Epidemiol. Infect. (2011), **139**, 718–727. © Cambridge University Press 2010
doi:10.1017/S095026881000172X

Occurrence and genetic relatedness of *Listeria* spp. in two brands of locally processed ready-to-eat meats in Trinidad

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(*Accepted 26 June 2010; first published online 22 July 2010*)

SUMMARY

Contamination of locally produced, ready-to-eat meats by *Listeria* spp. has been previously reported at one processing plant in Trinidad. However, the status of this pathogen in locally produced products sold at retail outlets is unknown. This study was conducted to establish whether there is a risk to consumers of locally processed meats caused by the presence of *Listeria* spp., and whether a link exists between the presence of the pathogen in retail products and the manufacturing plant of one brand (B). Four hundred and eighty ready-to-eat meat products of two popular local brands (A and B) were collected from retail outlets and analysed for the presence of *Listeria* spp. together with food samples and surfaces from one manufacturing plant (B). Eighty-eight of the retail products (18·3%) were contaminated with *Listeria* spp., of which, 52·3% were *L. innocua*, 44·3% were *L. monocytogenes* and 3·4% belonged to the *L. seeligeri–L. welshimeri–L. ivanovii* (Siwi) group. *L. innocua* was found in 15 in-process food samples and on three surfaces of equipment at plant B. Four in-process food samples were also contaminated with Siwi isolates. Repetitive extragenic palindromic PCR DNA fingerprinting showed a possible association between strains of different *Listeria* spp. and brand as well as with manufacturing plant B.

Key words: *Listeria*, processed meat.

INTRODUCTION

The presence of *Listeria* spp. in foods, particularly ready-to-eat products, is of significant public health importance. One member of this genus, *L. monocytogenes*, has been responsible for numerous outbreaks of foodborne illnesses with symptoms ranging from potentially deadly infections such as gastroenteritis and meningitis to septic abortions in pregnant women [1–3]. The biggest threat to food safety by this organism is due to its hardiness and ability to resist common methods of food preservation [4]. With the exception of the rarely occurring *L. ivanovii*, which is more often associated with animals than humans [3, 5], other species of *Listeria* are apathogenic including *L. seeligeri*, *L. innocua*, *L. welshimeri* and *L. grayi* [3, 6]. *L. innocua*, although harmless to humans, is of practical importance as it is closely related to *L. monocytogenes*, and is used as an indicator of the presence of the more harmful species [5, 7, 8].

The need to identify *Listeria* to the species level is therefore of utmost importance to food

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manufacturers. Biochemical and serological-based methods have been conventionally used to identify this organism at the genus and species levels. However, these traditional methods are labour intensive and time consuming. With the advent of polymerase chain reaction (PCR)-based diagnostic assays, it is now possible to cost-effectively and quickly confirm identification of different species of *Listeria* [9, 10]. Multiplex PCR utilizing primers that target specific markers such as the *iap* or *prs* genes allows for the simultaneous identification and differentiation of potentially pathogenic *L. monocytogenes* strains from other species [5, 11–13].

PCR-based techniques such as repetitive extragenic palindromic PCR (rep-PCR) can also be used to genotype bacterial isolates and give useful information on genetic relatedness of strains within a species [14]. Rep-PCR is based on generating multiple DNA fragments by amplification of regions between repetitive elements present in bacterial genomes. Distance measures among isolates can be obtained based on presence or absence of amplified fragments (loci) using cluster analysis methods [14]. Primers based on REP, BOX and, ERIC elements have been extensively used for genotyping bacteria, including *Listeria* [15].

The presence of *Listeria* spp. was previously reported in both raw meat samples and post-processed ready-to-eat products at a processing plant in Trinidad [16]. However, there is very little published data on the prevalence of this pathogen in locally produced, ready-to-eat meats sold at retail outlets. A previous study detected *L. monocytogenes* in one of 70 delicatessen meat samples collected from local supermarkets [17] but no information was given on the origin or description of the positive sample.

This study investigated the occurrence and genetic relatedness of *Listeria* spp. in retail products (chicken frankfurters, chicken bologna, bacon) of two locally produced brands (A and B) and the processing plant environment of one of the brands (B). These were the two most popular brands of locally processed meats in Trinidad based on a survey conducted by interviewing managers of supermarkets (data not shown) prior to sample collection and analysis. The plants producing these brands are located on different regions of the island, ~80 km apart. Both are privately owned and are among the largest meat-processing plants in Trinidad. Only the processing environment of plant B was included in the study since approval was not obtained from the management of plant A to conduct investigations at that plant.

