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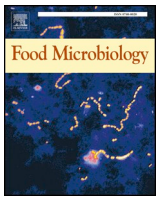

Quantifying the uncertainty of transfer of *Cronobacter* spp. between fomites and floors and touch points in dairy processing plants

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ABSTRACT

Cronobacter spp. are opportunistic pathogens that must be controlled in infant powder manufacturing plants. This study evaluated the spread of *Cronobacter* cells via contact surfaces within a dairy manufacturing environment. Transfer rates of *Cronobacter* spp. were determined from vectors for transmission including moveable fomites (e.g. trolley wheels and boots) and gloved hands to various types of recipient surfaces (stainless steel, linoleum and resin-coated concrete) typical for dairy manufacturing environments. Overall, with a starting inoculum of 10^6 CFU/mL, approximately 10^4 CFU/mL *Cronobacter* cells were transferred from each fomite onto each recipient surface during the initial transfer event. Gloved hands transferred the highest number of *Cronobacter* cells, followed by polyvinylchloride boots and then polyurethane trolley wheels. We demonstrate, using a combination of experimental data and uncertainty analysis, that if a movable fomite (boots or trolley wheels), or gloves became contaminated, *Cronobacter* could be spread over a wide area within a manufacturing plant. To the authors' knowledge, this is the first quantitative estimation of the spread of *Cronobacter* within a dairy manufacturing plant, that can also be practically applied as a tool for providing information in making risk management decisions. In particular, the estimation of spread suggests areas for cleaning and sanitation within a dairy manufacturing environment during a contamination event.

1. Introduction

Maintaining the hygiene of dairy manufacturing environments is of critical importance to prevent post-pasteurization contamination of dairy products. The presence of foodborne pathogens in manufacturing environments can compromise food safety resulting in foodborne outbreaks (CDC, 2015; CFIA, 2016; Jourdan-da Silva et al., 2018).

Particular niches in dairy manufacturing environments have been found to act as reservoirs for foodborne pathogens. For example, *L. monocytogenes* is commonly associated with drains and floors in manufacturing plants (Kabuki et al., 2004; Dzieciol et al., 2016; Rückerl et al., 2014). Foodborne pathogens are also well known to transfer from humans, and/or fomites to product (Todd et al., 2009). For example, a study from an oil meal factory showed that shoes and work gloves were contaminated with *Salmonella* within 1 day of disinfection, and that limiting the movement of operators also reduced the spread of *Salmonella* within the factory (Morita et al., 2006).

For *Cronobacter* spp., several studies have identified the reservoirs of contamination in milk powder manufacturing plants, including the infrastructure (roofs, drains), the product itself (powder residues or recycled powders), the general environment (air and floors), the manufacturing process (dust in the spray-drying, fluidized-bed, and packing areas); cleaning tools (vacuum cleaners) and also on movable fomites (trolley wheels and shoes) (Craven et al., 2010; Fang et al., 2015; Fei et al., 2015; Jacobs et al., 2011; Reich et al., 2010; Sonbol et al., 2013).

Several of these studies highlighted the potential movement of *Cronobacter* through the manufacturing environment, for example detection of this organism on the surfaces of workers' shoes (Fang et al., 2015). However, the extent of transfer of *Cronobacter* that occurs from such movable fomites (such as boots and trolley wheels) onto floors or onto touch points (from hands or gloves) within a dairy powder plant setting has not been studied in detail.

Cronobacter spp. are important opportunistic pathogens for dairy manufacturers who produce infant formula, or its ingredients (CODEX, 2008) and it is internationally recognised that all infants (< 12 months of age) are the population at risk for infections with *Cronobacter* species that might be present in dairy infant formulae (Iversen and Forsythe, 2003; Gurtler et al., 2005; CODEX, 2008; Jason, 2015). Thus, understanding the movement of microorganism in the dairy manufacturing environment is important.

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This study aims to measure the transfer rates of *Cronobacter* from fomite carriers (boots, trolley wheels and gloves) used in a typical New Zealand dairy manufacturing plant, to 3 recipient surface types including stainless steel, linoleum and a resin-coated concrete. Data from this study were incorporated into a model for quantifying the probability that *Cronobacter* would spread from such fomites. A practical table is also presented to help calculate the potential area that has been contaminated with *Cronobacter*, thus giving manufacturers confidence in the size of the area to be targeted for risk management activities (such as defining a cleaning strategy during a contamination event).

