The microbial ecology of high-risk, chilled food factories; evidence for persistent *Listeria* spp. and *Escherichia coli* strains

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ABSTRACT

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Aims: The intention of this study was to provide evidence of any *Listeria* spp. or *Escherichia coli* strain persistence, and to identify whether strains of these organisms adapt to specific environmental or product niches in food factories.
Methods and Results: A 3-year assessment of the microbial ecology of four, ready-to-eat food-processing factories was undertaken in which approx. 196 000 and 75 000 product and environmental samples were examined for *Escherichia coli* and *Listeria* spp. respectively. A total of 152 *E. coli* isolates (44 environmental and 108 product in 62 ribogroups) and 260 *Listeria* spp. isolates (174 environmental and 86 product in 30 ribogroups) were identified and ribotyped. The overall prevalence of *E. coli* (0·08%), all *Listeria* spp. (0·35%) and *L. monocytogenes* (0·23%) was very low. Some 10 *E. coli* ribogroups and 14 *Listeria* spp. ribogroups showed evidence for persistence, defined as the isolation of the same strain, from the same site, over a prolonged time period. The majority of *E. coli* strains were product niche oriented whilst the majority of *Listeria* spp. strains were environmental niche oriented.
Conclusion: Current UK high-risk food factory designs, personnel hygiene and cleaning and disinfection regimes are sufficient to control *Listeria* spp. and *E. coli* to very low levels.
Significance and Impact of the Study: Persistent strains of these organisms, however, can remain within factory high-risk production areas over considerable time periods, warranting an examination of the strain persistence mechanisms and alternative hygiene controls.

Keywords: ecology, *Escherichia coli*, *Listeria* spp., niche, persistence.

INTRODUCTION

Microbial growth on food industry surfaces can be defined as a consortium of micro-organisms (which may occasionally include pathogens) developing within a defined time period, depending on the availability of water and nutrients and the cycle of cleaning and disinfection programmes. Even after thorough cleaning and disinfection in high-risk chilled food factories, in which hygiene practices are most extensive, field trials in eight factories in which both open surfaces and poor hygienic design features of food-processing equipment were sampled, indicated an average count of 2500 CFU per swab (Holah 2000). It has always been assumed that such consortia, including pathogens and spoilage organisms, become one source of the food product flora. The difficulty with this assumption, however, is that the microbiologist's ability (until the last 10 years or so) to exactly characterize or 'fingerprint' micro-organisms beyond species, and thus potentially trace contamination from the food environment to the food product, was limited. In particular, there was little evidence that micro-organisms could survive and grow both on environmental

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surfaces and in product and that surface consortia were persistent (surviving for long time periods in the consortia) rather than transient (appearing in the consortia on a day-to-day basis from a range of sources).

Using molecular typing techniques, evidence that micro-organisms may differ in their ability to colonize various sites or niches was suggested by Michiels *et al.* (1997) who analysed 80 fluorescent pseudomonad strains obtained from minced turkey meat and a meat mincer. Random amplification of polymorphic DNA (RAPD) analyses demonstrated that 17 mincer surface strains were significantly different to 63 meat strains, i.e. there may be different strains colonizing product and environmental niches. In a similar study in a shrimp-processing plant, Destro *et al.* (1996) used RAPD and pulsed-field gel electrophoresis to identify 115 isolated *Listeria monocytogenes* strains. They also noted that none of the strains recovered from the environment, process water or utensils was recovered from the shrimps. A study by Lawrence and Gilmour (1995) isolated 289 *L. monocytogenes* strains from both the raw and cooked processing areas of a poultry plant which were subsequently typed by RAPD. The majority of strain profiles were found in the incoming raw poultry, in cooked poultry and on environmental samples from both raw and cooked processing areas. This suggests that it may be possible for some strains to grow both on product and on environmental surfaces, although six profiles present in the cooked product environment were not subsequently found in cooked product. Similar findings of presence on surfaces and in product, together with surface exclusive strains, were shown by Dodd *et al.* (1988) for *Staphylococcus aureus* strains differentiated using plasmid profiles on raw poultry at slaughter.

Evidence for strain persistence has been most frequently demonstrated for *L. monocytogenes*. Persistence has been noted for 'months' (Jacquet *et al.* 1995; Salvat *et al.* 1995; Ojeniyi *et al.* 2000; Chasseignaux *et al.* 2001; Norton *et al.* 2001), 8 months (Rørvik *et al.* 1995), 11 months (McLauchlin *et al.* 1990), 14 months (Johansson *et al.* 1999), 1 year (Lawrence and Gilmour 1995), 17 months (Pourshaban *et al.* 2000), 2 years (McLauchlin *et al.* 1991), 3 years (Brett *et al.* 1998), 40 months (Unnerstad *et al.* 1996), 4 years (Nesbakken *et al.* 1996; Aase *et al.* 2000; Fonnesbech Vogel *et al.* 2001), 7 years (Unnerstad *et al.* 1996; Miettinen *et al.* 1999) and 10 years (Kathariou 2003). These authors have, between them, demonstrated persistent strains in the following food products and processing facilities: cheese; mussels, shrimps and raw and smoked fish; fresh, cooked and fermented meats; pâté and pork tongue in aspic; chicken and turkey meat; and pesto sauce.

