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Prevalence of *Enterobacteriaceae* on Ready to Eat Salads, Drinking Water and Surfaces in Food Markets of Maputo, Mozambique

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Abstract Vegetable salads constitute an important component of many meals worldwide. However there is concern for their safety and microbiological quality because they have been implicated in outbreaks of many foodborne diseases, especially in developing countries. In Mozambique, the knowledge of the microbiological quality and virulence genes of bacterial isolates from ready-to-eat (RTE) salads is limited. This study aimed to evaluate the prevalence of *Enterobacteriaceae* on RTE lettuce, drinking water and surfaces in food markets of Maputo, Mozambique. A total of 35 samples of RTE lettuce salads and 42 drinking water samples were collected from 35 food vendors, in addition to 105 swabs of hands, knives and bowls from seven markets in Maputo City, Mozambique. The prevalence of *Enterobacteriaceae* bacterial isolates from the collected samples was determined using plate counts method following ISO 21528-2 and ISO 21528-1 (for drinking water). The purified isolates were identified using a matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF-MS). A total of 219 isolates were obtained. *Enterobacter* isolates (45.2%) were the predominant species. *Enterobacteriaceae* counts ranged from 0.52 to 6.98log CFU/g. There was no statistically significant correlation between bacteriological counts on RTE lettuce salads and swabs. However, there were significant differences among the numbers of *Enterobacteriaceae* detected in water for other samples. The prevalence of *Escherichia coli* was observed in fewer samples, a remarkable tendency of the presence of this bacterium was found in the utensils. The *E. coli* isolates obtained in this study tested negative for the presence of virulence genes (stx1F, stx1R, stx2F, stx2R). These findings provide valuable background information that can support food safety decisions and confirm that the vast majority of vendors do not sanitize utensils effectively.

Keywords: *food markets, indicators, MALDI-TOF-MS, hygiene quality, foodborne pathogens*

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1. Introduction

The consumption of ready-to-eat fresh vegetables has increased in developing countries due to changing lifestyle patterns [1]. Vegetables salads are regarded as an essential part of a nutritious and healthy worldwide [1,2,3]. Most salads are however consumed raw or after minimal processing, and generally do not receive heat treatment before consumption [4,5]. Salads derived from lettuce have been linked to numerous foodborne disease outbreaks associated with *E. coli* O157:H7 [6,7,8]. In 2008, the United Nations ranked green leaves as the "highest priority" for the number of outbreaks and the types of microbial hazards [7,9]. The Centers for Disease Control and Prevention (CDC) and the Food and Drug Administration (FDA) have investigated several multistate outbreaks involving vegetables, salad mix, in the past three years (2016-2018) and found Norovirus, *Salmonella* and *Escherichia* as the main cause of the foodborne diseases [7].

Contamination of RTE vegetable salads can occur through various production routes. Contamination may originate from human, animal, and environmental sources [1]. Food preparation facilities in food service are also responsible for contamination of salads which may affect the quality and lead to food safety issues [10,11].

Inaccessibility to safe water, lack of agricultural infrastructure largely contributes to contamination of vegetables salads in the developing countries [12].

Salads sold can be unfit for human consumption and could be deleterious to the health of consumers. Abakari et al., conducted a study in Ghana and found *Escherichia coli* in 96.7% of salad samples with levels ranging from 0 to 7.56 $\log_{10}$ CFU/g. *Salmonella* spp. and *Shigella* spp. were present in 73.3% and 76.7% of salads, respectively [13].

Members of the *Enterobacteriaceae* family are a gram-negative, non-spore forming bacterium that includes many bacteria that are found in human or animal intestinal tracts, as well as plants and the environment [14].

The *Enterobacteriaceae* may be superior to coliforms as indicators of sanitation indicated by good manufacturing practices because they have collectively greater resistance to the environment than the coliforms. However, coliforms constitute an important group within the *Enterobacteriaceae* family and constitute about 10% of the intestinal microbiota [14,15]. Important food pathogens in the *Enterobacteriaceae* family include *Cronobacter* spp, *Escherichia coli, Salmonella enterica*, *Shigella (boydii, flexneri, sonnei and disenteriae)* and *Yersinia* (*enterocolitica* and *pseudotu-berculosis*) [16,17,18]. In RTE salads, *Enterobacteriaceae* pathogens, including *Escherichia* and *Salmonella* have been implicated in disease outbreaks [19].

Species that are part of the coliform group include *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella* and *Escherichia*. These bacteria are used as indicators of food health quality because they are abundant in the feaces of warm-blooded animals and they are relatively quick and simple to detect [16]. The presence of coliforms in food points to failure to comply with proper good hygienic practices. Indicators are used for a variety of purposes in food systems including evaluating quality or safety of raw or processed food products and validating effectiveness of microbial control measures [18]. Other members of this family can be found in aquatic environments, soil, and vegetation [17].

