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Bacterial composition and Antibiotic Resistance in Ready-to-Eat Vegetables Sold in some Oyo State Markets.

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ABSTRACT

The demand for nutritionally rich, and convenient food products is increasing globally. Fresh produce get contaminated with enteric foodborne pathogens through multiple transmission routes. Fresh, ready-to-eat vegetables were collected at selected markets, namely Bodija, Sango, and Sabo Markets in Oyo state. Five samples each of carrot, lettuce, cabbage, spring onions, and cucumbers were collected from each market. Bacterial isolation was carried out using standard procedures, followed by Antibiotic susceptibility testing and metagenomic analysis to study the microbial communities represented in the vegetables. The highest occurring isolates were *Salmonella* spp (17 isolates), followed by *Serratia* spp (15 isolates), and the least isolated was *Klebsiella* spp. (6 isolates). One hundred percent resistance was recorded against cefixime, cefuroxime, and ceftazidime, however, 95.5% and 94.4% sensitivity, respectively, were recorded against ofloxacin and ciprofloxacin. Microbial communities that were identified in pooled fresh vegetable samples from the three markets in Oyo State showed that *Pseudomonas* was the highest occurring isolate. Other identified bacteria, in order of abundance, were *Janthinobacterium*, *Flavobacterium*, *Rheinheimera*, *Pantoea*, *Comamonas*, *Sphingobacterium*. This study reinforces the need for improved food safety practices, including proper handling, washing, and processing of vegetables before consumption.

KEY WORDS: Ready-to-eat vegetables, Metagenomic, Antibiotic-resistance, Microbial community

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INTRODUCTION

Ready-to-eat (RTE) vegetables are products subjected to minimal manufacturing processes after harvest, before being placed on the market, and ready for consumption [1]. The demand for nutritionally rich, convenient food products is increasing in both developed and developing countries. Fresh produce can become contaminated with enteric foodborne pathogens via different transmission routes, including pre and post-harvest sources [2]. The consumption of contaminated fresh produce, particularly minimally processed vegetables and fruits, is increasingly recognized internationally as a source of foodborne illnesses, posing a significant safety risk to human health [3].

About 90% of food-poisoning illnesses such as diarrhea, nausea, weakness, high body temperature are believed to be caused by species of *Klebsiella*, *Pseudomonas*, *Salmonella*, *Clostridium*, *Campylobacter*, *Listeria*, *Vibrio*, *Bacillus*, and *Escherichia coli* [4]. Antibiotic resistant bacteria contained in RTE fruits and vegetables is a concern due to the potential for these resistant bacteria to be transmitted to humans through the food chain [5]. Foodborne pathogens, such as *Escherichia coli* and *Salmonella*, have been found to harbor multiple antimicrobial resistance genes, enabling them to resist the effects of various antibiotics [6].

The emergence of antimicrobial-resistant strains in fresh produce is attributed to several factors, including the overuse and misuse of antibiotics in agriculture, as well as the ability of these resistant bacteria to spread through the environment and contaminate food sources [7]. It was estimated in 2019 that 1.27 million people died due to antimicrobial-resistant infections, and the global burden associated with antimicrobial-resistant infections across several pathogens–drug resistant combinations was estimated at 4.95 million cases [8].

As datasets become increasingly more complex and comprehensive, novel tools for analysis, storage, and visualization will be required. Metagenomics gives genetic information on potentially novel biocatalysts or enzymes, genomic linkages between function and phylogeny for uncultured organisms, and evolutionary profiles of community function and structure. Metagenomics is defined as the direct genetic analysis of genomes contained within an environmental sample [9].

This study was aimed at determining the bacterial diversity, through metagenomics analysis, in fresh vegetables from selected markets in Oyo state, as well as studying the resistance patterns of the Gram-negative bacterial isolates to commonly used antibiotics.

MATERIALS AND METHODS

Sample collection and preparation

Fresh ready-to-eat vegetables were collected at selected markets, namely Bodija, Sango, and Sabo Markets in Oyo state. Five samples each of carrot, lettuce, cabbage, spring onions, and cucumbers were collected from each market. Each sample was collected in sterile polyethylene bags and brought to the laboratory in ice coolers for analysis. Bacterial isolation was carried out on MacConkey agar and Nutrient agar using standard procedures. Morphological and biochemical identification of isolates was carried out as described [10].

