

Research Article

Resistance of Bacteria Isolates from Cabbage (*Brassica oleracea*), Carrots (*Daucus carota*) and Lettuce (*Lactuca sativa*) in the Kumasi Metropolis of Ghana

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Abstract: Despite the benefits humanity derives from vegetables, they are as well a good source of foodborne illnesses when contaminated with pathogenic microorganisms especially the antibiotic resistant ones. This study evaluated the antibiotic resistance of bacteria isolated from three commonly consumed vegetables; cabbage (*Brassica oleracea*), carrots (*Daucus carota*) and lettuce (*Lactuca sativa*) in the Kumasi Metropolis in Ghana. The bacteria isolates were subjected to antimicrobial susceptibility testing using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar. A total of 78 bacteria isolates were obtained from the three vegetables with the Gram-positive bacteria accounting for 23.08% (18/78) whilst the Gram-negative bacteria accounted for 76.92% (60/78). The Gram-negative bacteria consisted of *Acinetobacter spp.*, *Aeromonas salmonicida*, *Aeromonas spp.*, *Chryseomonas loteola*, *Citrobacter spp.*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Erwinia nigrifluence*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella spp.* and *Serratia spp.* while Gram-positive bacteria consisted of *Brevibacillus spp.*, *Brochothrix thermosphacter*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus spp.* All the Gram-positive bacteria isolates showed 100% resistance to penicillin, ampicillin and flucloxacillin, 88.2% were resistant to cefuroxime with 64.7% resistant to tetracycline and 17.6% resistant to cotrimoxazole but were all sensitive to gentamycin. For the Gram-negative bacteria, 96.7% were resistant to ampicillin with 90% being resistant to cefuroxime, 73.3% and 60% were resistant to chloramphenicol and tetracycline, respectively, but unlike the Gram-positives, 15% of the Gram-negative bacteria were resistant to gentamycin. All the Gram-positive isolates demonstrated multi antibiotic resistance (MAR), with *Staphylococcus aureus* and *Streptococcus spp.* being resistant to seven (7) out of the eight (8) tested antibiotics. For the Gram-negative bacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella spp.*, *Citrobacter spp.* and *Enterobacter* were resistant to all eight (8) antibiotics. The implication of the findings in this study presents vegetables as a potential vehicle for microbial food poisoning as well as a source of infectious diseases that cannot be treated with commonly used antibiotics. Thus, good practices should be undertaken to reduce if not eliminate contamination of vegetables.

Keywords: Bacteria, Ghana, Multiple Antibiotic Resistance, Prevalence, Vegetables

1. Introduction

Growing bodies of studies on vegetables have shown association of the commodity with high microbial load over

the world including Ghana [1-4]. Literature has emphasized the fact that contamination of vegetables results from the field of cultivation through contact with soil, contaminated water, wild or domestic animals, or inappropriately composted

manure used as fertilizers to enrich the soils. Contamination of the product also emanates from improper handling, storage and transportation by farmers, vendors and end users [5]. A key aspect of vegetable contamination with pathogenic microbes is the contaminants' resistant to antimicrobials. Individuals infected with drug-resistant organisms are more likely to remain in the hospital for a longer time and more likely to have a poor prognosis including increased frequency of bloodstream infections and increased mortality, hence the impact on particularly vulnerable patients is most obvious [6, 7]. The cost of treating antimicrobial resistance infections place a significant burden on society, a burden that is likely to grow larger as the number of cases of drug-resistant illness increases [6].