Working in close co-operation with selected UK chilled food manufacturers, the intention of this study was to closely monitor the microbial ecology of a number of high-risk food-processing areas (i.e. segregated zones following heat processing and up to primary packaging), in selected factories within a 3-year period, with particular attention to *Listeria* spp. and *Escherichia coli*. As such, the study had two main objectives: (i) to provide evidence of any strain persistence, and (ii) to identify whether strains of selected species adapt to specific environmental or product niches. In addition, it was recognized that in achieving these two objectives, an assessment of the prevalence of *Listeria* spp. and *Escherichia coli* in factory high-risk production areas could also be obtained.

MATERIALS AND METHODS

Selection of sites

Chilled food manufacturers were selected who were willing to provide both access for factory-based food product and food environment sampling, and the sharing of their own, routine, product and environmental sampling data. Two factories, sites 1 and 2 (both producing a large range of ready-to-eat meals), provided data for the whole of the 3-year study period. Site 4 (sandwiches) provided data for 24 months and site 3 (cooked meats) for 9 months. Each factory was visited on prearranged occasions to undertake product sampling and environmental swabbing: approximately every 3–4 months for the three long-term factories and on four occasions for the 9-month study. Together, the factories prepared a wide range of ready-to-eat meals (meat; sandwiches; dairy; meat or vegetable based with pasta, rice or potato) and thus the chance of detecting strains possibly associated with particular ingredients was maximized. In addition, their own or contract laboratories routinely analysed product and environmental samples for *Listeria* spp. and *E. coli*.

Product and environmental sampling

Product and environmental sampling was undertaken both by the authors and the factories' QA/Microbiology Departments. For the four factories, environmental and product sampling was undertaken as part of their routine sampling plans by operatives trained in microbiological sampling techniques. The methods of each of the four factories were all based on the use of cotton swabs and isotonic transport fluids containing a neutralizer appropriate for the disinfectant(s) used in the factories. Campden and Chorleywood Food Research Association (CCFRA) and factory staff undertook joint exercises with the companies at the start of the programme to ensure similarities in swab sampling and environmental sampling points were generally chosen to maximize the detection of the target micro-organisms. For all sites, sampling consisted of both open, flat surfaces and areas of poor hygienic design that were difficult to clean (e.g.

crevices and dead areas). Minor differences in sampling technique were not regarded as relevant to the detection of target micro-organisms. For CCFRA samples, sterile wooden swabs (F150CA, Bibby Sterilin Ltd, Stone, UK) were used. Where flat surfaces were sampled, an area of approx. 5×5 cm was swabbed. The swabs were premoistened in 9 ml of sterile maximum recovery diluent (LabM: LAB103) (LabM IDG, Bury, UK) and 1 ml universal inactivator (lecithin, 3 g l^{-1}; polysorbate 80, 30 g l^{-1}; sodium thiosulphate, 5 g l^{-1}; L-histidine, 1 g l^{-1}; saponin, 30 g l^{-1} in phosphate buffer 0·25 mol l^{-1} made up as 34 g KH_2PO_4 added to 500 ml water, pH adjusted to pH 7·2 ± 0·2 with NaOH 1 mol l^{-1} and made up to 1000 ml), transported in a cool box containing frozen 'ice packs' and processed within 6 h. During this 6 h time period, temperature monitoring by a Squirrel 1000 Series datalogger (Type 1025, Grant Instruments, Scientific laboratory Supplies, Nottingham, UK) indicated that the coolbox maintained an air temperature of 6°C (data not shown).

Product samples (for CCFRA and all factory sites) were taken from either individual ingredients (in the high-risk area) or the final ready meal. Food samples, transported and stored at 5°C prior to examination, were prepared following BS EN ISO 6887-1:1999 (Anon. 1999). The authors' swab samples were identified according to date, sampling site and factory. Product samples were similarly identified by date, ingredient and/or line (for ingredient samples), batch code (for final product samples) and factory. Any comments specific to the sample (e.g. taken during cleaning operations) were also recorded at the time of sampling. Food factory/contract laboratory-confirmed *Listeria* spp. or *E. coli* samples were identified by laboratory code, sampling site/product or ingredient type, date and factory.

Isolation and identification

The authors and the four laboratories of the factories and or contract laboratories all used isolation and identification methods that were approved by the relevant UK accreditation bodies. Swab diluent samples, after vortexing for 30 s, or product stomacher subsamples were added to appropriate selective and nonselective growth media for the detection of the target micro-organisms. Essentially, for both product and environmental samples, the same selective and nonselective growth media were used for the detection of target micro-organisms.

Detection of Listeria *species.* Detection of *Listeria* spp. was undertaken using the FDA, 30°C enumeration technique (Anon. 1988) for CCFRA and sites 2 and 4. This method involves a selective enrichment stage in Listeria Selective Enrichment Broth followed by isolation of colonies on Listeria Oxford Agar. Typically, colonies showing characteristic morphology were picked off appropriate plates and checked for purity by Gram stain, and catalase and oxidase reaction. Gram-positive, catalase-positive and oxidase-positive strains were re-streaked onto Oxford media. Sites 1 and 3 also used a selective enrichment stage in Listeria Selective Enrichment Broth but followed this with *Listeria* spp. presumptive identification by an ELISA technique according to AOAC Method No. 994·03 (AOAC 1998).