Antibiotic sensitivity testing

Antibiotic susceptibility testing of the gram-negative isolates was determined using the standard disk diffusion method. Each test isolate was grown overnight in nutrient broth. Each isolate was standardized to 0.5 McFarland which is equivalent to 1.5x 10^8 CFU/mL. The susceptibility of the various isolates was tested against 8 antibiotics namely ciprofloxacin, (10 µg), ceftazidime (30 µg), augmentin (30 µg), ofloxacin (10 µg), tarivid (10 µg), gentamicin (30 µg), nitrofurantoin (5 µg), cefuroxime (30 µg), and cefixime (30 µg) by the disk diffusion method. All inoculated plates were incubated at 37°C for 24 hours, and the diameter of zones of inhibition was measured and recorded [11].

Metagenomics analysis

DNA extraction method

Standard DNA extractions of the pooled RTE vegetables samples from the different markets was carried out following the ZymoBIOMICS™ DNA Miniprep Kit2 procedure. Cells suspended in lysis solution were transferred into the ZR BashingBead™ Lysis Tubes and mechanically lysed using the MP FastPrep-24™ lysis system with 1 minute of lysis at maximum speed and 5 minutes of rest for 5 cycles. Samples were then centrifuged at 10,000rcf for 1 minute. Four hundred (400µl) of supernatant was transferred from the ZR BashingBead™ Lysis Tube to a Zymo-Spin™ III-F Filter and centrifuged at 8,000rcf for 1 minute. The addition of 1,200 µl of ZymoBIOMICS™ DNA Binding Buffer was to the effluent was carried out, and mixed via pipetting. The transfer of 800µl of this solution to a Zymo-Spin™ IICR Column and centrifuged at 10,000rcf for 1 minute followed. This step was repeated until all material was loaded onto the Zymo-Spin™ IICR Column. DNA bound to the Zymo-Spin™ IICR Column was washed 3 times with 400µl and 700µl of ZymoBIOMICS™ DNA Wash Buffer 1 and then 200 µl of ZymoBIOMICS™ DNA Wash Buffer 2 with a 1-minute spin down at 10,000rcf for each, respectively. Washed DNA was eluted using 75µl of ZymoBIOMICS™ DNase/RNase Free Water following a 5-minute incubation at room temperature and a 1-minute spin down at

10,000rcf. The Zymo-Spin™ III-HRC Filter was prepared using 600 µl of the ZymoBIOMICS™ HRC Prep Solution and a centrifugation at 8,000rcf for 3 minutes. Eluted DNA was then purified by running the effluent through the prepared Zymo-Spin™ III-HRC Filter. Final DNA concentrations were determined via Qubit3 [12].

Amplification of the V3/V4 region by 16S analysis

Sequences were imported to Qiime2 for analysis. Primer sequences were removed using Qiime2's cutadapt2 plugin using the following degenerate primer queries:

Table 1: Primer sequence for Qiime2 analysis

Region	Forward Trim Sequence	Reverse Trim Sequence
V3/V4	CCTAYGGGNBGCWGCAG	GACTACNVGGGTMTCTAATCC

Sequences were then denoised using Qiime2's dada2 plugin3. Denoised sequences were assigned operational taxonomic units (OTUs) using the Silva 138, 99% OTUs full-length sequence database and the VSEARCH4 utility within Qiime2's feature-classifier plugin. OTUs were then collapsed to their lowest taxonomic units, and their counts were converted to reflect their relative frequency within a sample [13].

STATISTICAL ANALYSIS

The result of the study was subjected to statistical analysis using Excel and SPSS.

RESULTS

Table 1 revealed the frequency of occurrence and distribution of Gram-negative bacteria in RTE vegetables isolated from selected markets in Oyo and Osun states. The highest number of isolates was from Bodija market, followed by Sango and then Sabo market. The highest occurring isolates were *Salmonella* (17 isolates), followed by *Serratia* species (15 isolates), and the least isolated was *Klebsiella* species. (6 isolates). The antibiotic susceptibility testing revealed that the bacterial isolates from Bodija market recorded 95% sensitivity to gentamicin and ofloxacin respectively, followed by 90% sensitivity to ciprofloxacin. One hundred (100) percent resistance was recorded against cefixime, cefuroxime, and ceftazidime correspondingly (Figure 1). The isolates from Sango market were 100% sensitive to ofloxacin and ciprofloxacin, followed by 80% sensitivity to gentamicin and nitrofurantoin, but recorded 100% resistance to augmentin, cefixime, cefuroxime, and ceftazidime (Figure 2). The isolates from Sabo market were 90% sensitive to ofloxacin and ciprofloxacin, followed by 70% sensitivity to gentamicin, but they were all (100%) resistant to augmentin, cefixime, cefuroxime, and ceftazidime (Figure 3).