Antimicrobial resistant pathogens are a growing menace to all people, regardless of age, gender, or socioeconomic background and there are evidences of increased trend globally of prevalence of antibiotic resistance in foodborne bacteria [8-10]. A microorganism is resistant if it exhibits "significantly reduced susceptibility" when compared with that of the "original isolate" or a group of sensitive strains [11]. Similarly, an authority states, "antibiotic resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic" [12]. Antimicrobial resistance of microbes can be innate (intrinsic) or acquired (extrinsic) [1, 13-15]. Innate resistance of a bacteria may stem from the complexity of the cell wall, efflux mechanisms where microbes pump antimicrobials out of the cell, or enzymatic inactivation of the antimicrobial agent while acquired resistance results from genetic changes that occur through mutation of the antimicrobial's target site within the bacterium or acquisition of genetic material encoding resistance via plasmids [1, 13]. Drug-resistant infections have profound health consequences for the most vulnerable populace including patients, children, the elderly people, pregnant women as well as lactating mothers [15]. The problem of antibiotic resistance is not restricted to Ghana only but it is worldwide and the World Health Organization (WHO) in the year 2000 hinted of the world reaching a crisis point in relation to the level of resistance of microbes to drugs used in treating common infectious diseases [16, 17]. Antibacterial resistance involves bacteria that cause many common and life threatening infections as there are available literature showing that much of the problem of infectious diseases worldwide result from antimicrobial-resistant bacteria and more virulent strains of such pathogenic microbes have evolved [18-21]. Significant resistance has been reported for several bacteria. Clinically important microbes that have developed resistance to available antimicrobials include bacteria that cause pneumonia, ear infections, and meningitis (*Streptococcus pneumoniae*), skin, bone, lung, and bloodstream infections (*Staphylococcus aureus*), urinary tract infections (*Escherichia coli*), foodborne infections (*Salmonella* or *E. coli* acquired from meat, eggs, nuts, fresh produce), and infections transmitted in healthcare settings (Enterococci, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella* spp.) [8, 22, 23].

Many gaps remain in the efforts to contain antimicrobial resistance worldwide [9]. In Ghana, most antimicrobial resistance studies have largely focused on microbes isolated from health centers or similar settings [24-26]. Meanwhile, researches involving vegetables had little information on antimicrobial resistance [2-4]. Data on antibiotic resistance microbes isolated from vegetable in Ghana are limited. The aim of this study therefore was to isolate, identify and determine the antibiotic resistance of bacteria isolates from vegetables including cabbage (*Brassica oleracea*), carrot (*Daucus carota*) and lettuce (*Lactuca sativa*) cultivated in peri-urban areas of Kumasi Metropolis, in Ghana.

2. Materials and Methods

2.1. Study Area

The Gyinyase Vegetable growing site, covering an estimated 6 hectares which is equivalent to 10,000 square meters or 2.471 acres of cultivated land was chosen as the study area. It is one of the largest among all the urban and peri-urban vegetable growing sites in the Kumasi Metropolis [27]. The vegetable site is located in the moist semi-deciduous forest agro-ecological zone (belt) of Ghana [28]. The main vegetables produced at this locality include cabbage, lettuce, carrots, spring onions and green pepper. The land is often enriched with animal manure [27, 28]. To improve on vegetable yield, the vegetables are irrigated from dugout wells located on the farm. Irrigation is by overhead technique using watering cans and water hose. The farmers engage in crop rotation, use of beds/mounds and the use of simple farm implements like hoes and garden forks.

2.2. Sample and Sampling Technique

2.2.1. Sample Collection

Cabbage, carrot and lettuce are the major cultivated crops and the most consumed vegetables in the city. A total of nine (9) samples (three samples each) were randomly collected over a period of three months (February to April). The samples were collected from the farm by sectioning the farm into two equal parts. Three beds from each half were randomly selected from which the samples were taken diagonally on each bed. The samples were collected into different sterile containers and transported to the laboratory within two hours and analyzed the same day.

2.2.2. Sample Preparation, Isolation and Storage of Pathogenic Bacteria

Portions of sample weighing 25 g were inoculated into 225 ml buffered peptone water (Oxoid Ltd. Basingstoke, Hants, England) and incubated at 37°C overnight. The overnight culture was pelleted at $4300.8 \times g$ for 20 minutes. The pellets were streaked on xylose-lysine deoxycholate (XLD) agar (Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland) and MacConkey agar (Sigma-Aldrich Co., St. Louis, MO, USA) for the detection of *Salmonella*, *Shigella*, *E. coli*, and other Enterobacteriaceae, Baird Parkar agar (Fluka, Sigma-Aldrich

Chemie, Buchs, Switzerland) incorporated with egg yolk for selective isolation of Gram positive *Staphylococcus* spp., brilliant green bile glucose agar (Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland) to isolate lactose fermenting bacteria like *E. coli*, *Enterobacter* and *Klebsiella* as well as non-lactose fermenting bacteria like *Salmonella*, *Shigella* and *Pseudomonas aeruginosa*, and Nutrient agar (Mast Group Ltd., Mersyaside, U.K.) to isolate *Bacillus* spp. All inoculates were incubated at 37°C under aerobic conditions for 24 hours.

