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THE SURVIVAL AND CONTROL OF *SALMONELLA* IN LOW WATER ACTIVITY FOODS' ENVIRONMENTS

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ABSTRACT

A number of large scale *Salmonella* outbreaks have been associated with low a_w food products where the significant causal factor was most likely cross-contamination. These studies sought to both: assess *Salmonella* survival and survival mechanisms when dried onto stainless steel under simulated food factory conditions, and to develop enhanced factory control approaches for this pathogen.

Fifteen isolates of S*almonella* survived 30 days under desiccation and one strain survived for one year and potentially could survive longer. Differences in the survival between different *Salmonella* serotypes, and isolates of the same serotype (*S.* Typhimurium), were noted. A 2-population Weibull model was used to model survival, since following an initial reduction in viability (72 h), no further reduction was seen. There was enhanced survival of *Salmonella* at lower temperatures and relative humidity, with the presence of food debris and when water was added.

At a physiological level, attributes associated with osmoresistance (RDAR phenotype, motility, filamentation, EPS production) were not observed in all desiccation resistant strains, additionally survival was greater on surfaces rather than in hyperosmotic solutions. Desiccated cells did not show increased sodium hypochlorite and propanol resistance. To assess molecular survival mechanisms, three RNA extraction methods were compared and real-time RT-PCR was performed for 13 genes. All selected housekeeping genes showed change in expression therefore were not used. Genes in desiccation sensitive strain had higher fold change during desiccation than in resistant strain (*aceA, nifU* and *otsB*).

The principles of HACCP were reviewed and a prerequisite management programme, based on a risk assessment of sources and vectors, was designed and applied in a low a_w food factory, to help control *Salmonella* cross-contamination.

These results suggest the potential for extended survival of *Salmonell*a in food factories, describe the most suitable model to predict survival kinetics, give some insight into *Salmonella* survival mechanisms and stress responses under desiccation conditions and identify strains and methods for further research studies. The results also confirm the need to keep low a_w factories free of food debris and to eliminate water to reduce *Salmonella* survival. Given the potential for *Salmonell*a survival, the results also highlight the need for the control of cross contamination to the food product via an improved prerequisite management plan.

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CHAPTER 1
LITERATURE REVIEW

1.1 LOW WATER ACTIVITY FOOD CONTAMINATION

For many years, low moisture foods, such as chocolate, were regarded as microbiologically safe due to their inherent product characteristics. The first outbreak of *Salmonella* that could be traced back to low moisture products was registered in the 1970s (Beuchat *et al.*, 2013) and since then it has become the main pathogen of concern for these foods (Finn *et al.*, 2013). There is growing concern for the potential presence of *Salmonella* in low a_w products. The problem within the food industry is widespread, with reports from the Food Standards Agency (FSA, 2014) and the Rapid Alert System for Foods and Feeds (RASFF Portal, 2014) indicating the presence of *Salmonella* in such foods and ingredients as: whole egg powder, coriander, soy bean meal, Tahini, minced dehydrated white onions, sesame seeds, dried sage, peanut butter, black pepper, dried mushrooms, oat cereals, low a_w sausage, chocolate and flour. As many of these foods contain a thermal processing step, post process environmental contamination is thought to be the major vector for contamination. Indeed, the same *Salmonella* type has been isolated from products produced over a number of years (e.g. 10 years for Malt-O-Meal in the USA) from the same plant (Russo et al., 2013). The assumption has been that the particular *Salmonella* strain isolated had survived in the plant for this time period and had contaminated products on a periodic basis, presumably when Good Manufacturing Practices (GMP's) were not well controlled.

All of these facts underline the importance of understanding the mechanisms of *Salmonella* survival in low moisture environments, establishing the environmental factors likely to favour their long term survival and ways to control them.

1.2 WATER ACTIVITY

The working definition of water activity (a_w), described by Reid (2007) is the following: 'water activity is, at a given temperature, the ratio of the equilibrium partial vapour pressure of water in the system (p_w) to the equilibrium partial vapour pressure p^o_w of pure liquid water at the same temperature', which is expressed as:

$$a_w = \left(\frac{p_w}{p^{\circ}_w} \right)_T$$ Equation 1.1

The a_w can range from 0 to 1 and from the microbiological point of view, it indicates how much water is available for microorganisms to grow and is a major factor in preventing or eliminating microbial growth (Potter, 1968). As a generalisation, the majority of spoilage bacteria will grow down to about 0.95 a_w, other bacteria, including pathogens, may grow at values of 0.90 or even 0.85 a_w (Karel, Fennema and Lund, 1975). At lower a_w values down to 0.61, only some yeast and moulds can grow, with filamentous fungi being by far the predominant microflora. Below 0.61 a_w it is not likely that any microorganisms can grow (Reid, 2007, Table 1.1). For the purpose of these studies, low water activity foods are those with an a_w below 0.85. This also complies with the definition developed by Scott *et al*. (2009) and definition used by Codex Alimentarius Commission (2013). The food types which belong to this category are listed in Table 1.1 and whilst they are not supporting pathogen growth, harmful bacteria such as *Salmonella* could survive in such a food and cause illness (Section 1.4.6). The most common reason for pathogen presence in low a_w food is cross-contamination from the environment (Table 1.5) and the most widespread pathogen which causes outbreaks in this type of food is *Salmonella* (CDC, 2014a). Therefore, the described studies are focused on the survival and control of *Salmonella* in low water activity food's environment.

1.3 LOW WATER ACTIVITY FOODS

The types of foods with water activity below 0.85 are listed in Table 1.1. They include foods such as cereals, rice, confectionary, spices, seeds, nuts, powders, dry fruits and vegetables, chips etc. The exact information of the percentage of dry foods out of all foods produced in the world is not available. However, Figure 1.1 shows the general classification of food consumed between 1988 and 1990 (FAO, 2014). The figure does not have more detailed information about food types in each group, however it can be assumed that a high percentage of cereals-based products will have low water activity. The category of fruits, vegetables, pulses and nuts may also contain spices, seeds, nuts, dry fruit and dry vegetables. Oils, fats and sugars are also common ingredients of low water activity foods.

Table 1.1 Minimum a_w for growth of microorganisms and typical products associated with these a_w.

Range of a_w	Microorganisms response	Food products e.g.
>0.98	All grow well	Canned food, fresh foods: milk, vegetables, fruits, meat
0.98-0.92	Most bacterial pathogens (including *Salmonella*) grow	Processed cheese, white bread, mayonnaise, fresh pasta, tomato ketchup
0.91-0.85	*Staphylococcus aureus* is the only pathogen that can grow	Salami, chocolate syrup
0.85-0.80	Most moulds, some yeast	Fudge sauce, cake icing, whey concentrate
0.80-0.65	Most halophilic bacteria, some moulds and yeasts	Wheat grains, cashews nuts, sunflower seeds, black pepper, beef jerky
0.65-0.61	Osmophilic yeasts and a few moulds	Marshmallows, dry fruits, chewing gums
<0.61	No microbial proliferation	
0.6-0.5	No microbial proliferation	Biscuits, rice, chocolate, honey, ground cinnamon
0.5-0.4	No microbial proliferation	Flour, caramels, toffees
0.4-0.3	No microbial proliferation	Cereals, whole egg powder, cocoa
0.3-0.2	No microbial proliferation	Crackers, corn flakes, peanut butter, dry pet food
0.2-0.1	No microbial proliferation	Flavours, coffee powder, tea, potato chips, milk powder

Source: Adapted from: Tapia *et al*., 2007, Schmidt and Fontana, 2007, Aqualab, 2012

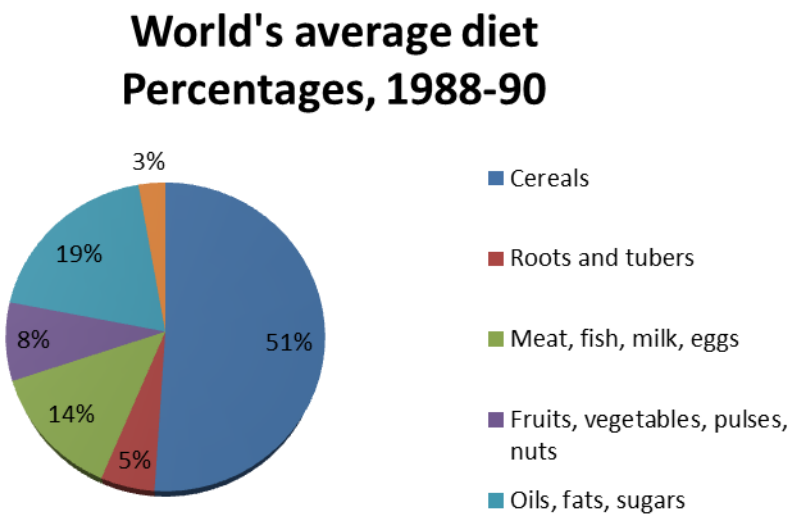


Figure 1.1 Pie chart of the percentages of the world's average consumption of different food commodities (FAO, 2014).

The complete data on daily consumption of low water activity food are not yet publically available, however the author of the thesis belongs to FAO/WHO working group on ranking of microbiological hazards associated with low moisture foods (FAO/WHO, 2014). The group used the WHO/FAO database named CIFOCOss (FAO/WHO Chronic Individual Food Consumption Database Summary Statistics) as being the most reliable individual food consumption database at global level. This database, which will soon be published on FAO/WHO websites, contains summary daily intakes statistics for different populations groups (i.e. toddlers, children, adolescents, adults, elderly and general population) based on 34 food consumption surveys on at least two days of consumption conducted in 23 countries from the last 10 years (Australia, Belgium, Brazil, Bulgaria, China, Cyprus, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Japan, Latvia, Netherlands, South Korea, Spain, Sweden, Thailand, United Kingdom). Table 1.2 summarises the range of daily consumption statistics (mean from whole population, 95 percent of consumers only and the estimation of the standard deviation) per population groups for low moisture broad food categories considered by the expert working group.

On average, each person consumes 341 g of low moisture foods per day which is a high proportion of a daily diet.

Table 1.2 Estimates of daily consumption of low moisture broad food categories at global level per general population (in g/person; FAO/WHO, 2014)

Food categories	General population (2 ->65 years)		
	Mean	SD	High consumption
Cereals and grains	223.8	189.9	532.7
Confections and snacks	67.4	258.4	542.3
Dried fruits and vegetables	21.1	-	295.5
Dried protein products	1.1	-	88.0
Honey and preserves	15.5	120.9	141.3
Nuts and nut products	2.1	-	154.9
Seeds for consumption	5.5	-	179.0
Spices, dried herbs and tea	4.4	-	49.1
Total	**340.9**		

Cereals and grains are the products which are mostly consumed, followed by confections and snacks. The outbreaks associated with these products have frequently occurred during the last decades (Table 1.5).

These consumption data clearly show that any hazards associated with such food products must be taken seriously and quickly resolved or reduced, otherwise they pose a high risk for public health.

1.4 *SALMONELLA* SPP.

Salmonella are facultatively anaerobic, Gram-negative, straight, small (0.7-1.5 µm x 2.0-5.0 µm) rods, which are usually motile with peritichous flagella (Bell and Kyriakides, 2002). They belong to the family *Enterobacteriaceae* and are chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources (Tindall *et al.*, 2005). Most species produce hydrogen sulphide, which can readily be detected by growing them on media containing ferrous sulphate, such as Triple Sugar Iron (Clark and Barret, 1987). The majority of the components of these bacteria are identical, which at the DNA level is between 95% and 99% (Virella, 1999). As a comparison, *E. coli* and *Salmonella*, which belong to the same family, are about 60% - 70% identical at the DNA level (Food Safety Net, 2014). To date, over 2500 serotypes of *Salmonella* have been identified (Grimont and Weill, 2007) and they are of major concern to nearly all sectors of the food industry (Section 1.4.5). The species and subspecies of *Salmonella* can be distinguished on the basis of differential characters such as listed in Table 1.3. Most of the serotypes cannot be distinguished by biochemical tests, and separation is based purely on differences in their antigenic formulae (Bell and Kyriakides, 2002). Serotyping is based on antigenic variability of lipopolysaccharides (O antigen), flagellar proteins (H antigen), and capsular polysaccharides (Vi antigen; Foley, Zhao and Walker, 2007).

1.4.1 *Salmonella* Nomenclature

Over decades, the results from DNA studies indicated that all *Salmonella* serotypes are probably a single bacterial species and, by international agreement, this was called *Salmonella enterica* (Threlfall *et al*., 1999). Seven subspecies were identified within the species. However, following more thorough research one of these subspecies, *S. enterica* subsp. *bongori*, has been found to be sufficiently

different from the other subgroups to be regarded as a separate species called *Salmonella bongori* (Grimont and Weill, 2007; Moreno Switt *et al*., 2009; Tindall *et al*., 2005; Table 1.4). At the moment, two species of *Salmonella* are recognised: *S. enterica* and *S. bongori*, and *S. enterica* is further divided into 6 subspecies.

The formal names for each serotype are therefore much extended e.g. *S. enterica* subsp. *enterica* serotype Typhimurium. For practical purposes this is shortened to *S.* Typhimurium. Therefore, for the purpose of this report, this current practical nomenclature style of serotype names has been used.

Table 1.3 Differential characters of *Salmonella* species and subspecies

Species	S. enterica						S. bongori
Subspecies	enterica	salamae	arizonae	diarizonae	houtanae	indica	
Characters							
Dulcitol	+	+	-	-	-	D	+
ONPG (2h)	-	-	+	+	-	D	+
Malonate	-	+	+	+	-	-	-
Galatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Growth with KCN	-	-	-	-	+	-	+
L(+)-tatrate	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	+	+
ɣ-glutamyltransferase	+(*)	+	-	+	+	+	+
B-glucuronidase	D	D	-	+	-	D	-
Mucate	+	+	+	-(70%)	-	+	+
Salicine	-	-	-	-	+	-	-
Lactose	-	-	-(75%)	+(75%)	-	D	-
Lysed by phage O1	+	+	-	+	-	+	D

(*) Typhimurium d, Dublin -.
\+ 90% or more positive reactions.
\- 90% or more negative reactions.
D different reactions given by different serovars

Adopted from Grimont and Weill, 2007

Table 1.4. Classification of *Salmonella* (Bell and Kyriakides, 2002)

Genus	Species	Subspecies	Serotypes examples
Salmonella	*enterica*	*Enterica*	Typhimurium
			Agona
			Muenchen
		Salamae	Helsinki
			Gilbert
		Arizonae	Arizonae
		diarizonae	Arizonae
		houtenae	Argentina
		Indica	Bornheim
	bongori	-	Balboa

1.4.2 *Salmonella* Infection

The incubation period of *Salmonella* in a host body takes from 4 hours to 72 hours after ingestion. The symptoms include typical ones associated with bacterial infection e.g. vomiting (can be bloody), nausea, diarrhoea and abdominal pain (Virella, 2000; Chen *et al.*, 2013). Sometimes fever is present which lasts for approximately 3 days. All the symptoms usually pass after 7 days of infection; however the pathogen can be present in faeces for up to 5 weeks, sometimes even longer (WHO, 2014). In 5%-10% of cases, bacteraemia occurs which might lead to a range of illnesses such as meningitis, and bone and joint infections (Hammack, 2012). In less developed countries, *Salmonella* can cause bloodstream infection and death (Reddy, Shaw and Crump, 2010).

1.4.3 Virulence Capabilities

The typical infectious dose of *Salmonella* spp. required to cause illness is assumed to be minimum 10^6 cells, this number can, however, be influenced by the immune state of the human's body and the physiological state of the cell and type of food carrier (Baird-Parker, 1990). Pre-exposure of cells to stressful conditions such as low a_w or low pH may improve the survival of cells in the harsh conditions of the gastric juices (Nojoumi, Smith and Rowbury, 1995). It has been suggested that lipids can protect *Salmonella* against these gastric juices (D'Aoust, 1997). Therefore foods with high fat content such as chocolate have been linked with low infectious dose of 1-10 cfu (D'Aoust, 1985; Kapperud *et al.*, 1990), especially in young children.

1.4.4 *Salmonella* in Relation to Food Safety

The subspecies of most concern in relation to food safety is *S. enterica* subsp. *enterica;* >99% of *Salmonella* isolated from infected people belong to this subspecies. *S.* Arizonae is also occasionally of concern to food safety but is more commonly associated with cold blooded animals e.g. reptiles (Old, 1992).

A foodborne outbreak is defined by CDC (2014a) as occurrence of two or more cases of similar illness resulting from consumption of common food. *Salmonella* was for decades the most significant pathogen of concern in terms of foodborne outbreaks. In USA it is still the pathogen which is causing the highest number of bacterial foodborne outbreaks (51%; Figure 1.2) and hospitalisation (64% out of all foodborne outbreaks hospitalisations; CDC, 2014a).

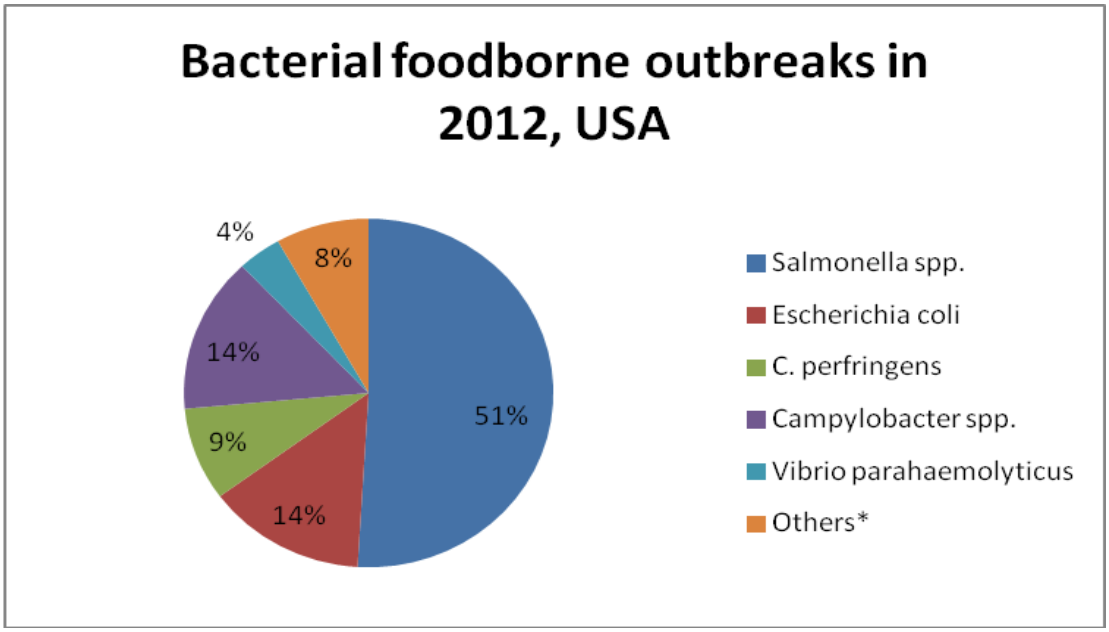


Figure 1.2 Bacterial foodborne outbreaks that occurred during 2012 in USA (CDC, 2014a)

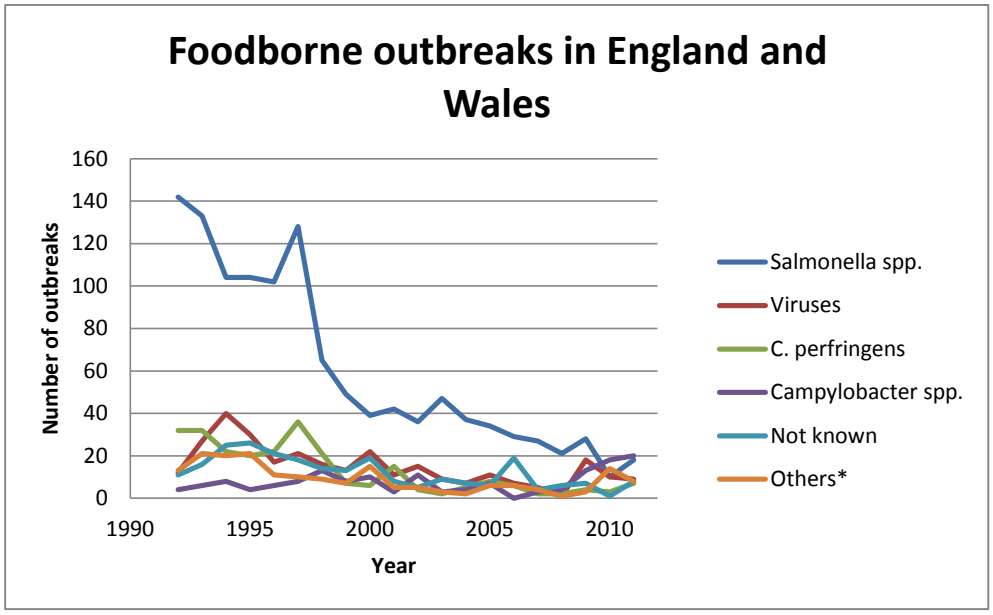


Figure 1.3 Foodborne outbreaks reported to the Health Protection Agency, England and Wales, 1992 - 2011 (by pathogen; HPA, 2012a).

However the recent records from England and Wales show that the number of *Salmonella* outbreaks in these regions significantly decreased and it is similar to a number of outbreaks caused by *Campylobacter* (Figure 1.3). The data from 1992-2011 show that over that time 51% of foodborne illnesses from outbreaks were caused by *Salmonella* (HPA, 2012a) but when looking at the data from 2011 only,

Salmonella caused 26% of outbreaks. Figure 1.3 also shows a general trend in the decrease in the number of reported cases of Salmonellosis in England and Wales. This is mainly due to the better handling and enhanced hygiene of the raw meat and products containing eggs, also better animal husbandry (including vaccination), movement restrictions, compulsory slaughter and disinfection procedures at poultry farms which have reduced the incidence of *Salmonella* in raw poultry.

The vaccination scheme began in breeding flocks in 1994 and in laying flocks in 1998 (Food Safety News, 2013) and it helped to reduce the number of outbreaks from 104 in 1994 to 39 in 2000. The most common serotypes, which were causing illness in England and Wales during 2000-2011, were *S.* Typhimurium and *S.* Enteritidis (HPA, 2012b). Both of the serotypes have been included in these studies. In USA these two serovars caused 39% of *Salmonella* outbreaks (CDC, 2014a).

The highest percentage of *Salmonella* infection in England and Wales is amongst infants and babies younger than one year old, followed by children of 1-4 years old (Figure 1.4). This is one of the most susceptible age groups to pathogens, together with the elderly population. The elderly, however, do not appear to be largely affected (HPA, 2012c). This may indicate that *Salmonella* is present in the food types which are most likely to be consumed by young children and infants. This food could potentially include infant formula, milk powder, confectionary and breakfast cereals.

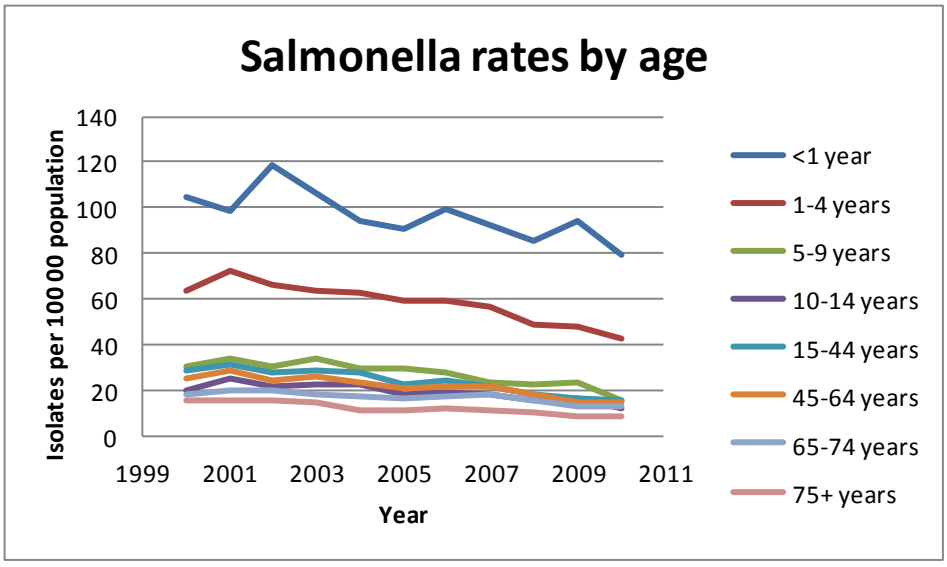


Figure 1.4 *Salmonella* by age group and year. All human isolates reported to the Health Protection Agency Centre for Infections. England and Wales, 2000-2010 (HPA, 2011c).

1.4.5 The Most Common Food Vehicles for Fooborne *Salmonella* in England and Wales

Data obtained by the Health Protection Agency (HPA, 2012d) indicate that between 1992 and 2011 the most common food vehicles for *Salmonella* were eggs and products containing raw eggs (31%). However 32 % of foodborne outbreaks were caused by food products which may include low a_w foods e.g. vegetables & fruits (including dried fruits and vegetables, seeds and nuts), desserts, cakes and confectionary (including chocolate etc.), mixed foods and other foods. Therefore almost one third of the outbreaks could potentially be caused by low a_w food products. Additionally in other categories such as meat products, the contamination could potentially come from the herbs and spices. Unfortunately, there are no data which show exactly how many outbreaks were caused by low a_w food products in England and Wales.

1.4.6 Outbreaks of *Salmonella* associated with consumption of low a_w food

During the last 40 years *Salmonella* outbreaks have been linked to a number of food products with low water activity including: cereal, seeds, nuts, chocolate, dried fruits and vegetables, halva, dried milk products, spices, salami, tahini or tea. These outbreaks are usually very specific and are characterised as widely geographically spread, affecting a large number of people and lasting for relatively long periods of time (CDC, 2014 and FSA, 2014, websites). Beuchat *et al*. (2011, 2013) and CDC (2014b) described that there have been around 40 large, documented outbreaks of *Salmonella* associated with dry foods. Table 1.5 shows the outbreaks associated with the serotypes used for this study and other serotypes involved e.g. *S.* Eastbourne in chocolate (D'Aoust *et al.*, 1975), *S.* Senftenberg in infant food (Rushdy *et al*., 1998), *S.* Oranienburg in black pepper (Gustavsen and Breen, 1984), *S.* Montevideo in chocolate (UK, 2006) and tahini (Unicomb *et al*., 2005) etc. The whole list also includes sources of *Salmonella* contamination and this is further described in Chapter 7. The highest number of outbreaks has been associated with peanut butter (Beuchat *et al.*, 2013), often part of a child's diet, and powdered infant formula. Assuming that the total tonnage of peanut butter and infant formula is not out of proportion to other commodities the reason behind it could potentially be the larger likelihood of children and infants becoming ill and the fat in peanut butter protecting the cells from gastric juices.

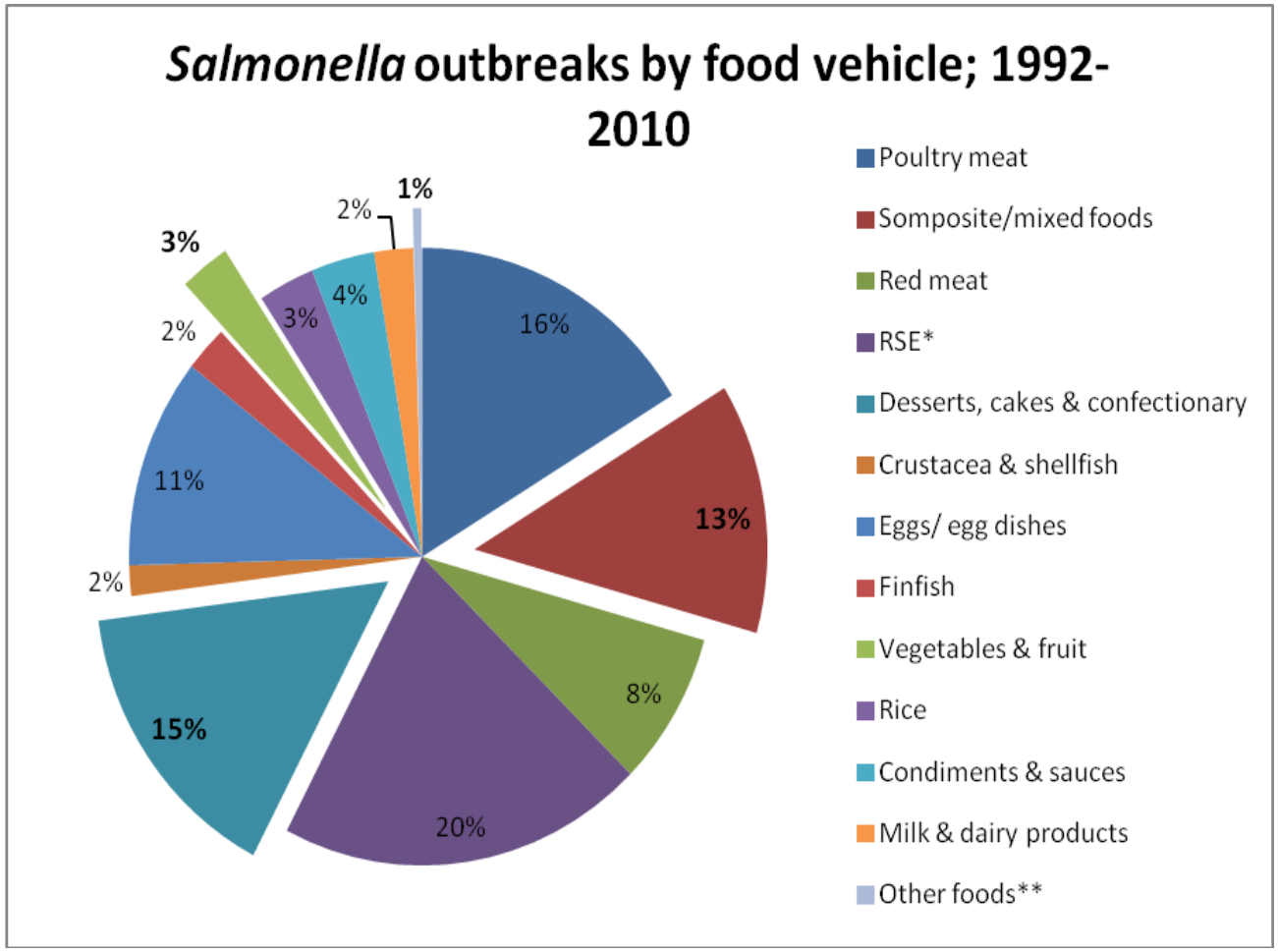


Figure 1.5 *Salmonella* foodborne outbreaks reported to the Health Protection Agency, England and Wales, 1992 - 2010 (by food vehicle). *Raw shell egg used as an ingredient in lightly cooked or uncooked food, **other foods include soup and pureed foods (HPA, 2012d).

