^4$ cfu/g)	Vegetable Salad Code	TCC ($x10^4$ cfu/g)
FS1	3.6	VS1	2.8
FS2	12.2	VW2	19.4
FS3	16.4	VS3	10.3
FS4	4.2	VW 4	8.4
FS5	14.3	VS 5	16.9
FS6	5.7	VW6	4.8
FS7	4.1	VS7	6.4
FS8	6.7	VW_8	18.8
FS9	14.3	VS_9	19.4
FS10	4.3	VW10	2.8
FS11	17.1	VS11	25.2
FS12	8.4	VW12	2.8
FS13	13.2	VS13	19.4
FS15	2.3	VW14	10.3
FS15	7.2	VS_{15}	8.4

Key: FS – Fruit salad, VS – Vegetable salad without cream, VW – Vegetable salad with cream

Table 3.2: Gram Stain Reaction and biochemical characterization of bacteria isolates from fruit and vegetable salads

									Kligler Iron Agar							
No of bacteria isolates	Gram Stain/Shape	Motility	Catalase	Citrate	Oxidase	Indole	Methyl Red	Urease	H_2S	Gas	Lactose	Glucose	Sucrose	Arabinose	Maltose	Probable Identity
34	- R	+	+	-	-	+	+	-	-	+	+	+	-	+	+	*Escherichia coli*
21	- R	+	+	+	-	-	+	-	+	+	+	+	+	+	+	*Citrobacter freundii*
10	- R	+	+	+	-	-	-	-	-	+	-	+	+	+	+	*Enterobacter cloacae*
9	- R	+	+	+	+	-	-	-	-	-	-	-	-	-	-	*Pseudomonas aeruginosa*
9	- R	+	+	+	-	-	-	-	-	+	+	+	+	+	+	*Enterobacter aerogenes*
8	- R	-	+	+	-	+	-	+	-	+	+	+	+	+	+	*Klebsiella oxytoca*
4	- R	-	+	+	-	-	-	+	-	+	+	+	+	+	+	*Klebsiella pneumoniae*
3	- R	+	+	+	-	-	+	+	+	+	-	+	-	-	-	*Proteus mirabilis*

R = Rod, C = Cocci, + = positive, - = negative

Table 3.3: Incidence of Bacteria in Fruits and Vegetables Salads

Bacterial isolates	Fruit salad	Vegetable salad		% Occurrence
		With cream	Without cream	
E. coli	13	11	10	34.7
C. freundii	10	6	5	21.4
E. cloacae	4	2	4	10.2
P. aeruginosa	3	4	2	9.2
E. aerogenes	2	4	3	9.2
K. oxytoca	2	4	2	8.2
K. pneumonia	2	1	1	4.1
P. mirabilis	-	-	3	3.1
TOTAL	36	32	30	100

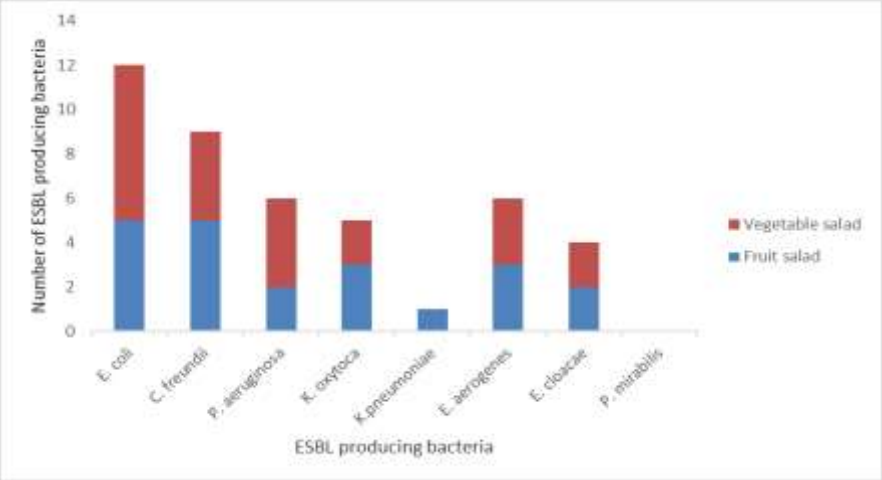

Figure 3.1: Occurrence of ESBL Producing Bacteria in Salads Samples with DDST

Table 3.4: Number of ESBL Producing Bacteria on Brilliance Agar

Gram Negative bacteria isolates	Colour Change on Brilliance ESBL Agar	No. of Positive Isolates in Fruits Salad	No. of Positive Isolates in Vegetables Salad	Total No. of ESBL Positive Bacteria
E. coli	Blue	5	6	11
E. coli	Pink	0	21	21
C. freundii	Green	1	0	1
C. freundii	Brown	4	12	16
K. pneumoniae	Green	2	2	4
E. aerogenes	Green	0	0	0
K. oxytoca	Green	1	0	1
K. oxytoca	Blue	0	5	5
E. cloacae	Green	0	0	0
P. mirabilis	Tan with halo	0	3	3
P. aeruginosa	Reddish brown	0	5	5