2. Materials and methods

2.1. Preparation of cultures

Five *Cronobacter* strains (four of them being *C. sakazakii*) stored in a culture collection at the Fonterra Research and Development Centre, Palmerston North, New Zealand, and previously isolated from a typical dairy manufacturing facility, were grown on Chromocult™ *Enterobacter sakazakii* agar (Merck) (CESA). A typical colony from each isolate was grown in reconstituted skim milk (RSM) medium overnight at 37 °C. The strains were combined to form a cocktail of $\sim 10^8$ CFU/mL. This mixture was then diluted in 9 mL RSM to $\sim 10^6$ CFU/mL and used as the inoculum for further work. Since a previous study showed that *Cronobacter* cells could reach levels of 6 log CFU/mL in infant formula at 22 °C in ~15 h (Parra-Flores et al., 2015), this level of cells represents a theoretical worst-case scenario for spread of the bacterium (e.g. hypothetically, if product in its liquid form were to spill during manufacture, and *Cronobacter* were to grow in this pool of liquid during a manufacturing run of 18 h).

2.2. Fomites and recipient surface types

Fomites included typical dairy processing boots (PVC sole gumboots, Bata Industrials, Wellington, New Zealand), trolley wheels used in dairy processing plants (polyurethane, 100 mm diameter, 30 mm width, Richmond Wheel and Castor Co., Auckland, New Zealand) and non-powdered natural rubber latex gloves (Labserv, ThermoFisher Scientific, Lower Hutt, New Zealand). Recipient surfaces (each ~15 cm × 30 cm) included 316 stainless steel, linoleum and resin-coated concrete (supplied by various dairy processing plant fabricators including BM Scott Ltd, Levin, New Zealand; Colespec Construction Ltd, Palmerston North, New Zealand; and Lee Builders, Palmerston North, New Zealand).

2.3. Transfer study from boots to recipient surfaces

A clean and dry dairy processing boot was inoculated with the *Cronobacter* spp. cocktail by spreading 1 mL of the inoculum, using a sterile spreader, onto the entire sole of the boot. The boot was allowed to air dry for 1 h. The boot was then pressed firmly from heel to toe onto a selected surface type (stainless steel, linoleum and the resin-coated concrete). Sequential "steps" onto the surfaces were taken. Each surface, where the "step" was taken, was swabbed using two Biolab FlexiSwabs™ (foam) (Biolab, Auckland, New Zealand) (Lindsay et al., 2010). The swabs were vigorously rubbed over the test surface, one after the other, and for 1 min each. This was to ensure that as many cells as possible were collected from the test surfaces (> 91% efficiency of removal). Both swabs were then placed in the same 9 mL peptone diluent (Fort Richard, Auckland, New Zealand), and processed in a stomacher for 3 min, the suspension was diluted in peptone, plated onto CESA plates and incubated at 37 °C overnight. Typical *Cronobacter* colonies were counted. In addition, at the end of the "steps" taken by the boot, cells remaining on the boot sole were recovered by rubbing a FlexiSwab vigorously over the surface of the boot for 1 min, accompanied by rinsing the boot surface with 9 mL peptone diluent into a stomacher bag. The swab and rinsate were processed in a stomacher and enumerated as previously described. Experiments were replicated on 6 separate occasions.

2.4. Transfer study from trolley wheels to recipient surfaces

A clean and dry dairy processing trolley wheel was inoculated with *Cronobacter* spp. by spreading 1 mL of the inoculum onto the outside surface. The wheel was allowed to air dry for 1 h. The wheel was then pressed firmly onto each surface type (stainless steel, linoleum and the resin-coated concrete) and rolled for one complete rotation. Sequential rotations of the wheel onto the surfaces were taken. *Cronobacter* cells on each surface where each rotation of the wheel was completed were recovered and enumerated from the surfaces as described previously for the boots. In addition, at the end of the wheel rotations, cells remaining on the wheel surface were recovered by vigorous swabbing and rinsing over the wheel surface as described for the boot. Experiments were replicated on 6 separate occasions.

2.5. Transfer study from gloved hands to stainless steel recipient surfaces

A clean and dry latex-gloved hand was inoculated with *Cronobacter* spp. by spreading 1 mL of the inoculum onto the palm and fingers of the glove. The inoculated gloved palm and fingers were then firmly pressed onto the stainless steel surfaces from wrist to fingertip. Sequential presses of the hands onto stainless steel surfaces were completed. Stainless steel was used as most touch points in New Zealand dairy manufacturing plants are stainless steel, such as stairwell banisters, door handles, and hand-holds on processing equipment. *Cronobacter* cells on the stainless steel surfaces were recovered and enumerated as described previously for the boots and wheels. In addition, at the end of experiment, cells remaining on the glove surface were recovered by vigorous swabbing and rinsing over the glove surface as described for the boot and wheel. Experiments were replicated on 6 separate occasions.