Table 1.5. *Salmonella* outbreaks associated with dry foods

Year	Food Product	People affected/ country	Serotype	Possible cause/comments	References	Potten-tial cause
2013	Tahini Sesame Paste	16, USA	Montevideo Mbandaka	FDA inspection identified: failure to store raw material to avoid contamination, failure to protect food transported by conveyor from contamination, not adequate plumbing and drainage	CDC, 2013	C
2012	Peanut butter	42, USA	Bredeney	FDA inspection identified: failure to manufacture foods under conditions and controls necessary to minimize potential growth and survival of microorganisms (e.g. failure with cleaning and disinfection, lack of appropriate hygienic design of the equipment and factory, water presence on surfaces, lack of appropriate segregation). Positive samples from the environment were obtained.	FDA, 2013	C
2011	Turkish pine nuts	43, USA	Enteritidis	Bulk bags contaminated (no information available about the source)	CDC, 2011	P
2010	Salami	272, USA	Montevideo	Black and red crushed pepper imported from Vietnam was a source of contamination; it was added after the lethality step.	CDC, 2010	P
2008	Cereal	28, USA	Agona	Presumably dust from the disruption of the previously intact wall (contaminated with *Salmonella* from 1998 outbreak) during plant maintenance activities, in combination with wet cleaning, may have reintroduced the desiccated outbreak strain of *Salmonella* Agona into the cereal production area.	Russo *et al.*, 2013	C
	Peanut butter	684, USA, Canada	Typhimurium	Route of contamination not known	CDC ,2009	ND
	Powdered infant formula	8, France	Give	Route of contamination not known	Jourdan *et al.* 2008	ND
2007	Snack (rice-corn)	65, USA	Wandsworth Typhimurium	The seasoning mix used in Veggie Booty may be the source of the contamination	CDC, 2007b	ND

Year	Food Product	People affected/ country	Serotype	Possible cause/comments	References	Potten-tial cause
2006	Peanut butter	715, USA	Tennessee	*Salmonella* was isolated from environmental samples from the manufacturing plant and environmental contamination is likely source of the outbreak. It was suggested that the contamination was persistent for prolonged period of time.	CDC, 2007a; Sheth *et al.* 2011	C
	Chocolate	37, UK	Montevideo	Leaking pipe has been implicated as a source of contamination. Waste water was dripping into the chocolate crumb.	Anon., 2006	C
2005	Almonds	15, Sweden	Enteritidis	Almonds were not treated	Müller *et al.*, 2007	P
	Cake mix	26, USA	Typhimurium	Route of contamination not known	Zhang, 2007	ND
2004	Powdered infant formula	141, France		The source of contamination in the manufacturing plant was not established, however a former and persistent environmental contamination was suspected.	Cahill *et al.*, 2008	C
2003	Almonds	29, USA, Canada	Enteritidis PT9c	Almonds were not treated. One environmental sample at the manufacturer premises and three samples from huller-shellers were positive for *Salmonella*	CDC, 2004	P
	Tea (aniseed)	42, Germany	Agona	Batch of tea was contaminated with aniseed cultivated in Turkey that have been fertilized with manure	Koch *et al.*, 2005	P
2002	Tahini	68, Australia, New Zeland	Montevideo	Exact contamination route not known. It is suspected that sesame seeds were contaminated during harvesting	Unicomb *et al.* 2005	P
2001	Chocolate	439, Denmark, AT, DE, Belgium, Finland	Oranienburg	Contaminated chocolate was produced during one week. Source was never determined. Hygienic violations were not observed in the factory. Milled cocoa beans received additional heat-steam treatment with 125-130°C. It is not clear if *Salmonella* survived heating process or contamination was introduced after.	Werber *et al.* 2005	ND

Year	Food Product	People affected/ country	Serotype	Possible cause/comments	References	Potten-tial cause
	Halva	70, AU, DE, UK Norway, Sweden,	Typhimurium DT104	Contamination was associated with sesame seeds produced in Turkey	de Jong *et al.*, 2001	P
	Peanuts	109, Australia, Canada, UK	Stanley, Newport	Asian-style peanuts were implicated as a source, some of them were roasted some of them just dried and flavoured.	Krik *et al.*, 2004	P
2000	Almonds	168, USA, Canada	Enteritidis PT30	*Salmonella* was isolated from raw almonds, factory equipment (6-7 months after being used), warehouse, orchards and distribution. It was recovered for prolonged period on frequent basis which suggest persistent contamination.	Beuchat *et al.* 2013	P
	Powdered infant formula	30, Korea	London	It was not possible to determine whether the PIF was contaminated during manufacture or after opening of the package for consumption, because the organism was only found in an opened package of formula.	Cahill *et al.*, 2008)	ND
1999	Coconut	18, UK	Java PT Dundee	Route of contamination not known	Ward *et al.*, 1999	ND
1998	Cereal	209, USA	Agona	Route of contamination not known	CDC, 1998	ND
1996	Infant dried milk	12, UK, France	Anatum	Salmonella was not found in environmental samples from the production plant. The product samples which were positive for Salmonella were produced from the same batch of raw ingredients	Anon, 1997	ND
	Peanut butter	54, Australia	Mbandaka	Route of contamination not known	Beuchat *et al.* 2013	ND
	Powdered infant formula	>48, Spain	Virchow	Salmonella was isolated from the milk powder however there were no positive samples from the factory environment. Exact route of contamination is not known	Usera *et al.*, 1996	ND

Year	Food Product	People affected/ country	Serotype	Possible cause/comments	References	Potten-tial cause
1995	Infant food	5, UK	Senftenberg	Cleaning remains from milling machinery were implicated as a source. The machinery was used to mill other products which may have not been heat treated.	Rushdy *et al.*, 1998	C
1994	Snack (savoury)	2200, UK, USA, Israel	Agona PT15	*S.* Agona was not isolated from any of the swabs taken from the production line, ingredients, or stool samples from workers. S Enteritidis was isolated from a reusable plastic bag in which the snack was stored for 48 hours before packaging. Contaminated batches were produced on at least 7 separate dates during four months.	Shohat *et al.* 1996; Killalea *et al.* 1995	C
1993	Potato chips	1000, Germany	Saintpaul, Rubislaw, Javiana	Contaminated paprika powder from South America was the source. The powder was applied to the roasted chips in the end of the production when the temperature dropped to 60°C	Lehmacher *et al.* 1995	P
	Powdered infant formula	3, Canada, USA	Tennessee	FDA isolated *S.* Tennessee from production equipment in the plant where the formula was dried	CDC, 1993	C
1989	Snack (corn)	47, UK	Manchester	Three *Salmonella* serotypes (including *S.* Manchester) were isolated from site producing yeast extract used for flavouring. These serotypes were also found in stream water used for cooling processing water in the factory.	Beuchat *et al.*, 2013	C
1987	Chocolate	361, Norway, Finland	Typhimurium	*S.* Typhimurium was not isolated from 11 different raw products and dry ingredients and environmental dust and debris from the factory, however different *Salmonella* serovars were isolated from environmental dust. Birds could access the plant and introduce contamination. Inspection of the factory revealed several routes of possible contamination of the product.	Kapperud *et al.*, 1990	C
1985	Chocolate	33, USA, Canada	Nima	Route of contamination not known	Hockin *et al.*, 1989	P
	Infant dried milk product	76, UK	Ealing	The source of infection was traced to the factory spray-drier, which had a hole in its inner lining, allowing escape of powder and its return from contaminated insulation material.	Rowe *et al.*, 1987	C

Year	Food Product	People affected/ country	Serotype	Possible cause/comments	References	Potten-tial cause
1982	Chocolate	245, UK	Napoli	Route of contamination not known	Beuchat *et al.* 2013	
1981	Pepper (black)	126, Norway	Oranienburg	Contaminated pepper was shipped from Brazil and it was not treated	Gustavsen and Breen, 1984	P
1973	Chocolate	95, USA, Canada	Eastbourne	Cocoa beans were most likely the source of contamination. The Centre for Disease Control (U.S.A.) found *S.* Eastbourne in roasted cocoa beans, in rooms dedicated to bean cleaning and roasting and on the Inside of a tempering tank. The inspection revealed numerous opportunities for cross contamination in the plant and also identified a number of deficiencies in the chocolate processing operations.	D'Aoust *et al.* 1975	P
	Powdered milk	3000, Trinidad	Derby	Workers may have been vehicles of *Salmonella* Derby.	Beuchat *et al.*, 2013	C
1972	Fishmeal	17, USA	Agona	Contamination spread from fishmeal to poultry and further to restaurant kitchen. The same strain was isolated from shrimps.	Beuchat *et al.*, 2013	P
1970	Cocoa	109, Sweden	Durham	Cocoa was contaminated and manufacturing process was not sufficient to kill possible Salmonella contamination.	Beuchat *et al.*, 2013	P

C – cross-contamination from the environment

P – not sufficient or lack of microbial reduction step; ND – cause not determined

1.4.7 Economical Implication for the Food Industry

The presence of pathogens, such as *Salmonella,* in low moisture foods can cause not only human health issues, it has also a huge impact on the businesses which produce contaminated food. In today's world all the information is very quickly spread via mass media, therefore the knowledge of any food poisoning incident can be transfer to a large number of people in a short period of time (CNN, 1998; BBC, 2006; The New York Times, 2009). If the source of an outbreak is traced to a specific product, or laboratory testing confirms presence of pathogens, the product must be recalled from the market (FDA, 2013). The cost of each recall is very high and it includes a number of financial exposures:

- Stop in production, therefore loss of profit during the time when the factory is closed for inspection or sanitation
- Cost of transporting, storing and destroying recalled product, and also supplying replacement goods
- Liability risk, therefore any potential legal actions taken by affected customers
- Reputation damage, which often is the largest loss associated with a recall or outbreak (GMA, 2011).

Loss of reputation is seen as having the largest impact on the business, which can be even devastating. Some companies which experienced the recall of contaminated foods went to bankruptcy (Peanut Corporation of America; FSN, 2013) or changed their names (Malt-O-Meal to Mom Brands; Mom Brands, 2012). The survey conducted by Grocery Manufacturers of America (GMA) indicated that 77% of companies which faced a recall during 2006-2011, had costs associated with it up to 30 million dollars. 23% of the companies had faced even higher financial impact (GMA, 2011). In Europe, only in 2013, the number of alerts associated with the presence of pathogens in low a_w food and feed was approximately 174, which also translates to a large economic impact on the businesses (RASFF, 2014).

1.4.8 Survival of *Salmonella* in Low a_w Food Products

The ability of *Salmonella* to survive for long periods of time in low a_w products e.g. dried almond hulls (Uesugi and Harris, 2006), halva (Kotzekidou, 1998) and peanut butter (Burnett *et al.*, 2000) has been documented. Juven *et al*. (1984) investigated survival of *S*. Montevideo and *S*. Heidelberg in dry milk, cocoa powder

and poultry feed at three water activities in the range of 0.4 to 0.75. After 14 weeks, survival was greater at an a_w of 0.43 and 0.52 rather than at 0.75 a_w. There were differences observed in survival between products equilibrated at a specific a_w value and different resistances to dry environments between two serovars was demonstrated (Juven *et al.,* 1984). Komitopoulou and Penaloza also studied the behaviour of Salmonellae in dry food and their work demonstrated that it can survive storage for 3-4 weeks in dry raw materials and that survival is dependent on the source of strains, cell preparation, inoculation methodology and storage temperature (Komitopoulou and Penaloza, 2009). The importance of storage temperature on desiccated cells was also examined by Hiramatsu *et al*. (2005) and it was proved that decreasing storage temperature from 25ºC - 35ºC to 4ºC increased the survival of *Salmonella* from 70 days to 24 months. Hiramatsu *et al.* (2005) reported that survival rates of *Salmonella* on dry foods and in a desiccation model system were significantly increased by the presence of sucrose. Similarly, dehydration of *Salmonella* in the presence of the compatible solutes trehalose and sucrose facilitated both dehydration tolerance and subsequent persistence during cold-storage (Gruzdev *et al*., 2012a).

1.4.9 *Salmonella* Presence in Plant Processing Environments

Whilst there is some information on the survival of *Salmonella* in dried products (Podolak *et al*., 2009), there is little information on the survival of *Salmonella* in the environment, both in terms of the length of survival and the possible factors contributing to such survival. Uesugi *et al*. (2007) demonstrated the potential for long-term environmental persistence of *Salmonella* in almond orchards. In their studies, 53 *Salmonella* isolates collected from the selected orchard during a 5 year period were identified as the same serovar and phage type: *Salmonella* Enteritidis PT 30 (Uesugi *et al.,* 2007).

The importance of contaminated surfaces in relation to the potential transmission of pathogens to food is apparent in food processing, catering and the domestic environment. Kusumaningrum *et al*. (2003) showed that *Salmonella* can remain viable on dry stainless steel surfaces and present the potential for contamination for considerable periods of time. *S*. Enteritidis was recovered from dry, highly contaminated stainless steel surfaces after at least four days and it was recovered after 24 hours from moderately contaminated surfaces.

Evidence of *Salmonella* survival has been found in plant processing environments. An outbreak of *Salmonella* Agona associated with toasted oats cereal showed a presence of the organism at low levels in a large part of the plant environment, including samples taken from the floor, production equipment and the exhaust system in the plant (Breuer, 1999; FDA, 1999). In a study involving contaminated chocolate by Craven *et al.* (1975), investigators found that the cause of the contamination was the uncontrolled airborne spread of dust due to inadequate zoning. It is evident, therefore, that *Salmonella* can be present in the environment and potentially contaminate the product. Samples taken in an oilmeal plant also detected *Salmonella* on the processing floor, in dust in the air and on equipment (Morita *et al*., 2006). Sandvang *et al.* (2000) isolated a *Salmonella* clone from a pig farm, which might both persist and survive in the farm environment. The same Typhimurium clone was sampled from the piggeries, conveyor belt, manure and soil spread with manure, over a period of 20 months. Other studies conducted by Baloda *et al*. (2001) confirm that *S.* Typhiumrium clones DT104 and DT12 can survive in soil up to 299 days. In several studies *Salmonella* was isolated from filters, processing floors, mixers, air and brooms in feed mills or crushing plants over a large period of time, indicating *Salmonella*'s ability to survive in dry processing environments (Binter *et al*., 2011).

In summary, survival of *Salmonella* in the soil, in water, and on a variety of surfaces provides the bacterium with an increased probability of infecting a new host (Winfield and Groisman, 2003). Additionally, there is an additional phenomenon to the importation of *Salmonella* contaminated products, which is the emergence and subsequent persistence or disappearance of serovars in human populations on a national scale (D'Aoust, 1994).

1.4.10 Heat Resistance of *Salmonella* in Low a_w Foods

It is also well known that as the water activity of foods becomes lower, the probability of microbial growth is reduced; however the ability of organisms to survive is greatly increased. Indeed the heat resistance of *Salmonella* was reported to increase from 27 seconds when heated in ground beef at 60˚C, compared to 14.6 hours when heated in wheat flour at 62˚C (Archer *et al*., 1998). Beuchat and Heaton (1975) also reported that heat treating *Salmonella* Senftenberg on pecans for 2 minutes at up to 93˚C (normally used during processing of pecans) was insufficient

to destroy all Salmonellae. In a case shown by McDonough and Hargrove (1968) *Salmonella* was able to survive at 76^{o}C for more than ten hours in dry milk powder. The effect of a_w on heat resistance of *Salmonella* in cocoa and hazelnut shells (Penaloza, Izurieta and Komitopoulou, 2012), chocolate (Goephert and Biggie, 1968; Barrile and Cone, 1970), peanut butter (Shachar and Yaron, 2006), liquid medium (Chiewehan *et al*., 2007), corn flour (VanCauwenberge *et al.*, 1981), egg products (Garibaldi *et al*., 1969), on surfaces (Kirby and Davies, 1990) and dried milk (McDonough and Hargrove, 1968) was also investigated and confirmed the increase of heat resistance of *Salmonella* with decrease of water activity.

1.4.11 Cross tolerance to other stresses

Development of cross-tolerance to diverse environmental stresses, following exposure to a single stress is widely common in bacteria (Abee and Wouters, 1999; Vorob'eva, 2003; Kultz, 2005). Similarly, cross tolerance was also reported in *Salmonella* following exposure to osmotic- or desiccation stress. These include, sodium hypochlorite (Hiramatsu *et al.*, 2005; Kieboom *et al*., 2006), and ethanol (Kieboom *et al*., 2006). In a recent study, the issue of cross-tolerance of dried *S.* Typhimurium to multiple stresses was studied (Gruzdev *et al.*, 2011). Dehydrated *Salmonella* developed increased tolerance to nine of ten tested stresses, including high NaCl concentrations, bile salts, UV irradiation, dry heat, ethanol, sodium hypochlorite, didecyl dimethyl ammonium chloride, and hydrogen peroxide. Surprisingly, the desiccated bacteria were more susceptible to organic acids, compared to control (wet) bacteria (Gruzdev *et al*., 2011).

1.5 *SALMONELLA* STRESS RESPONSES

1.5.1 Osmotic Shock Stress

Although bacterial cells may encounter an osmotic stress with other means than drying, during the process of dehydration, cells also face an osmotic stress due to loss of water. Exposure of *Salmonella* to osmotic or desiccation stress triggers an array of adaptive physiological responses in the bacterium. Hyperosmolarity outside the cell causes considerable shrinkage of the microbial cell due to the water loss (Csonka, 1989), consequently, the concentrations of all the intracellular metabolites will increase. Sudden plasmolysis may result in the inhibition of a variety of

physiological processes, ranging from nutrient uptake to deoxyribonucleic acid (DNA) replication (Csonka, 1989).

1.5.1.1 Transport of Osmoprotectants

In order to maintain turgor pressure the cells respond by increasing the concentrations of compatible solutes and osmoprotectants. In *Salmonella* these could be: glutamate, glutamine, proline (Csonka, 1981 and 1983), glycine-betaine (LeRudulier and Bouillard, 1983), choline or trehalose (Giaever *et al*., 1988). There is a hierarchy of osmotic solutes in their utilization and effectiveness; *Salmonella* prefer betaine over proline-betaine which, in contrast, is preferred over proline (Csonka and Epstein, 1996). Figure 1.6 shows a schematic of bacterial cell response to hyperosmotic shock. The initial response of bacteria to hyperosmotic shock is the increase of K^+ uptake. Three systems are responsible for this function: *Trk, Kdp* and *Kup*, with the first two being the most significant (Moat *et al.*, 2002). The *Trk* system consists of three components: *TrkA* (peripheral membrane protein), *TrkE* (membrane associated), and *TrkG* (membrane-spanning protein), and is expressed constitutively. The *Kdp* system has very high affinity for K^+, its expression highly depends on osmolarity of the medium (Csonka and Epstein, 1996) and it is also composed of three parts: *KdpA* (membrane-spanning protein), *KdpB* (integral membrane protein), and *KdpC* (peripheral membrane protein). In order to maintain electroneutrality, the cell next increases the glutamate pool by glutamate synthesis. This process is catalyzed by two enzymes: glutamate dehydrogenase (GDH) and glutamate synthase (GS; Csonka, 1989).

At a later time of the hyperosmotic stress, trehalose synthesis is induced by the products of the *otsAB* operon (Csonka and Epstein, 1996), and the levels of K^+/glutamate start to decline.

Trehalose production is dependent on the alternative sigma factor, RpoS for the induction of *otsAB* (Kempf and Bremer, 1998). RpoS synthesis is known to be induced during the osmotic shock (Rychlik and Barrow, 2005). If osmoprotectant molecules, such as glycine-betaine, proline, choline, are present externally, they might be taken into the cell by specific transporters, such as *ProP* (glycine-betaine, proline), *ProU* (glycine-betaine) and *OsmU* (betaine; Jovanovich *et al*., 1988). DNA supercoiling has been also shown to be important in the induction of *proU* (Higgins *et al*., 1988).

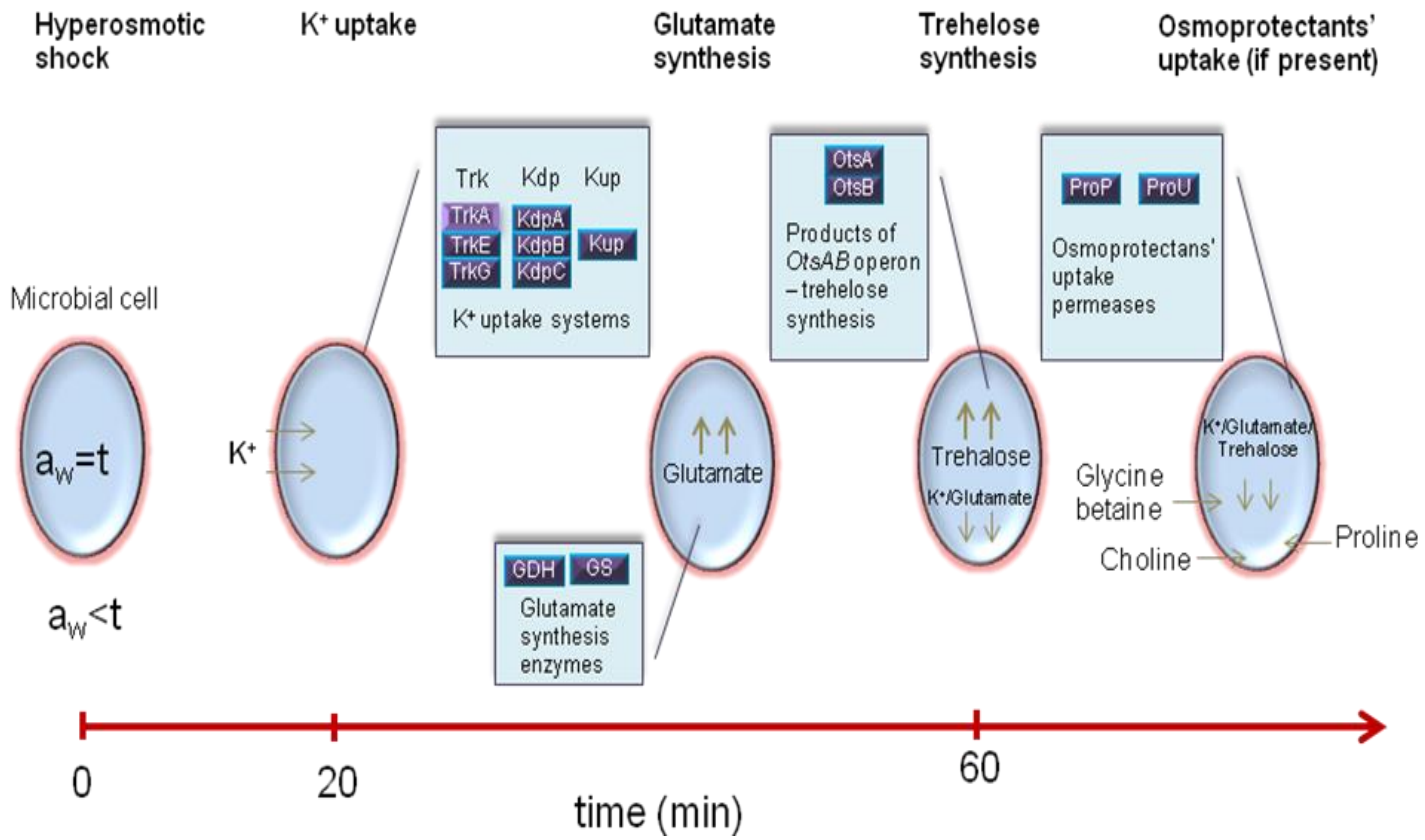


Figure 1.6 Synthesis and uptake of compatible solutes/osmoprotectants as a response to hyperosmotic shock. Based on Moat *et al.* (2002).

1.5.1.2 Passive Diffusion of Osmoprotectants

After initiating described mechanisms of osmoprotectants uptake or synthesis, cells which are placed in a high osmotic environment can start passive diffusion of osmoprotectants (Balaji *et al*., 2005). *S.* Typhimurium has two major porins which function as channels to facilitate the diffusion of small hydrophilic molecules: OmpF and OmpC. High osmolarity causes a decrease of OmpF and an increase of OmpC levels (Feng *et al*., 2003). This is mainly due to the smaller size of OmpC porin which blocks larger molecules and allows only smaller ones to come through. The transcription of *ompC* is mediated by a two-component regulatory system involving a membrane sensor protein, EnvZ, which activates the signal-transducing regulatory protein, OmpR, in response to some periplasmic signals (Feng *et al*., 2003). The total cellular level of these proteins in the cell is constant (Nikaido and Vaara, 1987; Csonka, 1989).

1.5.2 Physiological Responses to Desiccation and Osmotic Stress

Under desiccation, a portion of the *Salmonella* population may change into a physiologically dormant state and become viable but nonculturable (VBNC; Lesne *et al*., 2000; Podolak *et al*., 2009; Gruzdev *et al*., 2012). Transcriptome sequencing of *S.* Enteritidis in peanut oil showed that bacteria entered in a physiologically dormant state with <5% of the genome being transcribed compared to 78% in a rich nutrient medium (Deng *et al*., 2012). This phenomenon may result in under-estimation of *Salmonella* contamination in low-moisture foods and consequently poses a challenge to any food microbiology laboratory.

Mattick *et al. (2000)* demonstrated formation of filaments when *Salmonella* cells were placed in hyperosmotic solutions (a_w=0.92-0.96); however Eriksson de Rezende *et al*. (2001), found filamentation in a_w=0.95 but not in 0.92. Although, the two studies were performed in different setting and with different strains, it seems that filamentation is probably not a common mechanism for *Salmonella* persistence during dehydration and long-term storage as also studies conducted by Gruzdev and Sela (2014, unpublished results; Microbial Food-Safety Research Unit, Department of Food Quality and Safety, Institute for Postharvest Technology and Food Sciences, the Volcani Center, Bet-Dagan, Israel) did not show a relationship between these two mechanisms. These studies were focused on investigating an influence of *Salmonella* Typhimurium filamentation abilities on desiccation resistance on plastic surfaces.

Another response to a low a_w environment that was recently reported in *S.* Enteritidis cells, is the degradation of ribosomal RNA molecules (Deng *et al.*, 2012), which might serve as a potential source of nutrients required for adaptation of the bacterium to the water-less environment. This finding is in agreement with the need for increase biosynthesis of osmoprotectants and the observed increase in fatty acid catabolism (Li *et al*., 2012; Finn *et al.*, 2013).

Additional changes in cell metabolism upon exposure to a low a_w environment involve the cell membrane. An increase in the ratio of *trans* to *cis* unsaturated fatty acids was observed in cells exposed to high salt concentrations (Cronan, 2002). Also, the proportion of anionic phospholipid and/or glycolipids was increased (Russell *et al*., 1995).

Recent studies on air-dried *Salmonella* Tennessee (Li *et al*., 2012) desiccated in sterile quartz demonstrated massive accumulation of trehalose following the exposure to desiccation (Zhang and Yan, 2012). The role of extracellular polysaccharide matrix (EPS) and O-antigen capsule in long-term survival and persistence of *Salmonella* at dry conditions has been studied by several authors (Gibson *et al*., 2006; Garmiri *et al*., 2008). White *et al*. (2006) found that the expression of thin aggregative fimbriae (Tafi) and cellulose enhances the resistance of *Salmonella* to desiccation on dry plastic surfaces in the absence of nutrients. In contrast, Garmiri *et al*. (2008) observed no difference in *Salmonella* survival in dried blood droplets in a knockout mutant of the cellulose synthetase gene. Both strain and desiccation conditions varied in the two studies. Recently, Finn *et al*. (2013a) demonstrated the importance of the ABC transporter, *ProP* in desiccation tolerance of *Salmonella* on stainless steel, which also suggests that composition of the environment is an important factor in survivial abilities. Another study revealed the critical role of pathogenicity-related factor Sep22 in the ability of *Salmonella* to withstand desiccation (Tamura *et al*., 2009). However, the underlying mechanism remains unknown.

1.6 TRANSCRIPTOMIC ANALYSIS

1.6.1 Transcription of RNA

Expression of genes in living organisms is initialised by transcription of RNA from DNA template which leads to forming protein encoded by these genes (Turner *et al*., 2005a). The whole process of gene expression can be divided into

three stages: initiation, elongation and termination. In bacteria, transcription is initialised by the enzyme called RNA polymerase. This enzyme contains four catalytic subunits and one regulatory subunit known as the sigma factor (Clancy, 2008). Sigma factors are needed to initiate specific transcription from promoter DNA (Saecker, Record, & Dehaseth, 2011). The transcriptional machinery first unwinds the template DNA before RNA starts to be synthesised. This stage is referred to as open complex formation (Wang and Greene, 2011). RNA synthesis always occurs in a fixed direction: from 5'- to 3'- end of the RNA molecule. Free nucleotides are aligned on the DNA template by their complementarities with the template: adenine aligns with thymine in the DNA, guanine with cytosine, cytosine with guanine and uracyl with adenine (Griffiths *et al*., 2000). RNA polymerase also recognises a specific DNA sequence known as a terminator which terminates the transcription. Termination includes the release of the nascent RNA and polymerase from the template (Ryder & Roberts, 2003).

A number of methods is available to study transcriptomics, which is looking at the complete set of RNA transcribed from the genome in a specific cell, at certain conditions and time period. Transcriptomics is used to identify genes that are differentially expressed during e.g. different treatments or in different cells, therefore it shows mechanisms of molecular responses within a cell to specific conditions (Nature, 2014).

Additionally, there are two other methods which can be used to analyse molecular dynamics of the cell: proteomics (investigation into complete set of proteins in the cell) and metabolomics (analysis of complete set of metabolites generated by the proteins). Both of these methods are not as reproducible and standardised as transcriptomics is (Karakach *et al*., 2010).

