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Isolation and Characterisation of *Listeria* Species from Ready-to-eat Raw Pork Sausages Processed in Mauritius

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ABSTRACT---- ***The aim of this study was to evaluate the microbial safety, in particular, the presence and prevalence of Listeria species in ready-to-eat pork raw sausages sold in three main markets in Mauritius. Analyses were carried out over a period of six months (July - December 2013). A total of 90 samples were analyzed through standard methods for the isolation, phenotypic and molecular characterization. The antimicrobial susceptibility of identified Listeria isolates was also assessed. Statistical analyses showed that the prevalence of Listeria spp. was significantly higher (p< 0.05) in sausages stored at room temperature. 61.0% of samples were found contaminated with Listeria species, 23.1 % with L.monocytogenes and L.innocua, 7.7% with L.grayi and 7.1 % with other Listeria species. No contamination with L.ivanovii was noted..The susceptibility test showed that isolated L.monocytogenes, L.innocua, and L.grayi were susceptible to all five antibiotics tested. Highest susceptibility was observed against erythromycin and lowest to ciprofloxacin. Findings also suggested that the level of hygiene concerning the processing, storage and handling of raw pork sausages is relatively poor and there is potential risk of foodborne diseases. This study contributed to the prevalence and contamination levels of Listeria species in RTE raw pork sausages in Mauritius for the first time, providing information for authorities to improve the microbiological safety of RTE foods.***

Keywords---Ready-to-eat meat, pork sausages, *Listeria monocytogenes*, antimicrobial susceptibility

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1. INTRODUCTION

Listeria species are ubiquitous in the environment. *Listeria monocytogenes*is significant for cold-stored, Ready-To-Eat (RTE) foods and can grow at refrigerated temperatures. RTE food means products that are intended by the producer for direct consumption by humans without the need for thermal treatment such as cooking that will eliminate or reduce to an acceptable level, microorganisms of concern [1]. *Listeria monocytogenes*has been recognised as a human pathogen for several years [2, 3].*L. monocytogenes* can cause severe listeriosis infections resulting in meningitis, meningoencephalitis, septicemia, abortion, and prenatal infection in individuals with weakened immune systems, such as fetuses, infants, the elderly, and immunocompromised individuals [4, 5].The approximate fatality rate is 30% that may increase up to 75% in high risk groups, such as pregnant women, neonates, and immunocompromised adults [6, 7, 8]. *L. monocytogenes*is pathogenic for human, while *L. ivanovii*is rarely pathogenic for humans [9]. *Listeria* spp. are considered as an important cause of zoonoses infecting many types of animals such as domestic pets, livestock, avian species, rodents, amphibians, fish, and arthropods. *Listeriosis*is unique disease that represents a considerable public health concern because of its high mortality rate [10, 11, 12, 13]. Most cases of *Listeriosis*appear to be foodborne, including those acquired during pregnancy.

RTE meat products have been reported to be regularly contaminated with *Listeria* spp. and *L.monocytogenes* due to broad exposure to cross contamination during post-processing steps such as slicing, peeling, and packaging [14], in processing plant and equipment [15] or due to mishandling and improper cleaning of storage environments. RTE meat products represent a nutrient-rich environment for *Listeria* spp. *Listeria* can grow over wide temperature and pH ranges, withstand high salinity and water activity [16] and survive under mild preservation treatment [17, 18]. These features enhance their capacity to colonize food processing environments, contaminate food and make them good indicators of poor hygiene and cross contamination [19] in the food industry. The occurrence of the bacteria and especially *L.monocytogenes* in these products denotes a greater hazard that their incidence in raw meat as RTE meat products are often submitted to the wrong combination of temperature/time required to destroy the pathogens before consumption [20]. Once contamination occurs, the level of bacteria tends to increase along the food processing chain. Additionally, their ability to survive and multiply even at low temperature [21, 22] sustains contamination even when products are stored under refrigerated conditions [15].

