UJMR, Vol. 7 Number 1, June, 2022, pp 70-76 *E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668*

https://doi.org/10.47430/ujmr.2271.012

Received: 4th Jun, 2022 **Accepted:** 19th Jun, 2022

Molecular Detection of Virulence Genes in *Escherichia coli* and *Salmonella enterica* Isolated from Minimally Processed Foods Sold within Kaduna Metropolis

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Abstract

Minimally processed foods are widely consumed in Kaduna, Nigeria and the world at large. However, poor quality control during their processing make them prone to contamination by enteropathogens known to possess multiple virulence genes, possibly causing morbidities and mortalities. Investigating the prevalence and virulence genes of food-borne bacteria is therefore paramount. This study was aimed at detecting virulence genes in enteropathogenic bacteria associated with minimally processed foods sold within Kaduna metropolis. Samples of sliced watermelons, peeled sugarcanes, peeled and sliced pineapples and unshelled coconuts (n=140) were collected from Kaduna North, Kaduna South and Igabi LGAs. Upon enrichment, samples were inoculated onto Eosin-Methylene Blue (EMB) and Cefixime-Tellurite-Sorbitol MacConkey (CT-SMAC) agar and incubated for 18 hours at 35°C. Isolates were identified using Microbact™ 24E Gram Negative Bacteria Identification System for Enterobacteriaceae. Thereafter, E. coli and Salmonella enterica isolates' DNA was extracted and purified using AccuPrep Genomic DNA Extraction Kit; used as a template for the PCR amplification of the bundle forming pilus (bfpA) and invasion (invA) genes; and then the PCR products were visualised using agarose gel electrophoresis documentation system. The results of the Microbact™ analyses showed widespread contamination of the samples with E. coli (22 isolates) and Salmonella enterica (3 isolates). There was no statistically significant difference in the bacterial contaminants isolated from the various sampling areas (one-way ANOVA: p = 0.577); similarly, the type of the minimally processed food samples had no influence on the prevalence of E. coli and Salmonella enterica isolates (p = 0.345). PCR result revealed that the invA virulence gene (284bp) was present in one Salmonella enterica isolate. The presence of invA gene in the Salmonella enterica isolate indicated that the isolate is a virulent strain, which can cause food-borne infections. These results indicated the contamination of the minimally processed fruits with enteropathogens, hence, standards of quality control should be enshrined, towards safer foods and enhanced health of the consuming populace.

Keywords: E. coli; virulence gene; minimally processed foods; Salmonella enterica.

INTRODUCTION

The term 'minimally processed food' refers to any food, especially fruit/vegetable, or any combination thereof, which had undergone minimal processing, i.e. had been altered physically from its original form, but remains in its fresh condition (Bansal *et al.*, 2015). The minimal processing processes are mostly physical, including cleaning, portioning, removal of inedible fractions, grating, flaking, squeezing, chilling, vacuum and gas packing, and simple wrapping of foods, for eventual sale to consumers (Carlos *et al.*, 2010). Foods are minimally processed to make them safe chemically and microbiologically, to retain the desired flavor, color and texture of the food products and mainly to provide convenience to consumers (Monteiro *et al.*, 2010).

However, minimally processed foods are associated with residual microbial contamination (Gilbert, 2000), either from authoctonous microbiota, or from contaminants associated with the processing (Kabir *et al.*, 2019). These foods are hawked by street vendors throughout Nigerian cities and villages, and since most of the hawkers have little or no knowledge of basic food safety issues, concerns over the safety and quality of these foods have been raised (Oyeye and Lum-Nwi, 2008).

Globally, consumption of pathogenic bacteria found in these minimally processed foods leads to high health risks and subsequent episodes of food-borne illnesses, and corollary morbidities and mortalities (Musa and Akande 2003; Kabir *et al.*, 2020). In particular, bacteria in the *Enterobacteriaceae* family are major causative agents of food poisoning, diarrhea and many forms of gastrointestinal tract infections and associated morbidities and mortalities (Okeke *et al.*, 2001; Kaferstein, 2003; Todar, 2007).

