Journal of Dairy Research (2010) **77** 325–330. © *Proprietors of Journal of Dairy Research 2010*
doi:10.1017/S0022029910000282

Recovery of *Cronobacter sakazakii* from environmental surface swabbing materials using a 5-h enrichment procedure

Denise Lindsay*, Ben Somerton, Bruce Hill and Owen Shrubb

Fonterra Research Centre, Fitzherbert Science Centres, Dairy Farm Road, Palmerston North, New Zealand

Received 13 October 2009; accepted for publication 20 February 2010; first published online 9 June 2010

This study aimed to reduce the time taken to detect low numbers of *Cronobacter sakazakii* inoculated onto environmental swabs (100, 10 or 1 cfu per swab) using a simple plating procedure for application in a dairy testing laboratory. Three types of environmental swabs (Biolab FlexiSwab™, gauze swabs and the Whatman SwabCheck Polywipe™ sponge) were inoculated with either *Cron. sakazakii* in single culture or *Cron. sakazakii* together with *Citrobacter freundii*. A 5-h enrichment procedure of swabs in *Cronobacter* enrichment broth at 37 °C prior to plating was then compared with no enrichment or 24-h enrichment. The 5-h enrichment procedure was as efficient at detecting *Cron. sakazakii* on environmental swabs at low cell concentrations (100 cfu per swab), and in pure culture or in competition with other coliforms (*Citrobacter*), as pre-enrichment for 24 h. This protocol was also successful in detecting 10 cfu per swab 80% of the time. The results also indicated that the type of swab selected for use in environmental safety programmes is influential on the outcome, with the FlexiSwab™ and gauze swabs being the most efficient swabbing materials evaluated in this study.

Keywords: Enrichment, recovery, *Cronobacter sakazakii*, environmental swabs.

Cronobacter (*Enterobacter*) *sakazakii* is a Gram-negative rod belonging to the family Enterobacteriaceae. This mesophilic organism has recently emerged as a foodborne pathogen because sepsis, meningitis and necrotizing enterocolitis have occurred in neonates following the consumption of contaminated and rehydrated powdered infant milk formulae (Gurtler et al. 2005).

Enterobacteriaceae are common contaminants of raw milk (Martins et al. 2006). However, pasteurisation is effective for inactivating Enterobacteriaceae during dairy processing. In the case of *Cron. sakazakii*, even though strains may differ in their thermal resistance profiles (Edelson-Mammel & Buchanan, 2004), there is no documented evidence to suggest that *Cron. sakazakii* can survive pasteurization (Gurtler et al. 2005). Risk models have predicted that pasteurisation temperatures of 68 °C for 16 s should reduce *Cron. sakazakii* populations by 5-log cycles (Nazarowec-White et al. 1999).

There is widespread belief that re-contamination events between the spray drying step and the packaging step are the most likely cause of this pathogen being recovered from the dried product (Gurtler et al. 2005). Food-contact surfaces and food processing environments are considered to be two of the most significant sources of contamination by potential foodborne pathogens (Reij et al. 2004). In addition, evidence from a recent study indicates that *Cron. sakazakii* strains may persist in processing lines, that *Cron. sakazakii* strains may enter the process from air supply areas and that inefficient cleaning-in-place regimes may also allow *Cron. sakazakii* to survive in milk powder processing equipment (Hein et al. 2009).

As a consequence of the emergence of *Cron. sakazakii* as an important infant pathogen, and the recognition of the role of environmental sources in the contamination of the dairy process stream, robust and efficient testing protocols for monitoring dairy manufacturing environments for *Cron. sakazakii* have become a vital part of product safety risk management programmes. The aim of these protocols is to identify areas within the processing environment where *Cron. sakazakii* is present and from which process contamination could occur.