METHOD

Isolation of *Listeria* spp.

A total of 480 samples of locally processed bacon, chicken frankfurters and chicken bologna were collected (October 2005 to November 2006) from eight grocery stores in diverse areas in Trinidad and analysed for the presence of *Listeria* spp. using conventional biochemical and serological methods described by Pagotto *et al.* [18]. The samples collected were equally divided between the three products, two brands (A and B) and the grocery stores. Plant B was visited on two separate occasions during the processing of each of the three products. During each visit, raw ingredients, in-process food samples and finished products were collected and analysed for the presence of *Listeria* spp. together with swabs of environmental surfaces, equipment, workers' gloves and coats as indicated above. *Listeria monocytogenes* strain ATCC 7644 was used as a control in all experiments. A single isolate from each positive sample was stored as frozen culture at −80 °C in brain heart infusion broth containing 25% glycerol, for further analysis.

DNA extraction and PCR

After confirmation of the presence or absence of haemolysis in blood agar, a single colony of each isolate was selected and DNA was then extracted using the UltraClean™ Microbial DNA Isolation kit (Mobio Laboratories Inc., USA) according to the manufacturer's instructions. Confirmation of the genus *Listeria* was done by PCR amplification of a ~370-bp region of the *prs* gene using the *Listeria* forward (5′-GCTGAAGAGATTGCGAAAGAAG-3′) and *Listeria* reverse (5′-CAAAGAAACTTGGATTTGCGG-3′) primers [19]. Each reaction (25 μl) contained ~10 ng DNA, 2·5 μl 10× PC2 buffer (supplied by the manufacturer), 1·5 mM $MgCl_2$, 0·2 mM dNTPs, 1 pmol of each primer and 3·125 U Klen*Taq* polymerase (DNA Polymerase Technology Inc., USA). PCR was carried out in a Techne Touchgene Gradient Thermocycler (Techne, USA) using the following conditions: initial denaturation at 94 °C for 2 min followed by 30 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 45 s, with a final extension for 5 min at 72 °C. Reaction mixtures and conditions for

https://doi.org/10.1017/S095026881000172X Published online by Cambridge University Press

the determination of *Listeria* spp. by multiplex PCR were similar as above but primers targeting regions of the *iap* gene were used as described by Bubert *et al.* [5]: MonoA (5′-CAAACTGCTAACACAGCTACT-3′), an upstream primer for the detection of *L. monocytogenes*; Ino2 (5′-ACTAGCACTCCAGTTGTTA-AAC-3′), an upstream primer for the detection of *L. innocua*; Siwi2 (5′-TAACTGAGGTAGCGAGC-GAA-3′), an upstream primer for the detection of the *L. seeligeri–L. welshimeri–L. ivanovii* group (Siwi group); and Lis1B (5′-TTATACGCGACCGAAG-CCAAC-3′), a fixed downstream primer for all species. With these multiplex primers, strains of *L. monocytogenes* were expected to give an amplified fragment of ~660 bp, *L. innocua* ~870 bp and the Siwi group ~1·2 kb [5].

For rep-PCR, the BOXA1R primer (5′-CTACGG-CAAGGCGACGCTGACG-3′) [20] was used. Each PCR reaction (25 μl) contained ~10 ng template DNA, 2·5 μl PC2 reaction buffer (supplied by the manufacturer), 2·5 mM $MgCl_2$, 0·2 mM dNTPs, 5 pmol of BOXA1R primer and 3·125 U Klen*Taq* DNA polymerase (DNA Polymerase Technology Inc.). Cycling conditions were: an initial denaturation for 7 min at 95 °C, 30 cycles of 94 °C for 1 min, 51 °C for 1 min, 65 °C for 8 min, and a final extension at 65 °C for 15 min [20].

Products of *Listeria*-specific PCR (5 μl) and rep-PCR (9 μl) were separated by electrophoresis in 1·5% and 1·0% agarose gels, respectively, and photographed on a UV trans-illuminator [21]. Isolates were scored for the presence or absence of specific bands based on comparison to a 1-kb ladder (Invitrogen Life Technologies, USA).