Detection of E. coli. Detection of *E. coli* was undertaken following ISO 7251 (Anon. 1998) for CCFRA and sites 1, 3 and 4. This method uses Lauryl Sulphate Tryptone Broth as a selective enrichment broth for coliforms followed by *E. coli* Broth incubated at 45°C for 24 and 48 h as a selective enrichment broth for *E. coli*. Site 1 used a modification of this method using Fluorocult Agar and ultraviolet light to distinguish presumptive *E. coli*. Site 2 used a combined *E. coli*/coliform Petrifilm® (3M™ Healthcare, Loughborough, UK) method based on AOAC 991·14 (AOAC 1998).

Ribotyping. Isolates presumptively identified by the four laboratories of the factories as *E. coli* or *Listeria* spp. were held on refrigerated slopes until periodic collection and transporting to CCFRA. Factory and CCFRA isolates were coded with a unique CCFRA identification number and stored prior to ribotyping on slopes at 5°C, on the appropriate media: *Listeria* spp. (Oxford) and *E. coli* (NA). Prior to ribotyping, the isolates were streaked on to Brain Heart Infusion Agar (BHIA) (LabM: Lam 48). From the BHIA, one colony pick was touched onto the surface of a well-isolated colony and placed in a sterile microtube containing 200 μl lysis buffer (DuPont, Willington, DE, USA). The pick was vortexed in the buffer to produce a sample suspension using a BioVortexer (Biospec Products, Bartlesville, OK, USA) and 30 μl of suspension was transferred to a RiboPrinter® (Riboprinter™ Microbial Characterisation System, DuPont) sample carrier. A maximum of eight samples was processed in each batch. The sample carrier was incubated at 80°C for 10 min to denature the proteins in the bacterial cell before Lysostaphin (5 μl) and N-acetylmuramidase (5 μl) enzymes were added to lyse the cells. The sample carrier was then loaded into the RiboPrinter® and processed automatically using *Eco*RI to digest released DNA.

The RiboPrint® pattern of each isolate was compared with other patterns in the CCFRA database. Assignment to a particular ribogroup is based upon similarities in both band position and signal intensity. Each sample RiboPrint® pattern was given a ribogroup code, e.g. RIBO1 102-195-S-1, in which RIBO1 = sample database library, 102 = instrument number, 195 = batch number, S-1 = sample 1 in the batch (usually eight samples per batch). After the

isolates had been formally characterized, the organisms were placed on to beads (Protect, Technical Service Consultants Ltd, Heywood,UK) and stored at −80°C.

RESULTS

Sampling

Table 1 indicates the total number of samples that were analysed for *E. coli* and *Listeria* spp. over the 3-year duration of the project by CCFRA and the laboratories of the factory. The total number of samples taken for *E. coli* was 196 204 and *Listeria* spp. was 74 344. This is because factory 1, which processed 138 500 product samples for *E. coli*, only processed 16 640 for *Listeria* spp. The overall ratio of product to environmental samples in the study is 6·4 : 1.

A total of 479 environmental swab samples were taken by CCFRA. These included 82 product contact surfaces (conveyor belts, transfer vessels and product trays); 117 equipment (nonproduct contact including wheels on trolleys and maintenance equipment); 48 cleaning equipment (squeegees, scrapers and tray washers); 176 environment (floors, drains, doors, walls and light fittings); 36 personnel (hands, switches and footwear) and 20 chiller (blast chiller trays and fridge doors) samples. The number of isolates, ribogroups, samples taken and the percentage rate of isolation of *E. coli*, *Listeria* spp. and *L. monocytogenes* are shown in Table 2.

Table 1 Estimation of the total number of samples assessed by the authors and factory laboratories

	Sampling by CCFRA		Sampling by site	
Factory	Environment	Product	Environment	Product
1	210	6	15 600*	138 500†
2	150	11	2800	4200
3	50	5	3600	8640
4	69	3	4160	18 200
Total	479	25	26 160	169 540

*Estimates of factory site samples were based on the minimum number of samples taken on the factory's weekly product and environmental sampling plans.
†138 500 samples were taken for *E. coli* from site 1 but only 16 640 were taken for *Listeria* spp.

Table 2 Isolate, ribogroup and percentage of isolation rate details for the targeted strains sampled

	No. of isolates	No. of ribogroups	Total no. of samples	Rate of isolation (%)
E. coli	152	62	196 204	0·08
Listeria spp.	260	30	74 344	0·35
L. monocytogenes	170	18	74 344	0·23

Escherichia coli

The overall isolation rate of *E. coli* from CCFRA and factory samples was 0·08%. Of the 152 *E. coli* isolates, 44 were from the environment and 108 from food products. Of these, 119 isolates (78·3%), in 62 ribogroups, were positively identified by the RiboPrinter® as *E. coli*. Forty-nine ribogroups (79·0%) were found only once. For the other 13 ribogroups, the ribogroup, number of times isolated, site(s) from which the isolate was found, the time span from which the isolate was sampled and whether the isolate was sampled from the environment or from product, are shown in Table 3.