Environmental monitoring programmes generally involve the sampling of processing and environmental surfaces using a variety of commercially available swabbing materials. The numbers of pathogens within a manufacturing environment are likely to be low, and occur sporadically (Mullane et al. 2007). Therefore, sensitive test methods are required to increase the probability of detecting environmental contamination. Rapid results turnaround is also

*For correspondence; e-mail: denise lindsay@fonterra.com

https://doi.org/10.1017/S0022029910000282 Published online by Cambridge University Press

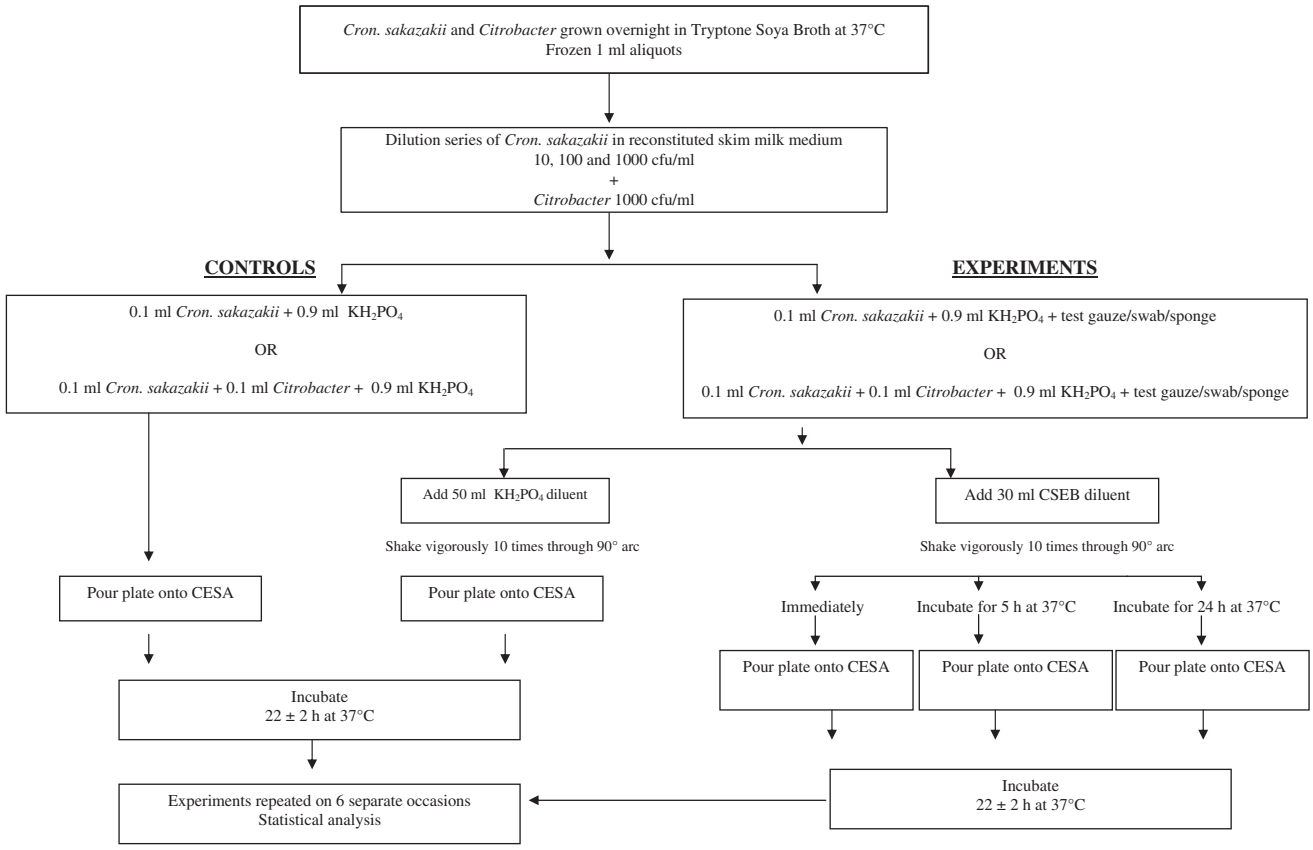

Fig. 1. Simplified flow diagram of experimental procedure

important to manufacturers so that environmental clean-up can be actioned as soon as possible after contamination has been identified.

The aim of this study was to reduce the time taken to detect *Cron. sakazakii* recovered on environmental swabs from the manufacturing environment using a simple procedure for application in a dairy testing laboratory. *Cron. sakazakii* in pure culture and in the presence of competing coliforms (*Citrobacter freundii* was used as a competitor) were tested using a modified enrichment procedure, and three types of swabbing materials were also compared.

Materials and Methods

Preparation of bacterial cultures

Cron. sakazakii NZRM50, a strain of dairy origin, and *Cit. freundii* NCTC 9750, were each grown in Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK) overnight at 37 °C (Fig. 1). Aliquots (1 ml) of the overnight cultures were frozen in the presence of 10% v/v sterile glycerol at −80 °C. Prior to each experiment, 1 ml aliquots were thawed and diluted appropriately.