Food poisoning caused by *Salmonella* (gastroenteritis) is the most common clinical manifestation of *Salmonella* infection worldwide followed by bacteremia and enteric fever (Iwalokun, 2001; Adeshina *et al.*, 2009; Majowicz *et al.*, 2010).

The virulence of these bacteria is associated with both chromosomal and plasmid factors (Thornbrough *et al.*, 2012). In *Salmonella,* the chromosomally located invasion gene *invA* is thought to trigger the invasion of Salmonellae into cultured epithelial cells (Silvia *et al.*, 2003). In atypical Entropathogenic *E. coli*, the bundle forming pilus (*bfpA*) gene is found, which is also another important factor in adhesion and pathogenicity (Contreras *et al.*, 2010; Stoppe *et al.*, 2017).

MATERIALS AND METHODS

Sample Area

This study was conducted in Kaduna metropolis (Latitude 10.53 °N and Longitude 7.44 °E), an area which covers about 268.35 km², in Kaduna State, North Western Nigeria. Kaduna State is located along the geographical coordinates: 10°31'23'' North and 7°26'25'' East, and is situated at an elevation of 626 meters above sea level (World Atlas, 2015). It is within the Guinea Savannah ecological zone, with annual wet and dry seasons (Denwe, 2014).

Sample Collection

The food samples in this research were purchased randomly from street hawkers in three (3) different Local Government Areas within Kaduna Metropolis: Kaduna South, Kaduna North and Igabi LGAs. Four (4) types of retail-sized, minimally processed food samples were included in this study. In total, 140 samples were collected, comprising of sliced watermelons (n=40), peeled sugarcanes (n=40), peeled and sliced pineapples (n=20) and unshelled coconuts (n=40), based on their availability during the sample collection period. The samples were thereafter put in new plastic bags, and analyzed within 24 hours of collection to avoid spoilage.

Isolation and Identification of Bacteria

E. coli and *Salmonella* species were isolated from the samples according to protocols outlined in the FDA Bacteriology Analysis Manual (FDA, 2013). All media and enrichment broth were prepared according to manufacturers' instructions. Each of the collected samples was enriched by adding 25g into 225ml of MacConkey broth, before being incubated for 20hrs at 37°C, respectively. Subsequently, enriched cultures were inoculated onto Eosin Methylene Blue (EMB) Agar and Cefixime-Tellurite-Sorbitol MacConkey (CT-SMAC) Agar plates, incubated for 18 hours at 35°C, and then observed for bacterial growth.

The colonies were identified biochemically using the **Microbact 24E Gram Negative Identification System Kit** (Oxoid, Hampshire, UK), which is intended for identification of *Enterobacteriaceae* based on the following 24 biochemical reactions: Lysine, Ornithine, Hydrogen-Sulphide, Glucose, Mannose, Xylose, ONPG (Ortho-nitrophenyl-ß-galactoside), Indole, Urease, Vogues-Poskauer (VP), Citrate, Tryptophan-deaminase (TDA), Gelatin, Malonate, Inositol, Sorbitol, Rhamnose, Sucrose, Lactose, Arabinose, Adonitol, Raffinose, Salicin, and Arginine (Mailafia *et al.*, 2021). Briefly, 5ml sterile saline solution was used to emulsify pure cultures of the bacteria, to match 0.5 McFarland standard (Riko *et al.*, 2021), which were then applied onto the kit and incubated for 48 hours before observing the results. The octal code results recorded for each isolate were interpreted using Microbact computer software identification package, according to the manufacturer's instructions.