Non-parametric χ^2 tests were applied to the multiplex data using SPSS software, version 15.0 (SPSS Inc., USA). The binary DNA fingerprinting data was subjected to Cluster analysis with average linkage and Euclidean distance (Minitab statistical software package, version 14.0; Minitab Inc., USA) to generate dendrograms showing relationships between isolates.

RESULTS

Ninety of the 480 (18·75%) retail samples, 24 food samples, and environmental surfaces from plant B were positive for the presence of *Listeria* spp. based on the conventional isolation and identification methods. Of the 114 single isolates obtained from positive samples, 110 (96·5%) isolates were confirmed as belonging to *Listeria* spp. by amplification of the ~370-bp *prs* gene fragment. Two isolates from retail samples and two from plant B did not give the expected ~370-bp band.

The results of the multiplex PCR showed a significantly (χ^2, $P<0·001$) higher number of isolates (64/110) having bands characteristic of *L. innocua* compared to *L. monocytogenes* (39/110) and members of the Siwi group (7/110). Six of the 39 isolates (15·4%) confirmed as *L. monocytogenes* were non-haemolytic on blood agar medium. All of these isolates were recovered from bacon products of brand A. Six of 64 *L. innocua* isolates (9·4%) were also haemolytic on blood agar. Of these, four were obtained from raw chicken bologna mixture from plant B and the remaining two isolates originated from two retail samples from plant A: one bacon and one chicken frankfurter.

Eighty-eight of the 90 isolates from retail samples gave positive PCR results for *Listeria* spp. and of these, 46 (52·3%) belonged to *L. innocua*, 39 (44·3%) to *L. monocytogenes* and three (3·4%) to the Siwi group (Table 1). Overall, *Listeria* spp. were detected in 18·3% of retail samples with prevalence rates of 9·6%, 8·1% and 0·6% for *L. innocua*, *L. monocytogenes* and the Siwi group, respectively. A significantly (χ^2, $P<0·001$) higher number of the isolates were from brand A (68/88) compared to brand B (20/88). There was no statistically significant (χ^2, $P>0·05$) difference in the distribution of species in plant A products; however, plant B products had significantly (χ^2, $P<0·05$) more samples which were positive for *L. innocua* than the other species.

The majority (66/88) of the *Listeria* isolates from retail samples were from bacon, which had a significantly (χ^2, $P<0·001$) higher prevalence of the organism in both brands (Table 1). Bacon also mostly accounted for the presence of *L. monocytogenes* from brand A products (χ^2, $P<0·001$) as well as *L. innocua* from both brands under study (χ^2, brand A: $P<0·05$; brand B: $P<0·001$). The Siwi group was only found in brand B bacon. No *L. monocytogenes* isolate was found in retail items from plant B and no isolate of the Siwi group was detected in plant A products. There were no significant (χ^2, $P>0·05$) differences in association of *Listeria* spp., *L. monocytogenes*, *L. innocua*, or Siwi group with grocery stores and locations.

Of the 24 isolates obtained from plant B using conventional methods, 91·7% (22/24) were confirmed as *Listeria* spp. by amplification of the *prs* fragment. Most of these isolates (18/22) were *L. innocua* while the remaining (4/22) belonged to the Siwi group

https://doi.org/10.1017/S095026881000172X Published online by Cambridge University Press

Table 1. *Distribution of* Listeria *spp. in retail samples of brand A and brand B products*

Brand	Product	*L. monocytogenes*	*L. innocua*	Siwi group	Total
Brand A	Frankfurters	3 (18·8%)	13 (81·3%)	0 (0·0%)	16
	Bologna	1 (50·0%)	1 (50·0%)	0 (0·0%)	2
	Bacon	35 (70·0%)	15 (30·0%)	0 (0·0%)	50
	Total	39 (57·3%)	29 (42·6%)	0 (0·0%)	68
Brand B	Frankfurters	0 (0·0%)	3 (100·0%)	0 (0·0%)	3
	Bologna	0 (0·0%)	1 (100·0%)	0 (0·0%)	1
	Bacon	0 (0·0%)	13 (81·3%)	3 (18·8%)	16
	Total	0 (0·0%)	17 (85·0%)	3 (15·0%)	20