Foodborne diseases are a major public health concern and costs billions of dollars losses every year [20]. The identification of bacterial pathogens from food has been traditionally done using culturing of the microorganisms on a selective media. However, traditional methods are time consuming, costly and not sensitive [20,21,22]. With the increased outbreaks of foodborne diseases, fast, reliable and accurate monitoring and detection of foodborne pathogens in food cannot be overemphasized. Recently, several rapid detections, identification, and monitoring methods like Immunoassays methods, DNA-based detection methods, MALDI-TOF MS biosensors methods; Electrochemical biosensors have been developed for foodborne pathogens [23-28].

Matrix-Assisted Laser Desorption Flight Time Mass Spectrometry (MALDI-TOF MS) has become one of the widely used and preferred methods for identification of food borne pathogens, since it allows rapid and accurate identification of microorganisms to the species level in clinical microbiology laboratories [29,30,31,32,33,34]. It has been successfully used in clinical diagnosis, food safety control, environmental monitoring [35,36,37,38,39]. Studies on microbiological quality and virulence genes in bacterial isolates of ready-to-eat salads provided by vendors in markets of Maputo, Mozambique are limited. However, Macaza found high counts of *Enterobacteriaceae* of *E. coli* in samples collected from food markets in the Nampula city [40], which indicates that the conditions are unsatisfactory. Food markets in Mozambique are described as establishments where people, in general, will have breakfast and lunch. Therefore, this study aims to determine the microbiological quality (based on hygiene indicator bacteria) and the prevalence of potential human pathogenic bacteria in RTE lettuce salads, drinking water and surfaces in food markets at Maputo, Mozambique. It is envisaged that the information from this research will be useful in providing recommendation on effective mitigation efforts toward enhanced food quality in vended food in Mozambique.

2. Materials and Methods

2.1. Sampling Design

Seven markets were visited and in each market twenty seven (n = 26) samples were collected in Maputo, Mozambique, over a 6-month period from March to August 2019. To obtain a representative sample for a given market, we randomly purchased the samples in different points of the markets. A total of 182 samples were collected in this study period (Table 1). Generally, in Maputo, Mozambique, food markets are open-air, made up of small establishments or small food service called "stalls." A sample from each vendor consisted of a RTE salad (lettuces salads), water used in the salad making process, and swabs from knives used for cutting vegetables, hands, and bowls used for mixing the salad ingredients. The salads considered in this study composed of lettuce, onions and tomatoes mixtures. Samples were collected from 5 vendors in each market. Samples were collected in sterile bags kept in ice chest, maintained at 0-4°C and taken to the Laboratory of Microbiology and Safety of the University Eduardo Modlane, Maputo campus, Mozambique and processed within 2-4 h for microbial analysis. Swab samples were done using SpongeSicle swabs with 10 ml neutralizing buffer. A verbal consent was obtained from officials responsible for the markets.

Table 1. Numbers of samples collected from seven food markets in Maputo

	Source of sample/Total No of sample					
Location/Market	Salad	Water fountain	Water reservoir	Swab hand	Swab knife	Swab bowl
Museu	5	1	5	5	5	5
Povo	5	1	5	5	5	5
Mandela	5	1	5	5	5	5
Estrela vermelha	5	1	5	5	5	5
Benfica	5	1	5	5	5	5
Xipamanine	5	1	5	5	5	5
Peixe	5	1	5	5	5	5
Total	35	7	35	35	35	35

2.2. Microbiological Analysis

2.2.1. Enumeration of *Enterobacteriaceae* in Drinking Water, Ready-to-eat Salads and Swabs

Enumeration of *Enterobacteriaceae* colonies in RET lettuce salads and swab samples was done using validated ISO methods. ISO 21528-2 Second edition (2018) was used for analysis of read to eat salads and swabs while ISO 21528-1 was used for analysis of drinking water. Briefly, violet red bile glucose (VRBG - Oxoid LTD, England) agar was prepared following the protocol recommended by the manufacturer. For RTE lettuce salads, a 25 g sample was aseptically cut from the lettuce salad using a sterile scalpel, and 225 ml of buffered peptone water (3M, St.Paul, MN) was added to a sterile polyethylene bag and macerated using a stomacher 400 circulator (Seward, London, UK) at 135 rpm for 3 min. Following the standard dilution method, 1ml from each of the macerated lettuce salad were added to buffered peptone, and total *Enterobacteriaceae* count was determined by plating in to VRBG agar plates in duplicates. Swab samples and drinking water were plated directly. Plates were incubated at 37°C for 18-24 h and enumerated following the ISO 21528-2 Second edition (2018). For each of the samples analysed two colonies showing red-purple halos (presumptive indication of the presence of *Enterobacteriaceae*) were selected, purified and preserved in glycerol at -20^{o}C. The isolate identities were determined using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS).