There are a number of methods used to study the whole genome or transcriptomics such as Northern blotting (Alwine *et al*., 1977), differential display (Liang and Pardee, 1992), serial analysis of gene expression (SAGE; Velculescu *et al*., 1995), dotblot analysis (Lennon and Lehrach, 1991), gene or RNA knockouts (Turner *et al*., 2005b) etc., however most commonly used at the moment are: DNA microarrays, real time RT-PCR and RNA seq.

1.6.2 Real Time RT-PCR

Real time PCR started to be wildly used after Higuchi *et al*. (1992) published their work on this subject. This technique has many applications such as genotyping

(Gipson, 2006), genetic diseases diagnosis (Ward *et al*., 2004) or assessing gene copy number in tissues with cancer (Kindich *et al*., 2005). However the most common use is to study gene expression which includes a reverse transcription (RT) of RNA (Gibson *et al*., 1996; Leutenegger *et al*., 1999, Ginzinger, 2002). Typical steps of real-time RT-PCR are shown in Figure1.7.

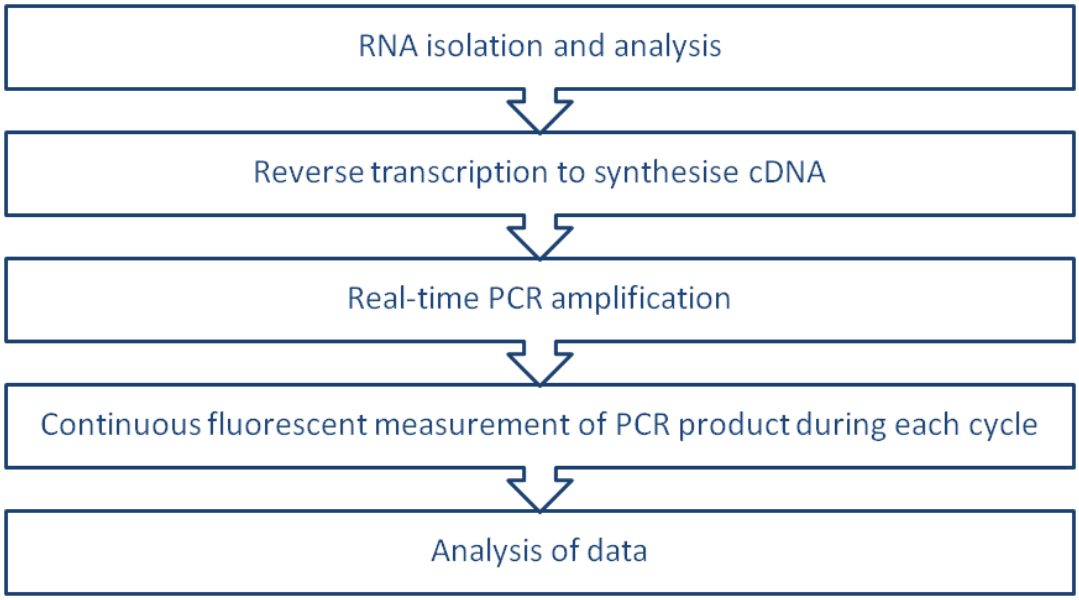


Figure 1.7 Procedure for real-time RT-PCR based on Fraga *et al*., 2008

A critical development of real-time PCR for studying gene expression was the ability to transform RNA populations into cDNA fragments which is called reverse transcription (RT). This was possible by discovery and development of purified reverse transcriptase (Fraga *et al*., 2008). After performing reverse transcription, the PCR reaction is performed by applying thermal cycling to a mixture of cDNA, two oligonucleotide primers, four nucleotide triphosphates, heat stable polymerase and buffer with magnesium ions (Kubista *et al*., 2006; Fraga *et al*., 2008). During this reaction short DNA sequences identified by primers are copied at each cycle. The whole process can be divided into three phases: exponential, linear and plateau (Klein, 2002). In the exponential phase the efficiency of reaction is the highest and theoretically each cycle should cause doubling of each DNA fragment (100% efficiency), however efficiency of >90% is usually recognised as optimal. During the linear phase the reaction slows down and during the plateau phase it will stop due to depletion of substrates and product inhibition. The amount of amplicon produced during each phase is measured using fluorescence- based technology in

real-time (Tichopad *et al*., 2003). This can be done by nonspecific fluorescent dyes e.g SYBR Green I or strand specific probes e.g. Taqman.

The difference in gene expression between samples is quantified by comparing the number of amplification cycles required for the samples' response curves to reach a particular threshold fluorescence signal level. The number of cycles required to reach threshold is called the CT value.

The final analysis of results is usually done using relative or absolute quantification methods (Valasek and Repa, 2005; Fraga *et al*., 2008).

1.6.3 DNA Microarrays

DNA microarrays follow the principles of Southern and Northern blot analysis, but in reverse, with the gene probes immobilised and a target sample in a solution. Microarrays are small slides to which are attached hundreds to tens of thousands of molecules of DNA which are immobilised target sequences, corresponding to a specific gene (van Hal *et al*., 2000). The DNA is able to hybridize complementary sequences created from mRNA transcripts labelled with a dye (fluorescent, Gibson and Muse, 2002). Usually mRNA extracted from test and control samples are used and they are labelled in different coloured dyes. They are mixed and hybridized to the microarray (Turner *et al*., 2005b). The hybridisation and washes are performed in highly controlled conditions and are followed by generating an image using laser-induced fluorescent imaging.

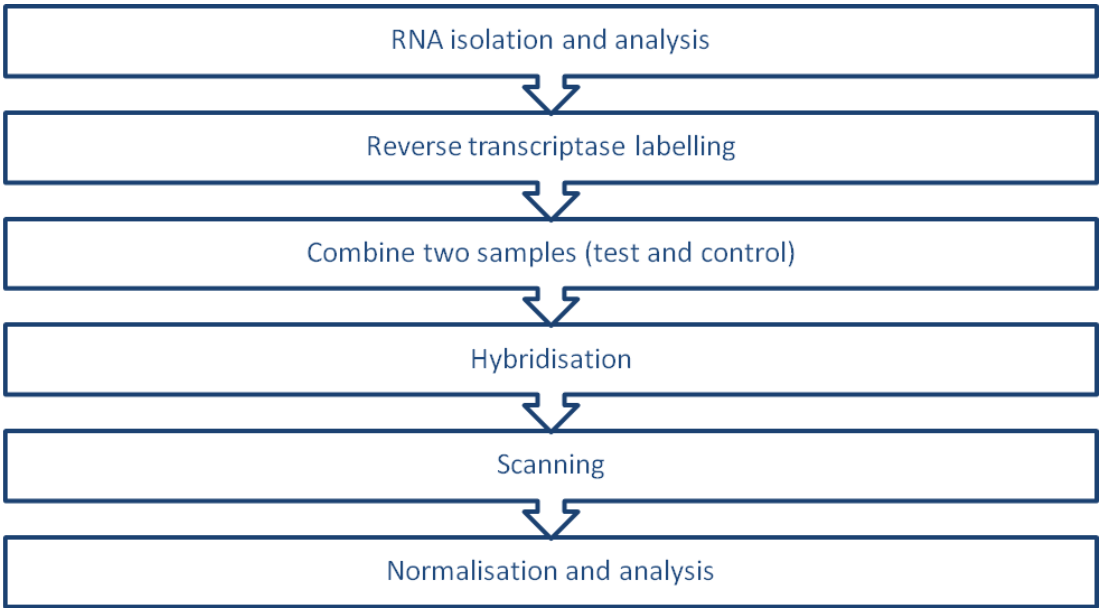


Figure 1.8 The steps required in a two-channel microarray experiment (Gibson and Muse, 2002)

The amount of fluorescence measured should be proportional to the amount of specific mRNA (Tarca *et al*., 2006). The results indicate how specific genes in test samples were expressed in comparison to genes in control samples.

The methods by which DNA microarrays are constructed and used can take various forms (Karakach *et al*., 2010). The schematic of steps for conducting DNA microarray analysis is shown in Figure 1.8.

1.6.4 RNA Seq

RNA sequencing is one of the transcriptomic methods which is based on directly determining cDNA sequences. Other methods based on this approach exist e.g. Sanger sequencing of cDNA, EST libraries (Boguski *et al*., 1994), serial analysis of gene expression (SAGE; Harbers and Carninci, 2005), cap analysis of gene expression (CAGE; Nakamura and Carninci, 2004; Kodzius *et al*., 2006) and massively parallel signature sequencing (MPSS; Brenner *et al*., 2000, Reinartz *et al*., 2002). These methods however have their limitations, therefore RNA-seq technology has been developed which is both mapping and quantifying transcriptomes. The principle of the method is that a whole population of RNA is converted into small fragments of cDNA into which adaptors are attached on one or both ends. Each of these fragments is then sequenced in a high throughput manner (Wang *et al*., 2009). The reads are typically 30–400 bp, depending on the DNA-sequencing technology used (Miller *et al*., 2013). Following sequencing, a transcript is produced by assembling raw sequence reads by using a de-novo method (Tinity, 2015; Grabherr *et al*., 2011) or aligning sequence reads to a reference genome or transcript.

The technology has many advantages over existing methods e.g. it is not limited to analysing transcripts that correspond to already existing sequences (Vera *et al*., 2008), it has high throughput, low error rate, ability to perform pair reads (Ozsolak & Milos, 2011; Metzker, 2010), ability to reveal sequence variations (Cloonan *et al*., 2008) etc., however it is still very expensive.

1.7 MATHEMATICAL MODELLING

Mathematical modelling using linear or nonlinear regression is an essential tool to analyse microbiological data. It is becoming more common to use kinetic parameters and models for ensuring food safety (Peleg and Cole, 1998). Models in general are used to mathematically describe a state of the process; they force

scientists to think through assumptions behind analysis (FDA, 2003). The regression models are used to define the outcome or different dependent variables as a function of independent variables and one or more model parameters. Parameters help to define the properties and behaviour of the model. The independent variables might be defined as time, temperature, humidity, weight, etc. If the model has more than one independent variable, a multiple regression method must be used. There might be different reasons to use mathematical models to interpret the data:

- Generating a standard curve that can be used to find out unknown values (empirical models)
- Model the number of successes in a sample of size n drawn with replacement from a population of size N (binomial distribution e.g. pass vs. fail, mutant vs. not mutant)
- Providing insight into the process that is thought to govern the phenomenon under study (mechanistic models, Motulsky and Christopoulos, 2003)

Choosing the best model should be based on a scientific decision based on understanding of scientific contents of the experimental data. There are a number of models which can be used to describe changes in microbial populations as a function of time. The traditional approach is to use a first-order kinetic model (Xiong *et al.*, 1999); often models described by FDA are used (FDA, 2000). The Weibull model (Peleg and Cole, 1998: Fernandez *et al*., 2002; Pérez-Rodríguez *et al*., 2013), multi-exponential (Geeraerd *et al*., 2000), Churchill model (Payne *et al.,* 2005), the stochastic Peleg model (van Boekel, 2003), Gompertz model (Xiong *et al*., 1999b) or logistic model (Baranyi and Roberts, 1994) have been used to describe different survival patterns of bacteria. The combination of equations can also be used (Ramos and Smith, 2009).

1.8 CONTROL OF CONTAMINATION AT THE FACTORY LEVEL

Currently all food manufacturers are obliged by law to ensure food they are producing is safe. In Europe, regulation No. 852/2006 on hygiene of foodstuff lays down requirements on safe food production. Food businesses should establish and operate food safety programmes based on the Hazard Analysis and Critical Control point (HACCP) principles. In USA, low moisture food production is largely

regulated by FDA (U.S. Food and Drug Administration; FDA, 2014). On January 4th, 2011 the Food Safety Modernization Act (FSMA, 2011) was signed into law and it enabled FDA to better protected public health by strengthening the food safety system. It forces food manufacturers to focus more on preventing food safety problems rather than relying primarily on reacting to problems after they occur. FDA therefore requires comprehensive, prevention-based controls across the food supply to prevent public health issues. The control of pathogens, including *Salmonella* in food factories is undertaken by a combination of Good Manufacturing Practices (Prerequisites programs) with an effective food safety system (usually HACCP).

1.8.1 Prerequisite Programmes (PRPs)

PRPs were defined by the World Health Organisation as ‘‘practices and conditions needed prior to and during the implementation of HACCP and which are essential for food safety’’ (WHO, 1993) such as:

- Construction and layout of buildings and associated utilities
- Hygienic design of the equipment
- Management of purchased materials and ingredients
- Cleaning and disinfection
- Personal hygiene
- Pest control
- Measures for the prevention of cross contamination
- Supplies of air, water, energy and other utilities
- Supporting services, including waste and sewage disposal
- Other relevant manufacturing operations (Bas, Ersun, & Kıvanç, 2006; PAS 220:2008)

Prerequisite programmes include Good Manufacturing Practices (GMP), Good Agricultural Practices (GAP), Good Hygiene Practices (GHP) etc. The implementation of an appropriate PRP is also seen as the foundation on which a good HACCP plan is built (Griffith, 2000); there are many examples of best practices to follow for PRPs at an international level, via retailers’ requirements (BRC, 2011) or from recognized food research bodies (IFST, 1998) or trade associations. A successful prerequisite programme as well as HACCP system is not achievable without well-conceived, well-written, and properly implemented Standard Operating Procedures (SOPs; Schmidt and Newslow, 2013). PRPs are

unique to each operation and there is the essential need of PRP in food businesses (Walker *et al*., 2003).

There are some prerequisites which after undertaking hazard analysis, show that they are more important for safety of food produced than others. They need control measures to acceptable levels but are not Critical Control Points (CCPs; Chua, 2014)

1.8.2 Hazard Analysis and Critical Control Points (HACCP)

HACCP is a food safety system aimed at controlling foodborne food safety hazards such as biological, chemical and physical hazards (Khandke & Mayes, 1998). It was developed in 1960s by Pillsbury Corporation which was requested by NASA to ensure food safety for the space missions. The idea of the system is the prevention of hazards rather that testing of the final product (Ropkins & Beck, 2000). The principles of HACCP were then described in 1969 by Codex Alimentarius Commission (CAC, 2003). They currently include seven principles:

1. Conduct a hazard analysis.
2. Identify Critical Control Points (CCPs).
3. Define critical limits for ensuring the control of each CCP.
4. Establish monitoring procedures to determine if critical limits have been exceeded and define procedure(s) for maintaining control.
5. Define corrective actions to be taken if control is lost.
6. Establish effective documentation and record-keeping procedures.
7. Establish verification procedures (ILSI, 1993; Savage, 1995; Mortimore and Wallace, 1998; Ropkins & Beck, 2000; FAO/WHO, 2007).

Additionally, before applying the 7 principles of HACCP, the management commitment should be obtained, the scope should be defined and the product and product process flow should be described (Gaze, 2009).

HACCP and PRPs are used globally throughout the whole supply chain (from farm to table; Sperber, 2013). While HACCP is working very well for controlling the processing part of food production, PRPs, which are controlling processing environment, are often overlooked and their neglect is the most common reason for outbreaks and recalls associated with food (Chapter 7).

1.9 OBJECTIVES

The aim of these studies was to investigate the following aspects:

- Determine *Salmonella* serovars' ability to survive on surfaces
- Assess the influence of the environmental factors such as: temperature, humidity, food debris, inoculum level and water access, on the survival of the selected *Salmonella* strains
- Assess the resistance to disinfectants of desiccated *Salmonella* cells that have been attached to surfaces for extensive time periods (e.g. 30 days)
- Analyse physical and molecular changes occurring in the desiccated *Salmonella* cells attached to the surfaces
- Develop control mechanism for *Salmonella* cross contamination from the environment to the product

The above objectives were achieved by conducting a series of trials and analyses and ultimately writing the research report that combines the author's understanding of the background to the problem and previous research with the results of the author's empirical research. Given the nature of the author's employment at Campden BRI, it was essential to give some solutions for controlling *Salmonella* in dry food factory environments.

CHAPTER 2
MATERIALS AND METHODS

Practical work was conducted at Campden BRI, the University of Nottingham and factories manufacturing low moisture foods.

2.1 CULTURE MEDIA, STRAINS AND CHEMICAL SOLUTIONS

All of the reagents made at the University of Nottingham were prepared using reverse osmosis (RO) water and at Campden BRI using deionised water according to manufacturing instructions, if not it is otherwise stated. If media required sterilization, it was performed by autoclaving at 121°C for 20 minutes on the liquid cycle. Most of the media were stored at the room temperature for 12 weeks, otherwise it is stated.

All agar media (if not stated otherwise), after sterilization were cooled at room temperature for approximately 1 hour and then placed in a 50°C±2°C incubator or 45°C±1°C water bath for a minimum of 1 hour and a maximum of 4 hours. They were then used directly to pour onto inoculated plates (pour plate technique) or they were used to pour into sterile 90 mm Petri Dishes. Petri Dishes were kept in sealed bags at 4°C for a maximum of 12 weeks.

2.1.1 Culture Media

The media prepared according to manufacturers' instructions are listed in Table 2.1.

2.1.2 Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn)

Iodine-iodine solution was prepared first by completely dissolving 25 g of potassium iodine (Analytical reagent, FSA Laboratory Supplies, Loughborough, UK) in 10 ml of sterile RO water and then adding 20 g of iodine (FSA Laboratory Supplies, Loughborough, UK) and making it up to 100 ml with sterile RO water. It was not heated.

MKTTn was prepared by dissolving 85.5 g of powder formula (Oxoid, CM1048) in one litre of RO water. The solution was brought to the boil and then cooled down to 45ºC±2°C. Just before use, 20 ml of Iodine-iodine solution and 5 ml of Novobiocine supplement (SRO181E) was added.

Table 2.1 Media and buffers prepared according to the manufacturing instructions

Media	Manufacturer	Amount (g) in 1L RO	Comments
Nutrient Broth (NB)	CM0001 Oxoid, Basingstoke, UK	13	-
Tryptone Soya Agar (TSA)	CM0131 Oxoid, Basingstoke, UK	40	-
Tryptone Soya Broth (TSB)	CM0129 Oxoid, Basingstoke, UK	30	-
Maximum Recovery Diluent (MRD)	CM0733, Oxoid, Basingstoke, UK	9.5	-
Phosphate Buffer Saline (PBS)	BR0014 Oxoid, Basingstoke, UK	One tablet in 100 ml of RO	-
Lurini-b Broth (LB)	Lennox, Molecular genetics powder, Fisher Scientific	20	-
Xylose Lysine Deoxycholate Agar (XLD)	Sigma-Aldrich	15	Brought to boil instead of sterilisation
Brilliant Green Agar (BGA)	CM0329 Oxoid, UK	52	Brought to boil instead of sterilisation
Buffered Peptone Water (BPW)	CM0509, Oxoid, , UK	20	-
Rappaport-Vassiliadis enrichment broth (RVS)	CM0669, Oxoid, Basingstoke, UK	30	Heated gently to dissolve before sterilisation

2.1.3 M9 minimal medium

M9 minimal medium agar base was prepared by mixing 250 ml RO water, 250 ml M9 minimal salts (2x, Gibco by Life Technologies, Ref: A13744-01) and 10 g agar (Fisher Scientific, BP1423). It was sterilized and then cooled down to 50°C±2°C and the following ingredients were added aseptically in a laminar flow cabinet:

- 1 ml 1M $MgSO_4$ – a solution was prepared using 120 g l^{-1} $MgSO_4$ (GPR Rectapur VWR Magnesium Sulphate, Prod. 291184P) in RO water and autoclaved.
- 0.05 ml 1M $CaCl_2$ – a solution was prepared using 110 g l^{-1} $CaCl_2$ (calcium chloride 96% extra pure powder, anhydrous, Acros Organics, Code 349610250) in RO water. The reagent was autoclaved.
- 2.5 ml 40% glucose – a solution was prepared by dissolving glucose (Fisher Scientific, D-glucose anhydrous, analytical reagent grade,

code: G/0500/53) in RO water. It was filter sterilised using a 0.45 µm filter (Sartorius Stedium, Minisart).

The agar medium was poured into sterile 90 mm Petri dishes (Bibby-Sterilin, Barloworld Scientific Ltd., London, UK) in 20 ml aliquots in a laminar flow cabinet.

2.1.4 Swim and Swarm Motility Agars

Swim motility agar was prepared using 20 mg l^{-1} LB broth base (2.1.1) and adjusted with 0.3% (w/vol) agar base (Fisher Scientific, BP1423).

Swarm motility agar was prepared using 20 mg l^{-1} LB broth base (2.1.1), along with 0.6% (w/vol) of agar (Fisher Scientific, BP1423) and 0.5% (w/v) glucose (Fisher Scientific, D-glucose anhydrous, analytical reagent grade, code: G/0500/53).

The agar mediums were sterilised and poured into sterile 90 mm Petri dishes (Bibby-Sterilin, Barloworld Scientific Ltd., London, UK) in 20 ml aliquots in a laminar flow cabinet. The plates were left overnight to dry.

2.1.5 Congo Red Agar (CR)

CR agar was prepared by dissolving 5 g of tryptone (Fisher Scientific Ltd, BP1421-2), 2.5 g of yeast extract (LP0021, Oxoid Ltd., UK) and 7.5 g of agar (Fisher Scientific, BP1423) in 500ml of RO water. This was then autoclaved at 121ºC for 20 minutes.

2.1.6 Water of Standard Hardness (WSH)

Solution A was prepared by dissolving 1.984 g ± 0.05 g of anhydrous magnesium chloride (≥98%, Sigma Aldrich) and 4.624 g ± 0.05 g anhydrous calcium chloride (96% powder, Acros Organics) and 100 ml of distilled water. It was then sterilized by passing through a 0.45 µm pore filter (Sartorius Stedium, Minisart) and stored at 4°C to 8°C for a maximum period of 1 week.

Solution B was prepared by dissolving 3.502 g ± 0.05 g sodium hydrogen carbonate (Powder/Certified ACS, Fisher Chemical, Fisher Scientific) and 100 ml of distilled water. It was then sterilized by passing through a 0.45 µm pore filter (Sartorius Stedium, Minisart) and stored at 4°C to 8°C for a maximum period of 1 month.

WSH was made of 6 ml of solution A and 8 ml of solution B and topped up to 1000 ml with sterile distilled water. The pH was adjusted to 7.0 $\pm$ 0.2, using 1 M sodium hydroxide (50% w/w/Certified, Fisher Chemical, Fisher Scientific). It was stored at room temperature for a maximum period of 2 hours.

2.1.7 Disinfectant Neutralisers

European standard neutraliser: The neutraliser used to deactivate propan-2-ol was chosen from the BS EN 13697:2001 list (Anon., 2001). The neutraliser was prepared according to the formula in Table 2.2.

Table 2.2 Formula used for European standard neutraliser preparation

Formula	g litre^{-1}
Lecithin (from egg yolk, Sigma-Aldrich)	3.0
Polysorbate 80 (Tween 80, Sigma-Aldrich)	30 ml
Sodium thiosulphate (pentahydrate, Sigma-Aldrich)	5.0
L-histidine (ReagentPlus, Sigma-Aldrich)	1.0
Saponin (Sigma-Aldrich)	30.0
Phosphate diluent (0.25 M)	10 ml

Sodium thiosulphate neutraliser: The neutraliser used for the sodium hypochlorite disinfectant was made up from 5 g of sodium thiosulphate (pentahydrate, Sigma-Aldrich) and 1000 ml of deionised water and sterilised.

2.1.8 Disinfctants

Propan-2-ol: Propan 2-ol (Fisher Scientific, 99.98%, HPLC grade, Code: P/7507/17, batch: 0898924) was diluted with WSH (2.1.6) to the following concentrations: 20%, 40% and 80% (volume/volume).

Sodium hypochlorite: Sodium hypochlorite of 12 % w/v active chlorine (GPR Rectapur, Product: 301696S, Batch: 11L070535; VWR, BDH, Probalo) was used. The concentration of the active chlorine in the sodium hypochlorite was measured before testing (see 2.5.1) and it was determined as 7.68% (76,800 ppm). The sodium hypochlorite solution was diluted with WSH (2.1.6) to provide test concentrations of: 50 ppm, 100 ppm, 250 ppm, 500 ppm and 1000 ppm of active chlorine (volume/volume).

2.1.9 *Salmonella* strains

Salmonella isolates were obtained from the Campden BRI culture collection (Table 2.3). They were received on Tryptone Soya Agar (2.1.1) slopes and a loopful of each strain was transferred to a separate 10 ml Tryptone Soya Broth (2.1.1) container and incubated for 24 hours at 37°C. After incubation, TSA plates (2.1.1) were inoculated with 0.1 ml of the incubated broth suspension and incubated for

another 18-24 hours at 37°C. The microbial growth was then placed in sterile vials containing cryobeads (Microbank^{TM}, Pro-Lab Diagnostics, Bromborough, UK) and the cultures were placed in a -80°C freezer. Identification and characterisation of the strains was carried out by ribotyping (Campden BRI, Dept. Microbiology, see Appendix A) prior to experimental use. The information about the phage types of most isolates was not available. Four isolates of *S*. Typhimurium were used (Table 2.3): multi-drug resistant *S*. Typhimurium DT104, *S*. Typhimurium NCTC 12023 (highly heat resistant; HR), *S*. Typhimurium, NCTC (PHLS), NC000 74-17 (used for disinfectant testing; DS) and *S*. Typhimurium isolated from whey powder. Six strains isolated from cereal manufacturing environments were also used: two *S.* Cubana , two *S*. Lomita and two *S.* Mbandaka.

Table 2.3 *Salmonella* isolates used for the thesis

No.	Given code	Name	Source
1.	FH/Sa/159	*Salmonella* Agona	Cotton seeds
2.	FH/Sn/161	*Salmonella* Napoli	Chocolate confectionary, refrigerated 7 years (1986 outbreak Italy)
3.	FH/Sm/164	*Salmonella* Muenchen	Cocoa bean environment
4.	FH/Se/162	*Salmonella* Enteritidis	Cereal
5.	FH/St/163	*Salmonella* Tennessee	Sesame seeds
6.	FH/St/165	*Salmonella* Typhimurium	Whey powder
7.	FH/St/167	*Salmonella* Typhimurium DT104	HPA, Colindale
8.	FH/St/168	*Salmonella* Typhimurium	NCTC 12023, heat resistant "HR"
9.	FH/St/68	*Salmonella* Typhimurium	NCTC (PHILS), NC000 74-17, European standard disinfectant test strain "DS"
10.	FH/S1/172	*Salmonella* Lomita	Cereals environment "1"
11.	FH/S2/173	*Salmonella* Lomita	Cereals environment "2"
12.	FH/S3/174	*Salmonella* Cubana	Cereals environment "1"
13.	FH/S7/178	*Salmonella* Cubana	Cereals environment "2"
14.	FH/S5/176	*Salmonella* Mbandaka	Cereals environment "1"
15.	FH/S6/177	*Salmonella* Mbandaka	Cereals environment "2"

2.1.10 Surfaces

Stainless steel discs (2 cm diameter, Grade 2B, 1.4301 (EN 10088-1, EN 10 088-2)) were used. They were soaked in hot water with detergent (Fairy liquid, Procter & Gamble) for 60 minutes. Each disc was then cleaned with a sponge and rinsed with sterile distilled water. They were then left to dry, wiped with alcohol wipes (Izo-wipes; Johnson Diversey), and autoclaved.

2.1.11 Controlled relative humidity (RH) chambers

In all experiments two different relative humidity (RH) levels were used, an RH of 33%±2% and an RH of 53%±2%, designed to be reflective of dry food processing environments. The humidity was established and maintained in sealed boxes (32 cm x 11 cm x 21 cm) by the presence of one of two salt solutions: saturated magnesium chloride salt (Fisher Scientific, Laboratory reagent grade, $MgCl_2 \cdot 6H_2O$, Batch: 0936578) for the lower humidity and saturated magnesium nitrate hexahydrate, 98+% salt (Acros Organics, ACS reagent, Lot: A0313935) for the higher humidity (see Appendix B for salt humidity values). The sealed boxes were held in an incubator at the desired temperatures: 25±1ºC, 15±1ºC or 37±1ºC. A calibrated, digital thermohygrometer (Rotronic; Hygrolog-D) was used daily for measurements of temperature and RH.

2.1.12 Humectants

- Sodium chloride (Fisher Scientific, >99.5%, code: S/3120/05) 4.8 M and 1.3 M
- Sucrose 5.8 M (calculated a_w=0.86, measured a_w= 0.84 at 22.8°C)
- Glycerol (BP Coustin & Warner Ltd., Batch: 002765) 100%

The humectants were diluted to their final concentrations with MRD and bacterial suspension.

2.2 ENVIRONMENTAL SURVIVAL STUDIES

2.2.1 Factory Visits

A number of visits to dry foods manufacturers was undertaken to establish environmental conditions in the factories and find out about manufacturing practices. A questionnaire was prepared to help gather the most important information (see Appendix C). Visits were undertaken to over 10 factories producing low moisture foods and the visited sites were situated in a number of different countries including:

UK, Ireland, Slovakia, USA and the Netherlands. The manufacturers of the following food products were visited:

- Chocolate (Slovakia, Belgium, Switzerland)
- Cereals (UK, Netherlands)
- Milk powder (USA, Poland)
- Wheat germ (UK)
- Dry pet food (Ireland)

2.2.2 Test Suspensions

Cultures were maintained at -80ºC in cryo vials (Microbank^{TM}, Pro-Lab Diagnostics, Bromborough, UK) containing glycerol. Working cultures of each serotype were sequentially prepared by adding a cryo bead to 150 ml Nutrient Broth (2.1.1) which was incubated for 24 h ± 1 h in a shaking incubator at 37°C and 100 rpm. The broth was then centrifuged at 3000 x *g* for 10 minutes (Hareus Megafuge 40R and rotor, Thermo Scientific).

After centrifugation, pellets were resuspended in 10 ml of Maximum Recovery Diluent (2.1.4) which formed ~10^7 - 10^8 cfu ml^{-1} suspensions. This was directly used to achieve ~10^7 cfu ml^{-1} inoculum on discs or it was diluted with MRD 1000 times to obtain a 10^4 cfu ml^{-1} inoculum or 100 times to attain 10^5 cfu ml^{-1} inoculum.