3.2 *Discussion*

The total coliform counts in the fruit and vegetable salads are in line with the findings of Osamwonyi [27] who reported coliform counts in salad samples as 10^4cfu/g while Pagadala [17] and Orji [13] reported higher count of 10^6 cfu/g in salad vegetable and vegetable salads respectively. Erkmen and Bozoglu [28] reported that bacteria count in fresh fruits and vegetables can range from $10^2 – 10^7$cfu/g.

The bacteria species recovered from the salads sample are in agreement with the findings of Richter [29] who recovered the same member of *Enterobacteriaceae* from vegetables retailed in Guateng Province, South Africa. Osamwonyi [27] and Itohan *et al*. [30] recovered similar bacteria species in salad vegetables in Abuja and vegetable salads in Edo State respectively. However, Orji *et al.* [13] and Amala and Agha [31] reported the presence of some of these bacteria from fruits and vegetable salads sold in different parts of the country.

The predominant bacteria in this study was *E. coli* with an occurrence of 34.7% while *P. mirabilis* was the least (occurrence 3.1%). This conforms to the findings of Adebayo-Tayo *et al*. [32], Mbae *et al*. [33], Abakari *et al*. [34], who reported *E. coli* as the predominant bacteria in vegetable salads. On the contrary, Amala and Agha [31], Ehimemen *et al.* [35], Udo *et al*. [36] reported *S. aureus* as the dominant bacteria.

Microorganisms can contaminate fruit salad through various sources such as unsanitary conditions, unhygienic handling, and processing, use of contaminated water to wash the fruits, cross contamination from other fruits, dirty processing utensils like knives, slicing trays and tables [37]. There is an increase in chances of microbial growth in fresh fruits and vegetables in the presence of external factors such as air, high humidity and temperature [28].

For the double disc synergy test, twenty-one (21) bacterial isolates in fruit salad and twenty-two (22) in

vegetable salads were positive, that is the cephalosporins showed a clear-cut extension towards the Amoxiclav.

On BEA, 68.4% of the isolates were ESBL producers with some having the expected colour change while others produced colonies with different colour change. This is in line with the findings of Kim *et al.* [19], Richter *et al.* [29], Ye *et al*. [39] who also detected ESBL producing bacteria in vegetables and foods of animal origin. *E. aerogenes* and *E. cloacae* did not show any colour change; *E. coli, K. pneumoniae,* and *P. mirabilis* strains produced the expected colour as described by the manufacturer. This is supported by the findings of Huang *et al*. [40] who also reported blue colony for *E. coli*, green for KESC, and tan with halo for *P. mirabilis* on Brilliance ESBL agar. More ESBL producing bacteria especially *E. coli*, *K. pneumoniae* and *P. mirabilis* were detected on the chromogenic agar than with DDST. Chromogenic agar proved valuable and showed high performance for the detection of ESBL-producing bacteria [40]. Also, Ongut *et al*. [41] reported that Brilliance ESBL gives an easier detection of ESBL producing *E. coli* as well as other members of the *Enterobacteriaceae*. Furthermore, Ezeanya *et al*. [38] opined that inclusion of cefpodoxime in Brilliance ESBL agar rather than ceftazidime and cefotaxime could account for its higher sensitivity over DDST. This justifies the claim by Vercauteren *et al.* [42] that cefpodoxime is the best substrate for screening ESBL producing bacteria.

K. oxytoca in vegetable salad grew as blue colonies instead of green. Huang *et al.* [40] reported that 11 strains of *K. oxytoca* grew as turquoise colonies on Brilliance ESBL agar; slightly different from the green colonies of the KESC group. Also, sixteen *C. freundii* isolates had brown colonies. Uyanga *et al*. [43] stated that the KESC produces green/blue to browny-green colour on CHROMagar (a chromogenic agar) due to β-glucosidase. *P.aeruginosa* produced a reddish brown coloration. The reddish brown reflects the presence of the species-related pyocyanin pigmentation [40].

4. Conclusions

The present study has revealed the presence of ESBL producing Gram-negative bacteria in fruit and vegetable salads. This suggests contamination due to improper handling and sanitation. Therefore, there is need for regulatory bodies to ensure that fruits and vegetables are properly handled by food processors to prevent any health hazard associated with consumption of contaminated foods.

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