2.6. Treatment of experimental transfer dataset

The transfer of a *Cronobacter* cell initially present on a fomite carrier (boot, wheel or glove) to a recipient surface (stainless steel, linoleum or resin-coated concrete) during one transfer event has been considered as a random process in this study. One transfer event can represent a single contact of a boot/wheel/glove on a surface. The *Cronobacter* cell can either remain on the fomite carrier or be transferred to the recipient surface during this transfer event. Every *Cronobacter* cell present on the fomite carrier has an equal chance of being transferred when there is contact with the recipient surface, and the probability of transfer in each transfer event is constant. Note, for this study, we have assumed that the transfer of a *Cronobacter* cell from the clean recipient surface to the fomite carrier during a transfer event did not affect the transfer probability.

The transfer of individual cells can therefore be modelled as Bernouli trials where the probability of transfer, p, is constant for each transfer event. Since the initial concentration of *Cronobacter* cells on the fomite carrier, n, is high ($n > 10^6$ CFU/mL), the concentration of cells, s, transferred to the recipient surface per transfer event (CFU/mL) can be approximated by a normal distribution, $N(\mu, \sigma)$, where the mean, μ, is equal to np and the standard deviation, σ, is equal to $\sqrt{np(1-p)}$. An initial estimate of the transfer probability, p, is s/n and the uncertainty of p will be estimated from data obtained during the transfer study.