2.2.3 Inoculation of Discs

A 50 µl volume of bacterial suspension was deposited onto stainless steel discs (2.1.10). Discs were placed in uncovered Petri Dishes and transferred to an incubator to dry at 30ºC for 80 minutes. After drying, Petri Dishes were covered and placed in air tight transparent plastic boxes (2.1.11) for a range of time periods, humidity and temperatures.

2.2.4 Determination of Viable Counts of Surface-Inoculated Bacteria

In order to assess the number of bacteria surviving on the discs, each disc was aseptically transferred into a sterile plastic universal container (diameter 4-5 cm) containing 10 ml MRD (2.1.4). Containers were left for 10 minutes at room temperature and vortexed for 1 minute to recover the remaining bacteria into suspension. Each sample was then serially diluted in MRD and 1ml volumes plated out in duplicate using Tryptone Soya Agar (2.1.1). This recovery method had been previously validated to maximise recovery from the surfaces.

2.2.5 Agar Incorporation Method

To verify the bacterial recovery process, and to detect any cultivable colonies left on the surface after recovery, a modified method of EN 13697:2001 (Anon., 2001) was used. Each disc from which bacteria had been removed was recovered from its container, rinsed with 10 ml of sterile distilled water and then placed onto a pre-poured TSA plate (2.1.1). A 0.1 ml volume of sterile, distilled water was pipetted onto the disc and rubbed over the top and the bottom surface with a pipette tip for 30 seconds. The discs were then over-poured with approximately 10 ml TSA and plates were swirled to ensure even mixing. All plates were incubated at 37°C ± 1°C for 48±4 hours. Colonies were then enumerated and the number of colony forming units (cfu) per test surface was calculated.

2.2.6 Food Debris

The following food debris were chosen due to the high number of *Salmonella* outbreaks associated with these commodities.

- High concentrations of food debris was achieved by using: chocolate powder [Cadbury hot chocolate; ingredients: sugar, cocoa, dried whey, glucose syrup, vegetable oil, dried skimmed milk, milk chocolate (4%; milk, sugar, cocoa butter, cocoa mass, vegetable fat, emulsifier E442, flavourings), dried glucose syrup, salt, thickener E466, milk proteins, anti-caking agent E551, emulsifier E471, stabiliser E339, Flavourings] and infant formula [Cow&Gate 1, first infant milk; ingredients: demineralised whey; vegetable oils; lactose; skimmed milk; galacto-oligosaccharides (GOS); whey protein concentrate; fructo-oligosaccharides (FOS); minerals and vitamins]. Each product was weighed (1.4 g, according to the instruction on the package) and added to 10 ml of bacterial suspension (~10^7 cfu ml^{-1}). This formed suspensions with a high concentration of nutrients and particles was used to inoculate the discs.
- Low concentrations of food debris were achieved by using: cocoa powder (Morrison's, ideal for drinking and baking) and dried skimmed milk [Morrison's; ingredients: dried skimmed milk (99.5%), vitamins A&D]. Each product was weighed (1.4 g, the same as for high concentration products) and added to 10 ml of bacterial suspension (~10^7

cfu ml^{-1}). This was directly used or then diluted 100 times with bacterial suspension which formed a suspension with a low concentration of nutrients and particles.

2.2.7 Periodic Wetting

Periodic wetting was conducted by opening the humidity chambers and depositing 100 µl of sterile distilled water onto the stainless steel discs previously inoculated with bacterial suspension (~10^7 cfu ml^{-1}). Water was introduced at different time periods after inoculation with bacterial suspension. After wetting, the humidity chambers were closed and returned to the incubation temperature. On average it took 2 days for the discs to become visibly dry.

2.2.8 Assessment of Bacterial Motility

Cryo beads of fifteen *Salmonella* isolates were placed on TSA plates and were incubated for 24 h ± 2 h at 37ºC ± 1°C. After the incubation period, one colony of each isolate was transferred to 50 ml of LB broth (2.1.1) and placed into a shaking incubator (37°C ± 1°C, 24 h ± 2 h, 200 rpm). The cultures were then centrifuged at 3600 x *g* for 10 minutes and re-suspended in 10 ml of PBS (2.1.1). Five micro littres of these suspensions were placed on the centre of a Petri Dish containing swim motility agar. The same was repeated for swarm motility agar. Swim motility was examined after incubation for 8 hours and swarm after 8 and 24 hours incubation at 37ºC ± 1°C. The diameter (cm) of the growth was measured as the degree of swarming and swimming.

2.2.9 Detection of Curli Fimbriae (RDAR Phenotype)

The expression of curli fimbriae was investigated using Congo Red (2.1.5) agar. After autoclaving and cooling the agar to approximately 50ºC± 2°C, the agar was supplemented with 40 µg ml^{-1} of Congo Red stain (Sigma, C.1.22120) and 20 µg ml^{-1} Coomassie Brilliant Blue R (Sigma, C.1 42660). Before adding the supplements to the agar, they were filter-sterilized (0.45 µm pore filter, Sartorius Stedium, Minisart). The plates were poured and left overnight for drying. These plates were then inoculated with five drops of 3 µl of suspensions described in 2.2.2 and incubated for 72 hours at 30ºC ± 1°C. The colony morphology was then examined and recorded.

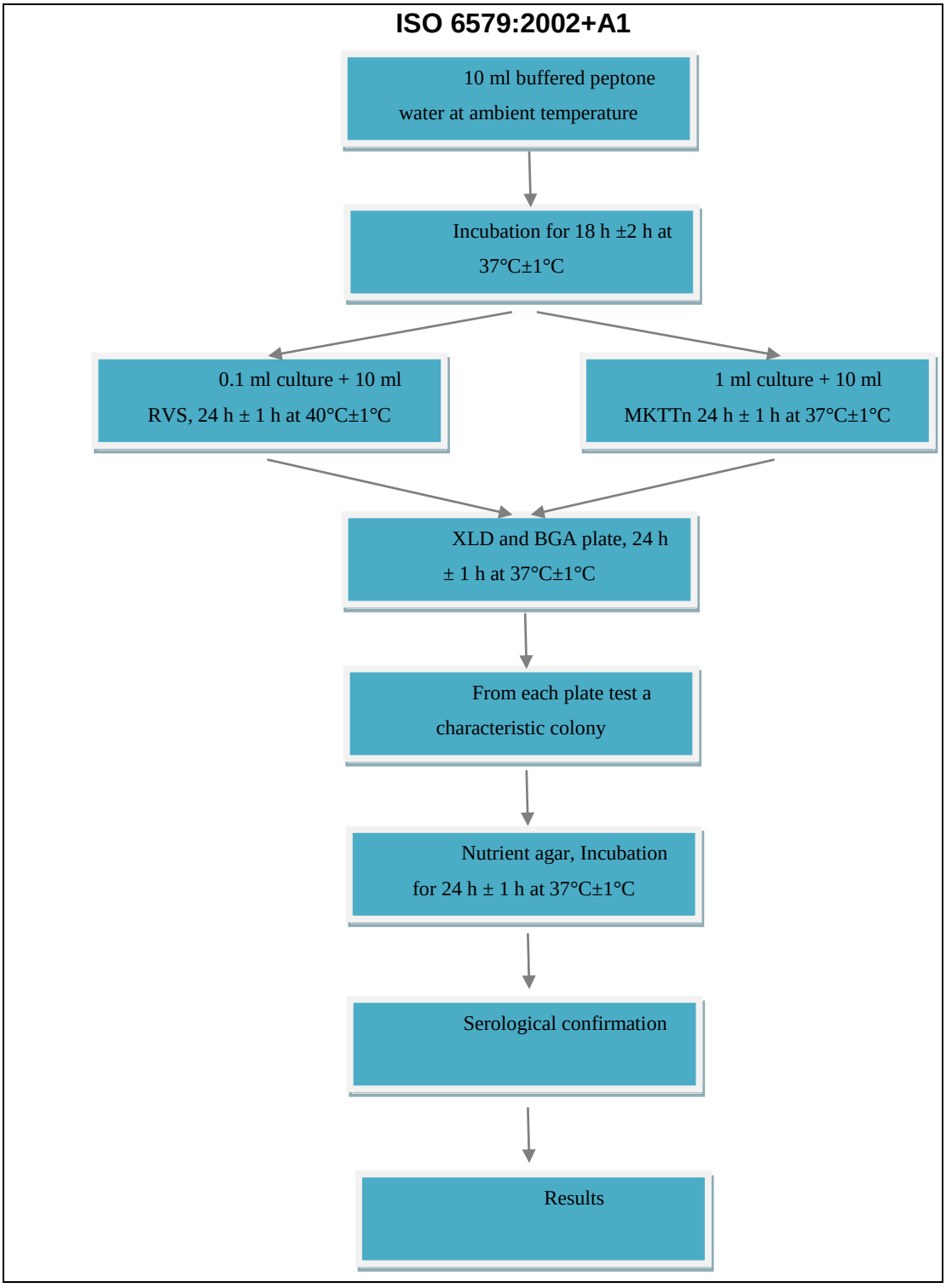


Figure 2.1 Schematic method for the detection of *Salmonella* spp. based on: Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella spp (ISO 6579:2002+A1, Anon., 2002)

2.2.10 ISO Recovery Method

Desiccated cells attached to discs were re-suspended in 10 ml of BPW (2.1.1), which was then incubated for 18 h ± 2h at 37°C ± 1°C. The next step was to inoculate two selective broths as shown in Figure 2.1.

After incubation for 24 h ± 1 h, using the culture obtained in the RVS (2.1.1) broth, by means of a loop, the surface of one 90 mm Petri dish containing the XLD (2.1.1) agar and one Petri Dish containing BGA (2.1.1) was inoculated, so well-isolated colonies could be obtained. The same was repeated for the culture from MKTTn broth (2.1.2). The Petri Dishes were inverted, so that the bottom was uppermost, and were placed in the incubator at 37 °C ± 1°C for 24 h ± 1 h.

The characteristic colonies on each media are:

- XLD- red colonies with black centre – *Salmonella* and *Edwardsiella*
- BGA - Red-pink-white opaque coloured colonies surrounded by brilliant red zones in the agar - most probably *Salmonella* (but not *Salmonella* Typhi).

The cultures were also identified by using two serological methods: *Salmonella* Test Kit for the Detection of *Salmonella* (Oxoid, DR1108A) (2.4.2) and Salmonella Agglutinating Serum for *Salmonella* Antisera (O' and H') (2.4.3).

2.2.11 Kinetic Model

Several equations were analysed to build the most suitable empirical model (Weibull, nth-order, exponential, Churchill model, and modifications of them in order to account for both the weak and resistant populations, see Appendix D).

$$\underbrace{\log_{10}(S) = \beta_1}_{\text{Subpopulation 1}} \cdot \underbrace{\left(\frac{t}{\delta_1}\right)^{\alpha_1} - (1-\beta_1) \cdot \left(\frac{t}{\delta_2}\right)^{\alpha_2}}_{\text{Subpopulation 2}}$$

Equation 2.1 Modified Weibull model

According to the best fitting parameters a modified Weibull equation considering two populations was selected (Equation 2.1). The subscripts 1 and 2 indicate the two different subpopulations (or the two different levels of resistance), S is the survival rate (cfu_t/cfu_0), β_1 is the fraction of subpopulation 1 in the total

population, δ is the time needed for the first decimal reduction, α is a shape parameter and t is the time in hours.

The goodness of fit for the obtained equations was analysed according to the coefficient of determination (R^2) and root mean square error (RMSE). In addition, the model's performance was tested with data generated in this study, by means of the bias factor, (B_f) and the accuracy factor (A_f) as described by Alvarez *et al*., 2003; Klotz *et al*., 2007; Meneses *et al*., 2013.

2.2.12 Root Mean Square Error (RMSE)

The error between predicted and experimental *Salmonella* survival values was estimated by using the Root Mean Square Error (RMSE), which is a statistical measure of the differences between values predicted by a model and the values actually observed from experiments and can be expressed by an absolute value, in this case $\log_{10}$:

$$RMSE = \sqrt{\frac{\sum_{i}^{n}\left(S_{observed} - S_{predicted}\right)^2}{n}}$$

Equation 2.2 Root mean square error (RMSE)

Where, $S_{observed}$ is the measured population, $S_{predicted}$ is the predicted population by the empirical model and n is the total number of experimental measurements.

2.2.13 The Bias Factor

The bias factor is a measure of over-prediction ($B_f > 1$) or under-prediction ($B_f < 1$) of the predicted values by the empirical model in comparison to the experimental values. A bias factor of 1 indicates perfect agreement between observed and predicted generation times.

The accuracy factor indicates how the predictions differ from the observed data. An A_f of 1 indicates perfect agreement between all predicted and observed S values. An A_f value higher than 1 (e.g. 1.2) shows that the prediction is on average, either 20% smaller or 20% larger than the observed values (Ongeng *et al*., 2010). The A_f should thus always be analysed in combination with the B_f. It is worth mentioning that the deviation percentage to the observed value (A_f) is in reference to the values as "$\log_{10}$" and not to the number of colonies.

2.3 STATISTICAL METHODS USED

All experiments were statistically designed and the results gained were analysed using Minitab 16 statistical software. For statistical comparison, the General Linear Model with Turkey test with 95 % confidence or the ANOVA test were used. Results with a p value <0.05 were considered as statistically significant.

2.4 ANALYTICAL METHODS

2.4.1 Gram Staining

Gram staining was carried out according to the method described by Rollins and Joseph (2004). A loopful of maximum recovery diluent (MRD, 2.1.1) was transferred to the surface of a clean 1-1.2 mm glass slide (BS7011/2, Thermofisher Scientific). One colony grown on XLD (2.1.1) agar was dispersed in the MRD and spread over a small area using a 10 µl sterile disposable loop (731171, Greiner Bio-One, Hungary). The film was allowed to dry for about 15 min at ambient temperature (~20°C) and heat fixed by briefly passing the slide through a Bunsen flame three to five times. The slide was flooded with the primary stain, crystal violet (PL8000, Pro-Lab Diagnostics), for 1 min, followed by washing with tap water and then treated for 30 s with the mordant, Lugol's iodine (PL8010, Pro-Lab Diagnostics). Excess iodine was washed off with water, the slide decolourised for 1 min with general purpose methylated spirit (M/4450/17, Fisher Scientific) and the excess washed off. Finally, the slide was counter stained for 30 s with carbol fuschin (PL8004, Pro-Lab Diagnostics), the excess rinsed off with water, and the slide allowed to dry for ~15 min at ambient temperature. Gram reaction and cell morphologies were examined using oil immersion microscopy (PBA Microscopes T250, England) at a magnification of 1000x. Gram-negative cells stained pink.

2.4.2 *Salmonella* Test Kit for the Detection of *Salmonella* (Oxoid, DR1108A)

Polyvalent antisera were prepared against a wide range of *Salmonella* flagellar antigens. When mixed with a suspension of *Salmonella* containing these antigens, the latex particles rapidly agglutinated to form visible clumps.

Reagent control was undertaken before analysing the samples. One drop of *Salmonella* Oxoid reagent was added to 1 drop of saline (supplied with the kit) in the same circle on a slide, it was mixed for 2 minutes and checked for agglutination. Positive controls were then prepared. A laboratory reference *Salmonella*

Typhimurium L2 strain was prepared and placed on a reaction card. It was observed for agglutination for 2 minutes, then 1 drop of the Oxoid *Salmonella* Test Kit Reagent was added to the same circle and mixed with a plastic loop. After 30 seconds the occurrence of agglutination was observed.

One drop of saline was placed within one circle on the reaction card. Using an inoculating loop the recovered colony was emulsified in the drop of saline to produce a thick smooth suspension. A drop of the Oxoid *Salmonella* Latex Reagent was added to the saline suspension. This was mixed for 30 seconds and the reaction card was gently rocked two or three times. Agglutination within two minutes is a positive result and indicates the presence of *Salmonella* in the sample.

2.4.3 *Salmonella* Agglutinating Serum for *Salmonella* Antisera (O' and H')

When a bacterial culture is mixed with this specific antiserum directed against bacterial surface components, the cells are bound together through antigen-antibody bonds to form aggregates (agglutination). This is usually visible to the naked eye as clumps in the suspension. On the basis of the observed agglutination pattern the serotype is determined using the Kauffmann-White Scheme.

A small drop of antiserum (approximately 20 µl) was placed on the glass slide. Several colonies of the culture grown on TSA plate were transferred to the drop of antiserum and mixed well. The amount of culture was sufficient to give a distinct milky turbidity. The slide was tilted for 5 - 10 seconds. The reaction was read with the naked eye by holding the slide in front of a light source against a black background (indirect illumination). A positive reaction was seen as a visible agglutination. A negative reaction was persistence of the homogeneous milky turbidity. A late or weak agglutination was considered negative.

Absence of reactions may be due to a strain expressing the Vi antigen, to a strain not covered by the antisera used or to a strain not being *Salmonella*.

2.4.4 Scanning Electron Microscope (SEM)

Sample analysis was undertaken in the Chemistry Department of Campden BRI (Chipping Campden, UK) using a Carl Zeiss EVO 60 EP Scanning Electron Microscope. The samples were sputter coated with gold, then transferred onto SEM sample holder and examined under vacuum.

2.4.5 Acridine Orange Staining

Acridine Orange (AO) is a cell-permeant nucleic acid selective metachromatic stain that emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA.

Discs with dried inoculum were flooded with acridine orange solution (10 mg ml^{-1}, Life Technologies) for 2 minutes, rinsed with RO water, dried at room temperature on the bench top and observed using phase contrast microscope under a UV lamp (2.4.7).

2.4.6 Live/Dead Baclight Staining

Bacterial cultures were grown as described previously (2.2.2). *S.* Typhimurium 'HR' culture was centrifuged at 5000 rpm at 10°C for 10 minutes. They were re-suspended in 20 ml of MRD (2.1.1) and centrifuged again under the same conditions as previously. Live/Dead Baclight (L7012) was used for staining, it was supplied with two solutions and 3 µl of each solution (A and B) were mixed together. Three micro litres of this suspension was added to 2 ml of bacterial suspension and incubated in the dark for 15 minutes (room temperature). The solution was then centrifuged for 5 minutes at 8000 rpm and inoculated onto a disc. This was observed on the Nikon phase contrast microscope (2.4.7).

2.4.7 Microscopy

A Nikon phase contrast microscope (61215, Japan) was used for observation of bacteria stained on stainless steel discs. The samples were observed under a range of magnification levels; from 100x to 1000x with immersion oil. The UV light (Nikon, super high pressure mercury lamp, model HB-10101AF) was used to visualise the bacteria.

2.4.8 Water Activity Measurement

The water activity of sodium chloride solutions were estimated first (FDA, 2012). The water activity of sucrose solution was calculated using the following equation:

$$A=1.00/(1+0.27n)$$

Equation 2.3 Water activity of sucrose solution (Karel and Lund, 2003)

In the equation 2.3, n is a number of moles sucrose in 100 g water, where 1 M sucrose weights 342 g. The actual water activity measurement of humectants used in the studies was undertaken using an AquaLab instrument (series 3 TE, Decagon Devices, Inc., Washington, USA). The sample cap was filled with approximately 7 ml of sample, with special attention not to overfill the cup. The sample cap was placed in the instrument drawer and the drawer knob was turned from open/load to read. The Aqualab displayed the sample's final a_w and temperature. RO water sample was measured for control purposes.

2.4.9 Spectrophotometer Reading

The OD of bacterial suspensions was measured using an Ultra Spec 2000 (UVNisible) spectrophotometer (GE Health Care, Little Chalfont, UK) at 600 nm.

2.5 DISINFECTANT TESTING STUDIES

2.5.1 Measurement of Active Chlorine

Active chlorine level in sodium hypochlorite (2.1.8) was tested at Chemistry Department at Campden BRI. A known volume of the sodium hypochlorite solution was measured and diluted with water to approximately 100 ml. Following this step, 20 ml of acetic acid (glacial, 99.85%, Sigma-Aldrich) and 1 g potassium iodide (puriss. p.a., reag. ISO, reag. Ph. Eur., ≥99.5%, Sigma-Aldrich) was added and the liberated iodine was titrated with a standard sodium thiosulphate (pentahydrate, Sigma-Aldrich) solution, using a starch indicator.

2.5.2 Disinfectant Test

BS EN 13697:2001 Chemical disinfectants and antiseptics - Quantitative non – porous surface test for the evaluation of bacterial and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas – test methods and requirements without mechanical action (phase2/step2) was used (Anon., 2001). Figure 2.2 shows a simplified schematic of surface disinfectant test. The bacterial suspensions (~10^7 – 10^8 cfu ml^{-1}) were prepared as described previously (2.2.2) and inoculated on stainless steel discs (2.2.3). The interfering substance described in the British Standard (BS EN 13697:2001) was not added to the bacterial suspension.

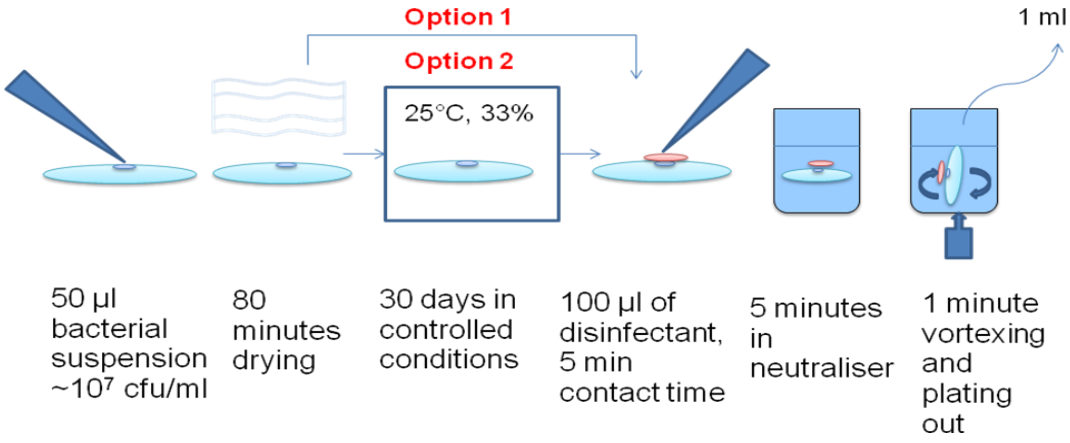


Figure 2.2 Simplified schematic of the modified standard European 13697:2001 surface disinfectant test

The inoculated discs (taken directly from the incubator after drying or taken from the controlled humidity chamber after 30 days at 25°C and 33% RH) were subjected to the lowest concentration of disinfectant by placing 0.1 ml of disinfectant (2.1.8) onto the surface of the disc to cover the dried test suspension.

This was held for a contact time of 5 minutes at the room temperature of between 18°C-20°C. At the end of the contact time, each disc was transferred into separate sterile 60 ml containers (Sterilin, metal cup, 125AB) containing 10 ml of the neutraliser (2.1.7) with approximately 5 g of glass beads (diameter ~ 5mm, Sigma-Aldrich). The discs were kept in the neutraliser for 5 minutes. Within this time, pots were agitated horizontally by hands for 1 minute ± 5 seconds. At the end of a 5 minute time period, 1 ml of suspension was transferred to 9 ml of MRD which formed a 10 fold dilution. The suspension was diluted further up to 10000 times and plated out in duplicate on TSA plates. This was repeated for each concentration, disinfectant and the strain used. An appropriate time plan was prepared to conduct the tests (Appendix E).

2.5.3 Water Control

A water control was performed to confirm that the water (WSH, 2.1.6) used to dilute the disinfectant itself did not have any antimicrobial effect. The same procedure was followed as for disinfectant testing (see 2.5.4), however instead of disinfectant, 0.1 ml of WSH was inoculated onto the surface of each disc.

2.5.4 Neutraliser Control

A neutraliser control was performed to confirm that the neutraliser did not have any antimicrobial effect. 60 ml sterile pots containing 10 ml of neutraliser and 5

g of glass beads were equilibrated in a water bath at 20°C. 0.1 ml of sterile distilled water was added to each pot which was mixed well and held in a water bath for 5 minutes. Each disc was transferred upside down into each pot. This was held for a contact time of 5 minutes at a room temperature of between 18°C - 20°C. Within this time, pots were agitated horizontally by hands for 1 minute ± 5 seconds. At the end of a 5 minute time period, 1 ml of suspension was transferred to 9 ml of MRD which formed a 10 fold dilution. The suspension was diluted further up to 10000 times.

2.5.5 Neutralisation Test

A neutralisation test was performed to check if the neutraliser was effective in stopping the disinfection process. 60 ml sterile pots containing 10 ml of neutraliser and 5 g of glass beads were equilibrated in a water bath at 20°C. 0.1 ml of the highest concentration of each disinfectant was added to each pot which was mixed well and held in a water bath for 5 minutes. Each disc was transferred upside down into each pot. This was held for a contact time of 5 minutes at a room temperature of between 18°C - 20°C. Within this time, pots were agitated horizontally by hand for 1 minute ± 5 seconds. At the end of a 5 minute time period, 1 ml of suspension was transferred to 9 ml of MRD which formed a 10 fold dilution. The suspension was diluted further up to 10000 times.

2.6 GENE EXPRESSION STUDIES

2.6.1 RNase-Free DNase (50; Qiagen, France)

DNase I stock solution was prepared according to the manufacturer's instructions, divided into portions of 100 µl and stored at -20°C±1°C. Each time one portion was thawed it was not returned to the freezer; however it was kept at 4°C for up to 6 weeks.

2.6.2 DNA Extraction

For each strain one cryobead of bacteria strain was transferred and spread onto a TSA Petri Dish which was then incubated for 20 hours at 37°C. After incubation a loop full of bacterial growth was transferred to 10 ml of Nutrient Broth. Cultures were grown at 37°C in a shaking incubator for 20 hours. After incubation they were centrifuged at 3800 g for 10 minutes and DNA was extracted using a Sigma-Aldrich extraction kit (GenElute Bacterial Genomic DNA kit, NA2110) according to the manufacturing instructions for Gram-negative bacteria.

2.6.3 Measurement of DNA and RNA Concentration

The concentration of the DNA and RNA extracts obtained was measured by spectrophotometry. A 1 µl sample was pipetted onto the pre-cleaned end of the lower measurement pedestal (receiving fibre) of the NanoDrop spectrophotometer (ND1000, England) whilst the second optic cable (sampling fibre) was open. The sampling arm was closed and spectral measurement performed using NanoDrop software v.3 (NanoDrop Technologies Inc., England). Sample carryover between successive measurements was minimised by wiping the sample from the pedestals using a Whatman lens cleaning tissue (2105918, Whatman, England). Nuclease free water (VWR, 436912C) was used as the control.

2.6.4 Calculation of Primers Annealing Temperature

The following equation was used to calculate the annealing temperatures of the primers used:

$$Ta = [(G+C) \times 4 + (A+T) \times 2]$$

Equation 2.4 Calculation of the annealing temperature of primers (Biophp, 2014)

The letters indicate the number of specific nucleobases in a primer sequence: A - adenine, C – cytosine, T - thymine and G – guanine.

The calculation was done separately for forward and reverse primers and then five degrees were taken out from the higher temperature (out of two calculated). If this result was lower than the annealing temperature of other primer, it was used in the experiments, if it was higher; the other primer temperature was used.

2.6.5 Preparation of Bacterial Cultures for Gene Expression Studies

2.6.5.1 Test Suspensions

Cultures of *S.* Typhimurium (whey powder) and *S.* Typhimurium HR (NCTC) were maintained at -80ºC in cryo vials (MicrobankTM, Pro-Lab Diagnostics, Bromborough, UK) containing glycerol. Working cultures of each serotype were sequentially prepared by spreading a cryo bead into 90 mm Petri Dish containing TSA. The plates were incubated at 37°C for 24 hours. Following this, a loop-full of bacterial culture was transferred to 50 ml LB (2.1.6) which was incubated for 20 h in a shaking incubator at 37°C and 150 rpm. The broth was then centrifuged at 3600 x *g* for 10 minutes (Hareus Megafuge 40R and rotor, Thermo

Scientific) and pellets were resuspended in 10 ml of PBS. This step was repeated 3 times with the final suspension being in the range of ~10^8 - 10^9 cfu ml^{-1}. Part of the suspension was directly incubated as non-desiccated controls and another part was used to inoculate stainless steel discs. The number of bacteria was assessed as described in 2.2.4.

2.6.5.2 Inoculation of Discs

A 50 µl, 2 x 50 µl or 3 x 50 µl volume of bacterial suspension were deposited onto stainless steel discs. Discs were placed in uncovered Petri dishes and transferred to a safety cabinet to dry at room temperature for 170 minutes. After drying, some of the discs were used to extract RNA and count attached cells, whilst otherwise kept in Petri Dishes which were covered and placed in air tight transparent plastic boxes (32 cm x 11 cm x 21 cm) for 22 or 68 hours at 33%±2% humidity and 25°C±1°C temperature.

2.6.6 RNA Extraction Using SV Total RNA Isolation System (Promega)

RNase AWAY™ (Invitrogen Ltd, Paisley, UK) and sterile RNase-free plastic ware and sterile RNase-free filter tips (Sarstedt; Biosphere® Fil. Tip 200 µl and 20 µl, long) were used to avoid contamination and RNA degradation. The technical manual for the SV Total RNA Isolation System (Products Z3105 and Z3100T; www.promega.com) was followed. Firstly, appendix 8B: the isolation of RNA from Gram-positive (*B. subtilis*) and Gram-negative (*E. coli*) bacteria was undertaken, followed by section 4E: RNA Purification by Centrifugation (Spin). Discs were inoculated with one drop of 50 µl of bacterial suspension prepared as described in 2.6.5.1 and they were placed in a safety cabinet for 170 minutes to dry, together with remaining bacterial suspension. Immediately after this, extraction using SV Total RNA Isolation System was undertaken with 3 different recovery methods:

a) Discs inoculated with 50 µl of suspension or 50 µl of suspension alone were recovered in 10 ml PBS containing 20% of stop solution: 5% H_2O phenol (Sigma, Phenol for molecular microbiology, EEC No 203-632) and 95% ethanol (96–100%, analytical grade reagent, Fisher Scientific, UK). This was incubated on ice for 30 min before centrifugation for 10 minutes at 4°C and 3800 *g*. The pellet was re-suspended in 100 µl of freshly prepared TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 0.4 mg ml^{-1} lysozyme (lysozyme from chicken egg white, Sigma Life Science, lyophilized powder

protein >90%). The solution was incubated at room temp for 3-5 minutes. After that 75 µl of RNA Lysis Buffer and 350 µl of RNA dilution buffer was added.

b) 50 µl of suspension inoculated on disc or 50 µl of suspension alone was recovered in 100 µl of freshly prepared TE containing 0.4 mg ml^{-1} lysozyme. The solution was incubated at room temp for 3-5 minutes. After that 75 µl of RNA Lysis Buffer and 350 µl of RNA dilution buffer was added.

c) 50 µl of suspension inoculated on disc or 50 µl of suspension alone were recovered in 75 µl of RNA Lysis Buffer and 350 µl of RNA dilution buffer.