The data in Table 3 shows that of the strains isolated more than once from site 1 three were also found in sites 2 or 3. Unique strains, isolated more than once, were not found in sites 2, 3, or 4. One strain, ribogroup 102-248-S-4, was dominant (35·5% of isolates), being isolated on 54 occasions and being present in three sites. Strains were isolated predominantly from the product with some strains, e.g. ribogroups 114-1132-S-5 and 114-1357-S-1, exclusively so. The other major strains, in terms of isolate numbers, were found predominantly (82%) in product and occasionally in the environment. Therefore, there is some evidence for *E. coli* being product niche specific with some strains also being able to survive in the environment. Evidence for persistence, defined as the isolation of the same strains from the same site over a prolonged time period, was observed within the strains isolated on more than three occasions, with some strains, particularly ribogroups 102-248-S-4 and 102-248-S-5, being regularly found over 15 and 11 month periods respectively. Persistence was only observed in site 1;

Table 3 Summary table of *E. coli* isolates

Ribogroup	No. of times isolated	Sites	Isolation time span (months)	Environment/ product isolate
102-248-S-4	54	1, 2, 3	15*	10/44†
102-248-S-5	11	1, 2	11	2/9
114-1132-S-5	7	1	14	0/7
114-1254-S-4	6	1, 3	3	1/5
114-1357-S-1	5	1	1	0/5
102-224-S-1	5	1	6	1/4
114-1232-S-4	3	1	8	1/2
114-1232-S-7	3	1	5	0/3
114-1242-S-4	3	1	2	0/3
102-204-S-1	2	1	*	1/1
102-248-S-7	2	1	3	1/1
114-1253-S-4	2	1	*	0/2
114-1311-S-5	2	1	*	1/1

*If no figure is indicated in the time span column, the isolate was only found on one sampling occasion but from two or more samples.
†The isolates were obtained from a total of 26 639 environmental and 169 564 product samples.

however, although this may be a reflection of the limited number of isolates found at the other sites. It is interesting to note that the three strains isolated from sites 2 and 3 were found in the environment only.

Listeria spp.

The overall isolation rate of *Listeria* spp. from CCFRA and factory samples was 0·35%. From both CCFRA and factory samples, 270 *Listeria* spp. isolates were identified and ribotyped. Of the 270 isolates, 260 isolates (96·3%), 174 from the environment and 86 from food products, in 30 ribogroups, were positively identified by the RiboPrinter® as *Listeria* spp. A total of 170 isolates in 18 ribogroups were identified as *L. monocytogenes*, 60 isolates in six ribogroups as *L. innocua*, 16 isolates in three ribogroups as *L. welshimeri* and 14 isolates in three ribogroups as *L. seeligeri*. Of the *L. monocytogenes* isolates, 110 were collected from site 1 (0·34% of samples positive), 44 (0·63%) from site 2, 4 (0·03%) from site 3, and 11 (0·05%) from site 4.

From the collected data, 12 ribogroups were found once (40·0%). For the other 18 ribogroups, the ribogroup, number of times isolated, site(s) from which the isolate was found, the time span from which the isolate was sampled and whether the isolate was sampled from the environment or from product, are shown in Table 4.

The data in Table 4 shows that, as for *E. coli*, the majority of *Listeria* strains were isolated from site 1. Unlike *E. coli* many *Listeria* strains were found in other sites as well. Only one ribogroup (114-830-S-7) was unique to other than site 1, being found in site 3. Again, as for *E. coli*, one *L. monocytogenes* strain, ribogroup 102-195-S-1, was dominant (31·2% of isolates), being isolated on 81 occasions and being present in all four sites. *L. monocytogenes* (67%) and *L. seeligeri* (85%) strains were isolated mainly from the environment, with some strains, e.g. ribogroups 114-997-S-7, 102-409-S-3 and 114-1249-S-5, exclusively so. Two of the *L. monocytogenes* strains, ribogroups 102-195-S-1 and 102-231-S-7, were found substantially in both product and environment niches. Strains of *L. welshimeri* and *L. innocua* were found in the environment and product. No *Listeria* spp. strain, isolated more than once, was found solely in product.

Of the 18 *Listeria* ribogroups, 14 showed evidence for persistence, with persistence ranging from 3 (102-251-S-3) to 25 months (102-231-S-1) with a mean persistence of 13·8 months. *Listeria* spp. were much more widespread across factory sites than *E. coli*, and the persistence of *Listeria* spp. strains over the sites is shown in Table 5. The results in Table 5 indicate that all *Listeria* species can be persistent in more than one factory site, although no one species appeared more persistent across sites than the others. No strain was persistent in a factory site if it was not also persistent in site 1.

In total, the RiboPrinter® was able to positively identify 78·3 and 96·3% of *E. coli* and *Listeria* spp. isolates respectively; the technique was therefore able to fingerprint the vast majority of isolates and hence maximize ecological information for these organisms.

Table 4 Summary table of *Listeria* spp. isolates

Species	Ribogroup	No. of times isolated	Sites	Isolation time span (months)	Environmental/ product isolate
L. monocytogenes	102-195-S-1	81	1, 2, 3, 4	21*	44/37†
L. monocytogenes	102-231-S-7	24	1, 2	15	15/9
L. monocytogenes	102-265-S-3	14	1, 4	21	10/4
L. monocytogenes	114-997-S-7	15	1	8	15/0
L. monocytogenes	102-409-S-3	9	1	11	9/0
L. monocytogenes	114-1249-S-5	7	1	13	7/0
L. monocytogenes	102-241-S-1	3	1	12	3/0
L. monocytogenes	114-1741-S-5	4	1, 4	*	2/2
L. monocytogenes	114-830-S-7	2	3	*	2/0
L. monocytogenes	114-1241-S-5	2	1	*	2/0
L. welshimeri	102-251-S-3	13	1, 4	3	4/9
L. welshimeri	102-234-S-3	3	1, 2, 4	*	2/1
L. seeligeri	102-197-S-3	11	1, 2	10	10/1
L. seeligeri	102-370-S-7	2	1	9	1/1
L. innocua	102-224-S-3	18	1, 4	19	12/6
L. innocua	102-350-S-7	18	1, 4	16	12/6
L. innocua	102-231-S-1	13	1	25	9/4
L. innocua	102-398-S-6	9	1, 2	13	8/1

*If no figure is indicated in the time span column, the isolate was only found on one sampling occasion but from two or more samples.