2.7. Validation of proposed model

The model described in section 2.6 is

$$P(S \leq s) = N(\mu,\sigma) \quad (1)$$

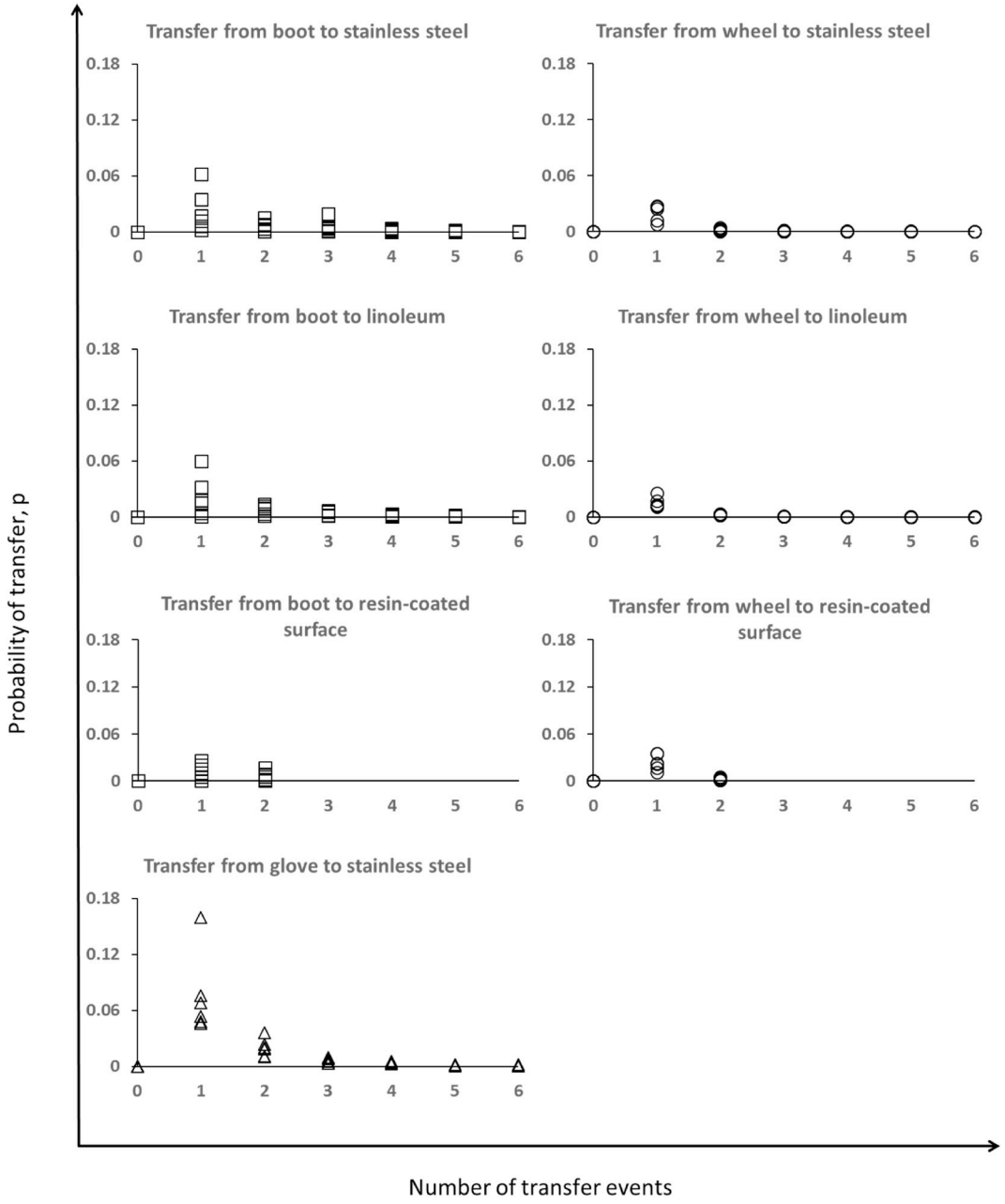

Fig. 1. Transfer of *Cronobacter* cells from a fomite (boots - square, trolley wheels - circle and gloves- triangle) to a recipient surface (stainless steel, linoleum and resin-coated concrete) during 6 experimental replicates. The probability of transfer, p, was estimated from the number of cells transferred per transfer event divided by an initial number of cells inoculated on the fomite carrier. For the resin-coated concrete surfaces, only the first 2 consecutive transfer events are shown, since the probability of transfer on the other surfaces indicated that the first 2 steps were the most relevant.

where S is the concentration of cells transferred (CFU/mL). The outcomes from this model, once the uncertainty in p was quantified using the experimental dataset, was compared to the outcomes from a validation dataset.

The validation dataset used cross-validation principles (Bro et al., 2008), and was generated using a boot inoculated with n *Cronobacter* cells (CFU/mL), as previously described, and was worn by one of the authors (~1.65 m and ~65 kg), and instead of pressing to the surface, was physically walked over resin-coated test surfaces. This was also repeated for linoleum surfaces. *Cronobacter* cells were enumerated from the recipient surfaces as previously described. This was replicated on 6 separate occasions for each surface type. The variable concentrations of *Cronobacter* cells from the 95 percentile of the model (equation (1)) were then compared to the actual concentration of cells transferred. The variable concentration of cells was computed using a simulation approach using the Microsoft Excel software.

3. Results and discussion

Fig. 1 summarizes the data on the transfer of *Cronobacter* cells from 3 fomite carriers (boots, trolley wheels and gloved hands) to 3 recipient surfaces (stainless steel, linoleum and resin-coated concrete). The probability of transfer from the fomite to recipient surface, p, was estimated from the number of cells transferred per each transfer event divided by the initial number of cells inoculated onto the fomite carrier (transfer rate). Three trends were immediately evident from the transfer events depicted in Fig. 1. Firstly, there was an appreciable difference between the probability of transfer between the 1st and subsequent

Table 1
Experimental data on the transfer of *Cronobacter* from a glove, sequentially, to 6 stainless steel surfaces. The transfer rate, tRate was obtained from the ratio of the cells transferred to the initial inoculum on the glove.

	Experiment 1 *Cronobacter* count (CFU/surface)	tRate	Experiment 2 *Cronobacter* count (CFU/surface)	tRate	Experiment 3 *Cronobacter* count (CFU/surface)	tRate
Inoculum on glove	1155000	0	980000	1.0000	980000	0
Transferred to surface 1	55500	0.0481	156500	0.1597	45000	0.0459
Transferred to surface 2	11800	0.0102	35500	0.0362	11000	0.0112
Transferred to surface 3	4200	0.0036	9150	0.0093	8250	0.0084
Transferred to surface 4	2650	0.0023	3500	0.0036	5400	0.0055
Transferred to surface 5	500	0.0004	1810	0.0018	1050	0.0011
Transferred to surface 6	445	0.0004	1610	0.0016	665	0.0007

	Experiment 4 *Cronobacter* count (CFU/surface)	tRate	Experiment 5 *Cronobacter* count (CFU/surface)	tRate	Experiment 6 *Cronobacter* count (CFU/surface)	tRate
Inoculum on glove	1510000	0	1470000	0	1700000	0
Transferred to surface 1	80500	0.0533	100000	0.0680	129500	0.0762
Transferred to surface 2	30500	0.0202	34500	0.0235	31500	0.0185
Transferred to surface 3	4850	0.0032	7900	0.0054	9650	0.0057
Transferred to surface 4	5700	0.0038	6100	0.0041	5150	0.0030
Transferred to surface 5	1875	0.0012	2050	0.0014	2105	0.0012
Transferred to surface 6	960	0.0006	2150	0.0015	1290	0.0008