2.6.7 RNA Extraction Using Trizol

RNase AWAY™ (Invitrogen Ltd, Paisley, UK) and sterile RNase-free plastic ware and sterile RNase-free filter tips (Sarstedt; Biosphere® Fil. Tip 200 µl and 20 µl, long) were used to avoid contamination and RNA degradation. Incubated bacterial cultures together with final bacterial suspensions were analysed by spectroscopy at OD 600 after 16 h, 20 h and 24 h of incubation (2.4.9). 150 µl of bacterial suspensions were diluted in 2850 µl of PBS (2.1.1) and this was checked for a number of *Salmonella* cells as described in 2.2.4. The suspension which was incubated for 20 h was used for further studies. Discs were inoculated with 3 drops of 50 µl bacterial suspension. At each time point discs were placed in 50 ml centrifuge tubes containing 3 ml of stop solution (2 ml of RNAprotect Bacteria Reagent, Qiagen and 1 ml of PBS). Also at each time point, three portions of 50 µl bacterial suspension were placed in 50 ml centrifuge tubes containing 2.85 ml of solution (2 ml RNA protect and 0.85 ml of PBS). This was incubated for 30 minutes at room temperature, discs were scratched with the end of a pipette tip to detach desiccated cells and vortexed for 30 seconds. The solutions were then centrifuged for 15 minutes at 3200 x *g*. The supernatant was removed and any remaining liquid was used to re-suspend the pellets and transfer the whole contents of the tube to a 2 ml Eppendorf tube. This was centrifuged again for 1 minute at 20800 x *g*. The supernatant was removed and the pallets were re-suspended on ice in 1 ml Trizol (Finn *et al*., 2013). The homogenized samples were incubated for 5 minutes on ice. Following this step, 0.2 ml of chloroform (analytical grade reagent, Fisher Scientific, UK) was added. Tubes were shaken vigorously by hand for 15 seconds and were then incubated at room temp for 2 to 3 minutes. The samples were centrifuged at 12,000 x *g* for 15 minutes at 4°C. Following centrifugation, the mixture separated

into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh Eppendorf tube and the RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol (Arcos Organics, UK). The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x *g* for 10 minutes at 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

The supernatant was removed and the RNA pellet was washed once with 1 ml of 75% ethanol. The sample was mixed by vortexing and then it was centrifuged at 7,500 x *g* for 5 minutes at 4°C. At the end of the procedure, the RNA pellet was air-dried for 10 minutes. The RNA was dissolved in 50 µl of RNase-free water (VWR, 436912C). 10 µl of Dnase Buffer and 2.5 µl of Dnase I enzyme (2.6.1) were added and then 37.5 µl of Rnase-free water was added to make up to 100 µl. After incubation at room temp for 10 minutes, 350 µl of Buffer RLT was added to each sample, mixed well, followed by 250 µl ethanol (96–100%, analytical grade reagent, Fisher Scientific, UK). The sample was transferred to an RNeasy Mini spin column placed in a 2 ml collection tube and it was centrifuged for 15 s at 8000 x *g*. The flow-through was discarded and 500 µl of Buffer RPE was added to the RNeasy spin column. This was centrifuged again for 15 s at 8000 x *g* to wash the spin column membrane. The flow through was discarded and again the same amount of Buffer RPE was added. This was followed by centrifugation for 2 minutes at 8000 x *g*.

The RNeasy spin column was placed in a new 2 ml collection tube and it was centrifuged at full speed for 1 min. The RNeasy spin column was again placed in a new 1.5 ml collection tube and 50 µl RNase-free water was added directly to the spin column membrane. This was followed by centrifugation for 1 min at 8000 x *g* to elute the RNA. RNA was stored at -80°C±1°C.

2.6.8 RNA Extraction Using RNeasy Mini Kit (Qiagen)

RNase AWAY™ (Invitrogen Ltd, Paisley, UK) and sterile RNase-free plastic ware and sterile RNase-free filter tips (Sarstedt; Biosphere® Fil. Tip 200 µl and 20 µl, long) were used to avoid contamination and RNA degradation. Discs were inoculated with two 50 µl droplets of bacterial suspension and were left to dry (2.6.5). At each time point, five inoculated discs were placed in 5 ml of RNAprotect Bacteria Reagent (Qiagen) mixed with 1 ml PBS. Discs were scratched (Anon., 2001) with the end of a pipette tip to detach desiccated cells and then they were vortexed

for 15 seconds. Bacterial suspension (10 times 50 µl) was placed in 2.5 ml of RNA protect reagent and vortexed for 15 seconds. The samples were left for 15 minutes on the bench top. Both suspensions were centrifuged at 3600 x *g* for 15 minutes. The supernatant was decanted and the pellets were resuspended in the remaining liquid. This was then transferred to 1.5 ml Ependorff tubes and centrifuged at 5000 x *g* for 10 minutes. After removing supernatant, 200 µl of TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg ml^{-1} lysozyme (Sigma, isolated from egg white) was added to the pelletes. These were mixed by vortexing for 10 s and were incubated at 37°C for 10 min. During incubation, tubes were vortexed for 10 seconds every 2 minutes. 700 µl of RLT buffer containing 10 µl b-mercaptoethanol per 1 ml buffer, was then added to each sample. In some of the samples particulate material was visible, therefore they were centrifuge for 2 minutes at 10000 x *g* and only the supernatant was used for further processing. This was followed by the addition of 500 µl of 99% ethanol (96–100%, analytical grade reagent, Fisher Scientific, UK) which included mixing by pipetting. 700 µl of the obtained lysate was transferred to an RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 15 s at 10000 x *g*. The flow-through was discarded and the step was repeated with the remaining suspension. 350 µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at 10000 x *g*. To perform DNase digestion, Dnase I incubation mix (70 µl RDD buffer and 10 µl Dnase I stock solution; see 2.6.1) was added to the column and it was incubated for 15 minutes at room temperature. At the end of the incubation period 350 µl Buffer RW1 was added to the RNeasy spin column. It was incubated for 5 min and then centrifuged for 15 s at 10000 x *g*. The RNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer RPE was added. The columns were centrifuged for 15 s at 10000 x *g*. 500 µl Buffer RPE was added to the RNeasy Mini spin column again and it was centrifuged for 2 min at 10000 x *g*. 30 µl RNase-free water was added directly to the spin column membrane, it was incubated for 1 minute at a room temperature and centrifuged for 1 min at 10000 x *g* to elute the RNA. This was repeated using another 30 µl of RNase-free water. After elution of RNA in RNase-free water, samples were stored in Eppendorf tubes at -80°C.

2.6.9 Purification of RNA with NaCl and ethanol

Samples of RNA were precipitated by adding NaCl (ACS reagent, ≥99.0%, Sigma-Aldrich) to a final concentration of 0.1 M. Following this step, 2.5 volumes

of 99% ethanol (96–100%, analytical grade reagent, Fisher Scientific, UK) was added. The samples were then incubated for 30 minutes at –20°C±1°C. The RNA was collected by centrifugation at 10,000 x *g* for 15 minutes at 4°C±1°C and it was resuspended in 50 µl of nuclease-free water.

2.6.10 Reverse Transcription

The reverse transcription was undertaken using Transcriptor First Stand cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany) which was designed to reverse transcribe RNA (mRNA, total RNA, viral RNA and in vitro transcribed RNA) from a variety of sources. The obtained cDNA could be used for studying gene expression levels via two-step RT-PCR.

The procedure was carried out according to the manufacturer's instructions with the specific conditions listed below.

The following reagents were added together:

10 µl RNA template (various concentrations)

1µl Anchored-oligo($dT)_{18}$ primer (final concentration: 2.5 µM)

2 µl random hexamer primer (final concentration: 60 µM)

=total volume 13 µl

The mixture was heated on the heating plate (Stuart Scientific SHT 1D, England) at 65ºC for 10 minutes and was cooled on ice.

To the mixture was then added:

4 µl transcriptor reverse transcriptase reaction buffer (final concentration: 8 mM $MgCl_2$)

0.5 µl protector RNase inhibitor (final concentration: 20 U)

2 µl deoxynucleotide mix (final concentration: 1mM each)

0.5 µl Transcriptor reverse transcriptase (final concentration: 10 U)

= final volume of 20 µl

The tubes were then placed in a thermal block (Stuart Scientific SHT 1D, England) and were incubated at 25ºC for 10 minutes followed by 30 minutes at 55ºC. The Transcriptor Reverse Transcriptase was inactivated by heating the mixture to 85ºC for 5 minutes. The samples were placed on ice and diluted to 100 µl with molecular biology grade water (VWR, 436912C).

2.6.11 Quant-iT™ OliGreen® ssDNA Reagent and Kit (Invirtogen)

Quant-iT™ OliGreen® ssDNA reagent is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA

(ssDNA) in solution. Quant-iT™ OliGreen® ssDNA reagent enables quantification of as little as 100 pg mL^{-1} oligonucleotide or ssDNA (200 pg in a 2 ml assay volume) with a standard spectrofluorometer and fluorescein excitation and emission wavelengths.

1 x TE buffer was made by diluting 20 x TE buffer (part of the kit) 1:20 in RNAse free water. The working reagent of OliGreen was prepared by diluting it 1:200 in 1 x TE buffer. Five micro litres of this reagent was added to 5 µl of cDNA sample in a 384 well plate. The plate was incubated in the dark for 5 minutes and run on a LightCycler® 480 (Roche).

Each sample was then checked to ensure that their Oligreen reagent reading was within 2 standard deviations of the mean Oligreen reagent reading of all samples. All samples with a crossing point outside of this were not used for the cDNA pool.

2.6.12 Primer Express 2.0

Primer Express® is a primer design programme from Applied Biosystems that also allows for an easy design of compatible TaqMan® probes, for use in real-time PCR or end-point PCR analyses. It can also be used for the design of primers alone when the user expects to analyze samples with SYBRGreen® only. The programme designs primers taking into consideration the basic principles of primers design:

- Avoiding the presence of two or more Gs or Cs in the final five bases at the 3' end of the sequence. The presence of these may facilitate formation of a GC clamp, which could interfere with the optimal function of the primers, by facilitating primer-dimer formation.
- Keeping the G-C content in the 30-80% range.
- Avoiding runs of an identical nucleotide. This is especially important for guanine, where runs of four or more Gs should be avoided.
- The annealing temperature should be 58-60°C.

The genes of interest sequence were inserted into the programme and the best primers were generated. Primers with the lowest number of penalty scores were chosen for the experiments (Table 2.4).

Table 2.4 Primers used in the studies

Gene	Forward Primer (5' –> 3')	Reverse Primer (5'-> 3')	Ref.
glpK	GACGAGCTGCAGGAAAAAGC	AGTTACGCTCGGTGGTTTCAA	Gruzdev *et al*., 2012
aceA	TGTACCCGGCAAACTCTGTTC	ATCTGATCTGCACGACGAAAAG	Gruzdev *et al*., 2012
hisD	CGGATGACCGTTCAGGAACT	CCGCCAATGTTTCAATGGTT	Gruzdev *et al*., 2012
ddg	CGGGCTGGACAACCTTACC	CATAAAATGCACGCCAACGA	Gruzdev *et al*., 2012
nifU	AGACGCGCGCTTCAAGACT	CACCCACTCCGTTACCAGAGA	Gruzdev *et al*., 2012
kdpA	TGGGACTGGGCATCCTGTT	GGTAGCCGCTGCGGATTTA	Gruzdev *et al*., 2012
rsuA – 16s	GGGCGTGAAATTCGTGGTAA	GCAGTTTGAAGGCCGCATT	Gruzdev *et al*., 2012
otsB	TTAACCGTATCCCCCGAACTC	CCGCGAGACGGTCTAACAAC	Primer Express
gltB	GCCTGCAGGACGACAAAGA	CGCCGGCATACATAGACCTT	Primer Express
prpE	CGTCCGGCGCATGTCT	TGCGGCGAAGCATCTTTC	Primer Express
nifU	AGACGCGCGCTTCAAGACT	CACCCACTCCGTTACCAGAGA	Primer Express
16s	AGTTCCACAACGCGATTGC	GCGAAGGCCGTTATCATCA	Primer Express
gapA	GGTGTTGACGTAGTGGCTGAA	AGCGTTGGAAACGATGTCCTG	Primer Express
agp	ATTCCAATATCGCGTCGCTGCT	TCATGCCAGCGTTGGAAGACAA	Primer Express
ffh	GCAGGTTGGCGTGGATTT	GCGGCGTTGACGATATCAA	Primer Express
proP	CGCTGGCCACGTTTTCC	ATTTATCGCCGAGCATACCAA	Primer Express
proU	CCAGGCGCAAGGGATTG	GGTTTGCGCATCAACGACTA	Primer Express
osmU	TCAGGACTGGCGCACTGTAC	AAAGCTCGGCGACCGTATC	Primer Express

2.6.13 Primer synthesis

Primers were synthesised by Eurofins MWG Operon (UK). They were re-suspended in an appropriate volume of nuclease free water (VWR, 436912C) to give 100 pmol as per the data sheet, and then they were diluted further to a 10 pmol concentration per primer. A small pool of template cDNA samples was made to check if the primers were working properly. The pool of samples was run on Light Cycler 480 with SYBR Green I as described in 2.6.16 to make sure the primers work, to determine what C_T (cycle threshold, the number of cycles required for the fluorescent signal to cross the threshold) they come up at, and to also check the melt curves for the primer dimers.

2.6.14 Polymerase Chain Reaction (PCR) amplification

Seven genes (Table 2.5) were tested with DNA extracted from all 15 *Salmonella* strains (2.1.9). The primers used are listed in Table 2.4 and PCR was undertaken using the DreamTaq Green PCR Master Mix (2x, Thermo Scientific) using 25 µl reaction mixture [2.5 µl DreamTaq Green PCR Master Mix, 1 µl forward primer (10 pmol μl^{-1}), 1 µl reverse primer (10 pmol μl^{-1}), 1 µl Template DNA (approx. 100ng μl^{-1}), 9.5 µl nuclease-free water] in PCR tubes (Fisher). The samples were amplified in a programmable thermocycler (Techne TC512, USA) using specific temperatures for different genes (Table 2.5).

Table 2.5 Thermal cycling conditions

Step	Temperature ºC	Time	Number of cycles
Initial denaturation	95	3	1
Denaturation	95	30 s	34
Annealing		30 s	
hisD	58		
rsuA	55		
kdpA	55		
nifU	60		
glpK	57		
aceA	59		
ddg	57		
Automated fluorescent extension	72	1 min	
Final extension	72	5 min	1

2.6.15 Gel electrophoresis of PCR amplicons

PCR product (10 µl) was mixed with 2 µl of 6X loading dye (G190A, Promega) and run on 2% (w/v) agarose (MB1200, Melford) gel containing ethidium bromide (0.2 µg ml^{-1}) in 1X TAE running buffer [40 mM Tris base, 20 mM glacial acetic acid (A/0400/PB17, Fisher Scientific] for approximately 2 h at 85 V to check the product size. A 1000 bp lambda DNA ladder (G210A, Promega) was used as the molecular size marker. The gel was visualised on a UV transilluminator (Bio-Rad) and images were recorded with Quantity one Gel Doc.

2.6.16 LightCycler® 480 (Roche) real-time RT-PCR

For a 384 well plate each reaction was undertaken in a final volume of 15 µl. A master mix of the constituents minus the cDNA template was made up.

The following was mixed well (no vortexing):

SYBR Master (2x, final conc. 1x)	7.50 µl
Forward primer (10 µM, final conc. 0.3 µM)	0.45 µl
Reverse primer (10 µM, final conc. 0.3 µM)	0.45 µl
Water (molecular grade)	1.60 µl
	10 µl total volume

The loading pattern for the 384 plate was planned and recorded as well as any subsequent alternations. Ten micro littres of each solution was transferred to a well plate and then 5 µl cDNA was added per well. The multiwall plate was sealed and centrifuged at 1500 x *g* for 1 minute.

LightCycler® 480 real-time PCR programme

1. Pre-incubation: 1 cycle
2. Amplification: 45 cycles (quantification)
3. Melting curve: 1 cycle
4. Cooling: 1 cycle

Block type: 384 wells

Detection format – SYBR Green I, Melt factor: 1.2, Quant factor: 30

2.6.17 Real-Time RT-PCR Results Analysis

The relative quantification with the Pfaffl method was used to calculate fold change of specific genes expression. A number of steps were performed for each gene to obtain the final results:

1. The comparison of C_T (cycle treshhold) values obtained from the control sample (suspension at the same time point as an experimental sample; $C_{T\ control}$) among the relevant experimental sample ($C_{T\ experimental}$) was calculated as:

$$\Delta C_T = C_{T\ control} - C_{T\ experimental}$$

2. To determine the PCR amplification efficiency, the serial dilutions of the template were performed (primarly 10-fold followed by 2-fold). The reaction amplification efficiency was then calculated using the following equaion:

$$E = 10^{(-1/slope)} - 1$$

slope refers to the slope of the plot of C_T value versus the log of the input template amount.

3. Each amplification efficiency was then converted to Pffafl efficiency (E_P)

$$E_p = (10^{(-1/slope)} - 1) + 1$$

4. The fold change in expression between the two samples was than calculated using the following equation:

$$\text{Fold change between samples} = (E_P)^{\Delta CT}$$

These calculations were performed for each target gene and a housekeeping gene.

5. To normalize the fold change between each sample set, the fold change of the target sequence was divided by the fold change of the housekeeping gene:

$$\text{Fold change (normalised)} = \text{Fold change}_{target}/\text{Fold change}_{housekeeping}$$

The normalised fold change was used to compare all studied genes expression in desiccation sensitive and resistant strain.

CHAPTER 3
SURVIVAL KINETICS OF 15 *SALMONELLA* STRAINS ATTACHED TO STAINLESS STEEL SURFACES AND KEPT UNDER LOW HUMIDITY CONDITIONS

3.1 INTRODUCTION

As discussed in Chapter 1, *Salmonella* is a major pathogen of concern for low water activity foods and understanding its persistence in dry food processing environments is important for producing safe food. Historically, low water activity foods (a_w<0.85) were of little concern with regard to microbiological safety due to their product characteristics. However, during the last 40 years *Salmonella* outbreaks have been linked to a number of food products with low water activity (CDC, 2014a). Beuchat and Komitopoulou (2011) and the Centres for Disease Control and Prevention (CDC, 2014) described at least 40 large, documented outbreaks of *Salmonella* associated with dry foods. There is, therefore, a growing concern for the potential presence of *Salmonella* in such products. As the manufacturing of many of these foods contains a thermal processing step, post process environmental contamination is thought to be the major source for contamination.

Evidence of *Salmonella* survival has been found in food processing environments. A study focused on contaminated chocolate by Craven *et al.* (1975), showed that the cause of the contamination was the uncontrolled airborne spread of dust in the factory environment. Samples taken in an oil meal plant also detected *Salmonella* on the processing floor, in dust in the air and on equipment (Morita *et al*., 2006). In several studies *Salmonella* was isolated from filters, processing floors, mixers, air and brooms in feed mills, equipment in dry sausage processing facility or crushing plants over a long period of time, indicating *Salmonella*'s ability to survive in dry processing environments (Gounadaki, 2008; Binter *et al*., 2011). It has been reported that once a production line is contaminated with *Salmonella spp*., the microorganism will establish itself on the equipment and facility surfaces. It is evident; therefore, that *Salmonella* can be present in the food processing environment and potentially contaminate the product.

Uesugi *et al*. (2007) demonstrated the potential for long-term (5 years) environmental persistence of *Salmonella* in almond orchards. However, this was an

open environment and may have been contaminated on an ongoing basis from an external source. Resistance of *Salmonella* strains to dry conditions was confirmed by Janning *et al.* (1994). In addition, Kusumaningrum *et al.* (2003) showed that *Salmonella* can remain viable on dry stainless steel surfaces and present the potential for contamination for considerable time periods.

The subspecies of most concern, in relation to food safety, is *S. enterica* subsp. *enterica.* More than 99 % of *Salmonella* isolated from humans belong to this subspecies (Old, 1992). The total number of *S. enterica* subsp. *enterica* serovars is approximately 1,531 (Grimont and Weill, 2007), however the number of serovars associated with outbreaks in low a_w foods, is only ~30 (Beuchat and Komitopoulou, 2011; CDC, 2014). Therefore, it is important to assess if these strains can also survive in the conditions similar to those present in low moisture factory environments so appropriate control mechanisms can be employed.

The studies aimed to (a) establish survival kinetics for 15 *Salmonella* strains using most suitable methods, (b) assess any differences between and within serotype isolates, (c) show if *Salmonella* cells attached to stainless steel surfaces, with traceable amounts of nutrients, can survive for prolonged periods of time, and (d) choose the best kinetic model to describe the survival. To achieve these aims it was necessary to choose and validate appropriate methods as recovery and analysis of microorganisms under desiccation conditions can be challenging. In order to ensure that the results would be as useful and applicable to the food industry as possible, the same *Salmonella* serovars as those from low water activity foods which caused recent *Salmonella* outbreaks were used. Additionally, heatresistant strain and a strain typically used for disinfectants testing (Anon., 2001) were used to investigate if they were also resistant to desiccation stress.

The chapter includes the results from trials conducted to support validity of selected methods and results from the core experiments on survival kinetics.

A schematic of the method, with references to the section numbers, is shown in Figure 3.1. The steps highlighted in blue are the basic steps used in the studies on survival of *Salmonella* strains during desiccation on surfaces, the steps highlighted in orange are the analyses conducted to verify the methods or to support achieved results. All the methods are described in details in Chapter 1.

3.2 RESULTS

3.2.1 Methods Verification

3.2.1.1 Degree of Required Cell Wash

An initial experiment was undertaken to assess the degree of cell washing necessary to remove any nutrients from the cell suspension used to inoculate stainless steel surfaces, which could have a growth or protection impact on subsequent cell survivability. Cells of *S.* Typhimurium 'HR' and *S.* Muenchen were thus prepared according to the methods described in Section 2.2.2.

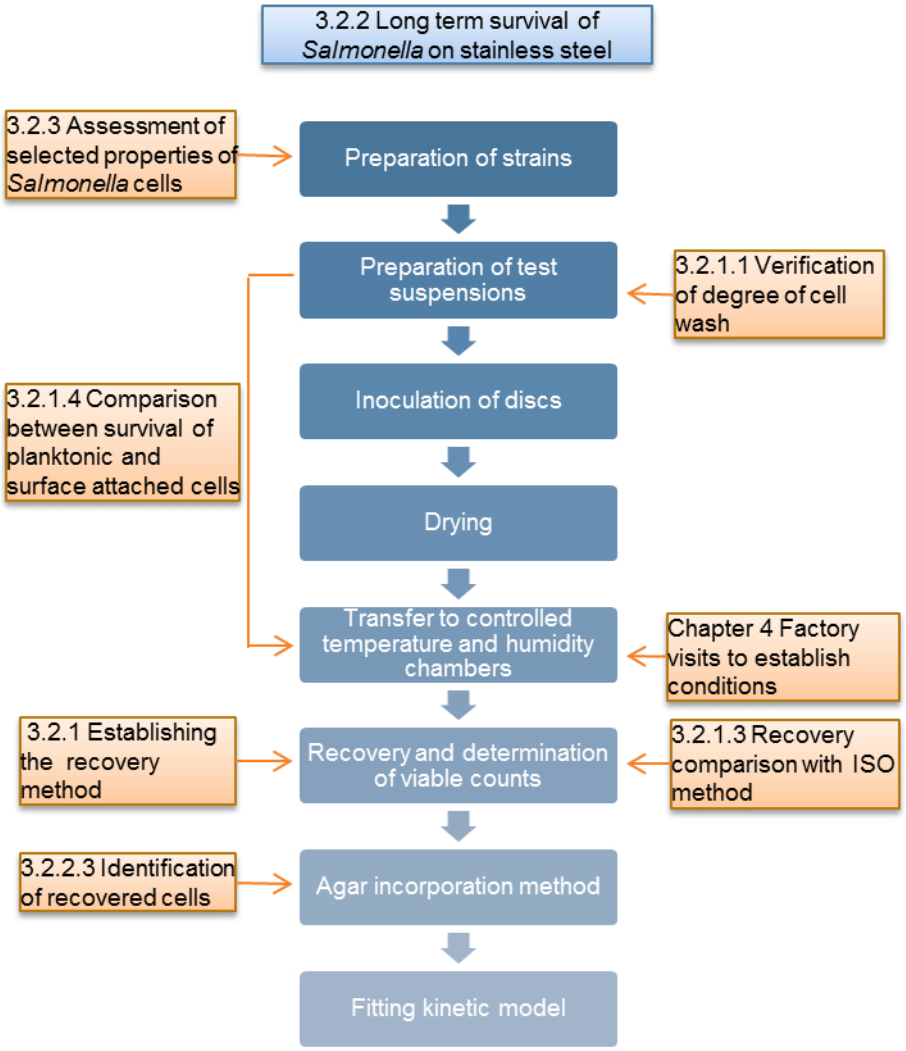


Figure 3.1 Schematic of tasks and methods used in this chapter

They were then washed and centrifuged once or twice with Maximum Recovery Diluent (MRD). Suspensions were inoculated onto stainless steel discs (Section 2.2.3), kept in controlled relative humidity and temperature conditions (33% RH and 25°C) and then recovered at different time points (n=3) to assess the survival numbers. The results for the two studied strains are shown in Table 3.1 . They were analysed for statistical differences using a one-way ANOVA method. A p value <0.05 was considered as a statistically significant difference.

No significant difference overall (p=0.960 for *S.* Typhimurium 'HR' and p=0.758 for *S.* Muenchen) was seen between cells washed once and twice in the survival of the two *Salmonella* strains on stainless steel surfaces, during the 30 days period (Table 3.1). Therefore for all subsequent trials pellets were re-suspended once in 10 ml of MRD which formed ~ 10^8 cfu ml^{-1} suspensions.

3.2.1.2 Establishing the Recovery Method

Four different recovery methods for cells desiccated on surfaces were tested before choosing the final method. The experiment was performed once but with 15 replicates of *S.* Typhimurium 'HR' for each method. For all methods, inoculated and desiccated discs (Section 2.2.2-2.2.3) were placed in a 60 ml container holding 10 ml of MRD.

Table 3.1 Survival of *S.* Typhimurium and *S.* Muenchen (log cfu $surface^{-1}$ ± SD), washed once or twice prior to inoculation, on stainless steel surfaces at different time intervals.

Time on surfaces (h)	*S.* Typhimurium 'HR' (log cfu $surface^{-1}$ ± SD)		*S.* Muenchen (log cfu $surface^{-1}$ ± SD)	
	1 wash cycle	2 wash cycles	1 wash cycle	2 wash cycles
0	5.08 ± 0.43	5.29 ± 0.14	5.78 ± 1.04	5.96 ± 0.13
1	5.74 ± 0.09	4.92 ± 0.30	5.94 ± 0.21	5.76 ± 0.16
3	5.41 ± 0.09	4.72 ± 0.51	5.38 ± 0.34	5.92 ± 0.12
6	4.84 ± 0.21	4.80 ± 0.58	5.81 ± 0.11	5.45 ± 0.44
24	4.11 ± 0.94	4.20 ± 0.38	4.89 ± 0.24	4.84 ± 0.35
30	4.28 ± 0.54	4.69 ± 0.15	4.50 ± 1.31	5.09 ± 0.16
48	4.01 ± 0.06	3.95 ± 0.35	4.81 ± 0.22	4.79 ± 0.06
72	4.06 ± 0.49	4.20 ± 0.49	4.78 ± 0.50	4.51 ± 0.43
168	3.42 ± 0.43	3.75 ± 0.11	3.94 ± 1.10	3.93 ± 0.64
240	4.09 ± 0.71	3.62 ±0.10	4.55 ± 0.07	3.82 ± 0.08
360	2.72 ± 0.55	3.39 ± 0.24	3.26 ± 0.26	3.07 ± 0.66
720	2.82 ± 0.39	3.18 ± 0.36	3.45 ± 0.52	3.30± 0.22

Further, the following recovery was performed:

a) Vortexing for 1 minute on highest speed (American Scientific S8223)
b) Shaking horizontally with glass beads (diameter ~ 5mm, Sigma-Aldrich) for 1 minute
c) Soaking for 1 hour in MRD (§2.1.1) and then vortexing for 1 minute
d) Sonicating in an ultrasonic bath (2 L, 35 kHz Low Frequency Ultrasonic Bath Cleaner UC-1200BT, Shanghai Lorderan Scientific Instrument Co., Ltd.) for 4 minutes

Table 3.2. *S*. Typhimurium 'HR' recovered using four different methods after 24 hours attachment

Disc No.	Vortexing with diluent	Shaking with glass beads	Soaking 1h and vortexing	Ultrasonic bath
1	2.86	*	4.69	3.81
2	4.77	5.08	4.75	2.18
3	4.89	3.34	4.88	3.15
4	4.04	5.11	4.56	2.96
5	4.34	5.15	5.34	4.18
6	4.08	5.08	4.65	3.25
7	4.32	5.15	4.41	4.11
8	4.30	4.26	4.94	2.34
9	4.36	4.92	4.81	3.97
10	4.77	3.79	4.92	3.94
11	5.15	4.26	3.98	4.20
12	5.08	5.11	5.18	3.26
13	4.67	5.08	5.40	3.15
14	4.62	5.18	5.00	2.88
15	3.98	3.34	4.85	3.74
Mean	**4.42**	**4.63**	**4.82**	**3.41**
SD	**0.55**	**0.67**	**0.35**	**0.63**

The recovered suspensions were then diluted and plated out as described in Section 2.2.4. The results are expressed in Table 3.2 as log cfu surface^{-1}.