The group of RTE meat products includes among others sausages subjected to thermal treatment by a producer and raw sausages.In meat products that support the growth of *L. monocytogenes* such as cooked sausages and raw sausages other than dry sausages and cured hams *L. monocytogenes* must be absent in 25 g of meat products before they left the

immediate control of the producer and lower than 100 cfu/g in products placed on the market during their shelf-life [1]. The prevalence of *L.monocytogenes*in RTE food is commonly reported in high rates in different parts of world. *L. monocytogenes*was detected in Spanish-style sausage (3.7%), blood sausage (11.1%), cooked meat samples (8.8%), different RTE foods (7.3%) and in in-store-packaged deli meat products (8.5%) [23, 24, 25]. A USDA-FSIS survey published in 2001 showed that 1-10% of retail RTE meat and poultry products were contaminated with *L. monocytogenes*[26]. However, a study in Australia reported a low prevalence (0.2%) of *L. monocytogenes*in these products [27]. No published information on the status of food borne listeriosis in Mauritius is available. The objective of this study was to investigate the presence of *Listeria* spp. and, in particular,*L.monocytogenes* in RTE pork sausages processed in Mauritius and to assess the susceptibility of the *Listeria* isolates to different antibiotics.

2. MATERIALS AND METHODS

2.1 Sample collection

The study was conducted on RTE raw meat sausages (raw smoked pork sausages). All products were classified as ready-to-eat and required no further cooking by the consumer. The sampling sites were distributed through three main markets in Mauritius situated in Port Louis, Rose Hill and Curepipe and, visited by a high number of consumers on a daily basis. The products were packed in individual packaging or were distributed without packaging. Also, some products were displayed at room temperature while others were refrigerated. Five replicates of pork sausages stored at room and refrigerated temperatures were collected from each market at intervals of two weeks between July 2013 and December 2013. All of the samples were kept below 4^0C during transportation and testing was initiated within 2 h after purchase. Information regarding storage temperature and conditions was recorded.

2.2 Qualitative detection

Samples were tested according to the protocol of selective enrichment and isolation of the United States Department of Agriculture (USDA)[28, 29] for the isolation of *Listeria* spp. from meat products. In brief, samples were submitted to qualitativeanalysis by homogenizing 25 g of the respective sample and 225 mL Listeria enrichment broth (UVM I formulation, OXOID), for 30 s in stomacher bags. Homogenates were incubated at 37°C for 24hr. Thereafter, 0.1 ml of enrichment culture was transferred to 10ml of UVM II enrichment broth (Fraser Broth Base, HIMEDIA) and incubated at 37°C for 24hr. A serial dilution in Fraser broth was carried out until a concentration of 10^{-6} was obtained. The enriched broth was inoculated on PALCAM agar [30] plates and incubated at 37°C for 24hr. The spread plate and total viable count techniques were used for the enumeration of *Listeria* spp. in samples and results were expressed as log cfu/g. Presumptive *Listeria* colonies on PALCAM agar plates were isolated on Tryptone Soy agar with 0.6% yeast extract [29, 31] and incubated at 37°C for 24hr. Isolated colonies were subjected to Gram stain and the following biochemical tests: catalase, oxidase, indole, motility, carbohydrate fermentative test (D-mannitol, L-ribose, D-rhamnose and D-methyl-α-mannoside)[12, 16, 32] 16, hemolysis and CAMP tests [33] .