2.2.2. Confirmation of Presumptive *Enterobacteriaceae* Colonies Using MALDI-TOF-MS

Purified bacterial cultures stored on Nutrient Agar media (NA-Mindrad) were transferred directly to the matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) steel polished target plate (Bruker, Bremen, Germany) and overlaid with the cyano-4-hydroxycinnamic acid matrix (Bruker). The target plate was subsequently analyzed using MicroFlex LT MALDI-TOF-MS (broker) in conjunction with Biotyper automation software and libray (Bruker). Duplicate score values were recorded and used to determine the accuracy of identification. A score value between 1.999 and 1.700, and value above 2.0 was used to determine the genus and probable species of the organism. Scores above 2.3 were used for highly probable species identification. The MALDI-TOF-MS test were carried at the Centre of Excellence in Food Security, Department of Plant and Soil Sciences, University of Pretoria.

2.2.3. Molecular Identification of Virulence Genes in *E. coli* Isolates

Virulence genes in *E. coli* (mdfR, mdhR, stx1F, stx1R, stx2F, stx2R) were detected using PCR. Total genomic DNA was from pure cultures was extracted using Zymo Kit - Quick DNA Minipre Kit following the manufacturer's protocol recommendation. The virulence genes were amplified using specific primers Amplification reactions were performed in a total volume 25ul of PCR green master volume of primers used. Amplification reactions were carried out in (c1000 Touch) thermocycler of the Centre of Excellence in Food Security, Department of Plant and Soil Sciences - Pretoria University. Amplification of the expected band size signifies the presence of virulence genes.

2.4. Statistical Analyses

Counts of colony forming units were done in duplicate and average means and standard deviation (±SD) for each of the sample and locations were calculated. Data analysis was performed using the Statistical Package for Social Sciences (SPSS, Inc. Chicago, IL,USA). Descriptive statistics (means, standard errors, percentages and frequencies) were calculated for all variables.

3. Results

3.1. Sample Collection

A total of 182 samples (n = 35 RET lettuce salads; n = 42 drinking water; n = 35 swab hands; n = 35 swabs bowls and N= 35 knifes swabs) were collected and 222 presumptive *Enterobacteriaceae* isolates of microorganisms were obtained and identified. None of the RTE salads were stored at refrigeration temperature at the point of sale. The isolates were identified up to the species level and included: *Enterobacter cloacae* (32.43%), *Klebsiella pneumonia* (20.27%), *Enterobacter asburiae* (12.16%), *Citrobacter freundii* (6.3%), *Klebsiella oxytoca* (4.95%), *Kluyvera ascobarta* (4.05%), *Escherichia coli* (3.15%). Other isolates like *Kluyvera*, *Kosakonia*, *Citrobacter*, *Pantoea*, *Aeromonas*, *Leclercia*, *Acinetobacter*, *Raoultella*, *Kosakonia*, *Pseudomonas*, and *Streptomyces* were isolated in low frequencies (Table 2).

3.2. Total Plate Count and Identification of *Enterobacteriaceae* on RTE Lettuce Salads

A total of thirty-five (n = 35) sample of RTE lettuce salads was analyzed in this study. The results show that, most (91.42%, n= 32) of the samples were found to be positive for *Enterobacteriaceae.* The identified species include: *Enterobacter cloacae* (34%), *Klebsiella pneumonia* (28%), *Enterobacter asburiae* (18%), *Citrobacter freundii* and *Klebsiella oxytoca* (6%). Other species like *Citrobacter koseri*, *Kosakonia cowanii*, *Raoultella ornithinolytica* and *Raoultella terrigena* were found in 2%. The Povo and E.V markets, were the markets that had the largest number of species identified (between 1 and 4), the remaining obtained between 1 and 2 identified, Table 3. The highest *Enterobacteriaceae* count obtained was 5.30 log CFU/g which was observed in E.V market, and the lowest was 1.70 log CFU/g which was observed in Xipamanine market. The mean counts ranged from 1.89 to 4.39 log CFU/g. (Table 4).