No difference was observed between the number of *Salmonella* recovered using the first three methods ($p>0.05$). However the recovery using the ultrasonic bath was significantly lower compared to the other methods ($p<0.05$). Vortexing for 1 minute was chosen as a recovery method for all of the experiments.

3.2.1.3 Recovery Comparison between Established Method (Section 3.2.1.3) and ISO 6579:2002 method

Recovery of *Salmonella* cells in these studies using the method described in Section 3.2.1.2 was compared with the method predominantly used in the food industry to detect *Salmonella* in food or environmental samples: *Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella spp (ISO 6579:2002+A1, Section 2.2.10).* This was conducted with two strains *S.* Typhimurium (whey powder isolate, FH/St/165) *and S.* Typhimurium 'HR' (FH/St/168). Suspensions were prepared and inoculated discs (n=3) were kept under desiccating conditions (33% RH and 25°C) for 290 days (Section 2.2.2-2.2.3). Bacteria attached to one half of the number of inoculated discs were recovered with the method described in Section 3.2.1.2 whilst the other half was recovered using the ISO method (Section 2.2.10).

Table 3.3 shows the results obtained when using both methods. The ISO method is qualitative and the method used in these studies is quantitative. *S.* Typimurium (whey powder isolate) was not detected with either method after 290 days under desiccating conditions. *S.* Typhimurium 'HR' was recovered after the attachment for long period of time.

The ISO method showed positive results with both broths (MKTT and RVS) and agars (XLD and BGA) used (Figure 3.2).

The method chosen for all of the experiments (Section 3.3.1.2) also showed high number of *S.* Typhimurium cells on the discs. Recovered microorganisms were identified using two antisera (Section 2.4.2 and 2.4.3) and they all showed agglutination which suggested they were *S.* Typhimurium cells.

3.2.1.4 Comparison between the Survival of Planktonic and Surface-Attached Cells

The survival of surface attached *Salmonella* cells was compared with a planktonic cell survival. This was not undertaken during every single experiment, it was undertaken during one set of trials to show that in general microorganisms in suspension survive better than desiccated microorganisms. The strains chosen for these experiments were *S.* Typhimurium (isolated from whey powder) and *S.* Typhimurium 'HR'.

Table 3.3 Comparison of results between the chosen experimental method (Section 3.3.1.2) and ISO 6579:2002 for cells recovered after 290 days under desiccation conditions

Strain	Test method				ISO method			
	Counts Log cfu disc^{-1}	Agar incorp.	O' and H' antisera	Flagellar antisera	XLD	BGA	O' and H' antisera	Flagellar antisera
S. Typhimurium (whey powder)	<0.7 (ND)	0	Negative	Negative	-	-	Negative	Negative
S. Typhimurium 'HR'	2.19 (±0.16)	TMC	Positive	Positive	Black colonies	Pink-white colonies	Positive	Positive

ND = not detected , limit of detection: 5 cfu disc^{-1} (0.7 Log cfu disc^{-1} as discs were recovered in 10 ml of MRD and 1 ml was plated out in duplicate)
Agar incorp. – agar incorporation method, see Materials and Methods 2.2.5
O' and H' antisera – Materials and Methods 2.4.3
Flagellar antisera - Materials and Methods 2.4.2
ISO method - Materials and Methods 2.2.10
TMC – too many to count

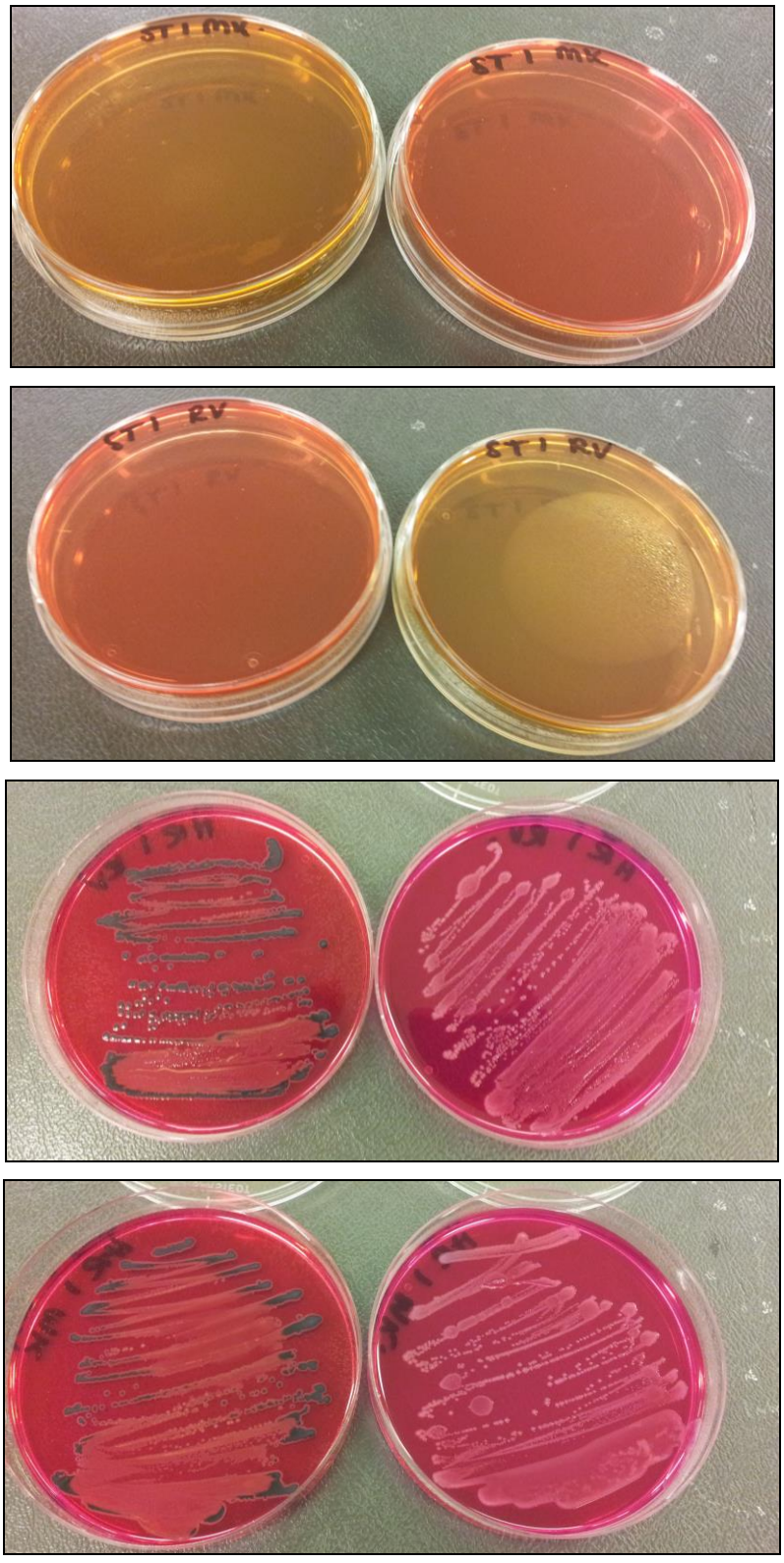


Figure 3.2 Growth of *Salmonella* on selective agars using ISO 6579:2002 (A) S. Typhimurium, inoculated from MKTT broth, left: XLD, right: BGA (B) *S*. Typhimurium, inoculated from RVS broth, left: BGA, right: XLD (C) *S*. Typhimurium HR, inoculated from RVS broth, left: XLD, right: BGA (D) *S*. Typhimurium HR, inoculated from MKTT broth, left: XLD, right: BGA

Discs were inoculated with the *Salmonella* suspension, were dried and moved to a controlled environment as described previously (§2.2.2-2.2.3). The remaining suspension was kept in tightly closed containers under the same conditions: 33% RH and 25ºC temperature. Viable cells were recovered and the results were presented as the reduction in numbers, expressed as $\log_{10}$ cfu surface^{-1} (or ml^{-1} for suspension) from the point directly after drying (time 0) to the specific time points (Figure 3.3).

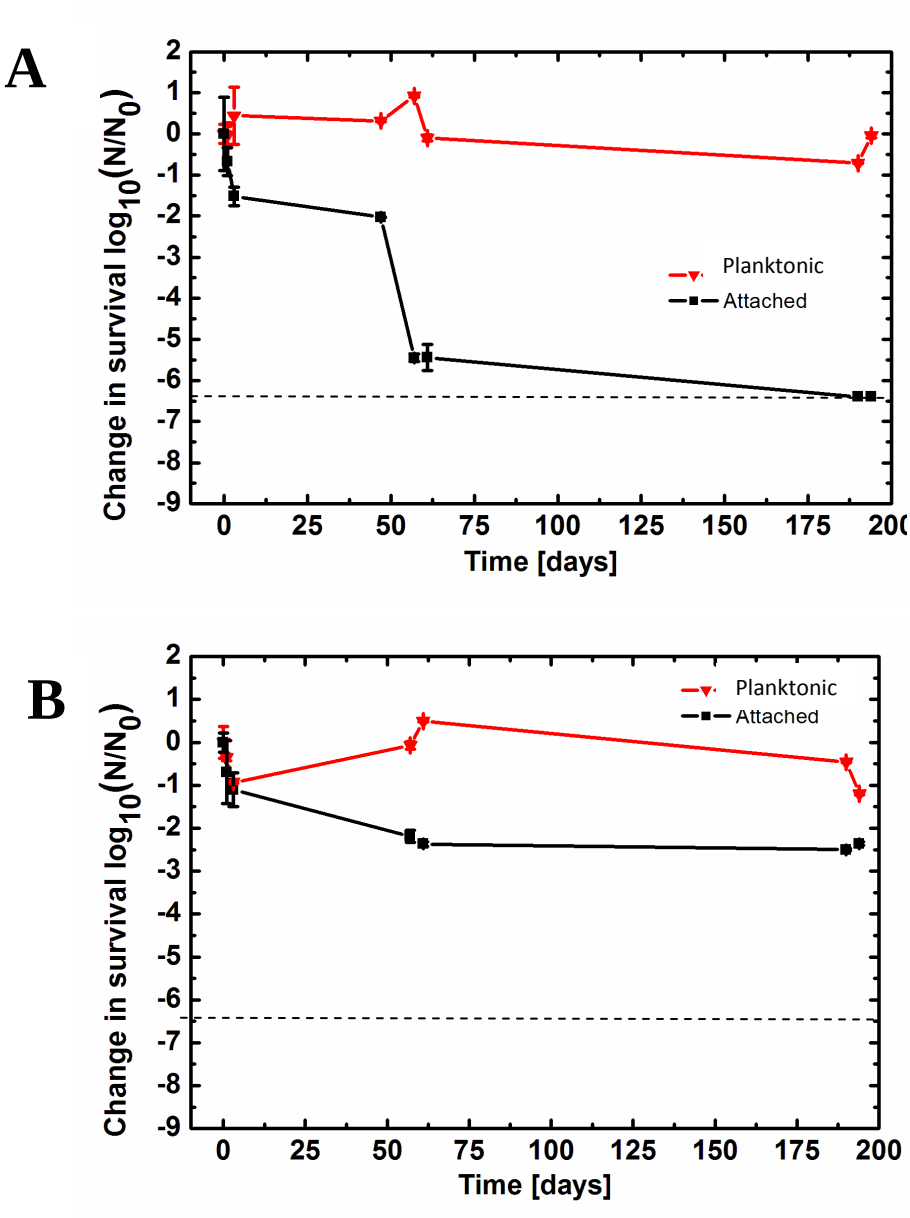


Figure 3.3 Comparison between survival of *Salmonella* suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33% RH and 25ºC for up to 194 days and *Salmonella* suspended in MRD and kept at the same conditions (A) *S.* Typhimurium (whey powder isolate) (B) *S.* Typhimurium 'HR' Bars represent one standard deviation. Dotted line shows limit of detection.

For both strains, the planktonic cells showed only a slight reduction with time. Surface attached cells however, declined significantly within time.

S. Typhimurium (whey powder isolate) cells were not detected after 190 days of desiccation (>6.4 $\log_{10}$ cfu surface^{-1} reduction) and *S.* Typhimurium 'HR' strains showed a maximum of 2.5 $\log_{10}$ cfu surface^{-1} reduction after 194 days under desiccating conditions (Figure 3.3).

3.2.2 Long Term Survival of *Salmonella* on Stainless Steel

3.2.2.1 Survival Kinetics of 15 Salmonella Strains

These studies were focused on the establishment of a survival model for all 15 *Salmonella* strains, comparing the differences between each serotype and the same serotypes, isolated from different products and environments. The environmental conditions which were used (RH=33 % and T=25 ºC) and all methods are described in Section 2.2.2-2.2.5.

A 50 µl volume of each test suspension was deposited onto the centre of stainless steel discs. Replicate discs (n=3) were placed in an uncovered Petri Dish and transferred to an incubator to dry at 30ºC for 80 minutes. After drying, Petri Dishes were covered and placed in air-tight, humidity and temperature-controlled, transparent plastic boxes (§2.1.11). The number of microorganisms surviving on the stainless steel surfaces was assessed at the following time points after drying: 0 h, 1 h, 3 h, 6 h, 24 h, 30 h, 2 days, 3 days, 7 days, 10 days, 15 days and 30 days.

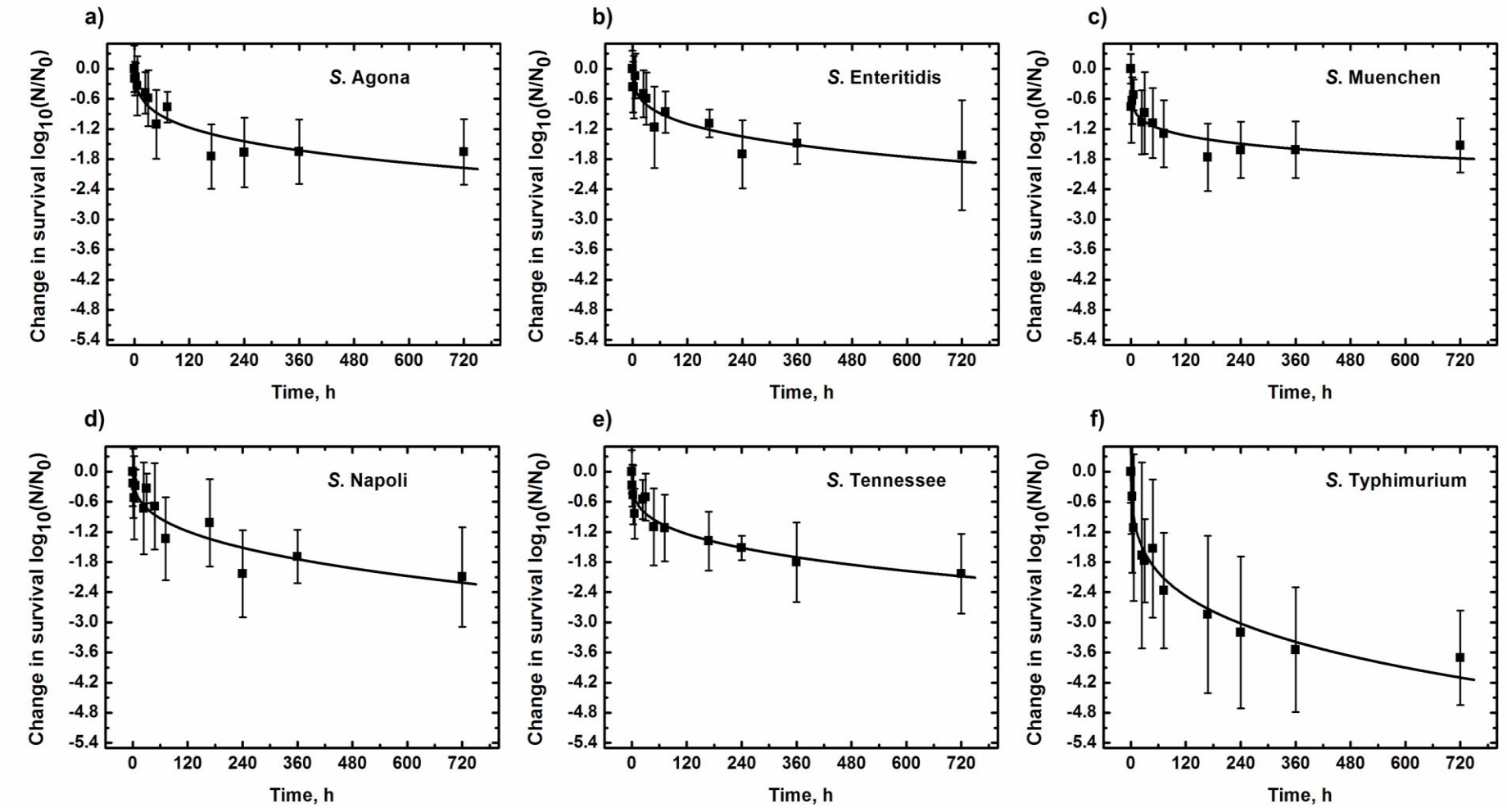
Experiments were repeated at least 4 times using 3 replicates each time for each test condition and these data were used to develop a kinetic model. Trials involving the strains isolated from a cereals processing environment had three technical replicates for each strain at each time point. These strains were tested as a final part of the study and according to the sample power and size calculations, there was no need to conduct more replicates to be able to notice statistical differences between strains.

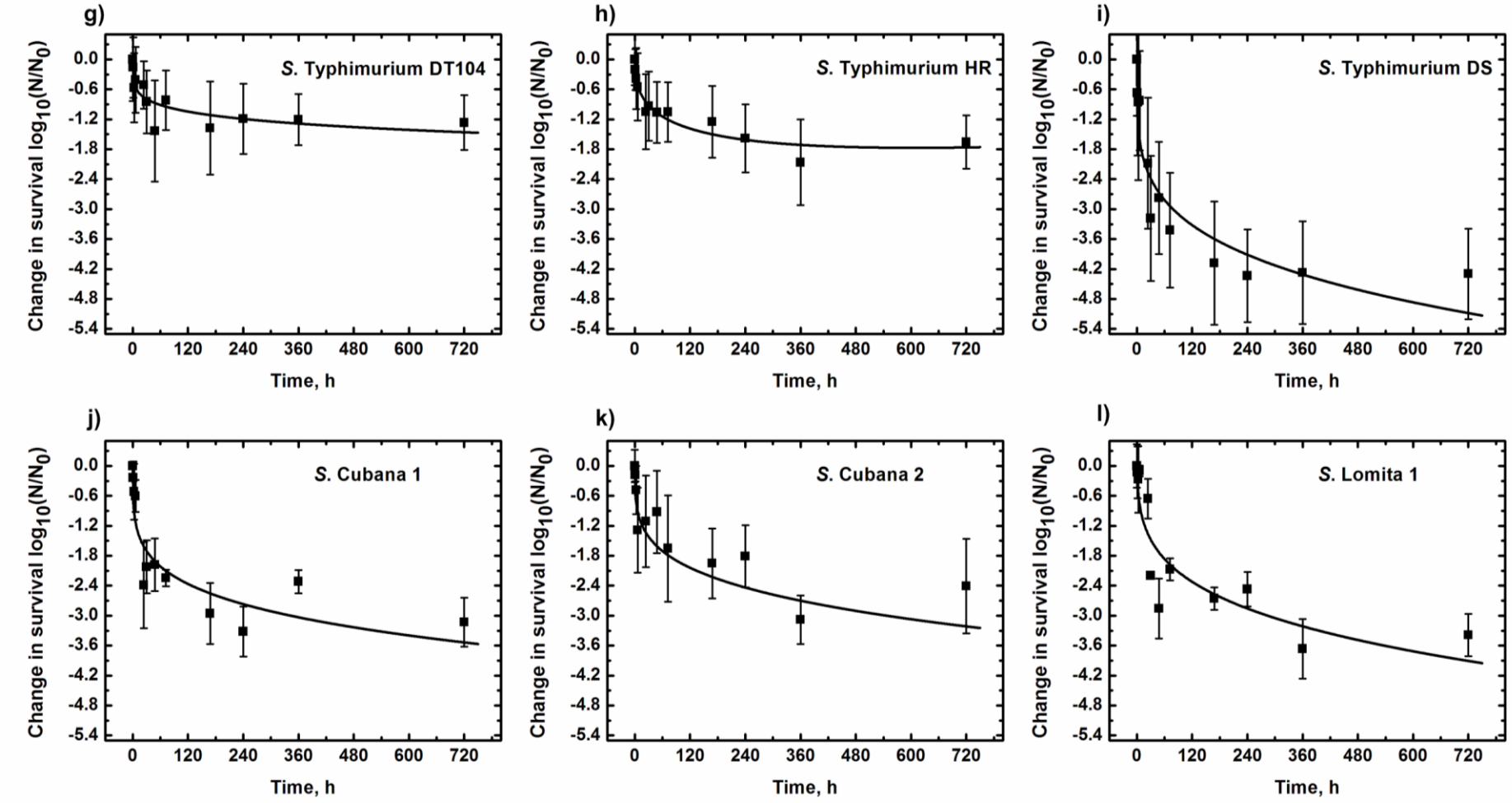
The results are expressed as the mean $\log_{10}$ cfu surface^{-1} reduction at each time point compared to time 0 (directly after drying). The points on the graphs (Figure 3.4) represent the mean of replicate experimentally obtained values and the lines represent predicted kinetics gained by fitting the modified Weibull model (Section 2.2.11). Fifteen different *Salmonella* isolates (Section 2.1.9) were used to compare their survival rate and assess their resistance to desiccation. The initial

bacterial level on the stainless steel discs ranged from 6.6 to 7.3 $\log_{10}$ cfu per surface. Strains had different sensitivities to initial drying (80 minutes at 30°C) which resulted in different starting levels when placing the discs in the controlled humidity boxes (Table 3.4). *S.* Typhimurium 'DS' and *S.* Typhimurium (whey powder isolate) were found to be the most sensitive to initial drying (a loss of viability of 2.08±0.92 and 1.84±0.57 $\log_{10}$ cfu per surface, respectively), and they were also most sensitive to the desiccating conditions under controlled humidity and temperature (Figure 3.4). The loss of viability after initial drying for the rest of the strains ranged from 0.65±0.29 to 1.44±0.82 $\log_{10}$ cfu per surface.

Table 3.4 Colony counts (mean $\log_{10}$ cfu surface^{-1}) and standard deviation values (SD) before and after initial drying for all 15 *Salmonella* serotypes

Serotype	Mean $\log_{10}$ cfu surface^{-1} (SD)	
	Before drying	After drying
S. Muenchen	7.11 (0.14)	6.42 (0.29)
*S.*Typhimurium DT104	7.07 (0.24)	6.12 (0.84)
S. Typhimurium (HR)	6.56 (0.31)	5.91 (0.52)
S. Agona	7.08 (0.20)	6.36 (0.47)
S. Enteritidis	7.03 (0.23)	6.37 (0.36)
S. Tennessee	7.19 (0.24)	6.47 (0.43)
S. Mbandaka 1	6.87 (0.00)	6.19 (0.58)
S. Cubana 2	6.91 (0.00)	6.15 (0.32)
S. Napoli	7.26 (0.40)	6.01 (0.69)
S. Mbandaka 2	6.79 (0.00)	5.34 (0.82)
S. Cubana 1	6.86 (0.00)	6.04 (0.03)
S. Lomita 1	6.81 (0.00)	6.03 (0.44)
S. Lomita 2	6.94 (0.00)	6.26 (0.14)
S. Typhimurium	7.28 (0.21)	5.44 (0.62)
S. Typhimurium (DS)	7.15 (0.29)	5.07 (1.13)


a)
S. Agona
b)
S. Enteritidis
c)
S. Muenchen
d)
S. Napoli
e)
S. Tennessee
f)
S. Typhimurium
Change in survival log10(N/N0)
0.0
-0.6
-1.2
-1.8
-2.4
-3.0
-3.6
-4.2
-4.8
-5.4
0
120
240
360
480
600
720
Time, h


g)
S. Typhimurium DT104
h)
S. Typhimurium HR
i)
S. Typhimurium DS
j)
S. Cubana 1
k)
S. Cubana 2
l)
S. Lomita 1
Change in survival log10(N/N0)
0.0
-0.6
-1.2
-1.8
-2.4
-3.0
-3.6
-4.2
-4.8
-5.4
0
120
240
360
480
600
720
Time, h

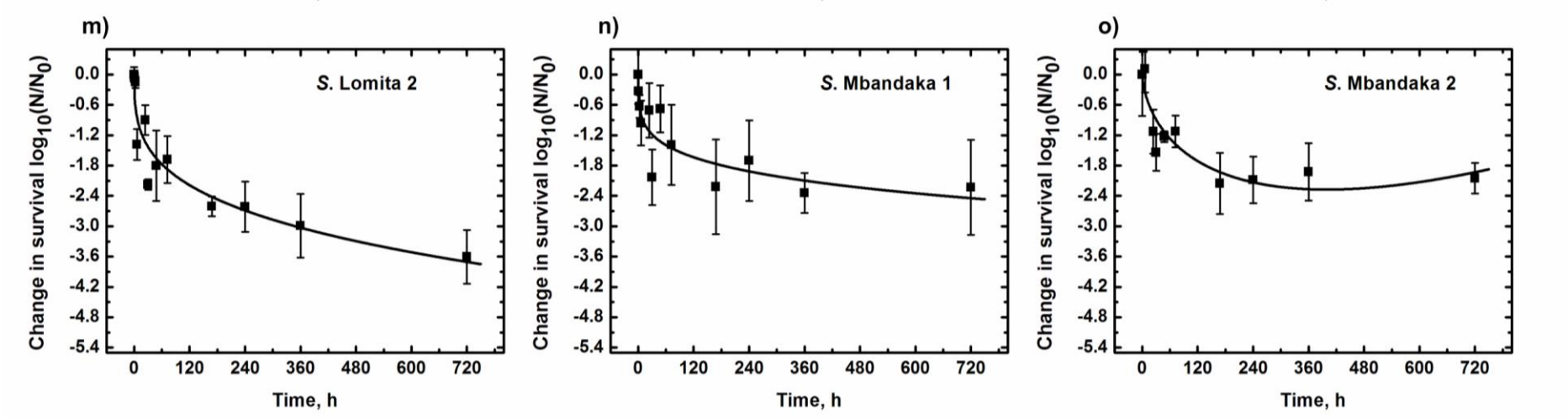


Figure 3.4 Modelled reduction kinetics of 15 strains of *Salmonella* suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33% RH and 25ºC for up to 720h. Bars represent one standard deviation. Points represent mean experimental values.

The highest $\log_{10}$ reduction was associated with *S.* Typhimurium (whey powder isolate) and *S.* Typhimurium 'DS' which, showed a 4.1±0.9 and 4.3±0.9 $\log_{10}$ cfu per surface reduction after 30 days, respectively. The lowest reduction rate, from the point after initial drying to 30 days, was associated with *S.* Muenchen, S. Typhimurium DT104 and S. Typhimurium 'HR', where the microorganism levels dropped by 1.5±0.50, 1.3±0.60 and 1.6±0.50 $\log_{10}$ cfu surface^{-1}, respectively. A similar reduction was also observed for both S. Agona and S. Enteritidis. The rest of the strains, after 30 days, showed reduction levels ranging from 2.1 to 3.6 $\log_{10}$ cfu per surface (see Table 3.4 and Figure 3.4). After obtaining broad experimental data, the most suitable model was selected and fitted to describe the mechanism of the reduction for each *Salmonella* strain. Table 3.5 shows the integrity of fit for the obtained equations. The coefficient of determination (R^2) is close to 1 for most of the strains, indicating that the suggested model fits relatively well to the experimental values. Nonetheless, the model may present deviations from the experimental data as it was not possible to fit the model to 100% of the experimental points. In order to find out whether this deviation created an under- or over-prediction, the B_f value was calculated. The B_f values are mostly above 1 indicating that the model is over predicting the reduction rates for 13 out of 15 strains.

Table 3.5 Values of the coefficient of determination (R^2), root mean square error (RMSE), bias factor, (B_f) an accuracy factor (A_f) for the obtained equation

Serotype	R^2	RMSE ($\log_{10}$)	B_f	A_f
S. Agona	0.94	0.22	1.03	1.29
S. Enteritidis	0.94	0.19	1.04	1.32
S. Munchen	0.93	0.18	1.01	1.15
S. Napoli	0.93	0.26	1.03	1.32
S. Tennessee	0.96	0.16	1.01	1.17
S. Typhimurium	0.98	0.22	0.99	1.12
S. Typhimurium DT104	0.87	0.22	1.08	1.29
S. Typhimurium HR	0.96	0.15	1.01	1.74
S. Typhimurium DS	0.94	0.52	1.11	1.26
S. Cubana 1	0.90	0.48	1.16	1.39
S. Cubana 2	0.85	0.36	1.33	1.37
S. Lomita 1	0.90	0.56	1.36	1.67
S. Lomita 2	0.94	0.37	1.31	1.60
S. Mbandaka 1	0.87	0.39	1.07	1.30
S. Mbandaka 2	0.88	0.32	0.82	1.60

R^2 = 1 indicates best fit
RMSE = 0 indicates smallest error
B_f >1 indicates over-prediction, B_f <1 indicates under-prediction, B_f =1 indicates best fit
A_f =1 indicates that prediction is the same as experimental data

This means that the real reduction is smaller than is plotted by the kinetic curve on the graphs and in some cases (S. Lomita '1') this is by 67% (A_f 1.672942 ~67%). The reduction kinetics are under predicted (i.e. the curve lies above the actual data points) only for *S.* Typhimurium (whey powder isolate) and *S.* Mbandaka '2'. An over-predicted result is not desirable since a higher reduction level will be estimated compared to the real value. This must be considered when using the model to predict the survival rates of *Salmonella* strains and, depending on the A_f values, the appropriate percentage should be added or reduced ($1 > B_f$ or $1< B_f$ respectively) from the predicted values.