2.3. Microbiological Examination

2.3.1. Identification and Microbiological Characterization of Isolated Microbes

The pure bacteria isolates were identified by examining their colonial and cell morphology, Gram reaction and by using standard biochemical test methods [29]. Further identification as well as characterization of the microorganism was done using analytical profile index (API) kits (BioMérieux, SA, France). All isolates were maintained in 1 ml of 50% glycerol in cryo-tubes and stored at - 80°C.

2.3.2. Susceptibility of the Isolates to Antibiotics

All the isolates were subjected to antimicrobial susceptibility testing using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Sigma-Aldrich Co., St. Louis, MO, USA). Briefly, bacteriological peptone water subcultures from between 16 and 18 hours cultures, which had attained the 0.5 McFarland turbidity standards were used to seed uniformly the entire surface of the Mueller-Hinton agar plates. The plates were air dried, antibiotic disks [Ampicillin (10 µg/disc), Penicillin (10 i.u./disc), Flucloxacillin (5 µg/disc), Erythromycin (15 µg/disc), Tetracycline (30 µg/disc), Gentamicin (10 µg/disc), Cotrimoxazole (25 µg/disc), Chloramphenicol (30 µg/disc), Ceftriaxone (30 µg/disc), Cefuroxime (30 µg/disc), and Cefotaxime (30 µg/disc)] were carefully placed on the surface of the plates in a sterile environment and incubated at 37°C for 16-18 hours. The diameters (in millimeters) of clear zones of growth inhibition around each antimicrobial agent disks were measured using a millimeter rule. Each bacterial species was classified as Resistant (R), Intermediate Resistant (I), or Susceptible (S) per guidelines of Clinical Laboratory Standard Institute [30]. The prevalence of the resistance levels was determined using results from the susceptibility determinations. All analysis was done in triplicates. The results were processed using Microsoft Excel 2007 and tabulated for easy interpretation.

2.4. Ethical Clearance

Consent was sought from the Head of the Vegetable Growers Association at the study site and ethical clearance and approval was obtained from the Committee on Human Research, Publication and Ethics of the Kwame Nkrumah University of Science and Technology, School of Medical Sciences/Komfo Anokye Teaching Hospital (CHRPE/AP/244/12).

3. Results and Discussion

From February to April 2013, 9 samples (3 each of cabbage, carrot and lettuce) were sampled from the Gyinyase vegetable growing site in the Kumasi Metropolis. The first set of analysis bordered on isolation and identification of bacterial contaminants of the samples after which the bacterial isolates were subjected to antibacterial susceptibility test where varied degrees of resistance were observed.

3.1. Isolated Bacteria

Figure 1 displays the percentage of bacterial isolates observed in all three samples based on Grams reaction. A total of 78 bacteria isolates were obtained from the three vegetables, Gram-positive bacteria accounting for 23.08% (18/78) whilst the Gram-negative bacteria accounted for 76.92% (60/78) (Figure 1). Cabbage had the highest number of isolates (30) followed by lettuce with 27 and carrot with the least number of isolates (21). Gram-negative bacteria have been reported as the dominant bacterial population on vegetables [5]. By virtue of their cultivation close to the ground, use of polluted water and improper handling, vegetables are highly exposed to microbial contamination.