Figure 4 shows the microbial communities identified in pooled RTE vegetable samples from the three markets in Oyo State. *Pseudomonas* was the highest (36.6%) occurring isolate. Other identified bacteria were *Janthinobacterium* (8.03%), *Flavobacterium* (11.5%), *Rheinheimera* (6.8%), *Pantoea* (4.6%), *Comamonas* (0.96%), *Sphingobacterium* (1.26%), amongst others. The metagenomic taxonomic profiles and the relative frequencies of the identified bacteria is revealed in Table 2.

Table 1: Frequency of occurrence of Gram-negative bacterial isolates in RTE vegetables from selected markets in Oyo State

s/n	Isolated bacterial genera	1	2	3
1	*Salmonella* spp.	6	8	3
2	*Serratia* spp.	8	1	6
3	*Aeromonas* spp.	3	2	2
4	*Klebsiella* spp.	4	2	0
5	*Citrobacter* sp.	3	1	4
6	*Escherichia coli*	2	4	2
7	*Pseudomonas aeruginosa*	3	2	2
	Total	29	20	19

KEY: Location 1: Bodija market; Location 2: Sango market; Location 3: Sabo market

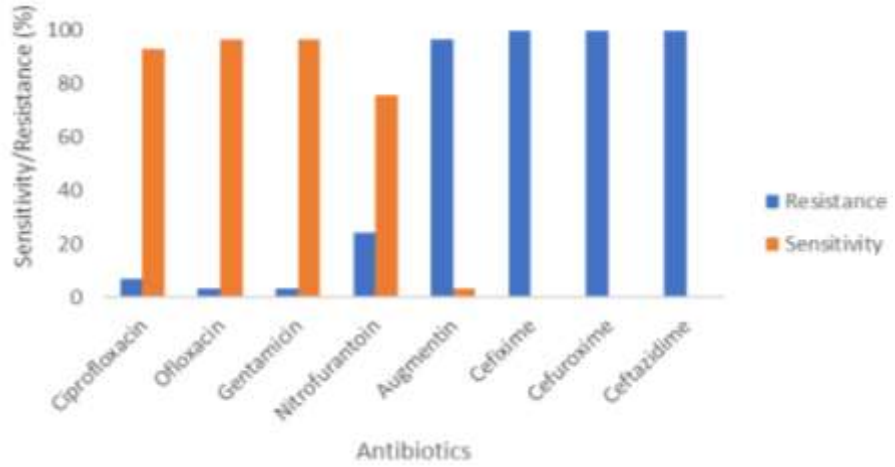

Figure 1: Resistance patterns of Gram-negative bacterial isolates from Bodija market in Oyo State

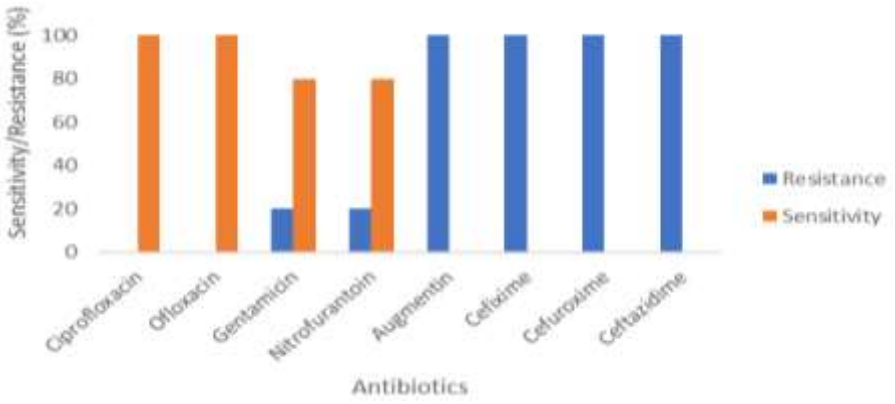

Figure 2: Resistance patterns of Gram-negative bacterial isolates from Sango market in Oyo State.