2.3 Molecular characterisation

Molecular techniques including PCR based assays were used as confirmatory analysis for the presence of *Listeria* spp. and in the identificationof isolates to species level. Identification of *Listeria* spp. with PCR was performed after selective enrichment to obtain more reliable results [32, 34, 35]. Genomic DNA was extracted according to a modified version of BAM protocol recommended for the DNA isolation of *Listeria* species. Bacterial isolates were sub cultured on Nutrient agar plates at 37°C for 24hr. Single colonies were cultured in 10 ml of Brain Heart Infusion broth (BHI) at 37°C for 20-24hr to mid-log phase.1 ml of cells was pelleted by centrifugation (7,000 x g for 5 min). The cells were washed with 1 ml of sterile distilled water, re-suspended in distilled water to give approximately 10^5 cells ml^{-1}, and heat lysed at 95^0C for 5 min. Cell debris was pelleted by centrifugation (7,000 x g for5 min), and the supernatant containing DNA was transferred to a clean sterile tube[36]. For DNA amplification, a final volume of 50µl was used containing 50 mMKCl, 10 mMTris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphates, 1.5 U of Taq polymerase, 10 µl of total cell lysate, and 0.5 µM (each) of primer for each reaction. Primers S1F (5'-AGTCGGATAGTATCCTTAC-3') and S1R (5'-GGCTCTAACTACTTGTAGGC-3') were chosen to amplify a 460 bp DNA fragment (S1) [37].PCR conditions were as follows: initial denaturation at 95ºC for 5 min, followed by 30 cycles, each with 1 min denaturation at 95ºC, 1 min annealing at 55ºC and extension at 72ºC for 1 min, before a final extension at 72ºC for 5 min. The PCR products obtained from DNA extracted from the samples were first analyzed by electrophoresis in 1.5% agarose gel and were stained with ethidium bromide and visualized under short-wavelength UV light. Once identified, *Listeria* isolates were subjected to additional PCR reactions with specific primers (Table 1.0) to differentiate between the isolated *Listeria* species [34, 38, 39, 40, 41].

Table 1: Primers used for Identification at Species Level

Primers	Sequences	Species name	Target
Hly 1(F) Hly 2(R)	CGGAGGTTCCGCAAAAGATG CCTCCAGAGTGATCGATGTT	*L.. monocytogenes*	Hly
lin 0464 F lin 0464R	CGCATTTATCGCCAAAACTC TCGTGACATAGACGCGATTG	*L.. innocua*	Lin 0464
liv22-228 F liv22-228R	CGAATTCCTTATTCACTTGAGC GGTGCTGCGAACTTAACTCA	*L.. ivanovii*	N-Acetylmuramidase
lgr20-246 F lgr20-246R	CTGCACGATCAAGGTCAATC CGTATTGCGCACCAGTGATA	*L.. grayi*	Oxidoreductase

Sequencing of the isolated strains was carried out by InqabaBiotec (South Africa). Sequences obtained were analyzed through software such as CLC bio and FASTA and compared to the non-redundant nucleotide database at the National Center for Biotechnology Information by using their World Wide Website, and the BLAST (Basic Local Alignment Search Tool) algorithm to find percentage homology of the isolates with known *Listeria* spp.

2.4 Antimicrobial susceptibility test.

The susceptibility of the identified *Listeria* species was tested using the disk diffusion technique as recommended by the Clinical and Laboratory Standards Institute [42] against chloramphenicol (15mg), Clindamycin (2mg), ciprofloxacin (5mg), erythromycin (15mg) and rifampicin (5mg). Bacterial isolates were cultured overnight in Full Fraser broth and spread on PALCAM plates. 5 antimicrobial disks from spring-loaded sealed cartridges containing 50 disks were placed on the plates 20 mm apart. Plates were incubated under microaerophilic conditions at 37°C for 24hr. Inhibition zone around each disk was measured and interpreted according CLSI [43] andFallahet al. [31].Zones of inhibition were measured with a precision caliper to the nearest 0.01 mm.

2.5 Statistical analysis

Minitab 17.0 was used to analyse the log cfu/g in samples and the inhibition zone (mm) of the different antibiotics. Mean and standard deviation were calculated through the Anova table. The Turkey's test was then used to compare mean differences at 5% for prevalence of *Listeria* in samples per region and for antimicrobial test. The differences were considered to be significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Qualitative and quantitative analysis