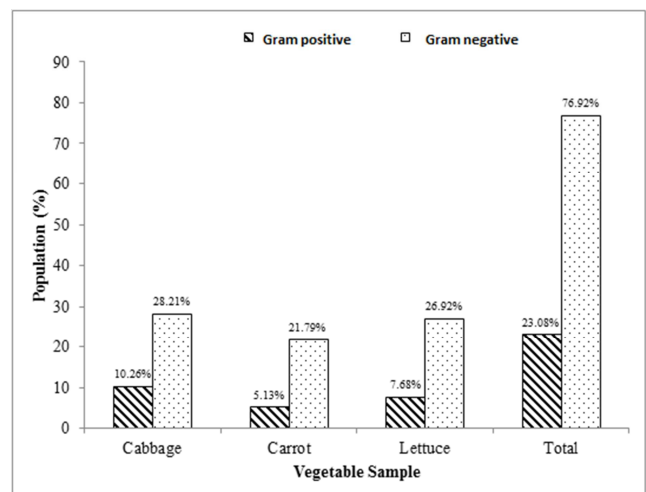


Figure 1. Percentage distribution of bacteria isolates based on Gram's reaction.

Values are in percentages of the total population of the bacteria isolates. The bars that have been shaded in stripes represent Gram-positive bacteria isolates and those shaded with dots represent Gram-negative bacteria isolates.

Table 1 and Table 2 exhibit the various bacteria genera that were observed in this study. The bacteria genera that were observed include; *Acinetobacter* spp., *Aeromonas salmonicida*, *Aeromonas* spp., *Brevibacillus* spp., *Brochothrix thermosphacta*, *Chryseomonas loteola*, *Citrobacter* spp., *Enterobacter cloacae*, *Enterobacter sakazakii*, *Erwinia nigrifluens*, *Escherichia coli*, *Klebsiella pneumoniae*, *Leuconostoc mesenteroides* spp. *cremoris*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Serratia* spp., *Staphylococcus aureus*, *Staphylococcus* spp., and *Streptococcus* spp. Some bacteria of human health importance isolated and identified

included; *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella* spp. (Tables 1 and 2). The isolation of these bacteria in the current study is noteworthy because these bacteria are responsible for various human ailments such as bloody diarrhoea, urinary tract infection, destructive changes to human lungs, lower respiratory tract infections, gastroenteritis or salmonellosis, boils, skin & soft-tissue infections, food poisoning, toxic shock syndrome, pharyngitis, intra-abdominal infections, septic arthritis, osteomyelitis, central nervous system infections, and ophthalmic infections [2, 3, 7, 28, 31, 32].

3.2. Antibiotic Susceptibility

We evaluated antibiotic susceptibility of the microbial isolates revealing high levels of antimicrobial resistant microbes in the vegetables (Table 1 and 2). Resistance to antimicrobial agents is an increasing global problem as it poses a threat to humans [17, 33]. Resistant bacteria could colonize vegetables for a number of reasons; the direct use of antimicrobials during cultivation of crops, the use of

contaminated animal's manure and irrigation with contaminated water en-route to the fork (consumer) [5].

3.2.1. Antibiotic Susceptibility of the Gram-positive Isolates

Table 1 shows the pattern of resistance observed in the Gram positive bacterial isolates. The overall resistance rates among the Gram-positive bacteria isolates were between 17.6% (cotrimoxazole) and 100% (flucloxacillin, ampicillin and penicillin) (Table 1). Remarkably, all the Gram-positive bacteria isolates demonstrated 100% resistance to penicillin, ampicillin and flucloxacillin, 88.2% were resistant to cefuroxime, 64.7% were resistant to tetracycline and 17.6% were resistant to cotrimoxazole while all the Gram positive isolates were susceptible to gentamycin. The none detection of gentamycin resistant Gram positive bacteria in this study is contrary to other research works which reported 100% gentamycin resistant Gram-positive bacteria from lettuce [34, 35]. Perhaps, variation in laboratory methods, sampling strategy, and sample handling practices and also geographical differences could be the reasons for the variation.

Table 1. Antibiotic Resistance Pattern of Gram-positive Isolates from the Three Samples.

Bacteria (no. of isolates)	% of bacteria isolates resistant to the antibiotics								
	PEN	AMP	FLX	ERY	TET	COT	CRX	GEN	MAR
<i>Brevibacillus</i> spp. (5)	100	100	100	80	60	0	100	0	6/8
<i>Brochothrix thermosphacter</i> (1)	100	100	100	0	0	0	100	0	4/8
<i>Staphylococcus aureus</i> (6)	100	100	100	100	40	20	80	0	7/8
<i>Staphylococcus epidermidis</i> (2)	100	100	100	100	100	0	100	0	6/8
<i>Streptococcus</i> spp. (4)	100	100	100	50	100	50	75	0	7/8
Overall Total % Resistance (18)	100	100	100	76.5	64.7	17.6	88.2	0	

KEY: PEN - Penicillin, AMP - Ampicillin, FLX - Flucloxacillin, ERY - Erythromycin, TET - Tetracycline, COT - Cotrimoxazole, CRX - Cefuroxime, GEN - Gentamycin and MAR - Multi antibiotic resistance.