Table 2. *Species of* Listeria *found in plant B's manufacturing environment during the processing of bologna and bacon*

Species	Product	In-process food	Equipment	Total
L. innocua	Bologna	9 (90·0%)	1 (10·0%)	10
	Bacon	6 (75·0%)	2 (25·0%)	8
	Total	15 (83·3%)	3 (16·7%)	18
Siwi group	Bacon	4 (100·0%)	0 (0·0%)	4
	Total	4 (100·0%)	0 (0·0%)	4

(χ^2, $P<0·05$). Of these isolates, most (19/22) originated from in-process food samples and 13·6% (3/22) were obtained from equipment during bacon and chicken bologna pre-cooking processes ($P=0·001$) (Table 2). No *Listeria* spp. was detected in the post-cooking environment and no *L. monocytogenes* was found in the entire plant environment. The Siwi group was only detected during bacon production: in meat on the injector during the first visit, in liquid cure on the second visit and in pumped meat during both sampling occasions.

BOX–PCR fingerprinting

Analysis of rep-PCR banding patterns revealed very diverse populations of the different *Listeria* spp. obtained from the retailed meats and the plant B environment. Isolates from the largest *L. innocua* group separated into two broad clusters in addition to two outlier isolates that were more genomically related to the *L. monocytogenes* reference isolate (Fig. 1). There were seven subgroups with 2–6 isolates having similarity levels of 100%. Most of the isolates (27/35) linked to plant B (retail grocery products, in-process food or equipment) were present in group 1, with the dominant product being bacon followed by bologna. The majority (14/18) of the isolates from in-process food and equipment clustered together in one subgroup, with approximately equal numbers of the isolates in this subgroup coming from samples collected during the processing of bacon and frankfurters. Most (19/29) of the isolates from plant A retail products were in group 2 (Fig. 1). The majority of these isolates were from bacon followed by frankfurters. There was no clear trend of distribution of isolates from retail products based on grocery stores from which the products were collected.

The *L. monocytogenes* isolates clustered into two major groups in addition to one outlier isolate G_1_h9_8 III A-8 and the *Listeria* control forming separate branches (Fig. 2). All the isolates originated from plant A grocery products, with the majority (35/39) from bacon and the remainder from frankfurters and bologna. Group 1 isolates were from six different grocery stores and included two subgroups with three and seven isolates that had identical DNA fingerprints. Group 2 isolates were from seven different grocery stores and had four groups which contained 2–9 isolates with identical DNA fingerprints. The subgroups containing isolates with identical fingerprints generally had isolates originating from different stores, for example, the seven isolates in the larger subgroup in group 1 came from four different stores and were mainly from bacon products, with the exception of one isolate from chicken frankfurters. Similarly, the largest subgroup in group 2 had nine isolates from four different stores which were mainly from bacon items together with one from frankfurters and one from bologna.

Within the Siwi group, only one subgroup contained two isolates with 100% similarity (Fig. 3). Both of these isolates originated from bacon food samples obtained from the plant environment, each from a separate sampling visit. The processing plant