This freezing and thawing approach was adopted for two reasons: to standardize the stage of growth of the cultures across all experiments, and to add an 'injury' step to simulate the damage that occurs during processing – the resuscitation of injured cells during subsequent enrichment steps is a key aspect of many microbiological methods (Jasson et al. 2007; Dupont & Augustin, 2009).

Preparation of test suspensions

Cron. sakazakii *in pure culture* – For each trial, 1 ml frozen aliquots containing *Cron. sakazakii* NZRM50 were thawed and diluted in reconstituted skim milk (RSM) to achieve three different levels of cells – approximately 10, 100 and 1000 cfu/ml. RSM was used to simulate the presence of the milk solids that are likely to be collected on swabs when sampling dairy manufacturing environments.

To determine the number of bacteria inoculated onto each swab, 0·1 ml RSM from each tube containing 10, 100 or 1000 cfu/ml was diluted in 0·9 ml phosphate solution (KH_2PO_4) in duplicate Petri plates. Pour plates were then prepared for counts of *Cron. sakazakii* NZRM50 using Chromocult™ *Enterobacter sakazakii* agar (Merck) (CESA) (Moore & Griffith, 2007). The plates were incubated for 22 ± 2 h at 37 °C (Fig. 1).

Cron. sakazakii *with simulated background flora* – *Cit. freundii* NCTC 9750 was used to evaluate the robustness of the test system for detecting *Cron. sakazakii* NZRM50 in the presence of other background coliforms. Aliquots (1 ml) of *Cit. freundii* NCTC 9750 were thawed and

https://doi.org/10.1017/S0022029910000282 Published online by Cambridge University Press

diluted in a similar way to *Cron. sakazakii* NZRM50 to achieve approximately 1000 cfu/ml. To determine the number of *Cit. freundii* NCTC 9750 inoculated onto each swab, 0·1 ml RSM from the tube containing 1000 cfu/ml was diluted in 0·9 ml KH_2PO_4 in duplicate Petri plates. In addition, 0·1 ml RSM from each tube containing 10, 100 or 1000 cfu/ml *Cron. sakazakii* NZRM50 was also added to the duplicate Petri plates. Pour plates were prepared and incubated as described previously (Fig. 1).

Recovery of bacteria from directly inoculated swabs

The following surface swabbing materials were tested: pre-moistened gauze (cotton) swabs (10 contained in a 70 ml sample bottle and pre-moistened with 20 ml diluent) (BHB Biosciences, Fort Richard, Auckland, New Zealand), the Biolab FlexiSwab™ (foam) (Biolab, Auckland, New Zealand) and the Whatman SwabCheck Polywipe™ sponge (Biolab). Each type of test swab was inoculated as described below.

Swabs inoculated with Cron. sakazakii *in pure culture*

For each trial, twelve sample bottles (70 ml volume) were prepared, four containing a gauze swab, four containing a FlexiSwab™ and four containing a Polywipe™ sponge. Each swab was inoculated with 0·9 ml KH_2PO_4 and 0·1 ml of the dilution of *Cron. sakazakii* in RSM containing 1000 cfu/ml, to achieve a final cell concentration of approximately 100 cfu per swab. The latter equated to 1–5 cfu/cm^2 if a 20–100 cm^2 surface was swabbed, as suggested in ISO Standard 18593 (International Organization for Standardization, 2004). A similar procedure was used to inoculate and prepare 12 sample bottles containing 10 cfu per swab (0·1–0·5 cfu/cm^2) and a further 12 sample bottles containing 1 cfu per swab (0·01–0·05 cfu/cm^2). In all cases, a total of 1 ml of liquid was added (Fig. 1). All experiments were carried out on six separate occasions.

To each sample bottle and swab, either 30 ml *Cronobacter sakazakii* enrichment broth (CSEB) (International Organization for Standardization, 2006) (Fort Richard) with vancomycin or 50 ml KH_2PO_4 (no enrichment, negative control) was added, and the sample bottle was shaken vigorously ten times through an arc of 90°.

For CSEB-containing sample bottles – Pour plates (1 ml) of CESA were prepared immediately (0 h) from one sample bottle for each dilution of cells and each swab type.

