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Research paper

Antimicrobial susceptibility patterns of *Listeria monocytogenes* isolated from fresh produce in KwaZulu-Natal Province, South Africa

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Fresh, ready-to-eat produce is frequently irrigated with untreated water, making it a leading cause of food-borne illness outbreaks worldwide. This study investigated the presence of *Listeria monocytogenes* in fresh produce that was grown using river water. Standard biochemical tests were used for the identification of *L. monocytogenes* isolated from river water used for irrigation, and from fresh produce including lettuce, spinach, and pumpkin. The *inlA* gene of *L. monocytogenes* was molecularly identified using PCR amplification. The susceptibility of *L. monocytogenes* isolates to antimicrobial agents was assessed using the Kirby-Bauer disc diffusion method. The presence of the amplified *inlA* gene (800 bp) indicated that all of the fresh produce and river water samples were contaminated with virulent *L. monocytogenes*. Lettuce and spinach exhibited higher quantities of *L. monocytogenes*, with lettuce recording 87 CFU/g and spinach recording 71 CFU/g. The *L. monocytogenes* isolates from spinach and lettuce sources showed significant resistance to colistin (56.2% and 53.3%, respectively) as well as ampicillin (68.8% and 53.3%, respectively). Moreover, lettuce (40%) and spinach (31%) exhibited a common resistance pattern of AMP-CHL-CT-KAN-PIP-ERY-TET, with a maximum MAR index value of 0.54. Our research demonstrates the transmission of multidrug-resistant *L. monocytogenes* from irrigation river water to fresh produce. Hence, the ingestion of ready-to-eat fresh produce carries the potential for human listeriosis, particularly among individuals with compromised immune systems.

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INTRODUCTION

Foodborne diseases (FBD), which mainly arise from the consumption of unsafe foods and water, have threatened the public health and economic status of human populations around the world (WHO, 2015; Jaffee et al., 2020). Furthermore, microorganisms (bacteria, viruses, and fungi) account for approximately 80% of the food-borne illness outbreaks recorded globally (Bintsis, 2017; Jaffee et al., 2018). Every year there are approximately 600 million reported cases of food-related illnesses globally, resulting in approximately 420 000 deaths. This is particularly true for individuals with weakened immune systems, the elderly, and children under the age of 5 (WHO, 2015). In Africa, about 135 million cases of foodborne diseases are recorded annually, resulting in about 180 000 foodborne-related deaths (Akhtar et al., 2014; Jaffee et al., 2020). In South Africa, 327 cases of foodborne disease outbreaks were documented between 2013 and 2017, resulting in 49 deaths (Shonhiwa et al., 2019). Consumption of food contaminated with pathogenic microorganisms such as *Salmonella, Clostridium perfringens, Bacillus cereus, Shigella* spp., and *L. monocytogenes* was the main cause of these outbreaks (Shonhiwa et al., 2019).

Listeria monocytogenes is a facultative anaerobic foodborne pathogen known to cause invasive and non-invasive listeriosis in humans (Allerberger and Wagner, 2010), mostly via the consumption of compromised foods and water (Kurpas et al., 2018). The ability of *L. monocytogenes* to grow in adverse conditions, including wide temperature ranges (–7 to 45°C), pH ranges (4.0 to 9.6), and salt concentrations (Junttila et al., 1988; Lado and Yousef, 2007), makes it a difficult pathogen to control and eradicate. Because of this, *L. monocytogenes* has been implicated in multiple FBD outbreaks worldwide. In South Africa, *L. monocytogenes* was implicated in a 2017–2018 FBD outbreak that claimed over 230 lives and was regarded as the worst outbreak to date (Smith et al., 2019). Most recently, *L. monocytogenes* was associated with the multi-state FBD outbreak from Dole packaged leafy greens, affecting 18 individuals in the USA (FDA, 2022). Most *L. monocytogenes*–associated outbreaks are a result of the consumption of ready-to-eat products, including green salads and vegetables (Hazards et al., 2018).

Although the incidence of human listeriosis outbreaks is very low compared to other foodborne illnesses, the unattended outcome of the disease is often more severe, making *L. monocytogenes* a priority. Furthermore, the *inlA* gene, one of *L. monocytogenes* virulence genes, can be used as a marker for the detection of *L. monocytogenes* in water and agricultural produce. The *inlA* gene was recently used for the identification of *L. monocytogenes* isolated from clinical sources, including vaginal swabs and faeces (Meghdadi et al., 2019).