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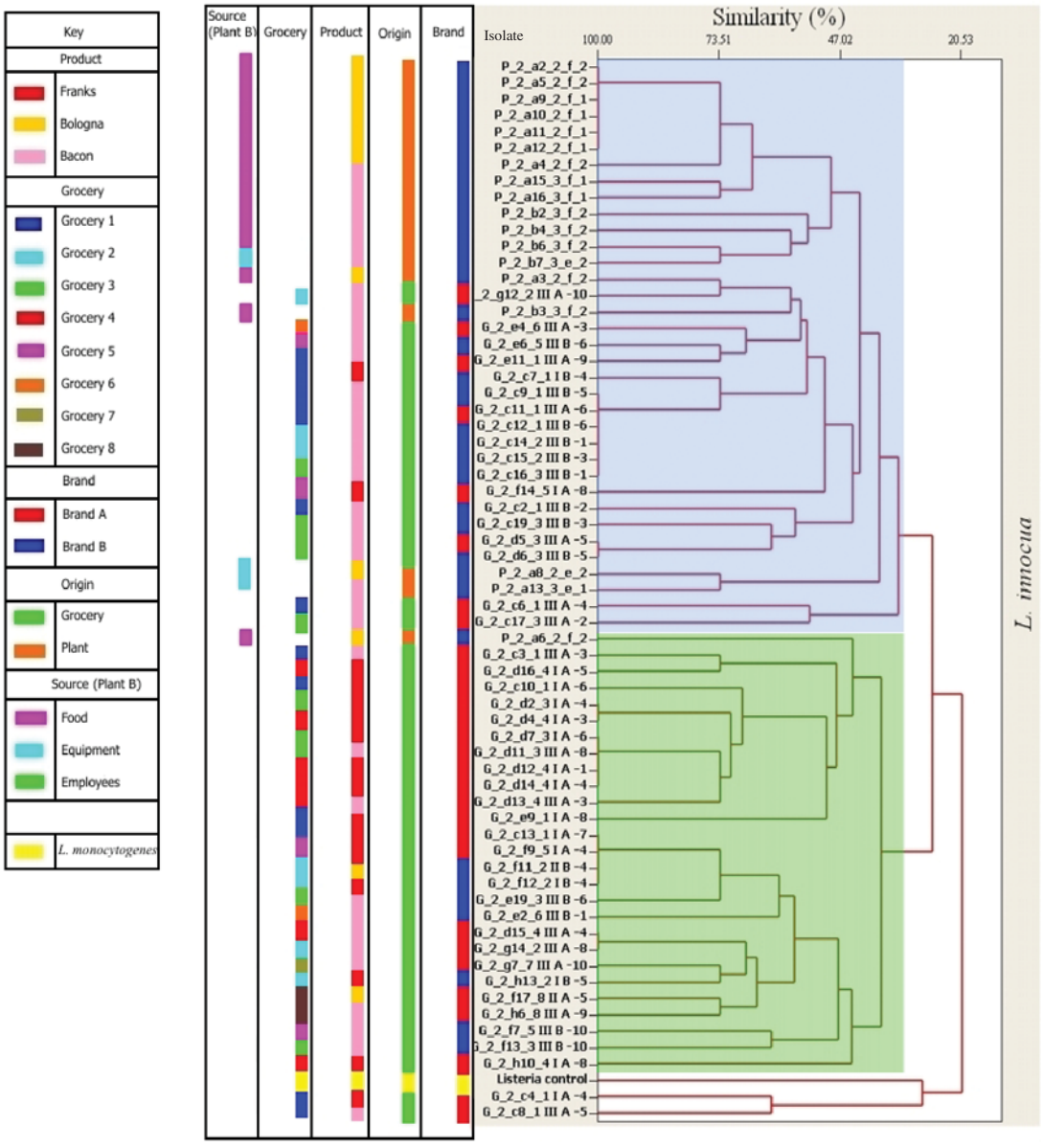

Fig. 1. Dendrogram showing clusters among *Listeria innocua* isolates. Group 1 isolates are coloured blue in the dendrogram and group 2 isolates green.

isolates generally clustered in a separate group from the grocery isolates, which were more diverse.

DISCUSSION

The results of the study show the occurrence of typical as well as atypical *Listeria* spp. in retail ready-to-eat meats and in-process food samples in Trinidad. Although there was a strong concurrence observed between haemolysis on blood agar and *L. monocytogenes* detected by molecular methods, certain strains were not haemolytic on blood agar plates. These strains may have been non-haemolytic variants which have been previously reported and attributed to a genetic mutation in the *listeriolysin* gene [22, 23]. A few *L. innocua* isolates also showed haemolysis on blood agar, which Volokhov *et al.* [24] suggested may be as a result of retention of genes from *L. monocytogenes*, its possible ancestral predecessor.

The study has shown that consumers of locally processed, ready-to-eat meats may be at risk due to contamination by *Listeria* spp. including *L. monocytogenes*. The detection of this pathogen led to a voluntary recall of chicken frankfurters, spice ham and turkey ham processed at one plant in Trinidad in 2003 [16]. The potential threat to public health due to