†The isolates were obtained from a total of 26 639 environmental and 47 705 product samples.

Table 5 Persistence of *Listeria monocytogenes* across factory sites and persistence of other *Listeria* spp.

	Location	No. of times isolated		Isolation time span (months)
		Environment	Product	
Listeria monocytogenes isolate details				
Ribogroup 102-195-S-1	Site 1	23	7	21*
	Site 2	15	28	10
	Site 3	2	0	*
	Site 4	4	2	21
Ribogroup 102-231-S-7	Site 1	14	9	15
	Site 2	1	0	*
Ribogroup 102-265-S-3	Site 1	10	2	13
	Site 4	2	0	3
Ribogroup 114-1741-S-5	Site 1	2	0	*
	Site 4	0	2	*
Listeria spp. isolate details				
L. welshimeri ribogroup	Site 1	1	9	4
102-251-S-3	Site 4	3	0	3
L. welshimeri ribogroup	Site 1	1	0	*
102-234-S-3	Site 2	1	0	*
	Site 4	0	1	*
L. seeligeri ribogroup	Site 1	1	1	8
102-197-S-3	Site 2	9	0	10
L. innocua ribogroup	Site 1	5	3	3
102-224-S-3	Site 4	7	3	19
L. innocua ribogroup	Site 1	7	3	21
102-231-S-1	Site 4	2	1	*
L. innocua ribogroup	Site 1	2	1	12
102-350-S-7	Site 4	10	5	19
L. innocua ribogroup	Site 1	4	1	13
102-398-S-6	Site 2	4	0	*

*If no figure is indicated in the time span column, the isolate was only found on one sampling occasion but from two or more samples.

DISCUSSION

This work reports on a collaborative study between CCFRA and four selected chilled food manufacturers. From the beginning of the work, the management of the factories and their own or contract microbiological testing laboratories were aware of the objectives of the study, i.e. the reporting and analyses of *E. coli* and *Listeria* spp. isolates, and were open with their microbiological sampling programmes and results. There is no suggestion that the degree of cleaning and disinfection prior to sampling and/or the nature of the sampling itself, by either CCFRA or the factories, was anything other than routine. From the 196 000 samples taken in four high-risk chilled food factories over the 3-year study period, the overall incidence of *E. coli* and *L. monocytogenes*, at 0·08 and 0·23% respectively, is very low. However, it should be noted that these *Listeria* spp. and *E. coli* product contamination rates are only estimates. The number of samples recorded (Table 1) was the minimum number of samples taken based on the factory's weekly product and environmental sampling plans. In effect, this is likely to be an underestimate of samples as, if *Listeria* spp. or high levels of *E. coli* were detected, sampling levels were typically increased for a short time period afterwards until the factory had demonstrated that the incidence of the pathogen was sporadic. Minor differences in the methods used by the authors and the factories may also have led to different isolation rates. Finally, and perhaps the largest variation, is that it is unlikely that CCFRA received all of the *Listeria* spp. and *E. coli* isolates from the laboratories of the factory during the 3-year project period. It is very unlikely that the number of isolates collected by CCFRA would outweigh the isolates not received. The error in the sample numbers and the method differences is likely to be low, although the error in the number of isolates collected from the laboratories could be as much as 25–50%. A range of the overall incidence of *E. coli* and *L. monocytogenes* would thus be 0·08% ± 0·04 and 0·23% ± 0·12% respectively.

From previous *Listeria* spp. troubleshooting exercises in high-risk, ready-to-eat product factories over the past

10–15 years, the authors have suggested a general 'rule of thumb' for *Listeria* contamination level. A level of *Listeria* product positives, described as the percentage of products positive for *L. monocytogenes* at end of shelf-life (measured by a presence/absence test), of above 5% is out of control. A level of 1–5% indicates room for improvement (this varies on whether the product is fully cooked or has undergone decontamination only, e.g. salad washing in chlorine) and a level of <1% should be what all high-risk chilled food factories should achieve. In all cases, the Public Health Laboratory Service in the UK recommends that the numbers of *L. monocytogenes* should be <100 per gram at the end of shelf-life (Gilbert *et al.* 2000). This rule of thumb is equally applicable to the level of *Listeria* recorded in high-risk area environmental swabs, although this can change, depending on the point of sampling. For example, it is more likely that *Listeria* will be found if predominantly more samples are taken of cleaning equipment and drains (*Listeria* collection points) than, for instance, walls and production equipment.

The overall incidence for *Listeria* spp. of 0·35%, together with the incidence levels for the individual sites, indicates that all factories were achieving levels of *Listeria* contamination of <1% and were hence 'in control'. It is unlikely, therefore, that any indication of persistent strains, or niche behaviour, could be attributed to poor manufacturing practices or poor cleaning and disinfection. It is difficult to put into perspective the level of *E. coli* contamination in the factories in terms of percentage of product positive, as traditionally it is the number of *E. coli* present in a sample that, if too high, is likely to trigger factory trouble-shooting procedures.