55 out of the 90 samples (61.1%) showed presence of *Listeria* spp. 23.1% samples positive for *L.monocytogenes* sausages with the same percentage for *L.innocua.* 7.7% were positive for *L.grayi* and 7.2 % for other *Listeria* spp. The high prevalence of *Listeria* in RTE raw pork sausages may be due to the ubiquitous nature of this microbe, which can contaminate products in slaughterhouses, before or after cooking, or through food contact surfaces and food handlers [44].As expected, the prevalence of *Listeria* species was observed to be significantly higher ($P<0.05$) in sausages stored at room temperature (Figure 1). Listeria species are known to be able to grow at temperatures between 1– 45^0C with an

optimum between 30-37^{0}C.The cfu/g of the samples was within the range 8 ± 0.1 × 10^3 – 8 ± 0.1 × 10^4cfu/g. This exceeds the limit of100 cfu/g [45] indicating a relatively high level of microbial contamination and poor sanitation.

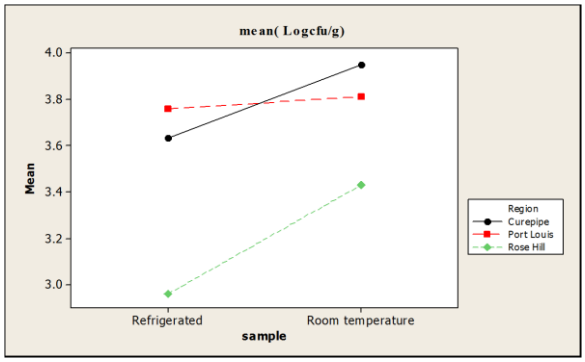

Figure 1: Mean log cfu/g of *Listeria* in two types of RTE pork sausages from the three different regions and at two storage conditions.

When the mean prevalence of *Listeria* spp. in RTE raw pork sausages per region was compared, the level of contamination was observed to be considerably higher in Port-Louis and Curepipe and lowest in Rose-Hill (Figure 2) for all samples. Several reasons may be attributed to this observation. The average ambient temperature at Port Louis for the months of July-December 2013 was the highest (28^{0}C) as compared to Rose Hill (25^{0}C). However, Curepipe had the lowest ambient temperature for these months with an average of 22^{0}C. The high number of samples containing the bacteria in this case could be related to the poor hygienic conditions both at the level of processing and storage of the products[46].Also, according to Chasseignaux et al [47], cross contamination are generally associated with presence of the bacteria on equipment, processing plants or by the workers themselves leading to variability in level of contamination.

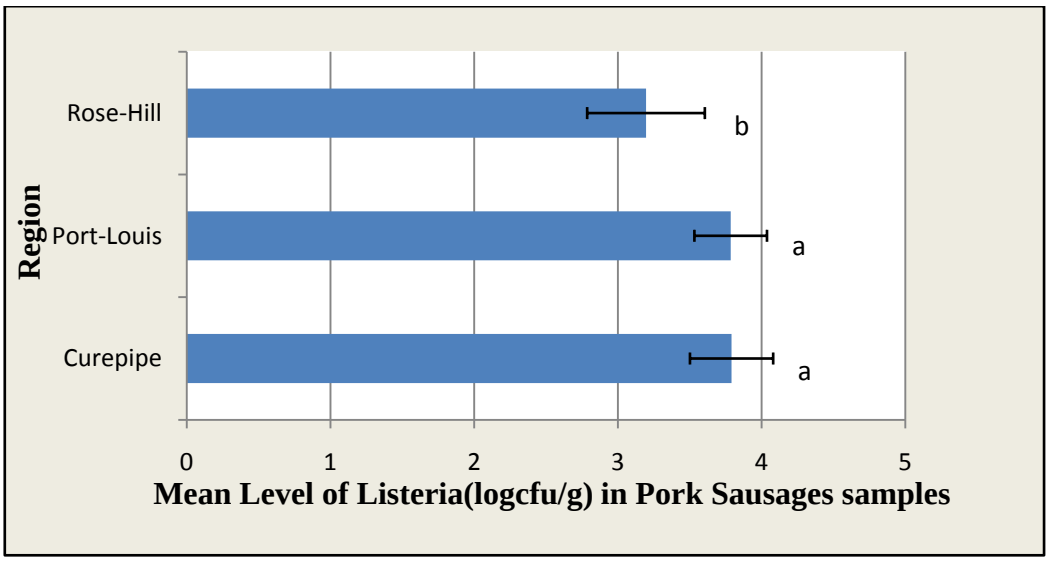

Figure 2: Mean log cfu of *Listeria* level in RTE pork sausages per region. Mean with same letters are significantly similar.