transfer events. The 1st transfer event deposited the greatest number of cells onto a recipient surface. For example, with a starting inoculum of 10^6 CFU/mL, approximately 10^4 CFU/ml *Cronobacter* cells were transferred from the trolley wheels onto each recipient surface during this first transfer event (equating to ~1%). For boots and gloves, this value was between 1 and 18%. Secondly there is a hierarchy of transfer efficiencies that depended on the type of fomite carrier. Comparatively, gloves were highly efficient at transferring *Cronobacter* cells, followed by boots and then trolley wheels (Table 1). Thirdly, the probability of transfer of cells reached a plateau after 6 transfer events.

This study highlighted gloved hands as the worst fomite tested for *Cronobacter* transfer (i.e. the most number of cells were transferred from gloved hands). Similar results have been found within healthcare settings for the transfer of pathogens between gloved hands and touched surfaces. For example, Koenig et al. (2016) showed that *Staphylococcus aureus* was efficiently transferred from gloved hands to metal surfaces during touching. Similarly, gloves are also known to efficiently transfer foodborne pathogens to food product surfaces (Wu and Ponder, 2018). This study highlighted the need to adhere to the hand hygiene guidelines in place in dairy manufacturing plants, usually consisting of handwashing, hand drying, spraying hands with sanitizer, donning gloves, and further spraying gloved hands with sanitizer. These practices are likely to minimize the spread of *Cronobacter* through the manufacturing plant on touch points if gloves were to become contaminated.

A probabilistic approach to quantifying the contamination of surfaces was appropriate given the transfer data (Fig. 2). Fig. 2. compares the uncertainty in p, when *Cronobacter* cells were transferred from a fomite to recipient surface during the 1st transfer event. The box and whisker plots summarizes the transfer rates from 6 experimental replicates of the 1st transfer event. The gloved hands transferred the most cells, and the spread of that transfer was greatest. Comparatively the median probability of transfer, p, were of the same order of magnitude for boots and trolley wheels. This means that for movable fomites, e.g. trolley wheels and boots, transfer rates to floor surfaces were similar overall, but shown to be influenced by the smoothness of the finished fomite carrier. Transfers from gloves showed the most uncertainty. Trolley wheels were shown to have a less variable transfer probability compared to the transfer probability for boots. This was likely due to the smoothness of the trolley wheel texture, compared to the ridges with deep grooves and more uneven texture of the boots.

Fig. 3 represents graphically the model outcomes for the transfer of *Cronobacter* cells between different fomites and surfaces, as given in equation (1). It has been assumed that the probability of transfer, p, was uncertain and that the experimentally determined estimates for p were equally likely (uniform) during a simulation. Therefore, the variability of contamination i.e. the variable concentration of *Cronobacter* cells, s (CFU/mL), contaminating a recipient surface after a transfer event, is approximated by a normal distribution N (μ, σ). The initial concentration of *Cronobacter* cells on the fomite carrier is n (CFU/mL) and the parameter, μ, is equal to np and the standard deviation, σ, is equal to √np (1-p). Note that this approximation has little validity when np and n (1-p) is less than 5. Further, Fig. 3 shows two examples of the probabilistic contamination model, one using gloves on stainless steel, and the other using boots on linoleum surfaces. Fig. 3a shows a cumulative distribution function [P (S > s |np, √np(1-p))] during the 1st transfer event when a glove contaminates a stainless-steel surface and where n is 10^3 CFU/mL. The concentration of cells contaminating the stainless steel surface at the 95th percentile is 89 CFU/mL. An alternate representation of the model is shown in Fig. 3b and c for *Cronobacter* transfer from the boot to the linoleum surface. In this representation, the contamination occurs from a central point, and can spread in any direction, over the 6 steps taken. Fig. 3b has an inoculum number on the boot of $n = 10^3$ CFU/mL *Cronobacter* cells, and Fig. 3c, $n = 10^4$ CFU/mL *Cronobacter* cells. Note that no distance scale is implied in the representation.

The probabilistic model of contamination of surfaces by *Cronobacter* cells developed in this study was validated by transfer experiments from boots to resin-coated concrete, and boots to linoleum surfaces and by taking two sequential steps (Fig. 4.). The concentration of cells inoculated onto the soles of boots, in this validation dataset, was 10^4 CFU/mL which was 2 orders of magnitude less than the laboratory dataset. This lower inoculum level tested the robustness of the model. Fig. 4 shows a comparison between modelled (by laboratory experiments) and validated (by actual steps) data for confirming the validity of the probabilistic modelling approach. The box and whisker plot summarizes the modelled number of *Cronobacter* cells transferred when n is reduced. In general, the data from the validation dataset is in agreement with the model predictions. The outlier in Fig. 4a for data representing the 1st transfer from boots to the resin-coated concrete surface is possibly linked to using a normal approximation to a Bernoulli or Binomial process when np approaches 5.