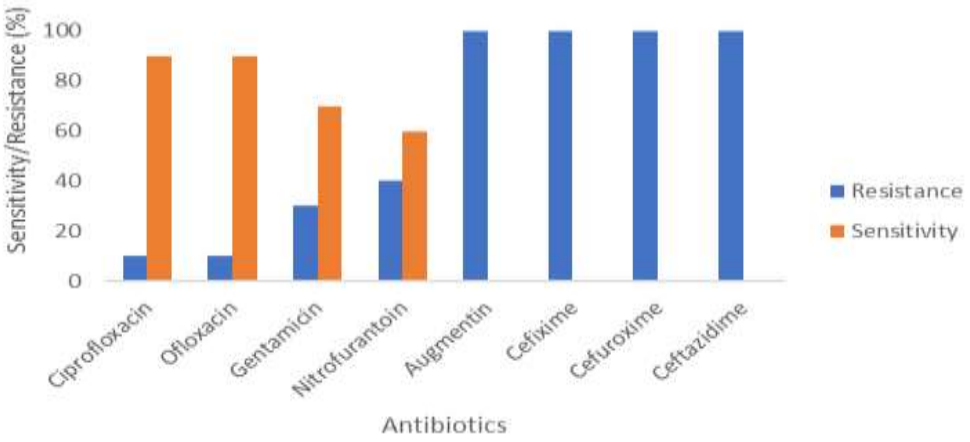

Figure 3: Resistance pattern of Gram-negative bacterial isolates from Sabo market in Oyo State.