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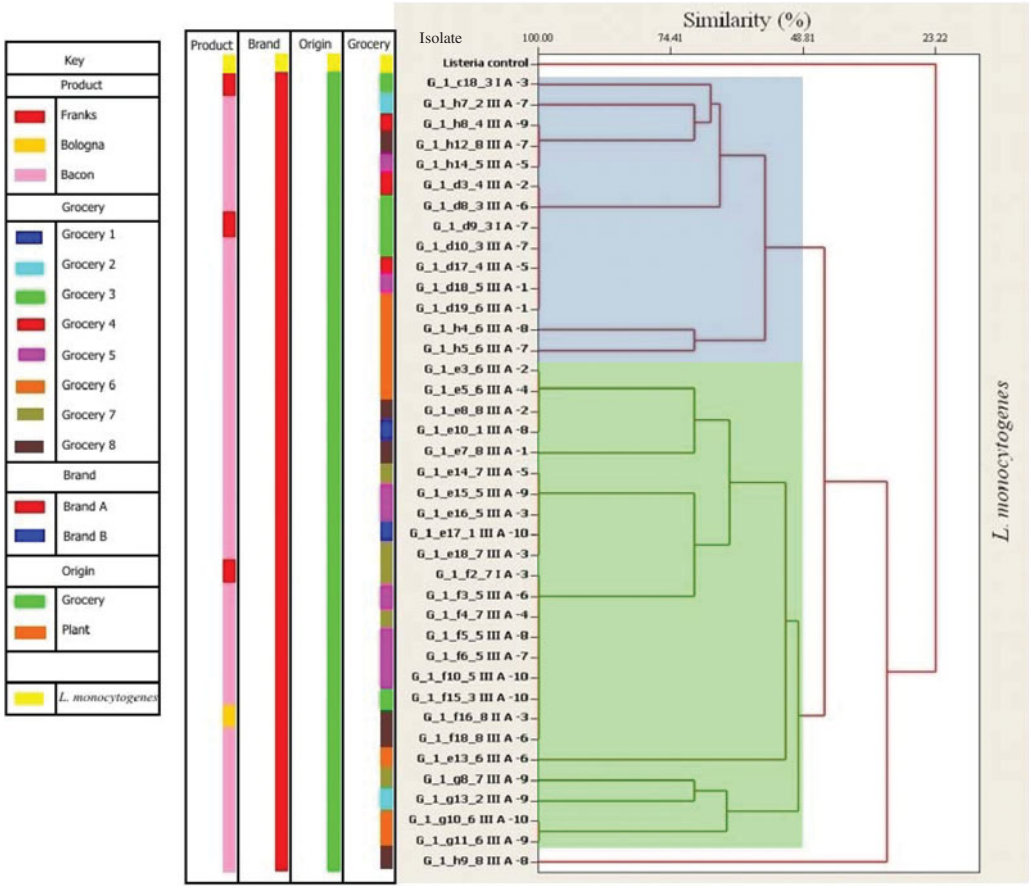

Fig. 2. Dendrogram showing clusters among *Listeria monocytogenes* isolates. Group 1 isolates are coloured blue in the dendrogram and group 2 isolates green.

contamination of ready-to-eat meats thus still exist since either *L. monocytogenes* or the indicator *L. innocua* [8] was found in all three product types from the two different brands investigated (Table 1). The 8·1 % prevalence rate of *L. monocytogenes* in retail samples from this study was similar to the findings of a study conducted in Greece, which also found that 8·1 % of samples of ready-to-eat meat products were contaminated with *L. monocytogenes* and that bacon had the highest level of association with the pathogen [25]. However, the prevalence rate determined in this study was higher than the 3–5 % contamination rates found for various categories of ready-to-eat meats from retail markets in Edmonton, Canada [26].

Both plants A and B, but particularly the former, need to revise their quality assurance programme. Of the three products investigated, bacon was the most prone to contamination by *Listeria* spp. This may be attributed to its high fat content which could have protected bacteria throughout manufacture, as well as poor quality assurance programmes implemented in the manufacturing operations [27–30]. Brand A products had a higher risk of exposing consumers to *Listeria* compared to brand B products. The fact that most of the *Listeria* spp. and all of the *L. monocytogenes* isolates came from products manufactured by plant A leads to the inference that the quality assurance programme in this plant was less effective than the programme in plant B. However, it must be noted that the study only included a limited number of samples over a limited period of time. Thus, further monitoring may be needed to ascertain whether there may be higher risks associated with products from plant B and other plants in Trinidad as well as imported brands.