A further sample bottle for each dilution of cells and each swab type was incubated for 5 h at 37 °C. Following incubation, and after gentle inversion of each sample bottle, 1 ml aliquots were pour plated using CESA (Fig. 1).

A final sample bottle for each dilution of cells and each swab type was incubated overnight (24 h) at 37 °C. Pour plates (1 ml) were again prepared using CESA. This served as a final positive control for the growth of *Cron. sakazakii*.

All plates were incubated for 22 ± 2 h at 37 °C and colony counts were determined (Fig. 1). Although the current ISO *Cron. sakazakii* method (International Organization for Standardization, 2006) stipulates an enrichment incubation temperature of 44 °C, for this study, 37 °C was adopted as a number of publications have indicated that several strains of *Cron. sakazakii* may not grow at 44 °C (Lehner et al. 2006).

For KH_2PO_4-containing sample bottles – Pour plates (1 ml) were prepared immediately from one sample bottle for each dilution of cells and each swab type using CESA as previously described.

Swabs inoculated with Cron. sakazakii *with simulated background flora*

Twelve sample bottles (70 ml volume) were prepared: four containing a gauze swab, four containing a FlexiSwab™ and four containing a Polywipe™ sponge. Each test swab was inoculated with 0·9 ml KH_2PO_4 and 0·1 ml of the dilution of *Cron. sakazakii* in RSM containing 1000 cfu/ml (this gave a final concentration of 100 cfu per swab), plus an additional 0·1 ml of the dilution of *Cit. freundii* NCTC 9750 in RSM containing 1000 cfu/ml (this gave a final background inoculum of 100 cfu per swab). A similar procedure was used to inoculate and prepare 12 sample bottles containing 10 cfu of *Cron. sakazakii*/ml and 100 cfu of *Cit. freundii* NCTC 9750/ml per swab, and a further 12 sample bottles containing 1 cfu of *Cron. sakazakii*/ml and 100 cfu of *Cit. freundii* NCTC 9750/ml per swab. The samples were incubated and plated as described previously (Fig. 1).

Calculating the percentage recovery of cells by each enrichment procedure

How effective each pre-enrichment procedure was for the recovery of *Cron. sakazakii* cells from test swabs was calculated using the following equation:

$$R = \left[\frac{N^a \times d}{N^b}\right] \times 100$$

In the equation, R is the percentage recovery of *Cron. sakazakii* for each enrichment step tested, N^a is the mean number of cfu counted on duplicate plates after each enrichment step, d is the dilution factor and N^b is the number of cfu inoculated onto each swab before the enrichment step, and estimated from control plates without swabs (Moore & Griffith, 2007). As cells were enriched in this study, values for percentage recovery above 100% were also recorded due to bacterial growth.

Statistical analysis

One-way analysis of variance (ANOVA) at 95 and 99% confidence followed by Tukey's test were used to analyse

https://doi.org/10.1017/S0022029910000282 Published online by Cambridge University Press

https://doi.org/10.1017/S0022029910000282 Published online by Cambridge University Press

Table 1. Percentage (%) recovery of *Cronobacter sakazakii* from inoculated swabs after incubation in *Cronobacter sakazakii* enrichment broth (CSEB) for 0, 5 and 24 h compared with controls without an enrichment step (KH_2PO_4). Results are an average of experimental data obtained on six separate occasions

	Without enrichment (KH_2PO_4)			0-h enrichment in CSEB			5-h enrichment in CSEB			24-h enrichment in CSEB		
Bacterial inoculum	FlexiSwab™	Gauze	Polywipe™ sponge	FlexiSwab™	Gauze	Polywipe™ sponge	FlexiSwab™	Gauze	Polywipe™ sponge	FlexiSwab™	Gauze	Polywipe™ sponge
Cron. sakazakii only												
100 cfu	0	0	0	0	0	0	58	29	20	308	308	308
10 cfu	0	1	0	0	0	0	58	31	29	3846	3846	3846
1 cfu	0	0	0	0	0	0	89	11	33	33 333	33 333	33 333
Cron. sakazakii with *Cit. freundii*												
100 cfu	2	1	1	1	0	0	191	49	26	303	303	303
10 cfu	1	0	0	1	0	0	67	24	19	2419	2419	2419
1 cfu	33	533	0	0	0	0	600	267	27	100 000	100 000	100 000