The modified version of the Weibull model suggested the existence of two subpopulations within each *Salmonella* strain population. It can be seen in Figure 3.4 that there was a relatively high rate of bacterial death at the beginning of the survival curves (subpopulation 2) for all isolates and a decrease in the death rate (except *S.* Typhimurium 'DS' and *S.* Lomita '2') from up to 72 h (subpopulation 1) under desiccating conditions. The fastest decline rate was associated with *S.* Typhimurium (whey powder isolate) and *S.* Typhimurium 'DS' where statistically significant differences could be seen between samples taken at time 0 and samples taken after 6 and 24 h in desiccating conditions, respectively. The slowest overall dying rate was shown for *S.* Muenchen and *S.* Typhimurium 'HR'. For these strains, a significant difference in microbial counts from time 0 could only be seen after 168 h under desiccating conditions. After the initial reduction in *Salmonella* count (reduction of subpopulation 2), the microorganism levels stayed constant (subpopulation 1). The strains which reached this state in the shortest time (after 24 h) were *S.* Muenchen, *S.* Typhimurium DT104 and *S.* Typhimurium 'HR'. Most of the strains, after reaching a certain reduction level, showed no further, significant viability loss for 27 days (Figure 3.4), suggesting time might not be a factor in prolonged survival of subpopulation 1. Significant differences were detected ($p < 0.05$) for all cases between the two sub-populations (or resistances).

Table 3.6 lists kinetic coefficients obtained using Equation 2.1 (Section 2.2.11) and these describe the behaviour of the surviving subpopulation (parameters δ_1 and α_1) and the more sensitive subpopulation (parameters δ_2 and α_2; Table 3.6). δ_1 and δ_2 are expressed in hours and indicate the time needed for the first decimal reduction; β_1 indicates the fraction of subpopulation 1 (surviving population) in the total population. It is worth mentioning that this fraction is in relation to the survival

when expressed as $\log_{10}$ cfu surface^{-1} and not to arithmetic values of the counts. α is a shape parameter and refers to the extent of the impact that time of exposure may have on the reduction rate. If α is lower, the reduction rate is less affected by time of exposure and thus the survival number of *Salmonella* is higher (however this value has to be analysed together with δ_1 and δ_2 to give an assessment of overall reduction rates). This can be seen for example with *S.* Agona. The first subpopulation (more resistant, surviving population is about 41% (β_1 fraction is 0.4148, which is equal to ~41%) of the whole population and is resistant to desiccation with numbers changing only slightly, between 120 and 720 h of desiccation (Figure 3.4a). This behaviour is represented in the kinetic equation by the low value for α_1 (Table 3.6). In addition, the kinetic parameter δ_1 shows how slowly this population dies (2.6 h to achieve the first decimal reduction). A high value of δ_1 would mean that a longer time of exposure to the inimical process is necessary in order to achieve a significant cell reduction. In contrast the more sensitive second subpopulation of *S.* Agona has a δ_2 of 1.5 showing the first decimal reduction of this subpopulation takes a shorter time and the higher value for α_2 in comparison to α_1 shows this subpopulation is more sensitive to time of exposure.

Table 3.6 Kinetic coefficients for Equation 2.1

Serotype	β_1	δ_1	δ_2	α_1	α_2
S. Agona	0.41	2.64	1.51	8.94^{-7}	0.22
S. Enteritidis	0.38	2.46	1.56	0.10	0.23
S. Munchen	0.18	2.20	1.77	2.21^{-5}	0.14
S. Napoli	0.48	2.37	1.48	0.23	0.29
S. Tennessee	0.35	2.38	1.43	0.28	0.28
S. Typhimurium	0.71	3.87	0.00	0.02	0.23
S. Typhimurium DT104	0.29	2.40	1.69	1.57^{-7}	0.15
S. Typhimurium HR	0.46	2.54	1.02	0.57	0.50
S. Typhimurium DS	-0.11	1.69	1.88	0.23	0.23
S. Cubana 1	0.66	4.49	0.01	0.06	0.18
S. Cubana 2	0.17	1.94	1.89	7.05^{-7}	0.23
S. Lomita 1	0.71	5.00	0.01	9.06^{-5}	0.23
S. Lomita 2	0.21	1.83	1.99	7.67^{-9}	0.27
S. Mbandaka 1	0.21	2.27	1.70	5.84^{-7}	0.20
S. Mbandaka 2	0.38	1.20	1.52	0.81	0.77

β_1 - indicates the fraction of subpopulation 1 (surviving population) in the total population; 1=100%;

δ - indicates the time (h) needed for the first decimal reduction;

α - shape parameter, refers to the extent of the impact that time of exposure may have on the reduction rate. Higher the value, higher the reduction rate is affected by time;

1 – subpopulation 1;

2 – subpopulation 2

'-' sign indicates subpopulation 2 accounts for 100 % of total population and one-population model should be used to describe the kinetics

The sign minus next to the β_1 value of *S.* Typhimurium 'DS' indicates there is only one population which is the sensitive population (number 2). This can be also seen at the graph (Figure 3.4 i) as it is clear that the number of cells declines rapidly and constantly. This is also seen by the low value of δ_1. It is apparent therefore that suppopulation 2 accounts for 100 % of total population. This suggests that the kinetic equation is not the most suitable to describe the reduction kinetics of *S.* Typhimurium 'DS' and most likely one-population Weibull model should be used in this case. The equation does not distinguish between positive values of β_1 and negative values and negative sign means that the subpopulation 1 is not present.

Considering *S.* Typhimurium (isolated from whey powder) as an example of one of the most sensitive strains, it could be concluded that the resistant subpopulation, which is 71% of the population (line 6, Table 3.6), is less affected by exposure time than the sensitive population ($\alpha_1 < \alpha_2$) and more time is needed to reduce the number of cells by one decimal reduction ($\delta_1 > \delta_2$) compared with the sensitive second subpopulation. However, this *S.* Typhimurium (whey powder isolate) strain is not very resistant to desiccation over the whole exposure time; this is because its level of resistance is dominated by the second sensitive subpopulation (Figure 3.4f) where the value of δ_2 is 1000-fold lower than for the more resistant first subpopulation, reflected in a very fast reduction in microbial cell counts at the beginning of the desiccation process. Analysing 'other sensitive strains, *S.* Typhimurium 'DS' seems to have a predominant sensitive subpopulation 2 (89%), *S.* Lomita '1' has a much lower δ_2 value than δ_1 and *S.* Lomita '2' has a high percentage of subpopulation 2 (79%) with considerably higher α_2 than α_1 value (i.e. with considerably less resistance). Therefore for each of these more desiccation sensitive strains it would suggest a different factor is determining their survival kinetics.

In addition, the better surviving strains have different factors which are influencing their resistance to desiccation. For example, *S.* Muenchen and *S.* Typhimurium DT 104 have a small amount of the population which is more resistant (18% and 29%, respectively); however these subpopulations have a very low α_1 factor (very modest influence of time of exposure) and need slightly more time for one $\log_{10}$ decline in cell numbers ($\delta_1 > \delta_2$). For these strains the α_2 values are also relatively low showing their sensitive populations are also less influenced by time of exposure. *S.* Typhimurium 'HR', however, has a much larger population which is

resistant (47%) but the α and δ values for both subpopulations are not very different, thus producing an overall more resistant population.

An example of a moderately desiccation resistant strain is *S.* Cubana '1' (Figure 3. and Table 3.6). The values for this strain indicate that most of the population (~66%) is resistant to harsh environmental conditions and no significant differences occur between 60 and 720 h. This resistant part of the population shows a very high value of δ_1 (4.5) in comparison to the sensitive population (δ_2 = 0.0007). Hence a longer time is needed to achieve a significant reduction in microbial counts of this resistant part of the population. The value of α_1 is smaller than the value of α_2, which means the resistant population is not much affected by exposure time and no significant changes are expected as a function of time. The sensitive population, although a smaller part (~34%) of the population has a very low δ_2 and this is reflected in the very rapid reduction seen during the first 0 – 60 h on the graph (Figure 3.4j), which is significantly different from the resistant population (p = 0.005).

Differences in survival kinetics and survival rates between different strains of the same serotype were noted, with *S.* Typhimurium DT104 and *S.* Typhimurium 'HR' (heat resistant) being in the high resistance group, and *S.* Typhimurium (isolated from whey powder) and *S.* Typhimurium 'DS' (a laboratory cultured strain used in European Standard disinfectant approval test methods) having low resistance to desiccation. In contrast there was no difference in the reduction levels between *S.* Lomita '1' and '2' (3.4±0.4 and 3.6±0.5 log cfu per surface reduction, respectively). However in contrast to the *S.* Typhimurium strains which were isolated from different environments, both *S.* Lomita strains were isolated from the same cereals environment, which may have selected specific desiccation tolerances in *Salmonella* strains harboured there.

3.2.2.2 Long Term Survival

Further studies were conducted to assess long term survival of *Salmonella* attached to stainless steel surfaces. *S.* Typhimurium 'HR' and *S.* Typhimurium isolated from whey powder were used. Bacterial suspensions were prepared and inoculated as described in Section 2.22-2.2.3. Discs were stored in desiccating conditions (25°C and 33% RH) for up to 12 months. The number of microorganisms was assessed at different time points during desiccation. Figure 3.5 shows the mean

$\log_{10}$ cfu surface^{-1} reduction at each time point compared to time 0 for two *Salmonella* strains.

S. Typhimurium (whey powder isolate) counts declined significantly over time, reaching undetectable levels after 87 days. The counts of *S.* Typhimurium 'HR' also decreased significantly, however after 360 days there were still approximately 2 $\log_{10}$ colony units per surface.

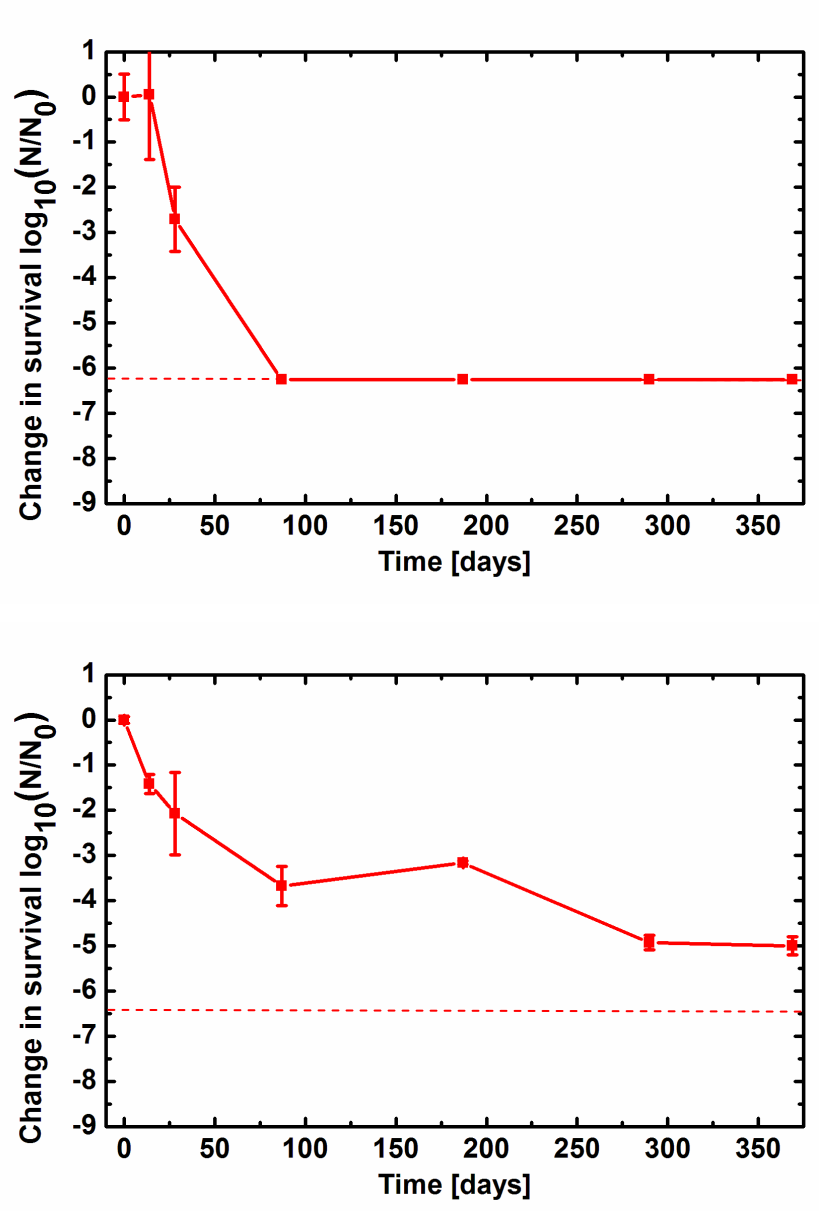


Figure 3.5 Reduction of *Salmonella* cells suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33% RH and 25ºC for up to 360 days expressed as $\log_{10}$ cfu surface^{-1}, vertical lines represent standard deviation; (A) *S.* Typhimurium (whey powder), (B) *S.* Typhimurium 'HR'. Dashed lines represent limit of detection.

3.2.2.3 Identification of recovered cells

It was necessary to confirm that cells recovered from stainless steel surfaces were *Salmonella* strains to rule out any contamination. Old colonies of *Salmonella* may give false negative results therefore fresh subcultures were always prepared for testing.

The *Salmonella* was recovered from desiccated discs as described in Section 2.2.4 and grown over night at 37ºC for 24 h on a TSA plate. In addition for visual assessment of the colonies on agar plates, a *Salmonella* test kit for the detection of *Salmonella* (Oxoid; Materials and Methods 2.4.2) was used. The Oxoid *Salmonella* Test Kit detects the majority of common *Salmonella* species including *Salmonella* Typhimurium.

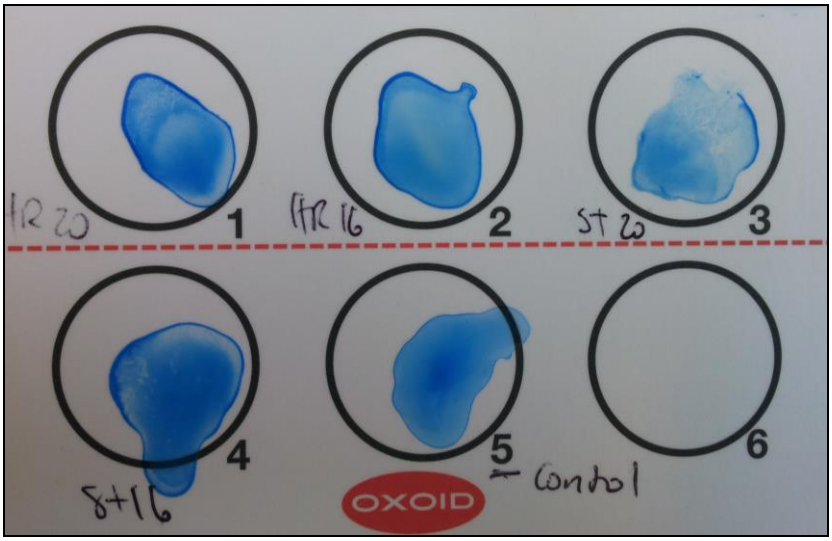


Figure 3.6 Agglutination of the recovered samples using the Oxoid *Salmonella* identification kit

Additionally, the *Salmonella* agglutinating serum for *Salmonella* antisera (O' and H') was utilised (Section 2.4.3).

All cells recovered after desiccation showed agglutination for both test methods. Figure 3.6 shows an example of the results using the Oxoid *Salmonella* detection kit.

3.2.3 Assessment of Selected Properties of *Salmonella* Cells

3.2.3.1 Screening Strains for Auxotrophic Mutation

M9 minimal medium agar plates (see section 2.1.3) were dried under laminar flow conditions. All 15 *Salmonella* cultures were taken from the -80°C freezer and

one cryobead was streaked on the agar surface. The plates were then incubated for 24 hours at 37°C and checked for growth. This was repeated 3 times for each strain. Auxotrophic mutants are not able to growth on minimal medium. All agar plates indicated microbial growth therefore it was concluded that there was no auxotrophic mutation and cells were able to utilise basic nutrients to grow.

3.2.3.2 Red-Dry-Rough (RDAR) phenotype

All 15 strains were tested for the RDAR phenotype (Section 2.2.9). The Congo Red Agar plates were inoculated with five drops of 3 µl of suspensions prepared as described in Section 2.2.2 and incubated for 72 hours at 30ºC. The colony morphology was then examined and recorded.

Figure 3.7 shows the photos of colonies of all 15 strains grown on Congo Red Agar and Table 3.7 describes the characteristics of grown cells.

3.2.3.3 Motility Abilities

Swim and swarm motility agars were prepared according to Section 2.1.4. Bacterial suspensions of 15 *Salmonella* strains were prepared as in 2.2.2. Five micro litters of these suspensions were placed on the centre of a Petri Dish. Swim motility was examined after incubation for 8 hours and swarm after 8 and 24 hours incubation at 37ºC.

Figures 3.8 and 3.9 show bacterial growth on the agar plates for swim and swarm motility abilities, respectively, for *S.* Typhimurium (whey powder) and *S.* Typhimurium 'HR'. The diameter of the growth area was measured for each inoculated strain and the results are shown in Table 3.8. The strains numbers in the table correspond to the strains listed in Table 3.7.

Only one strain did not show any swim ability. It was *S.* Typhimurium isolated from whey powder, which was also most sensitive to desiccation. S. Typhimurium DS showed the smallest growth zone which was 3.2 cm after 8 hours. The average growth zone for all strains which showed swim activity was 4.8 cm. The strains which did not show swarm activity after 8 hours were: S. Tennessee, S. Typhimurium (whey powder isolate), S. Typhimurium DS, S. Cubana 2, S. Mbandaka 1 and 2.

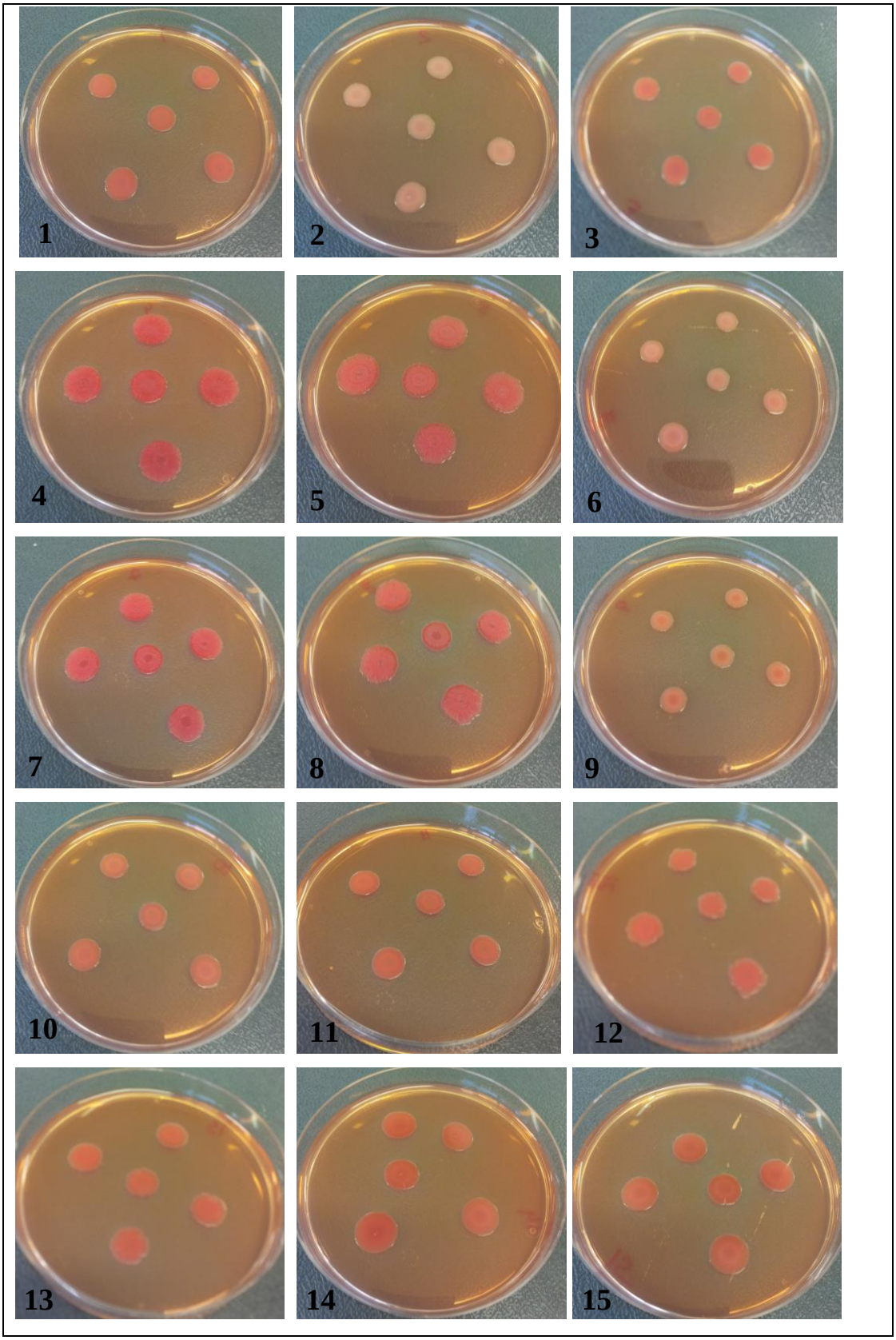


Figure 3.7 *Salmonella* colonies of 15 strains inoculated on Congo Red Agar. See Table 3.7 for strains corresponding to the pictures' numbers

Table 3.7 Morphology of colonies of 15 *Salmonella* strains grown on Congo Red agar

No.	Strain	Characteristics		
		Size	Colour	Roughness
1	*S.* Agona	Medium	Dark pink	Smooth
2	*S.* Napoli	Small	Pink	Smooth
3	*S.* Muenchen	Small	Pink	Smooth
4	*S.* Enteritidis	Large	Purple	Wrinkled
5	*S.* Tennessee	Large	Purple	Wrinkled
6	*S.* Typhimurium FH/St/165	Small	Pink	Smooth
7	*S.* Typhimurium DT104	Large	Purple	Wrinkled
8	*S.* Typhimurium HR	Large	Purple	Wrinkled
9	*S.* Typhimurium DS	Small	Pink	Smooth
10	*S.* Lomita 1	Medium	Dark pink	Smooth
11	*S.* Lomita 2	Medium	Dark pink	Smooth
12	*S.* Cubana 1	Medium	Dark pink	Slightly wrinkled
13	*S.* Cubana 2	Medium	Dark pink	Slightly wrinkled
14	*S.* Mbandaka 1	Medium	Dark pink	Smooth
15	*S.* Mbandaka 2	Medium	Dark pink	Smooth

Table 3.8 Growth zones of 15 *Salmonella* strains on swim and swarm medium

Strain	Swim agar – growth zone after 8h (cm)	Swarm agar – growth zone after 8h (cm)	Swarm agar – growth zone after 24h (cm)
1	5.6	1.5	Total coverage
2	5.0	1.5	Total coverage
3	4.2	1.8	Total coverage
4	6.2	1.7	Total coverage
5	3.8	None	0.6
6	None	None	3.0
7	4.5	0.9	Total coverage
8	5.5	2.2	Total coverage
9	3.2	None	0.9
10	5.0	1.4	Total coverage
11	4.5	1.2	Total coverage
12	4.8	1.2	Total coverage
13	5.0	None	Total coverage
14	5.0	None	Total coverage
15	5.5	None	Total coverage

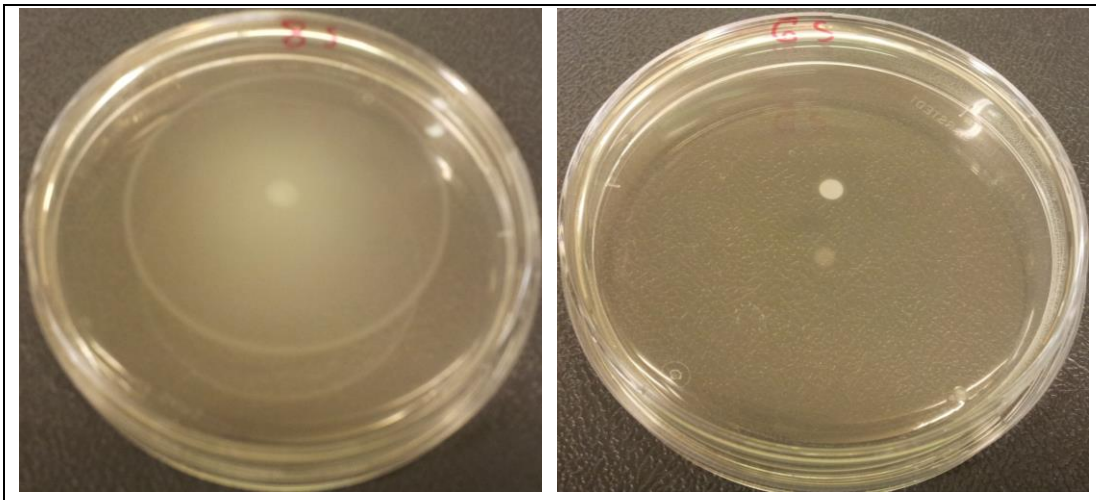


Figure 3.8 Swim abilities of (A) S. Typhimurium 'HR' and (B) S. Typhimurium (whey powder)

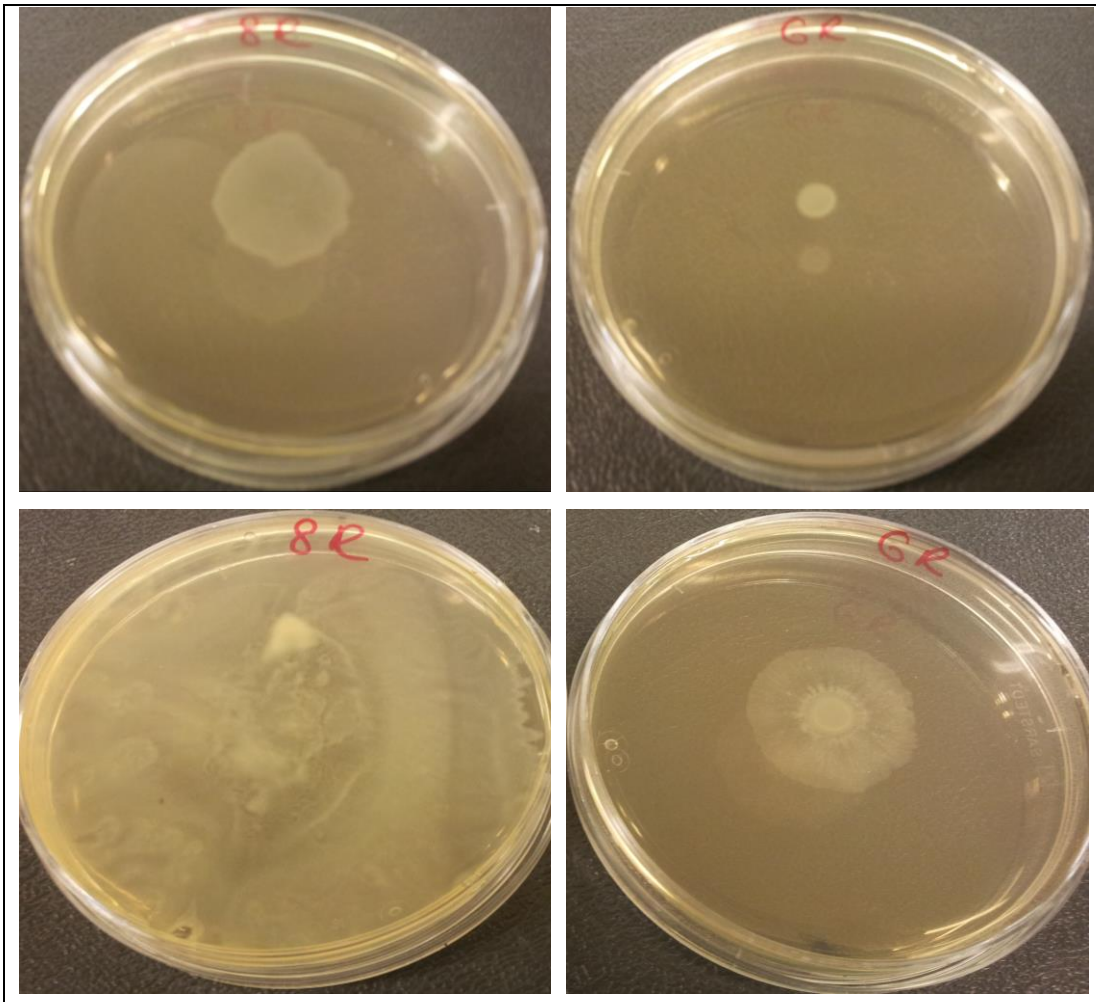


Figure 3.9 Swarm abilities of (A) S. Typhimurium HR after 8 h incubation, (B) S. Typhimurium (whey powder isolate) after 8 h incubation, (C) S. Typhimurium HR after 24 h (bottom) incubation and (D) S. Typhimurium (whey powder isolate) after 24 h incubation.

However after 24 h incubation there were only 3 strains which showed a significantly smaller growth zone than the rest and they were*:* *S.* Tennessee, *S.* Typhimurium (whey powder isolate) and *S.* Typhimurium 'DS'.

A typical for swarming bacteria dendritic fractal-like patterns on agar surfaces were not always observed, however these are not always clearly pronounced (Wook and Surette, 2005), therefore it does not rule out the swarming abilities of studied strains.

3.3 DISCUSSION

3.3.1 Methods Verification

These studies were focused on the reduction kinetics of 15 *Salmonella* strains during desiccation on stainless steel; however it was crucial to choose the most appropriate methods for this analysis.