Among grocery stores, the Siwi group was only found in brand B bacon products. The strains were found again in raw material and equipment during the manufacture of bacon in plant B, which suggests a linkage in contamination from plant to grocery. The persistence and survival of specific strains of this group in the plant environment, as well as their ability to contaminate different batches of product could also be inferred from the finding that two strains with

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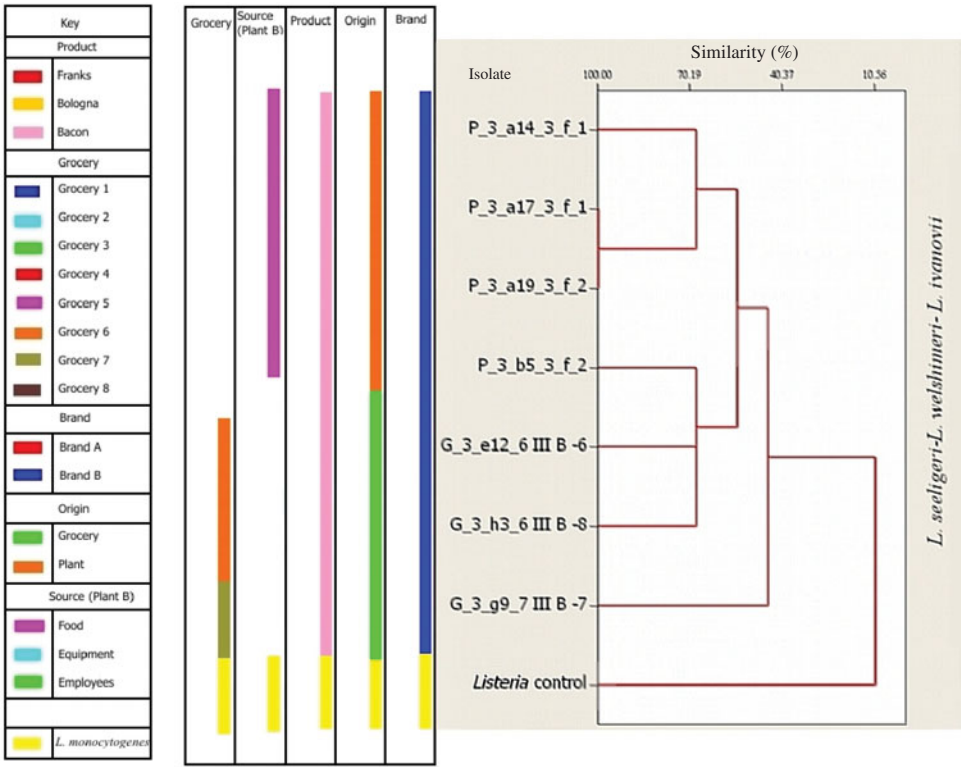

Fig. 3. Dendrogram showing clusters among isolates of the *L. seeligeri–L. welshimeri–L. ivanovii* group.

100 % similarity were obtained in food samples from different sampling visits.

The presence of *Listeria* spp. in raw materials such as the liquid cure and the raw chicken meat indicates the possibility that contamination of retail products could have originated from the raw material. Hinton *et al*. [31] reported survival of bacteria during processing as well as cross-contamination of post-processed broiler carcasses from raw materials. Contamination of sausages and cold cuts has also been attributed to the presence of pathogens in raw material [32, 33].

No finished products at plant B were positive for *Listeria* spp. despite the organism's presence on environmental surfaces, raw materials and retail items from grocery stores. If in fact there were low levels of contamination of finished products in the plant, it is also possible that the organism may not have been detected due to limited sensitivity of the analytical methods. However, bacterial levels could increase to within the limits of detection if unsuitable storage conditions existed in grocery stores since *Listeria* spp. is known to be able to grow at relatively low temperatures [34]. It must be noted that the existence of *Listeria* on surfaces is a serious risk for cross-contamination of finished products and is suggestive of the need for a better quality assurance programme. The minimum infective dose of *L. monocytogenes* in humans has not been established, although a review of the literature by Farber & Peterkin [1] indicated infection of healthy individuals occurred due to consumption of food contaminated with $2{\cdot}7 \times 10^6$ organisms/g and as little as 10^2–10^4 organisms/g for immunocompromised individuals. Thus, even low levels of contamination by *L. monocytogenes* at the plant may be a potentially serious health risk to susceptible individuals.