This study aimed to assess the prevalence of *L. monocytogenes* in fresh agricultural produce, including *Cucurbita* (pumpkin), *Lactuca sativa* (lettuce), and *Spinacia oleracea* (spinach) irrigated with untreated river water from a farm in Verulam, KwaZulu-Natal Province, South Africa. Several studies have already identified untreated water as the main route of introducing pathogenic bacteria onto agricultural produce (Pandey et al., 2014; Uyttendaele et al., 2015; Allende et al., 2017).

Recently, *L. monocytogenes* resistant to several antibiotics, including ampicillin, penicillin and trimethoprim-sulphamethoxazole, was detected in environmental waters in the Eastern Cape Province of South Africa (Mpondo et al., 2021). In the current study, the antibiogram of *L. monocytogenes* from fresh agricultural produce was also studied.

Surveillance of the carry-over of potential pathogens from contaminated irrigation water to agricultural fresh produce in South Africa is an area of research that is currently neglected. However, in a country with several immunocompromised individuals because of disease, lack of clean potable water and other factors, addressing water–fresh produce contamination in order to curb the spread of potential FBD pathogens is crucial. This work provides first-line data for antibiotic-resistant patterns of *L. monocytogenes* isolates from agricultural produce in the KwaZulu-Natal Province of South Africa.

MATERIALS AND METHODS

Sample collection

A volume of 1 L of each water sample and 200 g of each agricultural produce sample (i.e., lettuce, spinach, and pumpkin) were aseptically collected on a monthly basis (June–September 2021) from an agricultural farm in Verulam (29°36'47.2"S 31°02'11.7"E), KwaZulu-Natal, and stored in a portable ice chest during transportation. All samples were processed within 4 h of sampling.

Isolation of *L. monocytogenes* from agricultural produce and water samples

In this study, *L. monocytogenes* were isolated using a modified version of the protocol by Stea et al. (2015). Briefly, about 25 g of each composite sample of freshly harvested lettuce, spinach, and pumpkin obtained from the farm was immersed in 100 mL of sterile peptone water and vigorously homogenized by shaking for 5 mins in a water bath. A volume of 25 mL of the suspension (from spinach, lettuce, pumpkin, and river water sample) was mixed with 225 mL of *Listeria* enrichment broth (LEB; Sigma-Aldrich) and then incubated (37°C for 24 h). Following the overnight incubation period, a volume of 1 mL of *Listeria* enrichment broth (LEB; Sigma-Aldrich) was mixed with 9 mL of Fraser broth (Sigma-Aldrich, Fraser Broth Base) and incubated (37°C for 24 h) once more. Finally, the resulting enriched Fraser broth culture was streaked onto Oxford Agar (Sigma-Aldrich) in triplicate and incubated (37 °C for 24 h) further. The colonies that resulted were counted using a colony counter, and their quantity was measured in terms of colony-forming units (CFU) per 25 g of vegetables and CFU per 100 mL of water used. The colonies were subsequently purified and analysed using biochemical and molecular tests to determine their identity.

Identification of *inlA gene* in *L. monocytogenes* isolates by PCR

The crude DNA was prepared from the *L. monocytogenes* isolates using the DNA precipitation method according to Green and Sambrook (2016), and purified using the DNA purification kit (Gene-JET PCR Purification Kit, Thermo Scientific), according to the manufacturer's protocol. The pure DNA obtained was used as templates for the amplification of the *inlA* gene-specific in *L. monocytogenes* using the primer set 5'- AATCTAGCACCACTGTCGGG -3' and 5'- TGTGACCTTCTTTTACGGGC -3' (Rousseaux et al., 2004). The general thermocycling conditions used were 94°C for 3 min for initial denaturation, 35 cycles of 30 s denaturation at 94°C, 53°C for 1 min for annealing, and extension at 72°C for 1 min, with final elongation at 72°C for 5 min. The PCR reaction mixtures contained concentrations of the components including PCR mastermix (12, 5 µL), forward primer (2 µL), reverse primer (2 µL), double-distilled water (8, 5 µL), and DNA template (2 µL) to a final volume of 25 µL. The *L. monocytogenes* strain ATCC 7644 was used as the positive control. All samples display a fragment of 730 bp on 1% (w/v) TAE (40 mM TRIS base (w/v), 0.2 mM glacial acetic acid (w/v), 10 mM EDTA (w/v)), pH 8.0 agarose gel.