Table 2. Distribution of bacterial strains identified in sampled by locations

No. of identified strains/Locations	Museu	Povo	Mandela	E.V	Benfica	Xipamanine	Peixe	Total individual strains	% of individual strains
Acinetobacter junii	1	0	0	1	0	0	0	2	0.9
Acinetobacter pittii	2	0	0	0	0	0	0	2	0.9
Aeromonas caviae	2	2	0	1	0	0	0	5	2.25
Citrobacter freundii	1	2	1	4	1	1	4	15	6.3
Citrobacter koseri	0	0	0	0	0	1	0	1	0.45
Enterobacter asburiae	2	4	2	1	7	4	7	27	12.16
Enterobacter cloacae	10	14	4	17	10	11	6	72	32.43
Escherichia coli	2	2	0	1	0	0	2	7	3.15
Klebsiella oxytoca	0	5	2	2	1	0	1	11	4.95
Klebsiella pneumoniae	7	16	4	10	2	5	1	45	20.27
Kluyvera ascorbata	0	1	0	1	6	1	0	9	4.05
Kluyvera cryocrescens	0	0	1	0	0	0	0	1	0.45
Kluyvera georgiana	1	0	0	0	0	0	0	1	0.45
Kluyvera intermedia	0	0	2	0	0	0	0	2	0.9
Kosakonia cowanii	0	0	1	1	0	0	0	2	0.9
Leclercia adecarboxylata	0	0	0	3	0	1	0	4	1.80
Pantoea ananatis	1	1	0	0	1	0	0	3	1.35
Pantoea calida	0	1	0	0	0	1	0	2	0.9
Pantoea dispersa	0	0	0	0	0	2	0	2	0.9
Pantoea gaviniae	2	0	0	0	0	0	0	2	0.9
Pantoea septica	0	0	1	0	0	2	0	3	1.35
Pseudomonas mendocina	0	0	0	0	0	1	0	1	0.45
Raoultella ornithinolytica	1	1	0	0	1	0	0	3	1.35
Streptomyces violaceoruber	0	0	0	1	0	0	0	1	0.45
Total	32	49	18	43	29	30	21	222	100

Table 3. Distribution of *Enterobacteriaceae* bacterial strains identified on RTE lettuce salads vended in Maputo markets, Mozambique

No. of identified strains/Locations	Museu	Povo	Mandela	Estrela Vermelha	Benfica	Xipamanine	Peixe	Total Individual Strains	% of individual strains
Citrobacter freundii	0	0	2	0	0	0	1	3	6
Citrobacter koseri	0	0	0	0	0	1	0	1	2
Enterobacter asburiae	0	2	1	1	2	2	1	9	18
Enterobacter cloacae	1	2	3	4	4	1	2	17	34
Klebsiella oxytoca	0	1	0	1	0	0	1	3	6
Klebsiella pneumoniae	4	4	1	3	0	1	1	14	28
Kosakonia cowanii	0	0	0	1	0	0	0	1	2
Raoultella ornithinolytica	0	1	0	0	0	0	0	1	2
Raoultella terrigena	0	0	0	1	0	0	0	1	2
Total	5	10	7	11	6	5	6	50	100
% of individual strains	10	20	14	22	12	10	12	100	

Table 4. Prevalence of *Enterobacteriaceae* on RTE lettuce salads vended in Maputo markets, Mozambique

Location	Number of samples	% possitive	% unsatisfactory (≥ 4 Log CFU/g)	Mean Count (log CFU/g)	Min-Max
Museu	5	100	20	3.18 ± 0.93	2.00 - 4.08
Povo	5	100	40	3.93 ± 0.28	3.49 - 4.19
Mandela	5	100	20	3.18 ± 0.93	2.00 - 4.08
Estrela Vermelha	5	100	80	4.39 ± 0.70	3.51 - 5.30
Benfica	5	100	0	3.15 ± 0.74	1.82 - 3.58
Xipamanine	5	100	0	2.80 ± 0.89	1.70 - 3.60
Peixe	5	60	20	1.89 ± 1.89	0.00 - 4.41

3.3. Total Plate Count and Identification of *Enterobacteriaceae* on Drinking Water

Forty-two drinking water samples (35 from the manipulative reservoirs and 7 from the public supply) were analyzed in this research to see the prevalence of *Enterobacteriaceae*. All (n = 7) drinking water samples collected from the distribution source showed be negative for the *Enterobacteriaceae*. Thirty-one percent (n = 11) of the samples were found to be positive for *Enterobacteriaceae* of a total of 35 samples of manipulative reservoirs. The identified species included; *Enterobacter cloacae* (36.36%), *Enterobacter asburiae* (27.27%), *Klebsiella pneumonia* (18.18%) *Citrobacter freundii* and *Kluyvera ascobarta* with (9.09%), Table 5. The mean *Enterobacteriaceae* counts ranged from 0.24 to 2.01 log CFU/ml. The high count was registered in the market with 3.98 log CFU/ml and the lowest was recorded in the Povo market with 1.22 log CFU/ml. Notably all water samples collected from the Peixe market were negative, Table 6. Most (60%) of the samples from the Benfica and Xipamanine markets were positive for the presence of *Enterobacteriaceae.*

Table 5. Distribution of *Enterobacteriaceae* bacterial strains identified in drinking water used by vendors to clean salads in Maputo markets, Mozambique

Indentified strains/Location	Museu	Povo	Mandela	Estrela Vermelha	Benfica	Xipamanine	Peixe	Total individual strains	Relative %
Citrobacter freundii	0	0	0	0	1	0	0	1	9,09
Enterobacter asburiae	0	1	0	1	0	1	0	3	27,27
Enterobacter cloacae	1	0	0	1	0	2	0	4	36,36
Klebsiella pneumoniae	0	0	0	0	1	1	0	2	18,18
Kluyvera ascorbata	0	0	0	1	0	0	0	1	9,09
Total	1	1	0	3	2	4	0	11	100