Molecular Detection of Virulence Genes in the *Escherichia coli* and *Salmonella enterica* Isolates

DNA Extraction and Purification

The DNA of the bacteria isolates were extracted and purified using AccuPrep Genomic DNA Extraction Kit, according to the standard protocols recommended by the kit manufacturers (Qiagen USA, 2016). Initially, 20µL of Proteinase K was added to a clean 1.5mL Eppendorf tube, subsequently, a pure colony was inoculated into the tube, 200µL of binding buffer was added, and the mixture was then immediately mixed in a vortex mixer. The mixture was incubated at 60°C for 10 minutes, and thereafter, 100µL of isopropanol was added, mixed by pipetting, and then the mixture was briefly spun down to get the drops clinging under the lid. The lysate was carefully transferred into the upper reservoir of binding column tube without wetting the rim. The tube was closed and centrifuged at 8,000 rpm for 1 minute. The mixture was transferred into a new 2ml tube for filtration, where 500µL of washing buffer 1 was added carefully without wetting the rim. The centrifuging step above was repeated, and the solution was poured into a disposal bottle. Next, 500µL of washing buffer-2 was added and the previous centrifuging step repeated. Thereafter, it was again centrifuged at >12,000 rpm for 1 minute to completely remove ethanol. The binding column tube was transferred to a new 1.5ml tube for elution, and 200µL of elution buffer (nuclease free water) was added onto the binding column tube, and was centrifuged at 8,000 rpm for 1 minute to elute.

The eluted genomic DNA was obtained and the tube was left open for 10 minutes to dry out the DNA. The dried pellet was re-suspended in 20µL sterile water. The DNA was then used as the template for subsequent PCR amplification protocols.

Polymerase Chain Reaction (PCR) Amplification of the *E. coli* Bundle Forming Pilus (*bfpA*) virulence gene

The amplification process was carried out using 20µL Eppendorf tubes containing 16µL premix (Taq polymerase, dNTPs, $MgCl_2$, PCR buffer and distilled water), into which 2µL of template (extracted genomic DNA), 1µL of forward primer (5'-AATGGTGCTTGCGCTTGCTGC-3') and 1µL of reverse primer (5'-GCCGCTTTATCCAACCTGGTA-3') were added. The mixture was preheated at 94ºC for 5 minutes before it was subjected to the recycling steps. The amplification conditions for the PCR assay were 30 cycles at 94ºC, for 30 seconds each, followed by annealing at 55ºC for 30 seconds, and then extension at 72ºC, for 1 minute, followed by final extension at 72ºC for 10 minutes. The products were stored at 4ºC. Thereafter, the DNA samples were stained using Ethidium Bromide, and run on 2% w/v agarose gel for separation through electrophoresis. Finally, the amplified PCR products were visualised under ultraviolet light, and documented using gel documentation system (Nejman-Falenczyk *et al.*, 2015).

Polymerase Chain Reaction (PCR) Amplification for the *Salmonella enterica* Invasion (*invA*) Virulence Gene

The amplification process was carried in the same manner described for *bfpA* gene above, however, the primers used differed. For the *invA* gene, the primers used were forward primer (5'-GTGAAATTATCGCCACGTTCGGGCAA-3') and reverse primer (5'-TCATCGCACCGTCAAAGGAACC-3'). Similarly, a PCR protocol identical to the one used for *bfpA* gene was used, with the differences being that preheating at 95ºC was done for 2 minutes, annealing was carried out at 53ºC, and final extension was performed for 7 minutes. Visualization of amplification products was also carried out as described above.

Statistical Analyses

The variability in occurrence of *Escherichia coli* and *Salmonella enterica* with regards to the sites of sample collection and based on the type of food was evaluated using One-Way Analysis of Variance (ANOVA). All analyses were carried out using AnalyStat version 1.6.50, at 95% confidence interval, i.e. $p \leq 0.05$ is considered significant.