Thirteen *E. coli* and 14 *L. monocytogenes* ribogroups showed evidence for persistence, with a mean of 5·6 and 13·8 months respectively. The ratio of isolates to ribogroups for *E. coli*, at 1·92 : 1, was much lower than for *Listeria* spp., at 8·67 : 1. In addition, the number of ribogroups found once only, 79·0%, was much higher than for *Listeria* spp. (40·0%) and as such, there was more evidence for *L. monocytogenes* persistence. The presence of dominant strains, in this study *E. coli* ribogroup 102-248-S-4 and *L. monocytogenes* ribogroup 102-195-S-1, in food factories has also been noted by Gendel and Ulaszek (2000) and Chasseignaux *et al.* (2001).

The majority of *E. coli* and *Listeria* spp. isolates found more than once were found in site 1. Similarly, evidence for persistence was also most likely to be found in site 1. Site 1 is the largest and oldest of the chilled food sites sampled and produces the largest number of products from the largest number of raw materials. Its high-risk area may therefore have had the greatest chance of being contaminated from strains associated with raw materials; it also has the largest number of process lines and food operatives (most environmental niches), and has had the longest time frame in which strains could have become established. In addition, 79% of all the samples taken were from site 1.

While strain persistence has been demonstrated by a number of authors throughout the world, sometimes over considerable time periods, it is still difficult to prove that such persistent strains actually do survive within the factories during continuous production/cleaning cycles. It could still be argued that these 'persistent' strains merely enter the production area every (sampling) day, e.g. via raw materials, food operatives, the air, and the water supply, and are thus always present in the production area. Four key factors are apparent in this work, which give rise to the hypothesis of strain persistence.

First, the size of survey with analyses of over 196 000 product and environmental samples from four chilled food factory, high-risk areas, is perhaps the largest survey undertaken of its type, with the specific purpose of identifying strain niche or persistence. Secondly, this work was undertaken in four chilled food production sites, all having excellent low/high-risk barriers, designed using industry best practice to prevent daily pathogen access. The concept of low/high-risk areas and their management requirements is particularly well understood in the UK. If this work has also demonstrated the existence of persistent strains, under factory management conditions designed to prevent pathogen access, the case for persistent strains is therefore strengthened. Thirdly, the very low level of *Listeria* spp. and *E. coli* detection in product and the environment suggests that factory hygiene was well managed. If *Listeria* spp. and *E. coli* strains were routinely crossing the low/high-risk barrier, it would be likely that the detection level of *Listeria* spp. and *E.coli* would subsequently be higher. Similarly, if *Listeria* spp. and *E. coli* strains were routinely crossing the low/high-risk barrier, it would be likely that the number of ribogroups of these genera would be much larger. As the number of ribogroups identified (compared with the number of isolates) was low, particularly for *Listeria* spp., this suggests that the adaptation to, and persistence in, the high-risk area is the more likely scenario. Finally, and in agreement with Lundén *et al.* (2003b), this study has shown the persistence of the same strains in more than one food-processing site.

The nature of strain persistence is unknown but may be due to a number of factors affecting physical adaptation (surface attachment, biofilm formation, attachment strength, reduced growth rate, quiescence, cleaning and disinfection resistance) to the whole range of environmental conditions typical in chilled food factory environments (low temperature, wide pH range, fluctuating nutrient supply and moisture levels, frequency of cleaning and disinfection, etc.). Lundén *et al.* (2000, 2003a have demonstrated that

persistent *L. monocytogenes* strains may show enhanced surface adherence and increased disinfection resistance at very low disinfectant concentrations, although Holah *et al.* (2002) found no evidence of disinfection resistance of certain persistent strains to disinfectants at their normal in-use concentration.

It is difficult to define ribogroups as wholly environmental or from product, particularly if there were only low numbers of isolates from the ribogroup identified. For example, if a large population of *Listeria* spp. was found in the environment at the time of sampling, it is likely that any food produced in the vicinity of this population would become contaminated and, if sampled, would register *Listeria* as being of a product origin. Similarly if a large population of *E. coli* was found in product at the time of sampling, it is likely that the food contact surfaces over which it moved would also become contaminated and, if sampled, would register *E. coli* as being of environmental origin. The 6·4 : 1 ratio of product to environment samples taken in this study would also favour the detection of isolates in the product. This ratio of product to environmental sampling is normal for food factories as factories are required to take substantial product samples to verify their Hazard Analysis Critical Control plans.

The results from the *E. coli* and *Listeria* spp. studies have shown that the majority of *E. coli* strains were product niche oriented whilst the majority of *Listeria* spp. strains were environmental niche oriented. The two dominant *E. coli* and *L. monocytogenes* strains, however, were present in both niches. With respect to niche colonization, Chasseignaux *et al.* (2001) have described dominant *L. monocytogenes* genotypes that are present in both product and environmental niches, and environmental genotypes that are found only after cleaning and disinfection programmes. Similarly, Hoffman *et al.* (2003) have also described *L. monocytogenes* niche colonization and dominant strains.