Table 6. Prevalence of *Enterobacteriaceae* in drinking water used by vendors to clean salads in Maputo markets, Mozambique

Location	Number of samples	% possitive	Mean Count (log CFU/g)	Min-Max
Museu	5	20	0.48 ± 1.07	0.00 - 2.40
Povo	5	20	0.24 ± 0.55	0.00 - 1.22
Mandela	5	40	1.39 ± 1.90	0.00 - 3.56
Estrela Vermelha	5	20	0.48 ± 1.07	0.00 - 2.40
Benfica	5	60	2.01 ± 1.43	0.00 - 3.52
Xipamanine	5	60	1.57 ± 1.66	0.00 - 3.98
Peixe	5	0	0.00 ± 0.00	0.00 - 0.00

Table 7. Distribution of *Enterobacteriaceae* bacterial strains identified on hands surfaces used by vendors in Maputo markets, Mozambique

Indentified strains/Market Location	Museu	Povo	Mandela	Estrela Vermelha	Benfica	Xipamanine	Peixe	Total Individual Strains	Relative %
Citrobacter freundii	2	0	0	4	0	1	4	11	8,53
Enterobacter asburiae	0	2	2	1	4	0	6	15	11,63
Escherichia coli	2	2	0	1	0	0	0	5	3,88
Enterobacter cloacae	3	8	5	10	5	7	8	46	35,66
Kluyvera ascorbata	0	0	0	1	5	0	0	6	4,65
Klebsiella oxytoca	1	4	2	1	1	0	0	9	6,98
Kluyvera cryocrescens	0	0	1	0	0	0	0	1	0,78
Kluyvera intermedia	0	0	1	0	0	0	0	1	0,78
Kluyvera georgiana	1	0	0	0	0	0	0	1	0,78
Klebsiella pneumoniae	4	6	4	7	2	2	2	27	20,93
Kosakonia cowanii	0	1	1	0	0	0	0	2	1,55
Leclercia adecarboxylata	0	0	0	4	0	0	0	4	3,10
Raoultella ornithinolytica	0	0	0	0	1	0	0	1	0,78
Total	13	23	16	29	18	10	20	129	100,00

Table 8. Distribution of *Enterobacteriaceae* bacterial strains identified on drinking water used by vendors to wash salads in Maputo markets, Mozambique

Markets	Type of sample	Number of samples	% possitive	Mean Count (log CFU/g)	Min - Max
Museu	Hand	5	100	3.02 ± 0.36	2.65 - 3.49
	Knife	5	60	2.73 ± 0.64	0.00 - 3.18
	Bowl	5	80	3.31 ± 0.09	0.00 - 3.35
Povo	Hand	5	100	3.23 ± 0.64	2.18 - 3.88
	Knife	5	100	2.67 ± 0.29	2.40 - 3.06
	Bowl	5	100	3.08 ± 0.41	2.60 - 3.64
Mandela	Hand	5	100	3.23 ± 0.41	2.65 - 3.72
	Knife	5	80	2.90 ± 0.27	0.00 - 3.18
	Bowl	5	80	3.03 ± 0.38	0.00 - 3.31
Estrela Vermelha	Hand	5	100	3.69 ± 0.15	3.16 -3.86
	Knife	5	80	3.43 ± 0.20	0.00 - 3.62
	Bowl	5	100	3.44 ± 0.91	2.18 - 4.16
Benfica	Hand	5	80	3.75 ± 0.92	0.00 - 4.48
	Knife	5	80	2.88 ± 1.24	0.00 - 4.48
	Bowl	5	100	2.99 ± 0.96	2.18 - 4.48
Xipamanine	Hand	5	80	2.29 ± 0.90	0.00 - 3.60
	Knife	5	80	2.29 ± 0.90	0.00 - 3.60
	Bowl	5	80	2.67 ± 0.82	0.00 - 3.45
Peixe	Hand	5	100	4.27 ± 0.11	4.14 - 4.41
	Knife	5	60	2.77 ± 0.11	0.00 - 2.88
	Bowl	5	100	3.05 ± 0.27	2.65 - 3.34

3.4. Total Plate Count and Identification of *Enterobacteriaceae* on Surfaces Contact

In this study 105 swabs (35 hands; 35 bowls and 35 knifes) were collected. The *Enterobacteriaceae* isolate identities were determined using MALDI-TOF-MS analysis. The identified species include; *Enterobacter cloacae* (35.66%), *Klebsiella pneumonia* (20.93%), *Enterobacter asburiae* (11.63%), *Citrobacter freundii* (8.53%), *Klebsiella oxytoca* (6.98%), *Kluyvera ascobarta* (4.65%) and *Escherichia coli* (3.88%). Other species like *Citrobacter koseri*, *Kosakonia cowanii*, *Raoultella ornithinolytica* and *Raoultella terrigena* were found in were found in very low percentages, Table 7. Regarding the number of species of *Enterobacteriaceae* isolated, the E.V market was the one that registered the most with 29 isolates. On the other hand, the Xipamanine market was the one which recorded a smaller number of isolates (10). On surfaces (hands, knife and bowl), the counts of *Enterobacteriaceae* ranged from 2.18 to 4.48 log CFU/cm^2. In general, hand samples were the ones with the highest counts (2.18 - 4.48 log CFU/cm^2). The Benfica market recorded the highest (4.48 log CFU/cm^2) counts in all surfaces samples, Table 8. The Xipamanine Market recorded low mean counts of *Enterobacteriaceae*.