RESULTS

Bacterial Contamination of Minimally Processed Foods in Some Parts of Kaduna Metropolis

The results of the bacterial isolation showed that all the four minimally processed foods recorded the presence of bacterial contaminants, with coconut having the highest occurrence, i.e. 7.5% (n=3) and 37.5% (n=15) for CT-SMAC and EMB, respectively. For water watermelon, sugarcane and pineapple, the prevalence was 10% (n=4) and 37.5% (n=15); 7.5% (n=3) and 15% (n=6) and 10% (n=2) and 20% (n=4), respectively (Figure 1). Growth on EMB plates is statistically higher when compared to growth on CT-SMAC (one-tailed paired t-test: p = 0.029).

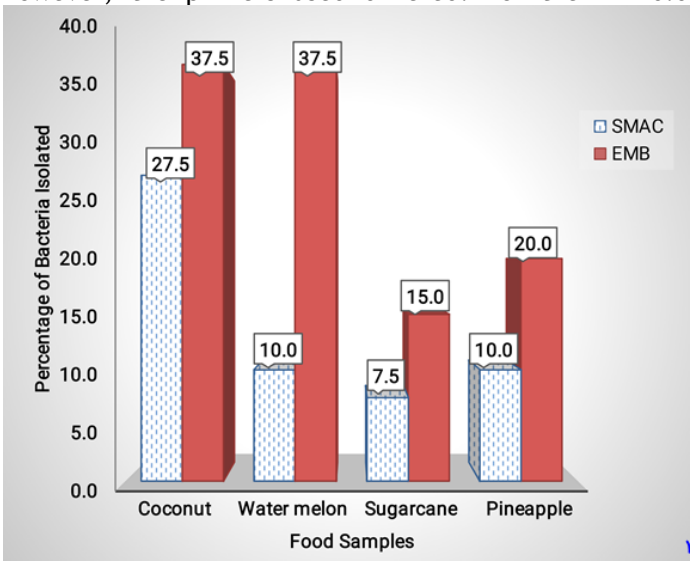

Figure 1: Distribution of Bacterial Contaminants on Some Minimally Processed Foods Sold in Some Parts of Kaduna Metropolis.

Furthermore, statistically, One-Way ANOVA showed equal contamination of the samples with regards to the area from where the samples were collected (p= 0.577), likewise, amongst the food samples positive for *E. coli* and *Salmonella enterica*, type of the minimally processed food sample had no influence on the prevalence of *E. coli* and *Salmonella enterica* isolates (p= 0.345).

Biochemical Identification of Isolates Using Microbact Gram - Negative Identification System.

The results of the bacterial identification as guided by the Microbact 24E Gram Negative Identification System Kit showed that twenty-two (22) *Escherichia coli* and three (3) *Salmonella* serotypes were confirmed by the system. *Escherichia coli* and *Salmonella enterica* were both confirmed in coconut and watermelon samples. In the sugarcane samples, only *Escherichia coli* were identified (Table 1).

Table 1: Occurrence of *Escherichia coli* and *Salmonella enterica* from the Food Samples Based on the Microbact Kit and Software

Samples	Media used for Isolation	Isolate code	Identified organism (% Probability value)
Coconut	CT-SMAC	C1	*Escherichia coli* (99.95%)
	EMB	C1	*Salmonella enterica* (99.95%)
	EMB	C8	*Escherichia coli* (96.5%)
	CT-SMAC	C6	*Escherichia coli* (99.5%)
	EMB	C12	*Escherichia coli* (95.5%)
	EMB	C10	*Escherichia coli* (90.5%)
	EMB	C14	*Salmonella enterica* (85.34%)
	CT-SMAC	C20	*Escherichia coli* (77.89%)
	EMB	C21	*Escherichia coli* (98.76%)
	EMB	C22	*Escherichia coli* (65.59%)
	EMB	C23	*Escherichia coli* (96.76%)
	EMB	C35	*Escherichia coli* (87.45%)
	EMB	C37	*Escherichia coli* (90.62%)
Watermelon	EMB	W2	*Escherichia coli* (77.98%)
	CT-SMAC	W4	*Escherichia coli* (81.95%)
	CT-SMAC	W6	*Escherichia coli* (89. 45%)
	EMB	W7	*Salmonella enterica* (90.67%)
	EMB	W21	*Escherichia coli* (79.54%)
	EMB	W34	*Escherichia coli* (90.56%)
	EMB	W39	*Escherichia coli* (87.53%)
	EMB	W40	*Escherichia coli* (91.52%)
Sugarcane	CT-SMAC	S2	*Escherichia coli* (75.57%)
	CT-SMAC	S7	*Escherichia coli* (90.85%)
	CT-SMAC	S30	*Escherichia coli* (82.35%)
	EMB	S31	*Escherichia coli* (94.54%)