The majority of the *Listeria* spp. isolates (65·4%) were identified as *L. monocytogenes*, followed by *L. innocua* (23·1%), *L. welshimeri* (6·2%) and *L. seeligeri* (5·4%). The ratio of isolates to ribogroups was similar for *L. monocytogenes* (9·44 : 1) and *L. innocua* (10·00 : 1) and was a lot higher than for *L. welshimeri* (5·33 : 1) and *L. seeligeri* (4·67 : 1). With respect to the number of ribogroups that were persistent, these were similar for all species (*L. monocytogenes* 56%, *L. innocua* 67%, *L. welshimeri* 67% and *L. seeligeri* 67%).

Listeria spp. strains differed primarily in their isolation rate, isolate to ribogroup ratio and environmental or environmental/product colonization niche basis. Therefore, it is not clear that as a microbiological pathogen sampling strategy, identification of *Listeria* isolates to *Listeria* spp. will give an accurate indication of the presence of persistent *L. monocytogenes* strains. In a study of 18 000 samples from 12 meat plants undertaken by Tompkin (2002), the ratio of *Listeria* spp. to *L. monocytogenes* was very different for each factory and varied from 1·12 : 1 to 63·6 : 1. Tompkin concluded that the likelihood that a sample containing *Listeria* spp. would contain *L. monocytogenes* was highly dependent on the unique ecology of each plant.

As 12 of 30 *Listeria* spp. ribogroups were sporadic, and seven of the 30 ribogroups were persistent *L. monocytogenes* strains, sampling for *L. monocytogenes* would probably be a better sampling strategy. If the number of *L. monocytogenes* isolates was constant and excessive, this could be followed by fingerprinting of the strains by a suitable molecular method to further elucidate the contamination source.

From a practical viewpoint, the UK chilled food industry pays significant attention to the design of the high-risk area and its barriers to prevent pathogen access, thorough personal hygiene practices and 'best practice' cleaning and disinfection programmes (Holah and Thorpe 2000; Anon. 2002). These have been shown to effectively control the presence of *L. monocytogenes* or *E. coli* in the chilled food factories sampled to a level of <1·0% of product or environmental samples. To control persistent strains requires additional cleaning and disinfection strategies. These could include the development of detergents focused on bacterial removal from surfaces, the use of alternative disinfectants, e.g. u.v. light, or the concept of 'whole room' disinfection using gases such as ozone or aldehydes.

In addition, the concept of potential persistent pathogenic strains, adapted to grow in both environmental and product niches, raises concerns over the possibility of major food poisoning outbreaks. Such persistent strains could grow in the factory to an extent that a large number of individual product items could be contaminated and then remain viable and/or grow during the shelf-life of the product. Therefore, there is clearly a need to establish the environmental (e.g. stress, attachment, biofilm formation, resistance to disinfectants and/or preservatives) or molecular foundation of persistence and so aid the development of novel intervention strategies.

REFERENCES

Aase, B., Sundheim, G., Langsrud, S. and Rørvick, L.M. (2000) Occurrence of and a possible mechanism for resistance to a quaternary compound in *Listeria monocytogenes*. *International Journal of Food Microbiology* **62**, 57–63.

Anon. (1988) *Listeria* isolation; revised method of analysis; notic. Bacteriological analytical manual. *FDA Federal Register* **53**, 44148–44153.

Anon. (1998) *Microbiological Examination of Food and Animal Feeding Stuffs: Enumeration of Presumptive Escherichia coli*: Most Probable Number Technique. ISO 7251/BS 5763: Part 8. London: British Standards Institution.

Anon. (1999) *Microbiology of Food and Animal Feed Stuffs – Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination – Part 1 – General Rules for the Preparation of the Initial Suspension and Decimal Dilutions*. BS EN ISO 6887-1:1999. London: British Standards Institution.

Anon. (2002) *High Risk Area Best Practice Guidelines*, 2nd edn. Kettering, UK: The Chilled Food Association.

AOAC (1998) *Official Methods of Analysis*, 16th edn. Gaithersburg, MD: Association of Official Analytical Chemists International.

Brett, M.S.Y., Short, P. and Mclauchlin, J. (1998) A small outbreak of listeriosis associated with smoked mussels. *International Journal of Food Microbiology* **43**, 223–229.

Chasseignaux, E., Toquin, M.-T., Ragimbeau, C., Salvat, G., Colin, P. and Ermel, G. (2001) Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry- and pork-processing plants. *Journal of Applied Microbiology* **91**, 888–899.

Destro, M.T., Leitao, M.F.F. and Farber, J.M. (1996) Use of molecular typing methods to trace the dissemination of *Listeria monocytogenes* in a shrimp processing plant. *Applied and Environmental Microbiology* **62**, 705–711.

Dodd, C.E.R., Chaffey, B.J. and Waites, W.M. (1988) Plasmid profiles as indicators of the source of contamination of *Staphylococcus aureus* endemic within poultry processing plants. *Applied and Environmental Microbiology* **54**, 1541–1549.

Fonnesbech Vogel, B., Huss, H.H., Ojeniti, B., Ahrens, P. and Gram, L. (2001) Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. *Applied and Environmental Microbiology* **67**, 2586–2595.

Gendel, S.M. and Ulaszek, J. (2000) Ribotype analysis of strain distribution in *Listeria monocytogenes*. *Journal of Food Protection* **63**, 179–185.

Gilbert, R.J., De Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C.D., Richards, J., Roberts, D. *et al.* (2000) Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. *Communicable Disease and Public Health* **3**, 163–167.