3.5. Prevalence of *E. coli* in the Sample

No *E. coli* was isolated from the potable water (n = 42) and the salad lettuce (n = 35) samples. On the other hand 5/182 samples were positive for *E. coli* isolates and it were isolated from bowls samples.

3.6. Virulence Genes in *E.coli* Isolates

The PCR assay was used for conforming the presence of virulence genes in *E.coli* but the virulence genes mdhF, mdhR, stx1F, stx1R, stx2F, stx2R, were not detected in any of the *E.coli* isolates.

Table 9. Correlation between the counts of *Enterobacteriacea*e founded in the samples

Level	Spearman's rho	Sig.*
Salads - water	0.410*	0.000
Salads - hand	0.199	0.000
Salads - knife	0.074	0.000
Salads - bowl	0.223	0.000
Water - hand	0.072	0.000
Water - knife	0.164	0.000
Water - bowl	0.337*	0.000
Hand - knife	0.218	0.000
Hand - bowl	0.276	0.000
Knife - Bowl	0.281	0.000

*correlation is significant at the 0.01 level (2 - tailed).

3.7. Correlation among the *Enterobacteriacea*e Counts in the RTE Lettuce Salads, Drinking Water and Surfaces

A summary of the correlation for the samples (RTE lettuce salads, drinking water and surfaces) is shown in Table 9. There was not a significant positive correlation found between the counts founded in RTE lettuce salads and drinking water, and the counts founded in RTE lettuce salads, hands and surfaces, and there were no significant differences in *Enterobacteriaceae* counts of swabs samples between the locations (P>0.05).

4. Discussion

4.1. Enterobacteriaceae Found

In the current study, we evaluated the prevalence of *Enterobacteriaceae* from ready-to-eat salads, drinking water and swabs in markets of Maputo by direct Matrix Assisted Laser Desorption/Ionization Mass Spectrometric - (MALDI-TOF-MS). The predominant species of *Enterobacteriacea*e found were: *Enterobacter cloacae* (34%), *Klebsiella pneumonia* (28%), *Enterobacter asburiae* (18%), *Citrobacter freundii* and *Klebsiella oxytoca* (6%). *E. coli* as one of the important public health strains were also found but in low counts. *Enterobacter* spp. in 18%, *Klebsiella oxytoca* in 8%, and *Escherichia coli* were not isolated in any of the samples. Recently, Shiningeni et al., reported high (83%) percentages of *Enterobacteriacea*e in RTE food vended in Windhoek, Namibia [41].

The *Enterobacteriacea*e family is a part of the normal gut microbiota but can also be found in the environment [16,42,43]. *Enterobacter*, *Citrobacter* and *Klebsiella* species are the mostly found in environments. For instance, water, salads, hands and utensils can be contaminated with these microorganisms. Many of the bacterial strains of *Enterobacteriacea*e family, are used to be dismissed as harmless commensals and usually considered by food manufacturers as hygiene indicators and therefore used to monitor the effectiveness of implemented preventive pre-requisite measures such as Good Manufacturing Practices and Good Hygiene Practices [14,44]. The presence of low levels of *Enterobacteriaceae* in foods is accepted and does not represent a direct safety concern. Members of the *Enterobacteriacea*e family are opportunistic pathogens responsible for a major health problems worldwide [45,46,47,48]. The genera *Escherichia*, *Klebsiella*, *Enterobacter*, as *Serratia*, and *Citrobacter* have been reported to be responsible for infections in humans and other animals. *Citrobacter* species are an uncommon cause of bacterial meningitis in neonates, but are associated with brain abscesses in the majority of cases [49]. Klebsiella species and E. coli can become carbapenem-resistant. *Klebsiella pneumoniae*, is responsible for pneumonia [50], and represent highest risk at the patients those with impaired immune systems, [51]. The important foodborne pathogens that are found in the *Enterobacteriaceae* family include Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Shigella spp., *Salmonella* (non-typhoid), *Salmonella* (Typhi/Paratyphi), *Yersinia enterocolitica* and Cronobacter spp [52]. In our study we did not found these pathogens foodborne.