The 100% overall total resistance by all the Gram-positive isolates in this study to penicillin, ampicillin and flucloxacillin (Table 1) is not unexpected as these antibiotics are commonly used in the Ghanaian society with no clear regulation controlling the use of antibiotics [36] and more so they belong to the same class of antibiotics.

Another important finding was that all the Gram-positive isolates demonstrated multi antibiotic resistance (MAR), with *Staphylococcus aureus* and *Streptococcus* spp. being resistant to seven (7) out of the eight (8) tested antibiotics. The resistance of *Staphylococcus aureus* to antimicrobials, particularly to β -lactam antibiotics and resistance of *Streptococcus* spp. to antibiotics have also raised high concerns as emerging problems in the food environment as documented by [8, 9, 22, 37]. The fact that the vegetables were sampled at the farm level without any washing or post-harvest reduction technique makes these results entirely

consistent with previous studies that had detected antibiotic-resistant bacteria on vegetable products at harvest or at the retail level [38-40].

3.2.2. Antibiotic Susceptibility of the Gram-negative Isolates

Table 2 demonstrates the resistant pattern of the Gram negative bacterial isolates observed in this study. From Table 2, concerning the susceptibility of the Gram-negative bacteria, the overall total resistance ranged from 15% (gentamycin) to 96.7% (ampicillin). Resistant to cefuroxime, chloramphenicol and tetracycline were 90%, 73.3% and 60% respectively. *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Citrobacter* spp. and *Enterobacter* were resistant to all eight (8) antibiotics. These results show that the vegetables harboured high levels of MAR bacteria.

Table 2. Antibiotic Resistance Pattern of Gram-negative Isolates from the Three Samples.

Bacteria (no. of isolates)	% of bacteria isolates resistant to the antibiotics								
	AMP	TET	COT	GEN	CRX	CHL	CTR	CTX	MAR
<i>Acinetobacter</i> spp. (2)	100	0	0	0	100	50	50	100	5/8
<i>Aeromonas salmonicida</i> (2)	100	50	50	0	100	100	100	100	7/8
<i>Aeromonas</i> spp. (3)	100	33.3	0	0	66.7	66.7	33.3	33.3	6/8
<i>Chryseomonas loteola</i> (2)	100	50	50	0	100	100	100	50	7/8

Bacteria (no. of isolates)	% of bacteria isolates resistant to the antibiotics								
	AMP	TET	COT	GEN	CRX	CHL	CTR	CTX	MAR
<i>Citrobacter spp. (1)</i>	100	100	100	100	100	100	100	100	8/8
<i>Enterobacter cloacae (5)</i>	100	40	40	20	100	100	20	20	8/8
<i>Enterobacter sakazakii (1)</i>	100	100	0	0	100	100	0	0	4/8
<i>Erwinia nigrifluence (1)</i>	100	100	0	0	100	100	0	0	4/8
<i>Escherichia coli (11)</i>	90.9	54.5	27.3	18.2	81.1	63.6	54.5	54.5	8/8
<i>Klebsiella pneumoniae (8)</i>	87.5	62.5	37.5	37.5	87.5	62.5	50	62.5	8/8
<i>Pseudomonas aeruginosa (12)</i>	100	83.3	83.3	16.7	100	83.3	100	100	8/8
<i>Salmonella spp. (11)</i>	100	63.6	18.2	9.1	81.8	72.7	63.6	72.7	8/8
<i>Serratia spp. (1)</i>	100	0	100	0	100	0	0	0	3/8
Overall Total % Resistance (60)	96.7	60	38.3	15	90	73.3	61.7	65	

KEY: AMP-Ampicillin, TET-Tetracycline, COT-Cotrimoxazole, GEN-Gentamycin, CRX-Cefuroxime, CHL-Chloramphenicol, CTR-Ceftriaxone, CTX-Cefotaxime and MAR- Multi antibiotic resistance.