Determination of AST, MAR, and MAR indices in *L. monocytogenes*

The Kirby-Bauer disc diffusion technique, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006), was used to perform antimicrobial susceptibility testing on the isolates obtained from agricultural products. The technique involved the use of Muller Hinton agar (Oxoid, Basingstoke, UK) supplemented with 7% defibrinated sheep blood. Furthermore, the susceptibility assay of *L. monocytogenes* isolates was tested against the following antimicrobial combinations and concentrations; chloramphenicol (CHL, 10 µg), ampicillin (AMP, 10 µg), kanamycin (KAN, 30 µg), fosfomycin (FOS, 50 µg), tetracycline (TET, 30 µg), pipemidic acid (PIP, 20 µg), gentamycin (GN, 20 µg), streptomycin (STR, 25 µg), erythromycin (ERY, 30 µg), and vancomycin (VAN, 30 µg). Furthermore, Krumperman's (1983) method was used to calculate and interpret the multiple antibiotic resistance (MAR) index. In this method, MAR = a/b, where a represents the number of antibiotics to which a specific isolate showed resistance, and b represents the total number of antibiotics that the isolates were exposed to.

Bioinformatics analyses and interpretation

We compared the resulting metabolic activities of the treatment groups and controls using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison post-test at $p < 0.05$. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Listeria monocytogenes in irrigation river water and agricultural samples

PCR analysis identified 59 *L. monocytogenes* isolates from river water and agricultural produce. All isolates showed the *inlA* gene (Fig. 1) which confirmed *L. monocytogenes* (Budniak et al., 2016). This finding supports previous studies that identified the presence of internalin genes in *L. monocytogenes* isolates from irrigation water and agricultural soil in South Africa's Eastern Cape Province (Iwu et al., 2022). Moreover, the *inlA* gene has long been known to be conserved in *L. monocytogenes* (Poyart et al., 1996), and has been implicated in the establishment of listeriosis (Chatterjee, 2006; Ingeborg, 2011; Casey et al., 2016). The surface internalin A (*inlA*) protein, a product of the *inlA* gene, plays an important role in the invasion of *L. monocytogenes* into mammalian cells (Werbrouck et al., 2006; Phelps et al., 2018). In humans, the internalization of *L. monocytogenes* into mammalian cells is brought about when the *inlA* binds to the transmembrane protein E-cadherin, leading to the emergence of the adherens junctions (cell-to-cell adhesion complex) in a Ca^{2+}-dependent manner (Bou Ghanem et al., 2012; Pizarro-Cerdá et al., 2012; Bonazzi et al., 2009). Therefore, molecular identification of the *inlA* gene in this study confirms the presence of pathogenic *L. monocytogenes* in agricultural produce.

In addition, statistical analysis of Oxford-agar culturable *L. monocytogenes* isolated from the river water sample revealed the highest number (74 000 CFU/mL) in June (Fig. 2).