Key: CT-SMAC = Cefixime-Tellurite-Sorbitol Mac-Conkey Agar, EMB = Eosin-Methylene Blue Agar, C = Coconut, S = Sugarcane and W = Watermelon.

The *invA* virulence gene (284-bp) was detected in only one isolate of the *Salmonella enterica*, which was obtained from coconut sample, as shown in Figure 2.

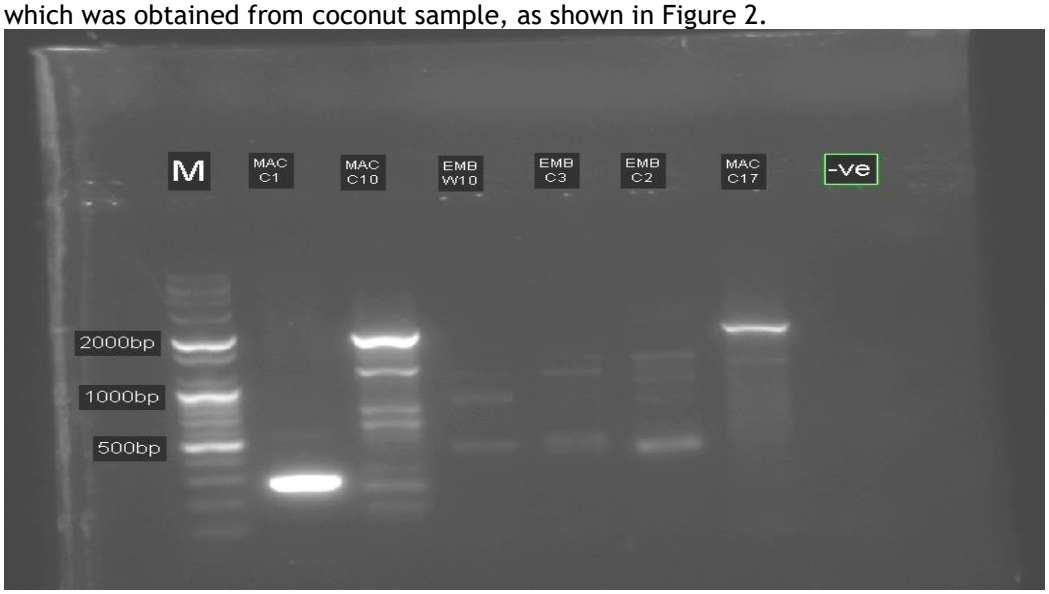

Figure 2: Visualisation of electrophoresis products from PCR amplification of *invA* gene from *Salmonella enterica*. Key: M lane = DNA marker of 100 bp increment, -ve = negative control.

DISCUSSION

The minimally processed foods used in this study (sliced coconuts, sliced watermelons, peeled/sliced sugar-cane and peeled/sliced pineapples) were found to be contaminated with potentially pathogenic bacteria. These foods were purchased directly from street hawkers within Kaduna Metropolis and have not undergone any further treatment such as heating or washing before consumption. The only hygienic process carried out was washing with tap water by the street hawkers before peeling or slicing them. Amongst the minimally processed foods, fruits and vegetables are more susceptible to contamination because cutting and slicing damage the natural protective barriers of intact food produce thereby releasing nutrients that facilitate the growth of microorganisms (Abadias *et al.,* 2008).