From a practical perspective, this model demonstrates that *Cronobacter* can be spread over a wide floor area within a

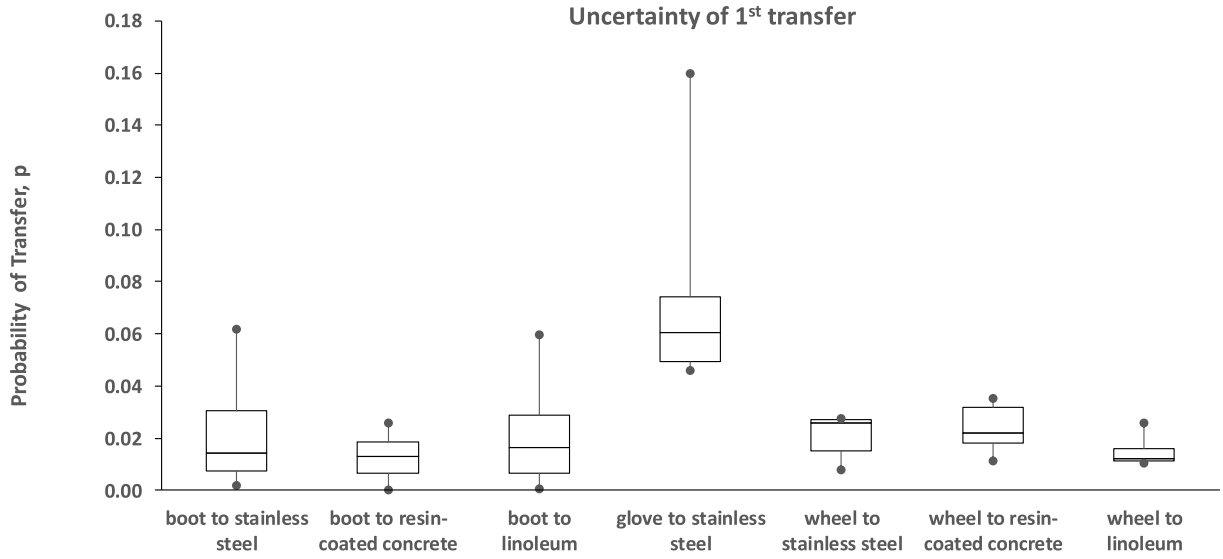

Fig. 2. A comparison of the uncertainty of the transfer rates of *Cronobacter* cells from 3 fomite (boots, trolley wheels, gloves) to 3 recipient (stainless steel, linoleum or resin-coated concrete) surfaces during the 1st transfer event. The box and whisker plot summarizes the transfer rates from 6 experimental replicates. The transfer rates are an initial estimate of the probability of transfer, p. and the transfer rate was derived from the number of cells transferred during the 1st transfer event divided by an initial number of cells inoculated on the fomite carrier.

manufacturing plant. Table 2 shows how this model could be applied in a practical way by a risk manager during a contamination event. To illustrate, the area covered by a worker transferring *Cronobacter* by, for example, walking across a contaminated area in a manufacturing plant was calculated. In this scenario, the step length can be used to calculate the possible area of contamination. A person with a step length of 0.6 m (defined as from one foot contact to the next opposite foot contact, or from the heel of one boot to the toe tip of the next boot) (Hunter et al., 2004) will travel 3.6 m after 6 consecutive steps since our model is based on six consecutive transfer events. Hence the circular area of contamination around the point source (simply using $A = \pi r^2$) can be calculated as 41 m^2

It should also be noted that the number of *Cronobacter* cells used to determine the transfer rates (inoculum of 10^6 CFU/ml) was atypical of the real levels found in dairy manufacturing environments. There is little literature describing counts of *Cronobacter* in such environments, as most studies use an enrichment method to detect this bacterium. However, Mullane et al. (2008) did estimate the count of *Cronobacter* in