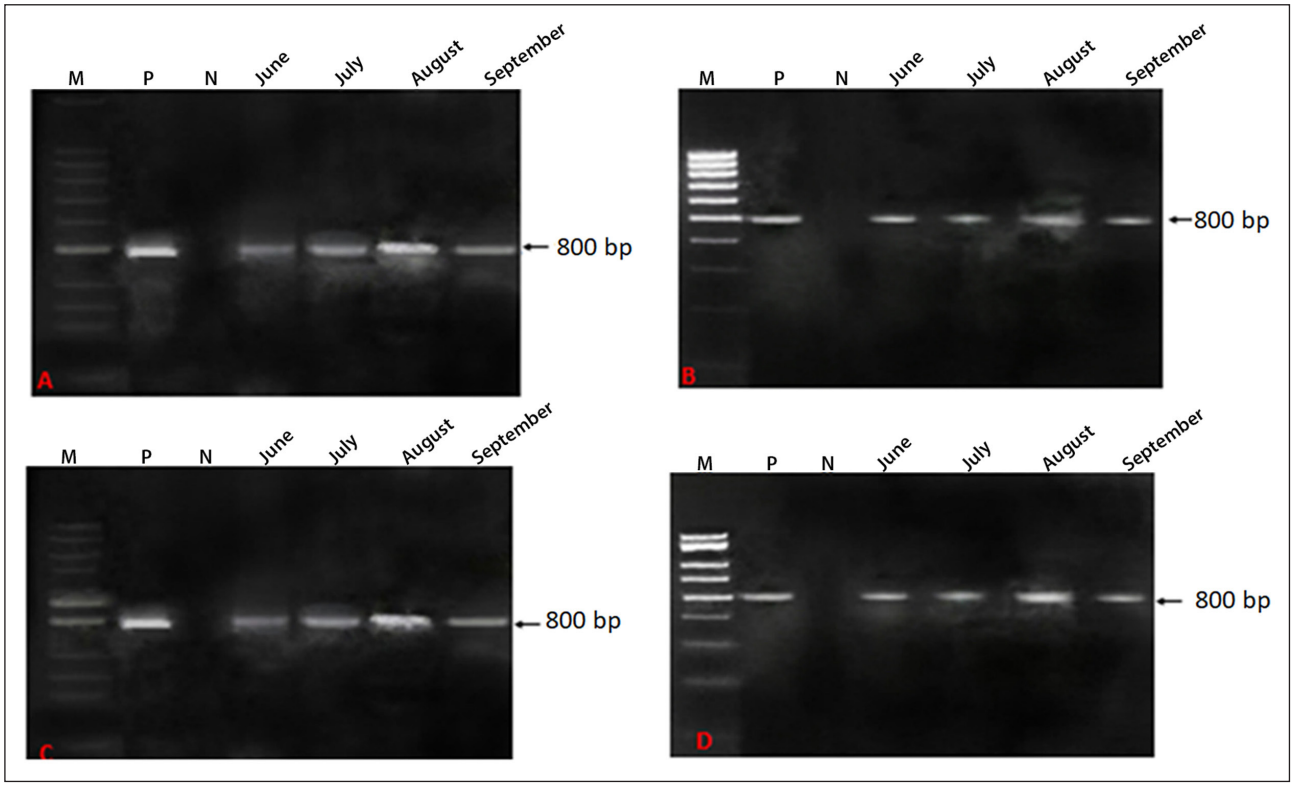

Figure 1. Agarose gel electrophoresis showing PCR products of approx. 800 bp from *L. monocytogenes inlA* gene: A – river water; B – spinach; C – lettuce; D – pumpkin; M – molecular weight marker; P – positive control and N – negative control. The DNA was resolved on a 1.0 % agarose gel.

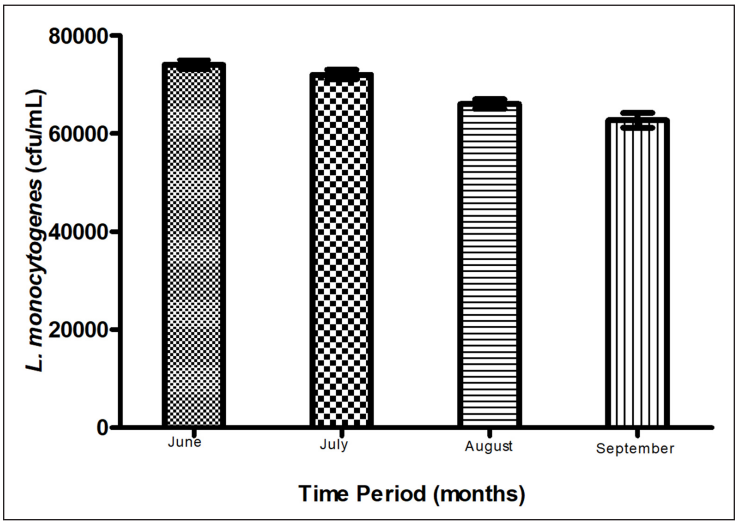

Figure 2. Enumeration of total presumptive *L. monocytogenes*. The number of CFU/mL is the sample means, which accounts for the dilution factor and number of replicates. Enumerations of total presumptive *L. monocytogenes* in river water (RW) are shown.

In a recent study conducted by Mpondo (2021), it was found that *L. monocytogenes* was present in 13% (9/69) of water samples collected from river and irrigation waters in the Eastern Cape Province of South Africa. Previously, Meghdadi et al. (2019) also detected *L. monocytogenes* 16.76% (16/180) in river waters. The high prevalence of *L. monocytogenes* in the river is likely due to anthropogenic inputs (Khatri and Tyagi, 2014). Several anthropogenic activities, including domestic and industrial sewage discharges, have been related to an increase in pathogenic microorganisms in rivers (Abraham, 2011). Concerning *L. monocytogenes*, infected individuals may excrete a high quantity of this bacterium in their faeces. Consequently, it can enter river waters through the release of untreated or partially treated wastewater (Manjur et al., 2016).