On observation of the DNA fingerprints, it was found that isolates which originated from the processing plant and those from retail samples did not generally have identical banding patterns. However, most of the *L. innocua* isolates segregated based on brand (Fig. 1). Additionally, isolates from the processing samples in plant B clustered together with isolates from retail items from this plant. This together with the fact that the retail samples came from several different grocery stores suggest that there is a link between the plant and contamination of the products. Other studies have also shown contamination of processed meats with *L. monocytogenes* and spoilage organisms have been due to cross-contamination from the manufacturing environment [35, 36]. Further

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influence of plant conditions on retail samples could be corroborated in this study as no *L. monocytogenes* was found in plant B and no *L. monocytogenes* species were found in brand B retail samples.

Some *L. innocua* strains obtained from brand A retail products were more closely related to isolates from plant B's samples, as seen in three subclusters which had isolates from brand A and brand B samples with 100% similarity (Fig. 1). Strains of *Listeria* are known to occur widely in different environments and could be commonly associated with raw meat from different areas and sources [37]. It is possible that both plants A and B may have common suppliers for imported raw materials resulting in the similar strains being present in both products.

The environmental tolerance of different *Listeria* spp., coupled with the nutritional composition of different meats may have also influenced which species or strains were found in a particular product. Boyer *et al*. [38] showed that *L. monocytogenes* was significantly more resilient to environmental stresses than *L. innocua*. This, coupled with the tendency of fat from pork to protect bacteria, could possibly explain in part, the relatively high prevalence of *L. monocytogenes* found in bacon [29, 30].

With respect to the cluster analysis of *L. monocytogenes*, the dendrogram (Fig. 2) indicates the possible existence and persistence of similar strains of the pathogen in plant A's manufacturing environment and the potential for these to contaminate several different products. In two subgroups with 100% similarity, strains which were mostly obtained from bacon were also present in chicken frankfurters and bologna. These particular strains may have survived in niches in the plant and could have been transferred to other products by employees or inadequately cleaned communal equipment. The fact that the *L. monocytogenes* with identical DNA fingerprints were recovered from different grocery samples (Fig. 2) further supports the link of product contamination to the processing environment.

There is also evidence of association of specific strains of *Listeria* spp. with plant B's environment. Strains of *L. innocua* showing 100% similarity were obtained on two separate sampling occasions about 1 week apart from pre-cooked bologna food samples. Considering that each piece of machinery is cleaned on a daily basis, it is possible that this specific strain of *L. innocua* could have persisted over time in biofilms [28] on equipment and the environment. Therefore, through either cross-contamination or contact with contaminated machinery bacterial cells could have been successfully transferred to new batches of food material during bologna manufacture. Alternatively, considering that sampling occasions were taken within a relatively close space of time, the same batch of raw material contaminated with that specific *Listeria* strain could have been used to manufacture bologna within a 2-week period. Similarly, one *L. innocua* subgroup with 100% similarity consisted of isolates which originated from two different brand B retail products: one chicken frankfurters and one bologna (Fig. 1). This supports the idea of bacterial *Listeria* surviving as biofilms [28] since the manufacture of both products utilized common plant equipment. These scenarios complement a similar study performed in a large meat-processing plant in Trinidad which attributed the presence of biofilms and lapses in good sanitary practices to the production of items contaminated with *Listeria* [16].

The Siwi group was the least prevalent in both retail and plant samples. This species group has been documented to occur very rarely in nature and this was reflected in its limited presence during this study [5]. However, the presence of the Siwi group may be of some concern as *L. ivanovii*, a member of this group, is considered pathogenic even though it rarely occurs in humans [3].

Further research is needed to determine the extent of *Listeria* contamination of ready-to-eat meats in Trinidad by including other locally produced brands as well as imported brands. However, from this limited study, a clear risk to consumers has been identified together with a possible link between processing plants and quality of retail products. Processors should improve the quality of their products by stringent implementation or reinforcements of programmes such as Good Manufacturing Practices, Good Hygiene Practices and Hazard Analysis and Critical Control Point programmes [32] in order to enhance the safety of consumers.

ACKNOWLEDGEMENTS

This study was supported by a financial grant from the School for Graduate Studies and Research, The University of the West Indies, St Augustine, Trinidad.

DECLARATION OF INTEREST

None.

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