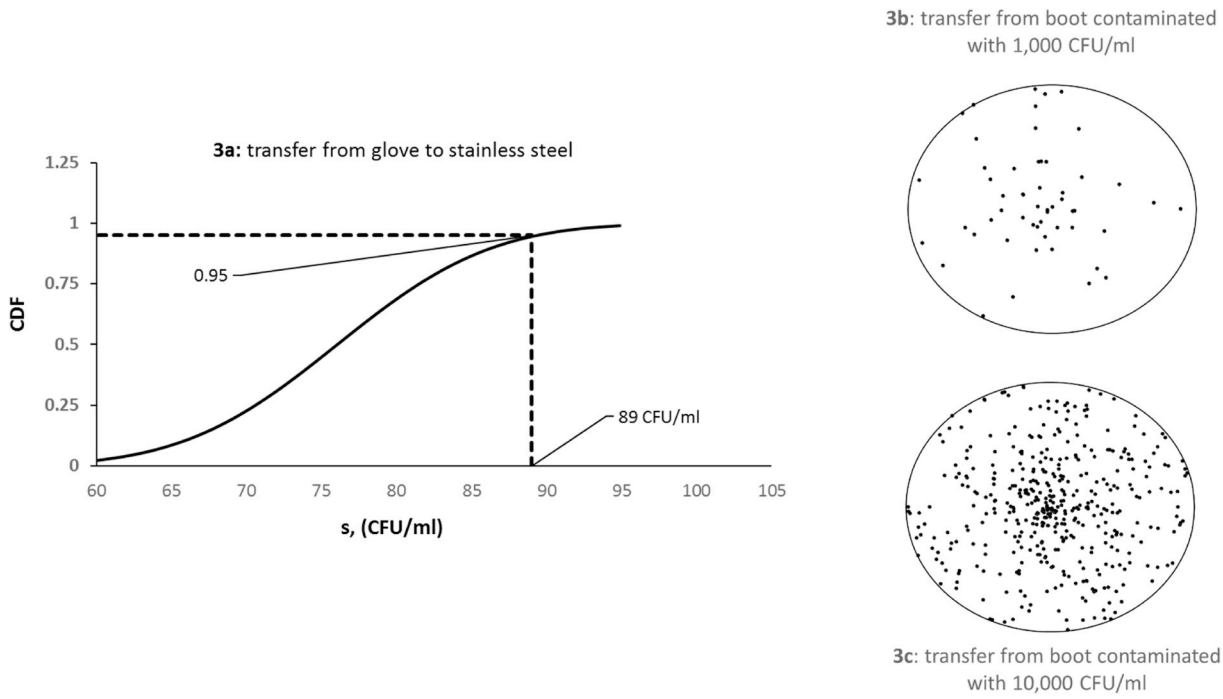

Fig. 3. Two representations of the probabilistic model for contamination of surfaces by *Cronobacter* cells. Fig. 3a shows the probability P(S ≤ s) of the numbers of cells transferred, cumulative probability distribution (CDF), during the 1st transfer event from a glove to a stainless steel surface given an uncertain transfer probability estimated experimentally, and where the initial number of cells present on the glove is 1000 *Cronobacter* cells per ml. In this figure, s is the number of cells transferred. Fig. 3b and c shows the modelled number of cells contaminating a linoleum surface given six consecutive steps or transfer events from a boot initially contaminated with 10^3 and 10^4 CFU/mL *Cronobacter* cells within an area represented by the discs.