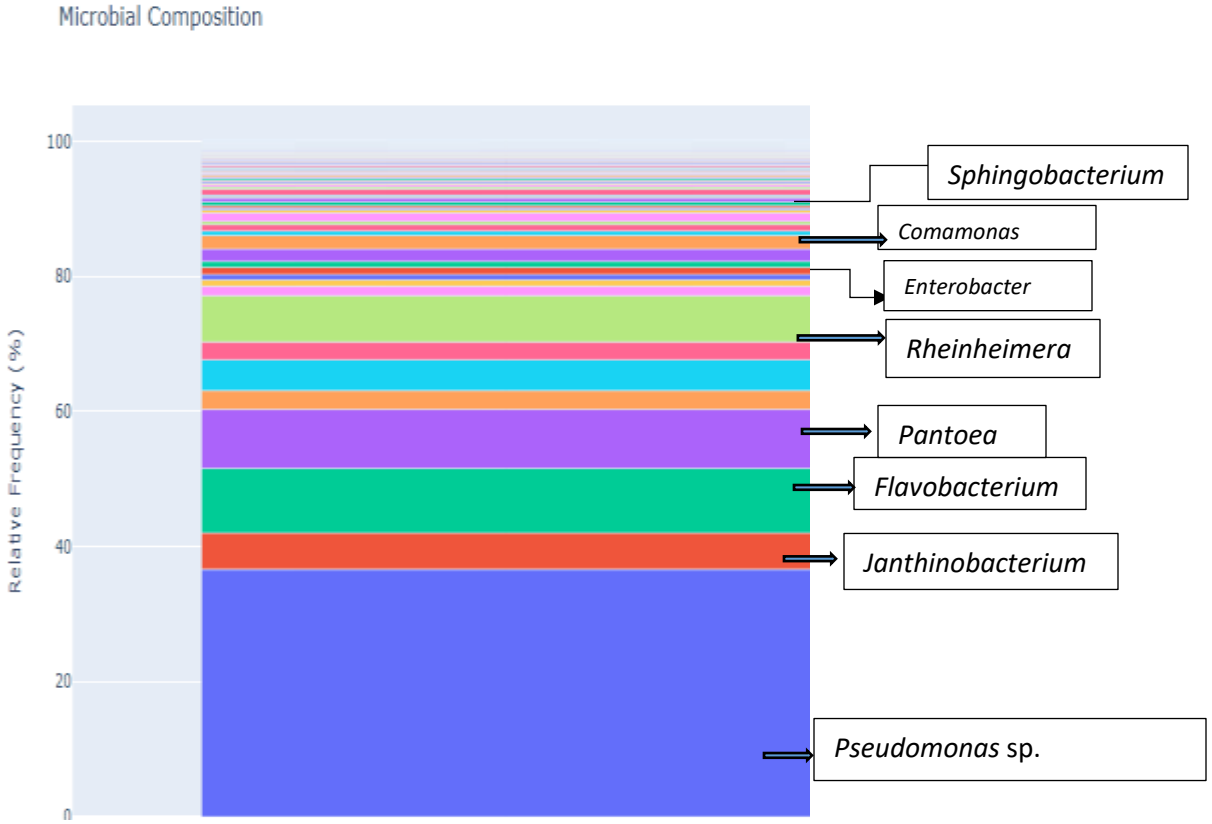

Figure 4: Microbial Communities identified in pooled RTE vegetable samples from the three markets in Oyo State.

Table 2: Taxonomic profiling of the pooled RTE vegetable sample from the three markets in Oyo state.

	Identified bacteria	Relative frequency (%)
1	*Pseudomonas* sp.	36.6
2	*Janthinobacterium* sp.	8.03
3	*Rheinheimera* sp.	6.8
4	*Flavobacterium* sp.	11.5
5	*Glutamicibacter* sp.	0.96
6	*Pantoea* sp.	4.6
7	*Chloroplast (cyanobacteria)*	1.06
8	*Pseudomonas putida*	0.85
9	*Cellvibrio* sp.	1.4
10	*Enterobacter* sp.	1.8
11	*Shewanella* sp.	0.65
12	*Aeromonas* sp.	0.56
13	*Klebsiella* sp.	0.56
14	*Paenarthrobacter nicotinovorax*	0.26
15	*Kosakonia sacchari*	0.24
16	*Sphingobacterium* sp.	1.26
17	*Pectobacterium carotovorum*	0.96
18	*Comamonas aquatica*	0.46
19	*Acidovorax* sp.	0.44

DISCUSSION

The results from this study show that many species of Gram-negative bacteria were isolated from the ready-to-eat (RTE) vegetables from the three markets sampled in Oyo State. In Nigeria, a study by [14] found contamination rates of 15%–40% in vegetables sampled from three different locations in Sango Ota, Ogun State with significant variation based on the location of the market and storage conditions. The intense pressure of increasing demand for healthy food is pushing farmers to depend on untreated wastewater for their production. Wastewater which is rich in organic matter, is readily available. However, wastewater has a high level of contamination with microbial pathogens and chemicals. A study reported high microbial contamination in RTE vegetables in some areas of Sub-Saharan of Africa has been attributed to organic matter present in waste water use [15].

Salmonella spp. were identified in all 3 locations, with the highest occurrence in Sango market. *Salmonella* spp is a major concern in vegetable contamination globally. Authors reported a prevalence of *Salmonella* spp in some RTE vegetables sampled from a market in Ilorin, aligning with the findings in this study [16]. *Serratia* spp. showed high variability, with the highest occurrence in Bodija market (8 isolates) and the lowest in Sango market (1 isolate). *Serratia* spp. are known to be opportunistic pathogens that survive in moist environments. Studies such identified 35% of *Serratia* sp. in RTE vegetable samples in a study in Johannesburg, correlating with their adaptability to humid conditions [17]. *Aeromonas* spp. were more evenly distributed across the locations, ranging from 1 to 3 isolates. A study [18] reported about 9% of *Aeromonas* spp. in combined vegetable samples from randomly selected locations, emphasizing their environmental persistence.

Klebsiella spp. isolates were prominent in Bodija and Sango markets (4 and 2 isolates, respectively), but absent in Sabo, the intermittent presence of *Klebsiella* spp. suggests contamination from irrigation water or post-harvest handling. It was observed that a 45% prevalence of *Klebsiella* spp. in RTE vegetables

in a study carried out in Saudi Arabia, highlighting the relevance of these findings [19].

Pseudomonas aeruginosa was found in all three markets. The opportunistic nature of *P. aeruginosa* mostly acts as a cause of spoilage and makes its presence in food a concern for immunocompromised individuals, it can cause fatal infections such as pneumonia, bloodstream infections and urinary tract infections. A study in Bangladesh found *P. aeruginosa* contamination in various vegetable samples [18].