Presumptive colonies were catalase positive, oxidase and indole negative. Motility was observed at room temperature. Identification to species level by carbohydrate fermentative tests, hemolysis and CAMP tests suggested the presence of four *Listeria* species. Fermentation of D-rhamnose, and D-Methyl-α-Mannoside suggested the presence of *L.monocytogenes* and this was further confirmed by a positive hemolysis and CAMP tests with *S.aureus*. Fermentation of L-ribose and a positive hemolysis test indicated the presence of *L.ivanovii*. Carbohydrates metabolized by other isolates suggested the presence of *L.grayi* and *L.innocua*.

3.2 Molecular characterisation

Standard culture methods EN ISO 11290-1 and ISO10560 are still being used to detect *L. monocytogenes*in food. Although these methods are quite far from being 100% effective [48], a primary ambition of an alternative, faster method should be to achieve equivalence with the current standard method. PCR-based methods have shown great potential to fulfill the requirements for fast, specific and sensitive detection of *L. monocytogenes*in food[49]. In this present study, PCR using primers S1 and S2 showed the presence of *Listeria* species in the analysed samples (Figure 3). PCR with primer Liv22-228F and Liv22-228R showed that there was no contamination with *L.ivanovii.*Analysis of the 23S rRNA sequence revealed that the isolates were related to the genus *Listeria*. Analysis of graphical data on CLC bio showed spaced, high and well-shaped peak for sequences from isolated *L.innocua*, crowded, short and irregular peaks for*L.monocytogenes*and *L.grayi*. Pairwise alignment with different species of the genus showed 97% sequence similarity with all the isolates as identified by the biochemical tests, namely *L. monocytogenes, L. innocua* and *L. grayi.*