Elevated incidence of *L. monocytogenes* in lettuce and spinach samples

The study found that the lettuce and spinach had the highest concentration of *L. monocytogenes*, while the pumpkin had the lowest (Fig. 3). A study by Willis et al. (2020) has also reported a higher prevalence of *L. monocytogenes* isolates in vegetables (69/673) than in fruits (3/340). Due to their structural characteristics, lettuce and spinach have the potential to retain a greater amount of water on their leaves compared to the pumpkin. As a result, this creates a favourable environment for *L. monocytogenes* to thrive and proliferate. A subsequent investigation conducted by Okeye et al. (2020) discovered a maximum count of 370 CFU/g of *L. monocytogenes* in lettuce, a

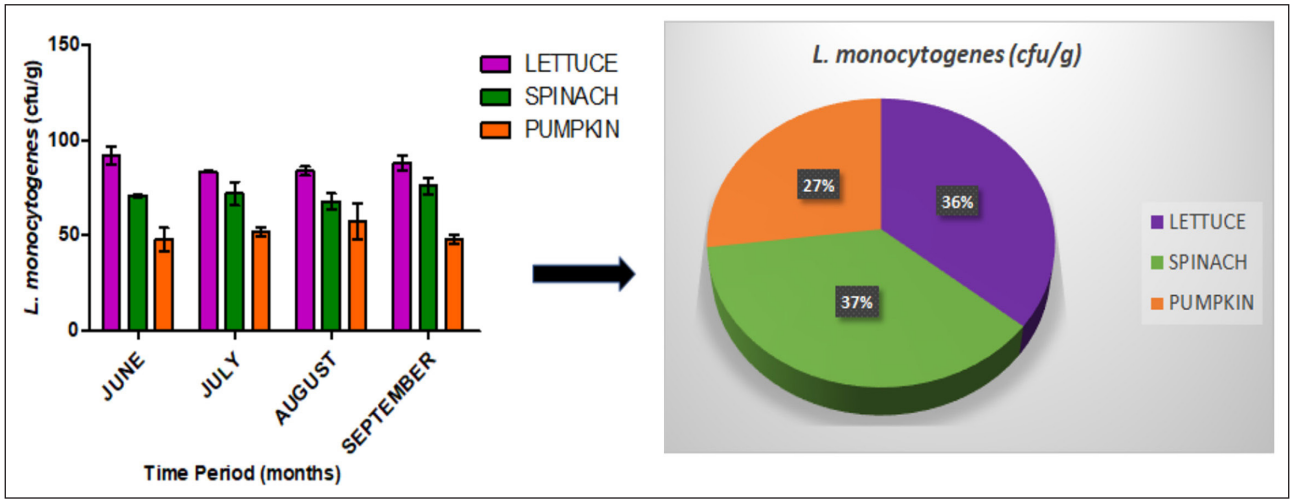

Figure 3. Total number of *L. monocytogenes* present in the three fresh produce samples without enrichment expressed in CFU/g. Lettuce contained the highest amount of *L. monocytogenes* followed by spinach and pumpkin.

Table 1. Susceptible rate of *L. monocytogenes* to antimicrobial agents

Fresh produce	No. of isolates tested	CT (10 µg)	AMP (10 µg)	KAN (30 µg)	FOS (50 µg)	TET (30 µg)	CHL (30 µg)	PIP (20 µg)	GN (20 µg)	STR (25 µg)	ERY (30 µg)	VAN (30 µg)
		No. (%) of resistant isolates										
Lettuce	15	8 (53.3%)	8 (53.3%)	6 (40%)	NT	2 (13.3%)	7 (46.6%)	2 (13.3%)	NT	2 (13.3%)	3 (20%)	NT
Spinach	16	9 (56.2%)	11 (68.8%)	7 (43.8%)	NT	3 (18.7%)	6 (37.5%)	4 (25%)	NT	3 (18.6%)	4 (25%)	NT
Pumpkin	14	4 (28.6%)	6 (37.7%)	3 (21.4%)	NT	2 (14.3%)	4 (35.7%)	1 (7.1%)	NT	2 (14.3%)	1 (7.1%)	NT