4.2. Prevalence of *Enterobacteriaceae in* RTE Lettuce Salads

The mean *Enterobacteriaceae* ranged from ranged from 1.89 ± 1.89log CFU/g to 4.39 ± 0.70 log CFU/g. The

highest mean was observed in E.V market and the lowest mean in Peixe market. The highest count level was 5.30 log CFU/g and was observed in E.V Market. Similar results were found in Rwanda, investigated kitchen scale salad preparation practices in a field study (food service establishments) [53]. Unsatisfactory levels of *Enterobacteriaceae* ((≥ 4 Log CFU/g) (ICMSF) were detected in 25.7% (9/35) RTE lettuce salads samples tested. Unsatisfactory levels of *Enterobacteriaceae* were the highest in E.V with prevalence of 80% (Table 4). The presence of the highest level of *Enterobacteriaceae* is indicative of unacceptable contamination during food preparation and inappropriate conditions such as prolonged storage at elevated temperature [44,54]. These findings are comparable with other studies done worldwide. In Namibia the highest mean counts were 4.10 log CFU/g. In Ruanda the highest mean *Enterobacteriaceae* count was 3.3 log CFU/g and in Zambia and Mashhad the counts ranged between 1.6 to 9.8 log CFU/g [41,53,55]. Although other authors, studied the prevalence *Enterobacteriacea* in fresh vegetables sold in retail of Canada over a period of four years (2009 - 2013), and found counts generally very low, with prevalence intervals ranging from 0 - 1.3 log CFU/g [56]. Unsanitary vending conditions, unhygienic practices act, insufficient food hygiene education and presence of reservoirs and vectors in or near the food production or service areas can contribute to increase the level of contamination of ready-to-eat foods [49], and it can be associated with the results.

The dominant identified species in this study included *Enterobacter cloacae*, *Klebsiella pneumonia* and *Enterobacter asburiae*, and can indicate poor food preparation, poor sanitary conditions as well as Also cross contamination. These species are genetically related bacteria used to assess the general hygiene status of a food product. These microbes can be introduced in food with cross contamination especially RTE salads. Recently authors, demonstrated the occurrence of various microbial pathogens which includes *Escherichia coli* in ready to eat vegetable salads in developing countries [1]. However, in this study *E. coli* were not detected in ready-to-eat lettuce salads, contrasting with other studies in other African countries like Namibia, Ghana and India, with where those bacteria were found in highest levels [13,41,57].

Although, no *E. coli* was enumerated from the RTE lettuce salads, we cannot ensure that this bacteria is not in the salads, as generally known *E.coli* is part of the normal microbiota in the digestive tract of both humans and animals [58]. This bacterium can be secreted, often in large numbers, through the feces into the environment [16]. The absence may be due the fact that the samples were collected during the dry season (no rain). Among the isolated, we cannot ignore the high counts obtained for the coliform bacteria such as *Enterobacter* spp, *Citrobacter* spp, *Klebsiella* spp been increasingly reported as important opportunistic pathogens [59].

The microbiological quality and safety of RTE lettuce salads sold in markets can be compromised at numerous points along a food system from farm to consumption. Since there is no step that kills pathogens during the production of RTE salads, a completely safe final product can never be guaranteed. In this perspective measures to reduce the contamination might be advised such as a proper handling and washing before consumption of these products as well as public education and awareness. Appropriate irrigation water is also important [16,60].

4.3. Prevalence of *Enterobacteriaceae* in Drinking Water

Tests for *Enterobacteriaceae* bacteria, as indicator of hygiene quality were done in 42 drinking water samples. It is well documented that fecal contamination of drinking water can cause numerous disease outbreaks [61]. In this study, *E. coli* was not enumerated from any of the drinking water samples indicating that that the water has not been contaminated with feces. Different results have been found in developing countries such as Kenya, India and Iran on what *E. coli* were found in 30%, 30% and 61% in drinking water respectively [62,63,64]. *Acinetobacter, Aeromonas, Enterobacter sakazakii, Helicobacter pylori, Klebsiella, Pseudomonas aeruginosa* bacteria, provides information on organisms that have been suggested as possible causes of waterborne disease [51]. In this study were not found all of this bacteria group. *Klebsiella pneumoniae* is a part of the group that was most isolated. According to the WHO, ideally, drinking-water should be free from known pathogenic micro-organisms capable of causing disease or any bacteria indicative of fecal contamination [51,65,66]. Although no isolates of *E. coli* were found in the drinking water samples, other *Enterobacteriaceae* species were found in 31% (11 of 35) of the sample, which means that almost half of the seller's reservoir water samples were contaminated. This may compromise the health of consumers seeking markets to meet their food needs because a lack of access to safe drinking water can lead to various health problems [67].

Waterborne diseases represent a major human health risk in many parts of the world, especially in developing countries. Mozambique as a developing country is well known for persistent and recurrent waterborne diseases [68,69]. The availability of safe drinking water in developing countries remains a major challenge due to poor sanitation condition [70]. Safe sanitation is essential for health, from infection prevention to improving and maintaining mental and social welfare [71].