Antibiotic sensitivity results for the isolated bacteria are presented in Figures 1, 2 and 3. A study found that 92% of isolates from RTE vegetables in Ilorin were sensitive to fluoroquinolones, and this observation is consistent with the results in this study [20]. It was reported that 100% resistance to third-generation cephalosporins (cefuroxime and ceftazidime) in Gram-negative isolates from vegetables in Ibadan markets [21], and observed resistance to augmentin in over 90% of *Escherichia coli* and *Klebsiella* sp. isolated from RTE vegetables in Zawi and Suman city in Libya, reflect trends similar to this study [22]. It was also reported that 90.9% of isolated bacteria from potable water sourced from hand-dug wells in Iwo, were resistant to cefuroxime [23].

A study reported 80% sensitivity to gentamicin by *Pseudomonas* spp. isolated from RTE vegetables [24]. The reduced efficacy was attributed to aminoglycoside-modifying enzymes. Similarly, it was observed that *E. coli* isolates showed 30% sensitivity to nitrofurantoin [25]. Production of aminoglycoside-modifying enzymes by the Gram-negative bacteria may have equally been responsible for the reduced activity to nitrofurantoin and gentamicin, as observed from this study.

This study also showed 100% resistance of the isolates to augmentin, cefuroxime, cefixime and ceftazidime. Beta-lactam antibiotics, including cephalosporins, are widely used medications in the health care system, but are often overprescribed, leading to the proliferation of beta-lactamase-producing bacteria [26]. A study carried out in Italy observed over 80% resistance to third-generation cephalosporins in *Enterobacteriacea* obtained from vegetables [27]. Similar reports of resistance levels of the isolates exceeding 70%, driven by widespread use in both healthcare and agricultural settings were observed [28].

The presence of various bacterial genera, such *as Pseudomonas, Sphingobacterium, Comamonas, Enterobacter, Rheinheimera, Pantoea, Flavobacterium,* and *Janthinobacterium*, highlights the microbial diversity and potential health risks associated with the consumption of contaminated vegetables. The largest proportion of the microbial population was represented by *Pseudomonas* spp. This genus is well-known for its adaptability to diverse environments and is frequently isolated from vegetables. The genus includes pathogenic species such as *Pseudomonas aeruginosa*, which can cause severe infections, particularly in immunocompromised individuals. *Pseudomonas* was the predominant genus in ready-to-eat vegetables, with a prevalence of over 45% [29].

Janthinobacterium was significantly represented in the microbial community and is commonly isolated from soil and water ecosystems in cold temperature including fresh water ecosystems. *Flavobacterium,* commonly found in soil and water, was moderately represented in the microbial community. Both *Comamonas* and *Pantoea* are noteworthy genera due to their environmental origins and occasional pathogenicity. *Pantoea*, for instance, is associated with opportunistic infections and has been isolated in studies [30] where it accounted for the bacterial population and effects on agricultural produce.

Sphingobacterium was another significant genus identified. It is known to be opportunistic hence its representation in the environmental diversity, with certain species capable of causing respiratory and bloodstream infections [31]. In a study conducted in Port-harcourt, *Sphingobacterium* was isolated from 20% of sampled fruits and vegetables [32].

The prevalence of *Enterobacter* is significant due to its association with nosocomial infections and antibiotic resistance. A study reported a 32% prevalence of *Enterobacter* in ready-to-eat

vegetables in various markets in Damietta Egypt, with resistance to beta-lactam antibiotics [33]. The findings of this study highlight the growing concern of antibiotic-resistant Gram-negative bacteria present in ready-to-eat (RTE) vegetables sold in specific markets in Oyo State, Nigeria. The detection of multidrug-resistant (MDR) strains underscores the potential risk of foodborne infections and the urgent need for stringent food safety measures. The microbial diversity analysis using 16S rRNA sequencing further revealed the complexity of bacterial communities associated with RTE vegetables, providing valuable insights into their ecological interactions and possible reservoirs of antimicrobial resistance (AMR) genes.

In conclusion, the presence of antibiotic-resistant bacteria in fresh produce poses a significant public health threat, as consumption of contaminated vegetables may lead to infections that are difficult to treat. This study reinforces the need for improved food safety practices, including proper handling, washing, and processing of vegetables before consumption. The high microbial diversity identified suggests that contamination may occur at multiple points, from cultivation to market display. Strengthening food safety policies, implementing antimicrobial stewardship programs, and conducting routine surveillance of AMR in food products are necessary steps to mitigate the risks.

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