Hoffman, A.D., Gall, K.L., Norton, D.M. and Wiedman, M. (2003) *Listeria monocytogenes* contamination patterns for the smoked fish processing environment and for raw fish. *Journal of Food Protection* **66**, 52–60.

Holah, J.T. (2000) Cleaning and disinfection. In *Chilled Foods: a Comprehensive Guide*, 2nd edn ed. Dennis, C. and Stringer, M.F. pp. 397–428. Cambridge, UK: Woodhead Publishing Limited.

Holah, J.T. and Thorpe, R.H. (2000) The hygienic design of chilled foods plant. In *Chilled Foods: a Comprehensive Guide*, 2nd edn ed. Dennis, C. and Stringer, M.F. pp. 355–396. Cambridge, UK: Woodhead Publishing Limited.

Holah, J.T., Taylor, J.H., Dawson, D.J. and Hall, K.E. (2002) Biocide usage in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*. *Journal of Applied Microbiology. Symposium Supplement* **92**, S111–S120.

Jacquet, C., Catimel, B., Goulet, V., Lepoutre, V., Veit, P., Dehaumont, P. and Rocourt, J. (1995) Typing of *Listeria monocytogenes* during epidemiological investigations of the French listeriosis outbreaks in 1992, 1993 and 1995. In *XII International Symposium on Problems of Listeriosis*, Perth, Western Australia, 2–6 October 1995. Canning Bridge, Western Australia: Promaco Conventions Pty Ltd.

Johansson, T., Rantala, L., Palmu, L. and Honkanen-Buzalski, T. (1999) Occurrence and typing of *Listeria monocytogenes* strains in retail vacuum-packed fish products and in a production plant. *International Journal of Food Microbiology* **47**, 111–119.

Kathariou, S. (2003) *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection* **65**, 1811–1829.

Lawrence, L.M. and Gilmour, A. (1995) Characterisation of *Listeria monocytogenes* isolated from poultry products and poultry-processing environment by random amplification of polymorphic DNA and multilocus enzyme electrophoresis. *Applied and Environmental Microbiology* **61**, 2139–2144.

Lundén, J., Miettinen, M., Autio, T. and Korkeala, H. (2000) Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surfaces after short contact times. *Journal of Food Protection* **63**, 1204–1207.

Lundén, J., Autio, T., Markkula, A., Hellström, S. and Korkeala, H. (2003a) Adaptive and cross-adaptive responses to persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. *International Journal of Food Microbiology* **82**, 265–272.

Lundén, J., Autio, T., Sjöberg, A-M. and Korkeala, H. (2003b) Persistent and non-persistent *Listeria monocytogenes* contamination in meat and poultry processing plants. *Journal of Food Protection* **66**, 2062–2069.

McLauchlin, J., Greenwood, M.H. and Pini, P. N. (1990) The occurrence of *Listeria monocytogenes* in cheese from a manufacturer associated with a case of listeriosis. *International Journal of Food Microbiology* **10**, 255–262.

McLauchlin, J., Hall, S.M., Velani, S.K. and Gilbert, R.J. (1991) Human listeriosis and paté: a possible association. *British Medical Journal* **303**, 773–775.

Michiels, C.W., Schellekens, M., Soontjens, C.F. and Hauben, K.J.A. (1997) Molecular and metabolic typing of resident and transient flora from a meat mincer. *Journal of Food Protection* **60**, 1515–1519.

Miettinen, M.K., Björkroth, K. and Korkeala, H.J. (1999) Characterisation of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed gel electrophoresis. *International Journal of Food Microbiology* **46**, 187–192.

Nesbakken, T., Kapperud, G. and Caugant, D.A. (1996) Pathways of *Listeria monocytogenes* contamination in the meat processing industry. *International Journal of Food Microbiology* **31**, 161–171.

Norton, D.M., McCamet, M.A., Gall, K.L., Scarlett, J.M., Boor, K.J. and Wiedmann, M. (2001) Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Applied and Environmental Microbiology* **67**, 198–205.

Ojeniyi, B., Christensen, J. and Bisgaard, M. (2000) Comparative investigations of *Listeria monocytogenes* isolated from a turkey processing plant, turkey products, and from human cases of listeriosis in Denmark. *Epidemiology and Infection* **125**, 303–308.

Pourshaban, M., Gianfranceschi, M., Gattuso, A., Menconi, F. and Aureli, P. (2000) Identification of *Listeria monocytogenes* contamination sources in two fresh sauce production plants by pulsed-field gel electrophoresis. *Food Microbiology* **17**, 393–400.

Rørvik, L.M., Caugant, D.A. and Yndestad, M. (1995) Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon

slaughterhouse and smoked salmon processing plant. *International Journal of Food Microbiology* **25**, 19–27.

Salvat, G., Toquin, M.T., Michel, Y. and Colin, P. (1995) Control of *Listeria monocytogenes* in the delicatessen industries: the lessons of a listeriosis outbreak in France. *International Journal of Food Microbiology* **25**, 75–81.

Tompkin, R.B. (2002) Control of *Listeria monocytogenes* in the food-processing environment. *Journal of Food Protection* **65**, 709–725.

Unnerstad, H., Bannerman, E., Bille, J., Danielsson-Tham, M.-L., Waak, E. and Tham, W. (1996) Prolonged contamination of a dairy with *Listeria monocytogenes*. *Netherlands Milk and Dairy Journal* **50**, 493–499.