Key: CT – colistin; AMP – ampicillin; KAN – kanamycin; FOS – fosfomycin; TET – tetracycline; CHL – chloramphenicol; PIP – pipemidic acid; GN – gentamicin; STR – streptomycin; ERY – erythromycin; VAN – vancomycin

significantly higher figure compared to the levels observed in this study in lettuce (87 CFU/g), spinach (71 CFU/g), and pumpkin (51 CFU/g) Nonetheless, these recorded values are considerably lower than the suggested infective dose of 100 CFU/g (Center for Food Safety and Applied Nutrition, 2017). According to previous studies, as few as 10 CFU/g of *L. monocytogenes* on agricultural produce have the potential to rapidly multiply and reach pathogenic levels within 8 days (Salvat and Fravalo, 2004). The findings of our study indicate that spinach and lettuce have a higher likelihood than pumpkin of containing *L. monocytogenes* transmitted from irrigation water.

Higher rate of resistance of *L. monocytogenes* isolates from lettuce and spinach sources to colistin and penicillin

In this study, we tested the susceptibility of 45 *L. monocytogenes* strains isolated from lettuce, spinach, and pumpkin to 11 antibiotics widely used in medical and veterinary practice. The findings indicated that the majority of *L. monocytogenes* isolates from lettuce and spinach sources exhibited comparable antibiotic-resistance patterns (Table 1), with colistin and ampicillin being the two antibiotics to which they displayed the highest resistance. The isolates originating from the pumpkin source exhibited remarkably low resistance to these two antibiotics. Penicillin is an antibiotic with a broad-spectrum effect, employed in the treatment of infections caused by *L. monocytogenes* and other clinically significant pathogens such as *Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae, and Haemophilus influenzae* (Kaushik et al., 2014; Peechakara et al., 2021). The lack of efficacy of colistin and ampicillin against *L. monocytogenes*, as stated in this study, aligns with the findings of Ennaji et al. (2008). Their study revealed resistance to these antimicrobials in *L. monocytogenes* isolates from poultry and red meat in Morocco. In contrast, Yan et al. (2019) conducted a 4-year study spanning from 2012 to 2015 which did not identify any cases of ampicillin resistance in 2 862 *L. monocytogenes* strains isolated from different food samples in China. Additionally, our study revealed a significantly low prevalence of resistance to pipemidic acid, streptomycin, and tetracycline among all *L. monocytogenes* isolates obtained from the three agricultural produce samples. In contrast to the research conducted by Harakeh et al. (2009) and Conter et al. (2009), this study revealed a low prevalence of tetracycline resistance. Moreover, the pumpkin isolates examined in this study exhibited a reduced occurrence of resistance to all of the antibiotics tested, including pipemidic acid (7.1%), erythromycin (14.3%), tetracycline (7.1%), and streptomycin (14.3%). In contrast to the findings of Jamali et al. (2013), who found that *L. monocytogenes* isolates were susceptible to gentamicin and vancomycin, our study revealed that all *L. monocytogenes* isolates obtained from lettuce, spinach, and pumpkin exhibited complete resistance to fosfomycin, gentamicin, and vancomycin. The widespread use of antimicrobials like gentamicin and vancomycin in the treatment of human and animal infections in South Africa may explain the high levels of resistance to these drugs.

The percentage resistance of *L. monocytogenes* isolates against the test antimicrobials is shown in Fig. 4. All the isolates showed 100% resistance to gentamicin, vancomycin, and fosfomycin. Conversely, for chloramphenicol (37.8%), ampicillin (53.3%), and colistin (46.7%) higher susceptibility rates were demonstrated.