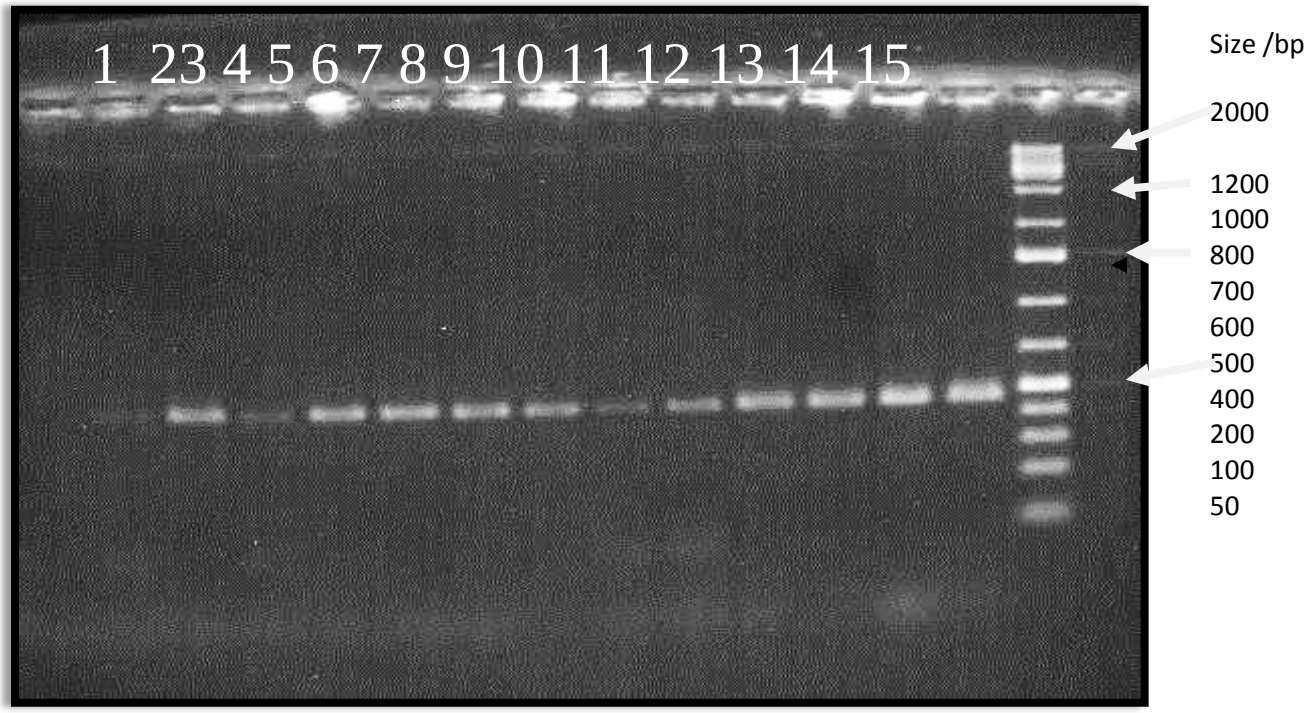

Figure 3: 1.5% Agarose gel electrophoresis of the products obtained from the amplification of the 23S rRNA of *Listeria species* with primer S1 and S2.

Lane 1, negative control. Lanes 2, 3, 6 & 7, samples from Curepipe (room temperature); Lanes 4 & 5, samples from Curepipe (refrigerated); Lanes 6 & 7 samples from Rose Hill (room temperature); Lanes 8 & 9, samples from Rose Hill (refrigerated); Lanes 10 &11, samples from Port Louis (room temperature); Lanes 12 & 13, samples from Port Louis (refrigerated). Lane 14, positive control: *Listeria innocua.* Lane 15, ladder.

3.3 Antimicrobial susceptibility test

Since the first antibiotic resistant and multidrug-resistant *L. monocytogenes* strains were isolated from patients in 1988 [50], antimicrobial resistant strains were commonly recovered from food, natural environment, and clinical cases of listeriosis. Surveillance of the emerging antimicrobial resistance profiles of *L. monocytogenes* in RTE foods is of utmost importance. The resultsof this study suggested no incidence of antimicrobial resistance in *Listeria* spp., including *L. monocytogenes* strains isolated from RTE raw pork sausages in Mauritius. Zhang et al. [51] reported that *L. monocytogenes*isolates from RTE meat products in the United States were sensitive to gentamicin and trimethoprim. Fallah et al [31] found that *L. monocytogenes*isolates from RTE poultry products sold in Iran were sensitive to ciprofloxacin and tetracycline, and Conter et al. [52] found that *L. monocytogenes*food isolates were sensitive to imipenem, gentamicin,teicoplanin, and fosfomycin and resistant to fusidic acid.All *Listeria* isolates were susceptible to the five antibiotics tested (Table 2). Statistical analysis showed that the susceptibility trend for the three isolates was

similar with *L.innocua* being less susceptible as compared to the other two (Figure 4). Although no antibiotic resistant strains were identified, 25 isolates exhibited intermediate resistance to Ciprofloxacin, 30 to Clindamycin and all isolates exhibited intermediate resistance to Rifampicin. The acquired intermediate resistance to Ciprofloxacin may be related to the excessive use of this antimicrobial in animal feed and as a drug of second choice in the treatment of human diseases [53].Several studies have elucidated that antimicrobial resistance maybe acquired by *L. monocytogenes*via conjugation [54, 55], self-transferable plasmids [50, 56], and vertical and horizontal gene transfer [54].