Table 2. Percentage (%) positive samples obtained after incubation of inoculated swabs in *Cronobacter sakazakii* enrichment broth (CSEB) for 0, 5 and 24 h compared with controls without an enrichment step (KH_2PO_4). Results are an average of experimental data obtained on six separate occasions

Swab inoculum	FlexiSwab™				Gauze				Polywipe™ sponge			
	KH_2PO_4	CSEB 0 h	CSEB 5 h	CSEB 24 h	KH_2PO_4	CSEB 0 h	CSEB 5 h	CSEB 24 h	KH_2PO_4	CSEB 0 h	CSEB 5 h	CSEB 24 h
Cron. sakazakii only												
100 cfu	42	17	100	100	0	8	100	100	0	0	100	100
10 cfu	0	0	83	100	0	0	83	100	0	0	83	100
1 cfu	0	0	50	100	0	0	58	100	0	0	25	100
Cron. sakazakii with *Cit. freundii*												
100 cfu	58	17	100	100	50	0	100	100	42	0	100	100
10 cfu	8	8	100	100	8	33	83	100	0	0	67	100
1 cfu	8	0	58	100	0	0	25	100	0	0	8	100

the resulting counts to determine which method recovered more *Cron. sakazakii* NZRM50 cells from each swab type tested.

Results and Discussion

The results of this study are shown in Table 1 (percentage recovery of *Cron. sakazakii* from the different swab types after each enrichment step) and Table 2 (percentage of samples that tested positive for *Cron. sakazakii* after each enrichment step). Swabs were successfully inoculated with approximately 100 cfu (97–98 cfu), 10 cfu (8–12 cfu) and 1 cfu (0·3–0·9 cfu) per swab, as determined by the numbers of colonies obtained when the inocula were plated onto CESA without swabs.

Procedures in which no enrichment was used (KH_2PO_4 or 0 h in CSEB) recovered the least *Cron. sakazakii* cells (Table 1). Furthermore, *Cron. sakazakii* was detected between 0 and 50% of the time when no enrichment was used (Table 2). In addition, no statistically significant differences ($P>0{\cdot}01$) were observed for counts of *Cron. sakazakii* obtained without enrichment. In contrast, the 5-h enrichment step in CSEB resulted in significantly higher counts ($P<0{\cdot}01$) of *Cron. sakazakii* compared with corresponding counts without enrichment (KH_2PO_4 or 0 h in CSEB). This was true for swabs inoculated with *Cron. sakazakii* only, and also for swabs inoculated with *Cron. sakazakii* and *Citrobacter* in combination.

Overall, enrichment of the swabs in CSEB for 24 h at 37 °C was the most efficient procedure for recovering *Cron. sakazakii* colonies (Table 1), and positive samples were obtained 100% of the time at all inoculation levels (Table 2). Similarly, the 5-h enrichment procedure resulted in the detection of positive samples 100% of the time for swabs inoculated with 100 cfu per swab (Table 2). This would mean that a surface contaminated with between 1 and 5 cfu of this pathogen per cm^2 could be detected using this 5-h enrichment step, if a minimum of 20–100 cm^2 were sampled according to the ISO procedure (International Organization for Standardization, 2004), and assuming that all cells are captured from the surface by the swab. This 5-h enrichment step would also detect <1 cfu/cm^2 (10 cfu per swab) 83% of the time using either FlexiSwabs™ or gauze swabs, and 67% of the time using the Polywipe™ sponge (Table 2). As speed is essential in method development for pathogen detection (Nugen & Baeumner, 2008), the 5-h enrichment step in this study proved to be more efficient for recovering *Cron. sakazakii* than no enrichment, and just as efficient as pre-enrichment for 24 h for swabs containing 100 cfu (Tables 1 & 2).

The 5-h enrichment step was also successful in detecting *Cron. sakazakii* in both single and mixed cultures, as no significant differences ($P>0{\cdot}05$) in counts between swabs inoculated with *Cron. sakazakii* in pure culture and swabs inoculated with *Cron. sakazakii* together with *Citrobacter* were recorded.

Previous studies have shown that different swabbing materials may influence the numbers of bacteria recovered from a surface (Moore & Griffith, 2007). No statistically significant differences ($P>0{\cdot}05$) were recorded in this study between counts obtained using the FlexiSwab™ and counts obtained using gauze swabs, or between counts obtained using gauze swabs and counts obtained using Polywipe™ sponges. However, counts obtained using Polywipe™ sponges were significantly lower ($P<0{\cdot}05$) than corresponding counts obtained using the FlexiSwab™. These results indicated that the type of swab selected for use in environmental safety programmes is influential on the outcome and consequently there is the need to carefully evaluate and select an appropriate swabbing material for use during environmental monitoring programmes.