Escherichia coli and *Klebsiella* spp. including *Salmonella* are all intestinal commensals of many mammals and are the Gram-negative bacteria most isolated from the vegetables. Unfortunately, they all exhibited 100% multi antibiotic resistance (MAR) to all the antibiotics used in this study (Table 2). This finding affirms the work of Laniewska-Trokenheim, Sobota and Warمیńska-Radyko [41]. *Escherichia coli* and *Klebsiella* spp. are among some common Gram-negative bacteria reported to be responsible for resistance infections that have been isolated [9]. It is important to note that our study site applies animal manure for better crop yield, this could be the source of some of these MAR pathogenic bacterial isolates. Resistance could also be as a result of the of the vegetable being in contact with the soil and ground water used for irrigation that are contaminated. Moreover, at the study site also, overhead irrigation technique using watering cans exists. This could have also contributed to the contamination of the vegetables as soil splashes onto to the vegetables during the process of irrigation.

Gentamycin susceptibility of Gram-positive bacteria isolates (0% overall resistance) and the relatively low gentamycin resistance of Gram-negative bacteria isolates (15% overall total resistance) in this study proves Gentamycin as one of the possible effective antibiotics. This finding is similar to [37, 39] but dissimilar to the findings of [41] who isolated gentamycin resistant Gram-negative rods from fresh vegetables. The variation could be due to difference in geography and farm practices as well as the use and handling of manure fertilizers [42]. The 15% overall resistance of the Gram-negative bacteria isolates to gentamycin in the current study may stem from the fact that in general, gram-negative bacteria are resistant to antibiotics or acquire resistance due to indiscriminate use of the antibiotic resulting in the outer cell membrane of the gram negative bacteria evolving as a protective mechanism against the antibiotic selection pressure [1, 43].

4. Conclusion

The purpose of the current study was to isolate, identify and determine the antibiotic resistance of bacteria isolated from cabbage (*Brassica oleracea*), carrot (*Daucus carota*) and lettuce (*Lactuca sativa*) cultivated on animal manure-enriched soils in Kumasi, Ghana. In this study, we found out that there was high prevalence of antimicrobial resistant bacteria on the

vegetables. All the Gram-positive bacteria isolated in this study showed 100% overall total resistance to penicillin, ampicillin and flucloxacillin but were sensitive to gentamycin with 15% of the Gram-negative isolates being resistant to gentamycin. Extensive multi antibiotic resistance (MAR) bacteria were also isolated and were observed showing some level of resistance to almost all the antibiotics tested. The implication of the findings in this current study presents vegetables as a potential vehicle for microbial food poisoning as well as a source of infectious diseases that cannot be treated with commonly used antibiotics. However, the outcome of this study, does not conclusively suggest that the resistance ability of the bacteria isolated is due entirely to animal manure being used since that correlation has not been established in this study. According to [35, 44] antibiotic-resistant bacteria are ubiquitous on vegetables, regardless of where they are produced, local climate conditions, farming practice, or vegetable type. Thus, the transfer of resistant bacteria or the risk of antibiotic resistance bacteria exposure of humans via vegetable consumption is inevitable. The situation, therefore, should best be managed by ensuring that good practices are designed to protect vegetables from contamination with pathogenic microorganisms and also protected from exposure to antibiotic-resistant bacteria. Farmers (crop and animal farmers alike) should be encouraged to establish good agricultural practices (GAP), marketers would need to undertake good distribution practices (GDP), manufacturers have to exhibit good manufacturing practices (GMP) and consumers on the other hand must ensure good hygienic practices (GHP) [45, 46] and as well undertake thorough postharvest reduction practices like washing vegetables with enough clean water incorporated with vinegar or salt where appropriate. These actions would ensure drastic reduction if not total elimination of antibiotic resistant microbes on raw commodities to guarantee safe consumption. It is therefore recommended that, further studies (molecular typing) should be conducted to determine the plasmids and/or genes responsible for the resistance in the various organisms to inform strategic mitigating actions.

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