Table 2: Results of antimicrobial resistance of *Listeria* spp. isolates in the study

	Breakpoints (mm)			Number of isolates		
Antimicrobial agent	Resistant	Intermediate	Susceptible	Resistant	Intermediate	Susceptible
Chloramphenicol(30µg)	≤20	-	≥21	-	-	55
Ciprofloxacin (5 µg)	≤16	17-19	≥20	-	25	30
Clindamycin (2 µg)	≤14	15-20	≥21	-	30	25
Erythromycin (15µg)	≤13	14-22	≥23	-	-	55
Rifampicin (5 µg)	≤23	24-29	≥30	-	55	-

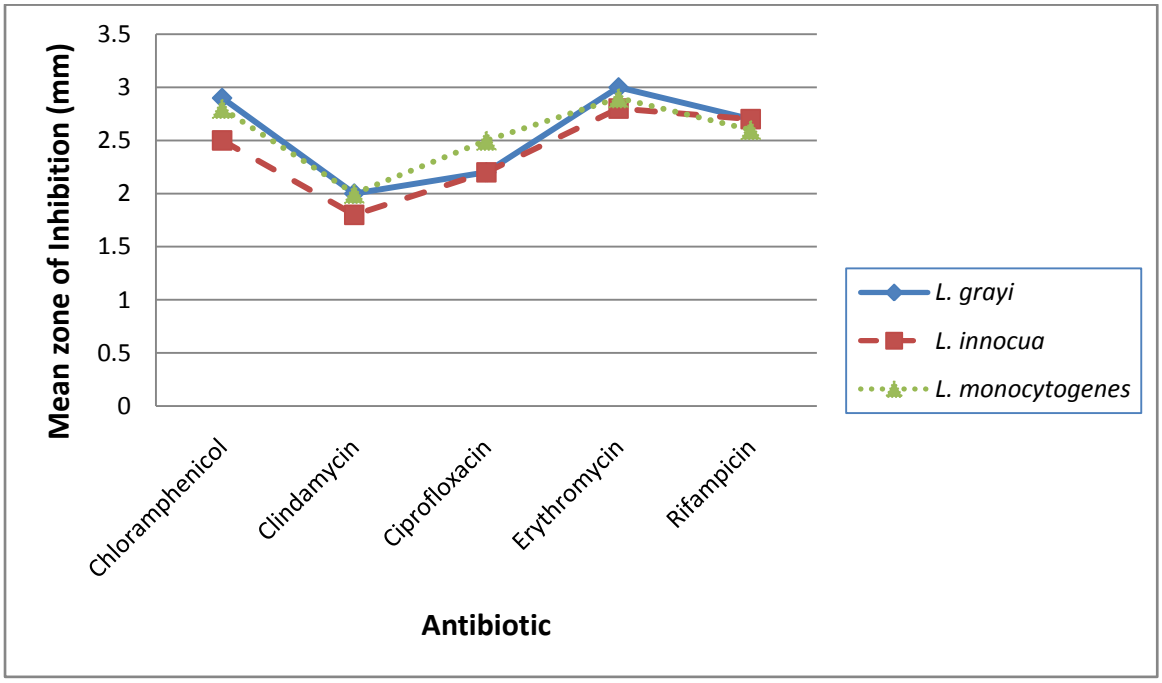

Figure 4: A 2-way analysis with two factors, microorganisms and antibiotics

5. CONCLUSIONS

In summary, the research findings of this study showed that ready-to-eat raw pork sausages processed in Mauritius are contaminated with *Listeria* spp. Including,*L.monocytogenes*. The high prevalence of the bacteria detected in the analysed samples may suggest poor hygiene practices at the processing of RTE raw pork sausages a retail level. These foods may also serve as potential vehicles for transmission of virulent *L.monocytogenes* with potential risk of food borne infection and Listeriosis. Results also suggest high level of cross contamination and poor hygiene in food processing and storage environments. Measures to control cross-contamination such as the Hazard Analysis and Critical Control Point (HACCP) should be implemented to identify sources of contamination and to reinforce food safety measures. Regulatory authorities should take measures to improve the microbiological safety of RTE foods, including raw pork sausages.

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Conflict of Interest: None

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