The presence of bacterial pathogens indicates contamination of the samples, which can be from many sources, mainly pre-harvest contamination, or post-harvest contamination (i.e. during the minimal processing). The variability in detection of bacteria from the collected samples showed lack of consistent sanitation and inadequate hygienic practices. Most of the locations where these minimally processed foods were purchased within Kaduna metropolis lack proper hygiene. The hawkers keep their stands mostly near open gutters where waste materials are disposed. Some hawkers also are stationed near heaps of foul-smelling waste products. It was observed that the hawkers use the same transparent polythene bags to cover sliced fruits for days without replacing with new ones until they are torn. Furthermore, there is an increased potential for fruits and vegetables to become contaminated with pathogenic species during processing as there are no systems of microbiological control for raw or minimally processed fruit foods (Ayicicek *et al.*, 2006).

Escherichia coli was detected in about 73.3% of the coconuts, watermelon and sugarcane samples. About 2.1% of coconuts and watermelon samples harbored only *Salmonella enterica*. The presence of *E. coli* and *Salmonella enterica* indicates faecal contamination and poor quality of fresh fruits and vegetables (Abidias *et al.*, 2008). A number of previous surveys had reported the isolation of *E. coli* and *Salmonella* from fresh fruits and vegetables (Jay, 2000; Kabir *et al.,* 2020). Similarly, Feroz *et al.* (2013) posited that the occurrence of *E. coli* on fruits and vegetables not only poses a threat to life for its consumers but can also reduce the shelf life of the foods. This suggestion is also in agreement with the submission of Subramanian *et al.* (2009).

The presence of *Salmonella* in foods is alarming due to its rate of development of antibiotic resistance, which appears to have accelerated in the past decades. Today, multi-drug resistant *Salmonella* spp. constitute a global problem (Adeleye *et al.,* 2019). It has also been observed that antibiotic susceptibility of *Salmonella enterica* isolates is not constant but dynamic and varies with time, environment and source (Denwe, 2014).

The molecular detection of the *invA* gene in a *Salmonella enterica* isolate indicates that it is a virulent strain which can cause food-borne infections to consumers of these minimally processed foods. This result is consistent with the findings of Mohammed (2013), who reported the detection of the invA virulence gene in *Salmonella* isolated from meat and poultry products. The *InvA* gene codes for a protein found in the inner membrane of bacteria, which is necessary for invasion to epithelial cells (Darwin and Miller, 1999). The control of invasion involves a number of genetic regulators and environmental stimuli in complex relationships (Craig, 2005). In addition, several environmental factors such as pH, osmolarity, oxygen tension, bile, magnesium ion concentration, and short chain fatty acids interact with genetic regulators leading to productive infection (Altier and Lawhon, 2000).

The absence of the *bfpA* gene in all the *Escherichia coli* isolates indicates that the samples were not contaminated with atypical Enteropathogenic *Escherichia coli* (aEPEC), which is an emerging diarrheagenic pathogen in developing countries like Nigeria. This result is consistent with the findings of Stoppe *et al.* (2017) and Contreras *et al.* (2010), who reported detection of phylogenetic group patterns of *Escherichia coli,* including aEPEC from commensal human and wastewater treatment plant isolates throughout the globe, and the allelic variability of eae, bfpA, and perA, marked as 'critical virulence genes', in Perusian Children.

CONCLUSION

This study found out that minimally processed foods (liced watermelons, peeled sugarcanes, peeled and sliced pineapples and unshelled coconuts) sold within Kaduna metropolis were contaminated with enteropathogens, mainly *E. coli* (88%) and *Salmonella enterica* (12%). The study also reported the molecular detection of the invasion virulence gene (*invA*) in *Salmonella enterica* isolated from the samples. These results indicate the contamination of the minimally processed foods with virulent enteropathogens.

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