In conclusion, a 5-h enrichment step in CSEB at 37 °C, prior to plating on CESA, could be used as a more rapid recovery procedure for *Cron. sakazakii* from environmental swabs in a dairy testing laboratory. However, because the sensitivity of the 5 h enrichment method requires ⩾100 cfu *Cronobacter*, it may be suggested that the 24 h enrichment procedure be performed simultaneously. The benefit of a dual enrichment procedure would be that a 5 h enrichment method provides results 19 h sooner for rapid notification of highly contaminated samples. In these experiments, the FlexiSwab™ and gauze swabs were the most efficient swabbing materials evaluated for the detection of *Cron. sakazakii* from dairy environments.

The authors wish to thank Barbara Kuhn-Sherlock for carrying out the statistical analyses in this study.

References

Dupont C & Augustin JC 2009 Influence of stress on single-cell lag time and growth probability for *Listeria monocytogenes* in half Fraser broth. *Applied and Environmental Microbiology* **75** 3069–3076

Edelson-Mammel SG & Buchanan RL 2004 Thermal inactivation of *Enterobacter sakazakii* in rehydrated infant formula. *Journal of Food Protection* **67** 60–63

Gurtler JB, Kornacki JL & Beuchat LR 2005 *Enterobacter sakazakii*: a coliform of increased concern to infant health. *International Journal of Food Microbiology* **104** 1–34

Hein I, Gadzov B, Schoder D, Foissy H, Malorny B & Wagner M 2009 Temporal and spatial distribution of *Cronobacter* isolates in a milk powder processing plant determined by pulsed-field gel electrophoresis. *Foodborne Pathogens and Disease* **6** 225–233

International Organization for Standardization 2004 *Microbiology of food and animal feeding stuffs – Horizontal method for sampling techniques from surfaces using contact plates and swabs*. Geneva: ISO (ISO Standard 18593 : 2004)

International Organization for Standardization 2006 *Milk and milk products – Detection of* Enterobacter sakazakii. Geneva: ISO (ISO/TS Standard 22964 : 2006)

Jasson V, Uyttendaele M, Rajkovic A & Debevere J 2007 Establishment of procedures provoking sub-lethal injury of *Listeria monocytogenes,*

https://doi.org/10.1017/S0022029910000282 Published online by Cambridge University Press

Campylobacter jejuni and *Escherichia coli* O157 to serve method performance testing. *International Journal of Food Microbiology* **118** 241–249

Lehner A, Nitzsche S, Breeuwer P, Diep B, Thelen K & Stephan R 2006 Comparison of two chromogenic media and evaluation of two molecular based identification systems for *Enterobacter sakazakii* detection. *BMC Microbiology* **6** 15 doi:10.1186/1471-2180-6-15

Martins ML, Pinto CLO, Rocha RB, de Araújo EF & Vanetti MCD 2006 Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. *International Journal of Food Microbiology* **111** 144–148

Moore G & Griffith C 2007 Problems associated with traditional hygiene swabbing: the need for in-house standardization. *Journal of Applied Microbiology* **103** 1090–1103

Mullane NR, Whyte P, Wall PG, Quinn T & Fanning S 2007 Application of pulsed-field gel electrophoresis to characterise and trace the prevalence of *Enterobacter sakazakii* in an infant formula processing facility. *International Journal of Food Microbiology* **116** 73–81

Nazarowec-White M, McKellar RC & Piyasena P 1999 Predictive modelling of *Enterobacter sakazakii* inactivation in bovine milk during high-temperature short-time pasteurization. *Food Research International* **32** 375–379

Nugen SR & Baeumner AJ 2008 Trends and opportunities in food pathogen detection. *Analytical and Bioanalytical Chemistry* **391** 451–454

Reij MW, Den Aantrekker ED & ILSI Europe Risk Analysis in Microbiology Task Force (2004) Recontamination as a source of pathogens in processed foods. *International Journal of Food Microbiology* **91** 1–11

https://doi.org/10.1017/S0022029910000282 Published online by Cambridge University Press