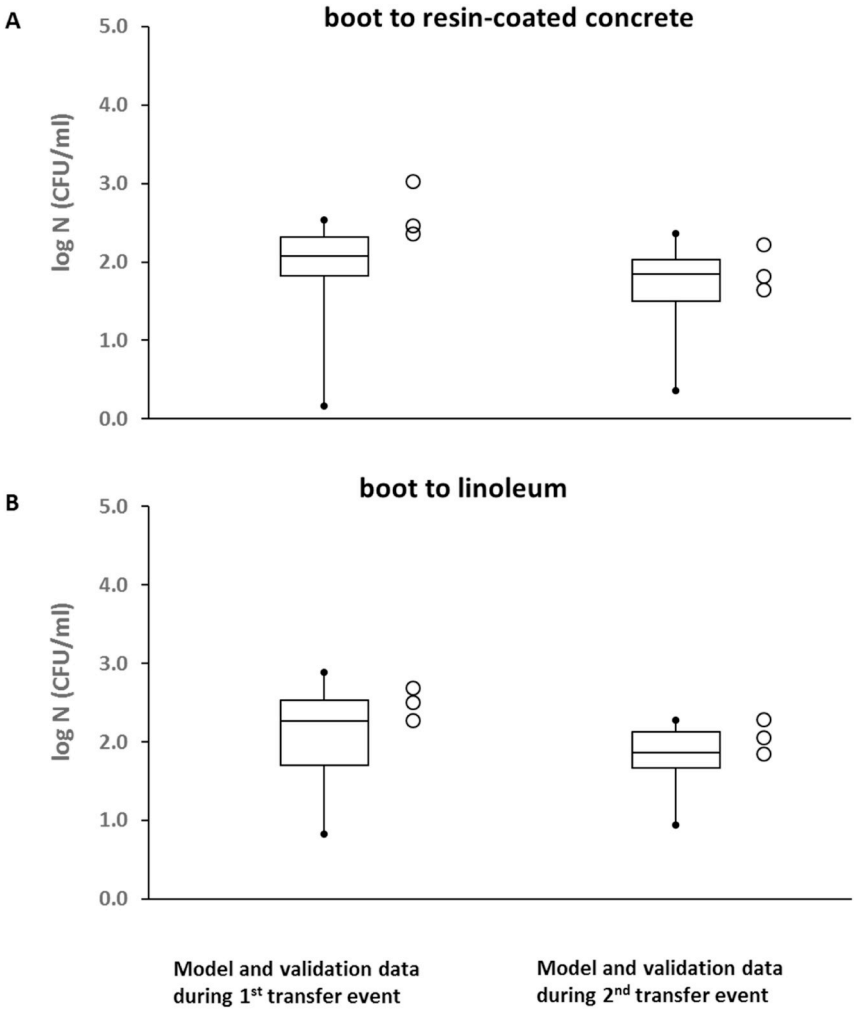

Fig. 4. Comparison of model predictions (●) and validation data (○) for the transfer of *Cronobacter* cells from boots to a resin-coated concrete surface (A) and to a linoleum surface (B). The box and whisker plot summarizes the modelled variable number of cells transferred (95 percentile) during the 1st and 2nd transfer events from the boots to the recipient surfaces. The number of *Cronobacter* cells initially inoculated on the boot ranged from 10^3 to 10^4 CFU/mL.

Table 2
Suggested areas for cleaning during a *Cronobacter* contamination event, based on risk to the final product, and when the bacterium is spread in the manufacturing plant by fomites such as boots, trolley wheels or gloved hands, based on the model presented here. The minimum (0.42 m), median (0.70 m) and maximum (0.98 m) stride lengths for humans at 95% confidence (Mullarney and Archbold, 2013) were used in our model to define these areas for boots. Wheel sizes of 50 mm, 100 mm and 125 mm were used in our model to define these areas for trolley wheels.

Fomite	Zone 1[a]	Zone 2	Zone 3
Boots	20 m^2	55 m^2	100 m^2
Trolley wheels	3 m^2 per wheel (for 4 wheels = 12 m^2)	12 m^2 per wheel (for 4 wheels = 48 m^2)	18 m^2 per wheel (for 4 wheels = 72 m^2)
Gloved hands[b]	50% of the touch points present and most closely associated with the original positive sample	50% of the touch points present and most closely associated with the original positive sample	100% of the touch points present

[a] Hygiene zones 1–3 have been defined as per (Ministry for Primary Industries, 2006). Zone 1 is the low risk hygiene zone and furthest away from product; zone 2 is the standard hygiene zone; and zone 3 is the care/critical hygiene zone, closest to the product.
[b] For gloved hands, a map of the area detailing the touch points needs to be understood.

the dust from a primary filter for a spray dryer as ~ 111 MPN *Cronobacter* cells/g dust. If we apply this lower level of cells to our probability model e.g. for gloves, then a level of 111 CFU/g (assuming 1g of dust on the glove), would result in ≤14 cells being transferred during the initial transfer event, and ≤1 cell still being transferred during the last (6th) transfer event (95th percentile of the probability model for transfer from gloves). Being able to calculate the potential area that has been contaminated with *Cronobacter* gives manufacturing plants a better idea of the size of the area to be targeted for risk management activities during a contamination event.

4. Conclusions

If a movable fomite (boots or trolley wheels) became contaminated, *Cronobacter* could be easily spread within a manufacturing plant over a wide floor area. Contamination of a gloved hand with *Cronobacter* could

easily result in the transfer of *Cronobacter* to numerous touch points (handles, handrails, operational switches) within a manufacturing plant due to the higher probability rate of transfer. Strict hand hygiene would minimize the spread of *Cronobacter* within a manufacturing plant, and regular cleaning and sanitizing of movable fomites, such as boots and trolley wheels, would further decrease the risk of spreading *Cronobacter* on flooring materials. Environmental monitoring would identify niches of *Cronobacter* within the manufacturing plant and this newly developed model could be applied in a practical way to better manage the risk of *Cronobacter* spread from those identified niches within the manufacturing environment.

Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103256.

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