4.4. Prevalence of *Enterobacteriaceae* in Swabs

The purpose of collecting swabs samples is to trace the sources of and evaluate the extent of the contamination [9]. In this study, the identified species included *Enterobacter* spp, *Citrobacter* spp, *Klebsiella* spp. and E. coli. The counts ranged from 2.01 to 4.39log CFU/g in the samples. The lowest (2.18log CFU/m^2) counts were observed in Peixe market and the highest (4.39 log CFU/m^2) were observed in E.V market. These findings concur with other study [72] in which they found 44% of *Enterobacteriaceae* in hands of food handlers. Other study founded *C. sakazakii* from 26.9% of 78 domestic kitchens visited in United States [73].

The species of *Enterobacteriaceae* identified in this study are responsible for cross-contamination and could signify unhygienic conditions during food handling and preparation [54]. From 105 swabs collected, five (5/105),

isolates of *E.coli* were recovered. These findings concur with a study conducted in Zahedan who found total coliform and *E. coli* in dishes (86. 67%, and 33. 3%) and spoons and forks, (79% and 30%) in establishments [74].

In fact, food contact equipment is an important factor of microbial contamination of ready-to-eat products such as lettuce. Microorganisms can be transferred during food preparation such as cutting and grinding, specifically when the same equipment is used for raw material, meat and RTE foods [75]. Food handlers with poor personal hygiene could be potential sources of infection due to pathogenic bacteria [76], and can be a source of foodborne contamination and they can cross-contaminate raw and processed food stuffs [9,77]. Developing countries have high problems of food borne diseases [78], due to the difficulties in adopting optimal hygienic practices during food handling [79]. In this study we did not find *E. coli* isolates in samples from the food-handlers, despite that, we cannot conclude that the hands of the handlers are safe. Food handlers may constitute a reservoir of virulent strains of *Staphylococcus aureus* and may be vehicles of their transmission to food [80,81]. The presence of the *E. coli* in the utensils is probably due to the fact that the utensils have not been washed properly. Not only that, but contamination may be due to the presence of flies that are prevalent in these unclean environments. On the other hand, high contamination of the hands may be related to improper hand washing and disinfection. Since food retailers in the markets do not have water pipes, the sellers store water in containers. The contamination could also be attributed to substandard cutting and preparation practices, particularly poor hygienic conditions of the premises that may result from rubbish, sewage and other noxious substances present in the vicinity

4.4. Virulence Genes in *E.coli* Isolates

Several studies have focused on determining the virulence genes in *E.coli* isolates of fresh produce sold at open hair markets, supermarkets and street venders from selected areas in a specific country [82,83,84,85], because it has been recognized as the leading causes of human food born infections throughout the world with fatal complications such as hemolytic uremic syndrome that ends in renal failure. The real-time PCR assay used for pathogen detection confirmed that the isolates of *E.coli* obtained using MALDI-TOF analysis were positive but the virulence genes stx1F, stx1R, stx2F, stx2R, were not detected in any of the *E.coli* isolates. These findings concur with other studies who collected vegetable salads samples from restaurants and market respectively and *E. coli* O157:H7was not detected in any of the samples analyzed [86]. In contrast *Escherichia coli* O157:H7 and *Listeria monocytogenes* were founded in different salad vegetables [87]. The negative results for the virulence genes of *E. coli* in the samples especially in the RTE lettuce salads can be explained by the fact that salt and vinegar are used to temper the salads in Mozambique. Acetic acid alone or combined with salt can inhibiting *Escherichia coli* O157:H7 for example [88,89,90]. Besides, in Mozambique, farmers do not use organic fertilizers basically, cattle and sheep are the major animal reservoir of STEC. Furthermore, we cannot ensure that enteric pathogens are not present as the survival and growth characteristics of different strains of *E. coli* and enteric pathogens can vary, [58]. Further studies are required to cover more numbers of samples and to investigate the presence of non shiga-toxin producing *E. coli*.

5. Conclusion

This study found high *Enterobacteriaceae* counts in RTE lettuce salads and other samples (what other sample, please list them) that could serve as an indicator for the need to promote improvement in sanitary and good hygienic practices in food markets of Maputo. *E. coli* was isolated in 4.76% of the surfaces samples and non-virulence's genes were found. However, we cannot disregard the importance of the *Enterobacteriaceae* isolates since some of them are used to indicate the sanitary conditions and many of them become pathogenic due to the acquisition of virulence-associated genes. The results are little evidence that those salads represent an important risk for transmission of pathogenic microorganism in in Maputo, Mozambique, and it can be a potential hazard for public health. Proactive research to ensure food processing in particular salads and hygiene controls are needed in Maputo markets to ensure food safety and preserve consumer health.

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Conflicts of Interest

The authors declare that there are no conflicts of interests.

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