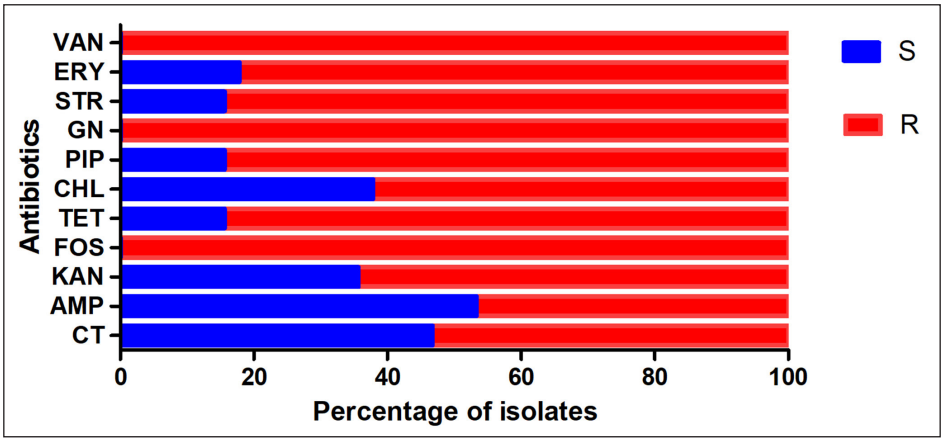

Figure 4. Percentage of *L. monocytogenes* isolates resistant or susceptible to each of the 11 antimicrobials tested. CT – colistin; AMP – ampicillin; KAN – kanamycin; FOS – fosfomycin; TET – tetracycline; CHL – chloramphenicol; PIP – pipemidic acid; GN – gentamicin; STR – streptomycin; ERY – erythromycin; VAN – vancomycin.

Table 2. MAR patterns of *L. monocytogenes* isolated from lettuce, spinach, and pumpkin irrigated with river water

Fresh produce	Antibiotic-resistant profile	Occurrences	MAR index
Lettuce	AMP-CHL-CT-KAN	3 (20%)	0.36
	AMP-CHL-CT-KAN-PIP	5 (33.3%)	0.45
	AMP-CHL-CT-KAN-PIP-ERY-TET	7 (46.7%)	0.63
Spinach	AMP-CHL-CT-KAN	3 (18.9%)	0.36
	AMP-CHL-CT-KAN-PIP	5 (31%)	0.45
	AMP-CHL-CT-KAN-PIP-ERY-TET	6 (37.5%)	0.63
Pumpkin	AMP-CHL-CT-KAN	2 (14.2%)	0.18
	AMP-CHL-CT-KAN-PIP	3 (21.4%)	0.27
	AMP-CHL-CT-KAN-PIP-ERY-TET	4 (28.5%)	0.63

The MAR patterns and MAR indices of *L. monocytogenes*

All isolates of *L. monocytogenes* obtained from lettuce, spinach, and pumpkin exhibited resistance to a minimum of 3 antimicrobial agents. Analysis of our data uncovered four distinct MAR patterns in the *L. monocytogenes* strains obtained from the agricultural produce. Among these, the combination of AMP-CHL-CT-KAN-PIP-ERY-TET was the most prevalent, accounting for 40% of lettuce and 31% of spinach (Table 2). According to a recent study conducted by Mpondo et al. (2021), it has been discovered that *L. monocytogenes* isolates obtained from environmental samples exhibit resistance to a maximum of 15 antimicrobials, resulting in a MAR index value of 1. In Malaysia, previous reports have documented a lower MAR index value of 0.56 in *L. monocytogenes* strains obtained from vegetable farms and retail markets (Huan et al., 2017). In this particular study, however, the *L. monocytogenes* isolates demonstrated a maximum MAR index value of 0.54, a value that remains significantly higher than the recommended MAR index value of 0.2 (Krumperman, 1983). The findings of this study demonstrate a high MAR index value (0.54), which signifies extensive contamination of the three agricultural products with multi-drug resistant *L. monocytogenes*.

In summary, this study has shown that there was a notable transfer of multidrug-resistant *L. monocytogenes* from the contaminated irrigation river water to the agricultural produce. The elevated levels of *L. monocytogenes* in lettuce and spinach are a cause for concern as these raw vegetables are commonly eaten in salads and could potentially lead to food-borne listeriosis, particularly for those who are more vulnerable to infection. Moreover, the MAR index values exceeding 0.2 indicate that the irrigation river water utilized on the farm poses a significant risk of contamination. Therefore, consistently monitoring the presence of foodborne pathogens and the emergence of antimicrobial resistance in food is crucial. This is vital to minimize the chances of exposure and effectively manage agricultural produce to prevent or minimize contamination by such pathogens in South Africa.

AUTHOR CONTRIBUTIONS

F Tshabuse – conceptualisation and methodology of the study, data collection and fieldwork, sample/data analysis, interpretation of results, writing of the initial draft, revision after review; NK Cele – sample/data analysis, interpretation of results, writing of the initial draft; AR Opoku – writing of the initial draft; R Basson – writing of the initial draft; MS Mthembu – writing of the initial draft; MF Swalaha – conceptualisation and methodology of the study, interpretation of results, writing of the initial draft.

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