The *Salmonella* strains chosen were associated with low a_w food outbreaks, were isolated from dry foods or their production environment or were known as resistant to other stresses such as heat or disinfection. Stainless steel was chosen as the most common surface used in the food handling environment (BSSA, 2013; Holah and Thorpe, 1990; factory visits), and the temperature and humidity conditions were chosen to reflect dry foods processing environments as observed during factory visits (Chapter 4). It is known that the exposure to a single stress condition e.g heat (Humphrey, 1995) or acid (Leyer and Johnson, 1993) is commonly associated with the development of cross-tolerance to other stress conditions (Abee and Wouters, 1999). Therefore the conditions used for the experiments were chosen to obtain an otherwise optimum environment for *Salmonella* cells (e.g stationary phase, ambient temperature, nutrients not limited etc.), so, most likely, only desiccation resistance mechanisms were involved in the survival of serotypes. Stationary phase grown microorganisms were used rather than log-phase bacteria as it is known that they have greater resistance to multiple stresses (Aldsworth *et al*. 1998; Rychlik and Barrow, 2005) and this is thought to more closely reflect the growth phase cells are in in food environments. The survival of cells in suspension was not carried out every time when each experiment was performed, it was only done during one set of experiments to prove that cells in suspension have greater survival abilities then desiccated cells attached to surfaces. Strains sensitive and resistant to desiccation did not differ in response when they were tested in suspension.

The appropriate recovery method is crucial for any studies focused on surface-attached cells. Use of a soaking method to recover previously desiccated cells has already been reported. Andrews *et al*. (1983) showed that a soaking method enhanced recovery of *Salmonella* from non-fat dry milk. In studies conducted by Wilson *et al*. (1985) the soaking method improved recovery from soy flour but not with onion powder or dried active yeast. In studies described in this thesis, there was no evidence that 1 hour soaking was more effective than the other two methods. Use of glass beads is also a commonly used method to recover surface attached cells, especially in the form of biofilms (Trachoo, 2004). This method, however, was not used because there was a possibility that glass beads could further damage *Salmonella* cells, which were already weakened by desiccation. In other studies sonication was found to be superior to other methods in recovering biofilm cells from stainless steel surfaces (Bjerkan *et al*., 2009; Kobayashi *et al*., 2009). The studies described in this chapter did not confirm this hypothesis. This could be because of the different frequencies used (30 kHz in other studies and 40 kHz in this studies) and possibly the temperature increase during sonication in this work could adversely affect recovery, causing cells damage and death.

Considering the fact that all three tested methods (Table 3.2) were equally good (ANOVA test), vortexing for one minute was chosen for further experiments. The method which used soaking was more time consuming and the method which used glass beads could potentially damage already weakened cells, if desiccated for even longer periods of time.

After long term survival of microorganisms on surfaces, it is important to make sure that recovered cells are the same as inoculated strains. This can be done by different methods e.g enrichment in selective media (Grounta *et al*., 2013; Perez-Rodrigueza, 2014), or e.g. serological confirmation (Van der Linden *et al*., 2013). Serological testing was done to confirm the identity of cells, additionally the method was compared with that commonly used in the food industry ISO 6579:2002 (Anon., 2002; Piknováa *et al*., 2002; Oliveira *et al*., 2012), and both analysis gave similar results. All recovered cells were identified as *Salmonella* spp. which ensures that the studies measured true survival of the evaluated microorganism, rather than external contamination.

3.3.2 Long Term Survival of *Salmonella* on Stainless Steel

The studies showed that the strain recognised as resistant to disinfectants (and so resistant to one stress factor) was not resistant to drying; in contrast the heat resistant strain of S. Typhimurium "HR" did show desiccation tolerance. Differences in the survival between different isolates of the same serotype (*S.* Typhimurium) were noticed. For most of the studied strains, an initial reduction in viability occurred in the first few days, with no or relatively little further reduction seen over the subsequent part of the 30 day period. This suggests that the length of the period of desiccation the strains are exposed to is not the most important factor in reducing the microbial population. During the first hours of exposure (<72 h), the cells may be more susceptible to desiccation. After 72 h, most of the serotypes seem to have developed a more resistant subpopulation possibly through fully expressed adaptive mechanisms; it is this subpopulation that can now persist for an extended period and shows a very slow death rate. This coincides with the findings from food borne outbreak investigations and factory sampling trials, which imply that some *Salmonella* strains have survived for years in factory environments (Russo *et al*., 2013).

The modified kinetic Weibull model fitted well with experimental data (R_2>0.85) and gave some insights into the survival behaviour for each *Salmonella* strain studied. The objective of analysing the model performance was to ensure a proper mathematical correlation between survival and time. However, it is not recommended to apply this mathematical model to predict the survival rate within another experimental set-up. The main objective of this kinetic model and the model performance analyses was to gather insights into the *Salmonella* desiccation and change in survival mechanisms. It is not expected that this kinetic model will predict survival of *Salmonella* within other experiment set-ups. The mathematical model should be validated with external data, obtained at other or similar experimental set-up, prior to be used.

It is important that, for each strain, each parameter obtained by the model is analysed together with the other parameters obtained for that particular strain and it is not recommended that individual kinetic parameters are compared between strains since their meaning is strongly dependent on the rest of the values (e.g. comparing only time needed for first detimal reduction - δ_2 or fraction of resistant population - β_1). This kind of comparison could only be possible if a 1-population Weibull model

with the same α values for every strain (or the same β value for each strain) was used. The kinetic parameters obtained from the modified Weibull model, in combination with the graphs (Figure 3.4), are considered to explain the kinetics of *Salmonella* survival for each single strain, but the kinetic parameters should not be used to range or group the strains in terms of most or least resistant. For these purposes, the graph for each strain can be used as a comparison reference. For most of the strains (except *S*. Typhimurium "DS" and *S.* Lomita "2") the second population was more susceptible to desiccation, which is reflected in the graphs by the values obtained from the early hours of desiccation. This population, therefore, would need less time for reduction in microbial counts ($\delta 2 < \delta 1$). Thus during the first hours of desiccation, microorganism levels declined faster (2^{nd} subpopulation) and during the late period of desiccation, microorganism levels stayed constant or declined slightly (1^{st} subpopulation – surviving subpopulation). The added value of the model is the possibility to calculate exactly the respective percentage of subpopulation 1 and 2 and the rates at which these subpopulations were declining. The kinetic parameters obtained suggested that each strain had a different response to desiccation in terms of the percentage of the population which was resistant, reduction levels, reduction rate and/or influence of time of exposure.

In previous studies, Kusumaningarum *et al*. (2003) reported that *S*. Enteritidis survived for at least 96 h on stainless steel surfaces at 20 – 25ºC and 40 – 45 % RH and Broennum Pedersen *et al*. (2008) found that *S*. Senftenberg attached to ELISA wells for 35 days at 40 % humidity were reduced in microbial population by 3.45 log10 cfu ml^{-1}. This is a similar result to other serotypes investigated in this study. However, the reduction was much higher in the Kusumaningarum *et al*. (2003) studies. These differences could be caused because of different strains, different culturing methods prior to inoculation, different drying methods or different humidity levels. The difference in survival rate between isolates of the same *Salmonella* serotype seen in the current study was also observed by Humphrey *et al*. (1995).

The ability of *Salmonella* to survive for long periods of time on surfaces was demonstrated by Hiramatsu *et al*. (2005) and Gruzdev *et al*. (2012a). Hiramatsu *et al*., studied long term survival of a selection of *Salmonella* serovars (Oranienburg, Chester, Enteritidis, Litchfield, Anatum, Virchow, and Typhimurium) inoculated onto paper discs and desiccated. The survival was significantly affected by storage

temperature with cells kept at 25°C surviving up to 70 days and cells stored at 4°C up to 24 months. The 4°C temperature was also used in Gruzdev *et al*. (2012a) studies and cells inoculated onto polystyrene surfaces survived for more than 100 weeks (2 years). This thesis focused on stainless steel surfaces as this is the most commonly used material in the food industry and the chosen temperature reflected real dry food factory conditions.

Studies conducted by Gruzdev *et al*. (2012a) also showed large variability in desiccation tolerance and long term persistence (up to 12 weeks) among different *Salmonella* serotypes as well as within a single serotype (*S.* Typhimurium). This confirms the findings of the present study and highlights the importance of choosing an appropriate serotype/isolate when conducting any specific challenge testing. Muller *et al*. (2012) investigated the survival of 25 selected *Salmonella* strains attached to surfaces after prolonged exposure to controlled humidity and temperature. A strong decline in survival trend was observed and only three *Salmonella* strains survived at desiccation conditions for up to 49 days, Variations in survival of all 25 isolates could be observed, including amongst several *S.* Typhimurium strains. The 25 strains were mainly porcine and clinical isolates, which may reflect their reduced resistance in comparison with the 15 strains used in this study, primarily isolated from dry environments. The serotypes which are isolated more often from dry processing environments may have been selected to survive because they had acquired mutations or genetic information that enhanced their desiccation tolerance (Binter *et al*., 2011). The number of mutated strains among *Salmonella* is reportedly high (Jorgensen *et al*., 2000); therefore it can be argued that the more frequent the appearance of mutators, the better the ability of this organism to generate isolates better adapted to survive in different environments. As a consequence very closely related *Salmonella* serovars can have very different responses to stress (Jorgensen *et al*., 2000).

It is not yet clear from the present study why some *Salmonella* strains survive better than others or what the differences are which makes one subpopulation decline more rapidly at the beginning of the desiccation process and the other subpopulation reach a plateau phase. One way *Salmonella* survives severe environmental conditions may be because surface inoculated cells dry towards the outer ring of the inoculum (Chapter 4) thus forming a multilayer of cells on the edge of the ring. The outer layer may then die off faster, protecting the inner layers. This could be one way

that the two subpopulations could arise. Additionally, initial decline of the population level, followed by no decline, could be explained by possible heterogeneity of the bacterial populations (differences in growth phases of cells; Booth, 2002). The single *Salmonella* inoculum may contain cells with different levels of expression of a particular cellular component which helps them cope with the stress e. g. protein in their cells; therefore the ones which contain this specific component are able to survive, and the ones which lack it, die off. This might explain the 'nonlinear' death kinetics of the analysed *Salmonella* strains. It may be argued that some of the cells entered a viable but not culturable (VBNC) state and could not be detected with the methods used. The recovery method however was validated and additionally an agar incorporation method was used to pick up low cell numbers. The LIVE/DEAD stain was used to establish the relationship between cultured cells and live cells on the surfaces; however the stain did not work well with dried, attached cells (results not shown). These hypotheses suggest why there may be two subpopulations (with different resistances) within one *Salmonella* strain population and support the findings suggested by the kinetic model used in this paper. Additionally, LeJean *et al*. (1994) and Bunning *et al*. (1990) observed similar occurrence in *Bacillus stereothermophilus* and *Salmonella* Typhimurium respectively, in regard to response to heat were the cultures studied contained a mixture of heat sensitive and resistant populations.

3.3.3 Assessment of Selected Properties of *Salmonella* Cells

Observation of rugose phenotype in *S.* Typhimurium DT104 was also undertaken by Anriany *et al*. (2001). The presence of rugose colonies was associated with the formation of aggregates and presence of extracellular substance (ES) closely associated with the cells in the pellicle. The ES in rugose colonies appeared to have an important protective function, allowing the cells to survive in the presence of acid and hydrogen peroxide and possibly other adverse conditions such as desiccation (Rice *et al*., 1992). In this study *S.* Typhimurium (whey powder) did not appear to have rugose colonies; however the S. Typhimurium 'HR' which is more resistant to heat and desiccation appeared to have a RDAR phenotype, therefore possibly also ES. Also another weak strain, S. Typhimurium 'DS', did not show rugose phenotype which is in line with the hypothesis. However, strains such as *S.* Napoli and *S.* Muenchen which were resistant to desiccation during the 30 day period, did not appear to have dark, wrinkled colonies. It is concluded, therefore, that presence of

ES (indirectly indicated by rugose phenotype) is not necessarily needed for these particular strains to survive desiccation and the mechanism of survival is not directly associated with the protective effect of ES.

Motility of bacteria may play a crucial role in the first stages of infection (Josenhans and Suerbaum, 2002) and may be significant during drying of droplets and when water is introduced in the factory environment (Finn *et al*., 2013). All of the strains studied were isolated from dry food environments, however most of them were found to be motile. The strain which did not show any swim ability, S. Typhimurium isolated from whey powder, was also most sensitive to desiccation. The other strain sensitive to desiccation, S. Typhimurium 'DS', showed the smallest growth zone which was 3.2 cm after 8 hours. This suggests that desiccation survival ability might be controlled by the same mechanisms as swimming ability. When analysing swarming abilities, it was shown that most of the strains which had strong or medium sensitivity to desiccation did not show strong swarming abilities after 8 hours (*S*. Typhimurium isolated from whey powder, *S*. Typhimurium DS, *S*. Cubana 2, *S*. Mbandaka 1 and 2); however one resistant strain (*S*. Tennessee) did not show swarming abilities. The results suggest that desiccation survival ability might be associated with swimming ability and in most cases, swarming ability. It could be because cells are moving in groups (Butler *et al.*, 2009), therefore create higher density which might be beneficial for survival. Additionally, the bacteria might avoid hash condition of desiccation by moving from the top of the droplet to the bottom layers, therefore dead cells could potentially protect living cells. The studies also suggests that most of the strains which can survive in the factory environment during desiccation, can also disseminate quickly when water is introduced to the environment and they may also have a higher chance of infection (Josenhans and Suerbaum, 2002). It is important therefore to prevent water access to the dry food factories environments and control *Salmonella* survival.

It was also necessary to investigate strains for auxotrophic mutation. Auxotrophs lack one or more enzymatic step in one or several biosynthetic pathways (Boeke *et al*., 1984). When placed on minimal media, they are unable to grow because all molecules of biomass (amino acids, nucleotides, enzyme cofactors, phospholipids, etc.) cannot be synthesized using C, N, P, and S from a single organic compound and inorganic ions (Pronk, 2002). Most natural isolates of *Salmonella* are prototrophic, however it had to be verified that all the studied strains were also

prototrophic, therefore their sensitivity to desiccation and further gene expression analysis is not affected by this mutation. Recent studies on transcriptomics of *S.* Typhimurium SL1344 during desiccation published by Gruzdev *et al*. (2012) give detailed insight into gene expression during desiccation, however the strain chosen for the studies is an auxotrophic mutant (Finn *et al*., 2013a) therefore it is not reflecting general responses to desiccation of *Salmonella*. The strains chosen in this work grew well on minimal media, therefore they can synthesise all the components needed for growth and they could be used for further work.

3.4 CONCLUSIONS

Essentially, with respect to the manufacturers of low-moisture foods, this study has shown that *Salmonella* strains survived for at least 30 days under desiccation conditions on stainless steel and potentially could survive much longer, which was shown with *S.* Typhimurium HR example and its 1 year survival. It is also concluded that some phenotypic characteristics which were suggested to be associated with desiccation resistance are not necessarily related to increased desiccation survival. This work also provides a validated method to study death kinetics of desiccated *Salmonella* cells and enable selection of the best strains to conduct any research with attached and dried cells of this pathogen. In addition, the results give an insight into the survival time and death kinetics for *Salmonella* in dry processing environments, which can be used to choose appropriate timeframes for further gene expression studies.

The results suggest that once this pathogen is introduced to the factory environment, there is a serious risk of its survival. Good control measures are necessary, therefore, in dry food factory processing environments, to avoid the entry of *Salmonella* and to prevent any cross-contamination from such factory environments to the food product.

A greater understanding of the ability of *Salmonella* strains to survive in the environment is now necessary to control this pathogen effectively. Further chapters will focus on investigations of different factory environmental conditions and practices (external factors) and their influence on desiccation resistance and an examination of *Salmonella's* molecular responses to desiccation.

CHAPTER 4
RESISTANCE OF DESICCATED CELLS TO DIFFERENT ENVIRONMENTAL CONDITIONS

4.1 INTRODUCTION

The results in Chapter 3 showed that different strains of *Salmonella* can persist on stainless steel surfaces for up to a year. The rate of decline was strain related, however there may be other factors contributing to different survival rates. Potentially, survival could be dependent on environmental conditions and gaining a detailed knowledge of the relationship between the number of surviving cells and conditions such as: temperature, nutrient level, water supply, relative humidity level etc. would be very beneficial for the dry food industry. This knowledge could also help to investigate the mechanisms responsible for survival and which could establish better control mechanisms within factory environments.

The literature also reveals evidence of long term persistence of *Salmonella* in factory environments. Nesse *et al*. (2003) showed that fish feed factories harboured some *S*. enterica clones which persisted for at least 3 years in the factories, most likely, due to inadequate decontamination procedures. In studies conducted by Davis and Wray (1997), samples from animal feed milling equipment taken in nine animal feed mills gave *Salmonella* isolation rates which ranged from 1.1 % to 41.7 %. The most frequently contaminated sampling sites were intake pits, where lots of raw materials and dust were present, and the cooler area where the moisture was often introduced. Observations undertaken in a poultry house suggested that *Salmonella* could survive for a 30-month period, despite the fact that the environment of the poultry house was exposed to detergents, disinfectants and subsequent desiccation (Broennum Pedersen *et al*., 2008).

Salmonella, therefore, can survive on factory surfaces for months (Alvarez, 2003; Kramer *et al*., 2006) and it has been shown that *S.* Typhimurium can even survive in samples of dust on school floors for a period of greater than 50 months (Robertson, 1972).

Additionally, in the dry food industry, removing all food residues from all factory surfaces is often not feasible, therefore the production environment is rich in nutrients (Moretro, 2012) which could potentially be available for microorganisms

to grow, if water is not available, or could protect the cells from other stresses. The importance of the influence of these food residues on the survival of *Salmonella* must, therefore, be investigated.

The challenge however is to use laboratory conditions which can reflect the real production environments. This will be simulated in this chapter by using food debris, water and range of temperature and humidity levels; however the method of inoculation is also crucial. Studies are often criticized when using 'unrealistically' high inoculum levels, partially because often less survival of the pathogen is observed in the case of lower inoculum levels (Richert *et al*., 2000). This suggests that the behaviour of pathogens at high contamination levels could be different from their behaviour at low contamination levels. Additionally, the way bacterial suspensions are deposited onto a surface could potentially influence their survival rate. Depending on the droplet size and the evaporation process, the cells can be arranged in concentrated areas (the outer circumference of the droplet as it dries on the surfaces) or randomly. The evaporation of small amounts of liquid such as droplets and thin films with suspended particles is an often studied phenomenon in physics (Winnik and Feng, 1996; Routh and Russel, 1998; Deegan, 2000a). Daily examples are the formation of coffee stain, or the drying of water colour paints, where a higher concentration of particles are observed on the edge of the droplet.

Conditions in different dry food factories may vary and some of them may favour the survival of *Salmonella* whilst others could make it more challenging. The objectives of the studies reported in this chapter were to characterise the environmental conditions in dry food factories, then test these conditions with *Salmonella* cells in a laboratory settings. Additionally, the droplet drying mechanisms of a suspension of *Salmonella* were studied to investigate if the way a surface is artificially contaminated with droplets of *Salmonella* suspension could affect their survival abilities. The cells were also dried on surfaces and observed under SEM directly after drying and 30 days after initial drying to compare the morphology of desiccated cells.

4.2 RESULTS

4.2.1 Factory Visits

A number of visits to dry foods manufacturers were undertaken to establish the environmental conditions in the factories and investigate their food

manufacturing practices (Section §2.2.1). A questionnaire was developed and used to establish a knowledge base for further studies (see Appendix C).

Generally, low water activity food production was considered as a dry operation and water was only used if wet cleaning was absolutely required. Water was usually controlled in the dry parts of processing plants to inhibit the growth of microorganisms, especially because dry food processing facilities and processing equipment are not designed to handle water without a risk of creating microbial niches. It was very challenging to control the unintentional introduction of water into the environment from uncontrolled sources (e.g., waterline leaks and condensation). If wet cleaning was required the use of water was minimised. Water was applied to small areas at a time and immediate and complete drying was usually ensured. Dry food plants could run for a very long time without stopping operations and undertaking a wet clean. Occasionally there were special circumstances, such as finding environmental sites positive for *Salmonella*, which required whole site decontamination. In this case equipment not primarily designed for wet cleaning had to be wet cleaned. Extreme care was taken to understand the risks and to formulate a plan that could successfully eliminate the contamination without spreading and enhancing the problem. All of the visited factories had issues with finding *Salmonella* positive samples in the factory environment.

The humidity in the finished product processing environment was usually low (~30 %) during production activities, however it was largely dependent on the humidity of the outside environment. The temperature depended on the process parameters and the weather conditions of the surrounding area. Some details related to specific manufacturing plants are summarised in the following sections.

4.2.1.1 Chocolate Manufacturing Plant

The plant visited was out of the production mode and was subjected to manual cleaning activities. The typical processing length between dry cleaning lasted approximately 6 weeks, which is the incubation period for *Ephestia* larvae. This insect is one of the most damaging insects in the chocolate industry. Only some parts of the processing line were subjected to out-of-place wet cleaning e.g., moulds, trays, utensils etc. Out-of-place cleaning is an alternative to CIP cleaning which is using small amount of water for cleaning which is done in separate areas, outside of the production zone. The rest was dry cleaned only or not cleaned at all. There were two

general guiding principles that the producers tried to address in the chocolate production facility:

- Physical separation of areas of the plant that processed raw cocoa beans and the areas of the plant post bean decontamination that processed the resulting chocolate. This means that any area of the plant after roasting had to be protected from any cross contamination, by air or any other means, from the raw bean areas of the plant;
- The chocolate processing area of the plant had to be continuously monitored by environmental and finished product microbiological testing and the use of water or water contamination of the environment was strictly avoided.

Chocolate splashes were seen on the factory walls and ceiling. The production was not running, therefore environmental temperatures were lower than the usual 18°C to 22°C. The humidity was not checked. The food contact materials used in production were mostly stainless steel and the material of the conveyor belt was PVC. Temperers were made of cast metal rather than stainless steel. In the chocolate processing facilities, the decontamination step of the cocoa beans was undertaken at the start of the processing (de-bacterisation step, roasting), therefore after that, there were many opportunities for cross contamination from the environment.

4.2.1.2 Breakfast Cereals Factories

Similar observations were made as for the chocolate manufacturing plant. The production should be kept dry however the factories did not separate the wet cleaning activities from the production area, therefore aerosols could be transferred to ready-to-eat product and surrounding surfaces. There was no air filtration system and the temperature and humidity changed depending on the weather conditions. The breakfast cereals were baked, however additional processed ingredients (though not necessarily pasteurised) e.g. raisins, seeds etc. were added after baking. The lines were open and therefore more exposed to the external environment.

4.2.1.3 Milk Powder Factories

Some of the factories did not have segregation between raw and finished product (unpasteurised liquid milk and pasteurised products), therefore there was a risk of cross-contamination with pathogens. The equipment which was handling milk powder was mainly cleaned-in-place (CIP) with dry cleaning implemented only

in the filling and packing rooms. Equipment was enclosed and CIP cleaned from milk pasteurisation to spray drying and the fluidised bed. In some circumstances, such as cleaning the magnets and sieves or removing the plug-ups, the equipment was opened by the operatives which could potentially introduce contamination from the factory environment. Some of the openings and powder filling areas were classified as higher hygiene control zones. Water was not completely eliminated from the environment and it was present on the floors. The temperature in the filling area was approximately 20ºC with relative humidity of 30 %. The other areas, where dried powder was handled, were very warm, up to 40ºC. The surfaces in the packing area had a high amounts of milk powder present.

4.2.1.4 Wheat Germ Factory

The factory tried to keep a dry environment policy, however parts of the equipment were cleaned using steam, which introduced moisture to the environment.

4.2.1.5 Dry Pet Food Factory

Some of the areas of the factory were not completely dry (e.g. next to the conditioner where water is added to the dry raw material) and this moisture could be transferred to further processing areas and become a source of contamination. The air was not filtered.

Most of the dry food processing plants visited experienced occasional problems with environmental *Salmonella* contamination. The most difficult part for the food manufacturers was to choose the best method of product decontamination and to then avoid post-process re-contamination. The observations were made based on over 10 factories visited in eight different countries.

4.2.2 Droplet Drying Mechanism

A droplet drying mechanism was investigated using a LiveDead dye (Section 2.4.6) and Nikon phase contrast microscope (Section 2.4.7) to check if cells accumulate and potentially create a multilayer of microorganisms on the edge of a droplet. In order to investigate the movement and location of the cells within a dried droplet, *Salmonella* Typhimurium `HR` cells were prepared and stained as described in Section 2.4.6. The stain mixed with inoculum was not centrifuged or washed before inoculation. After inoculating 50 µl droplets on surfaces and drying for 80 minutes at 37°C±1°C, they were observed under a phase contrast microscope under x100 magnification.

Most of the particles were accumulating on the edge of a droplet (results not shown). It was not clear however if these were the residues of the dye itself or microbial cells. It was, therefore, further investigated to analyse if the bacterial cells were accumulating on the edge or whether the rings were dye residues only. Cells were stained as described previously and centrifuged for 5 minutes at 8000 x *g* and re-suspended in 10 ml of MRD. 50 µl of bacterial suspension was then inoculated on each disc which were then placed into a 37°C±1°C incubator for drying.

The cells were observed under phase contrast (400 fold magnification) microscope at different drying time points: after 30, 40 and 60 minutes of drying (see Figure 4.1). It was difficult to observe droplets at the beginning of the drying stage (before 30 minutes of drying) as the surface tension of the droplet was too high which made it impossible to fit it underneath the microscope objective. The concentration of bacterial suspension inoculated was 4.2 x 10^8 cfu surface^{-1}.

The thickness of the rim of a droplet increased over the time of drying, tripling in size between 30 and 60 minutes. The colour of the droplet may vary depending on the time the sample is exposed to light. A video was also recorded of particles 'floating' towards the edge of a droplet during the drying period (see Appendix F).

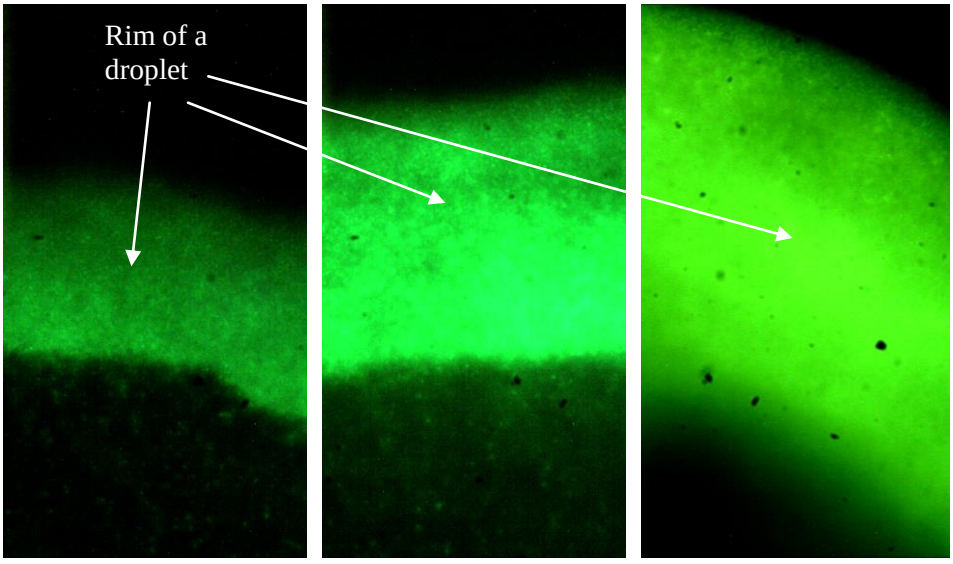


Figure 4.1 The rim of the edge of the S. Typhimurium 'HR' suspension (10^7 cfu disc^{-1}) droplet stained with LiveDead stain, observed during drying under 400x magnification with Nikon phase contrast microscope and UV lamp: a) 30 minutes, b) 40 minutes, c) 60 minutes of drying

4.2.3 Resistance of Desiccated Cells to Different Environmental Conditions

Following the studies described in Chapter 3, four *Salmonella* strains were selected to investigate the influence of environmental conditions on *Salmonella* survival. These included two of the strains which showed one of the highest survival rates: *S*. Typhimurium NCTC 12023 (high heat resistant; HR) and *S*. Muenchen isolated from a cocoa bean environment. The rationale for choosing these strains was to check if the resistance could be lowered by changing environmental conditions. The other two strains which were chosen showed medium resistance to desiccation: *S*. Cubana (2) and *S*. Mbandaka (1), respectively and they were both isolated from cereal production environments. They were chosen to see if resistance could be increased dependent on the environmental conditions.

A series of experiments was conducted to analyse the survival rate of the four strains under different conditions. The standard conditions were as follows: temperature 25°C±1°C, relative humidity 33%, inoculum level ~10^7 cfu ml^{-1}, absence of food debris and absence of water. The following variables of these conditions were used; however only one variable was changed during each experiment, the rest always stayed the same as the standard:

- The relative humidity levels of 33% and 53%, represent the lower and higher humidity range which may be seen in factories producing low a_w food products (personal observations from the factory visits described in Section 4.1, for method see Section 2.1.1).
- The temperatures 15°C±1°C, 25°C±1°C and 37°C±1°C represent a temperature range which may be seen in dry food production (personal observations from the factory visits described in Section 4.1).
- Addition of concentrated milk chocolate powder and infant formula powder to investigate the effect of high levels of protective material and nutrients on potential general and specific protective survival mechanisms (Section 2.2.6).
- Addition of diluted skimmed milk powder and cocoa powder to investigate the effect of low levels of protective material and minimal levels of nutrients on potential general protective survival mechanisms (Section 2.2.6).
- Periodic wetting with sterile distilled water every seven days, which simulated periodic wet cleaning in the factory environment (Section 2.2.7).

- A range of inoculum levels: 10^4, 10^5 and 10^7 cfu ml^{-1}, to investigate the possibility of dead cells protecting living cells.

A 50 µl volume of each bacterial suspension (section 2.2.2) at different starting levels (10^4, 10^5 and 10^7 cfu ml^{-1}) or containing different food debris was deposited onto stainless steel discs. Discs were placed in uncovered Petri dishes and transferred to an incubator to dry at 30ºC±1°C for 80 minutes. After drying, Petri dishes were covered and placed in controlled transparent plastic boxes (Section 2.1.11). The samples which were designed to investigate the influence of water access to the cells were wetted every seven days. The number of microorganisms surviving on the stainless steel surfaces was assessed at the following time points after drying: 0 hours, 2 days, 10 days and 30 days. For detailed methods see chapter 2 (Sections 2.2.2-2.2.5).

In Figures 4.2-4.5, counts at time 0 indicate microbial numbers at the time immediately after 80 minutes drying at 30°C±1°C.

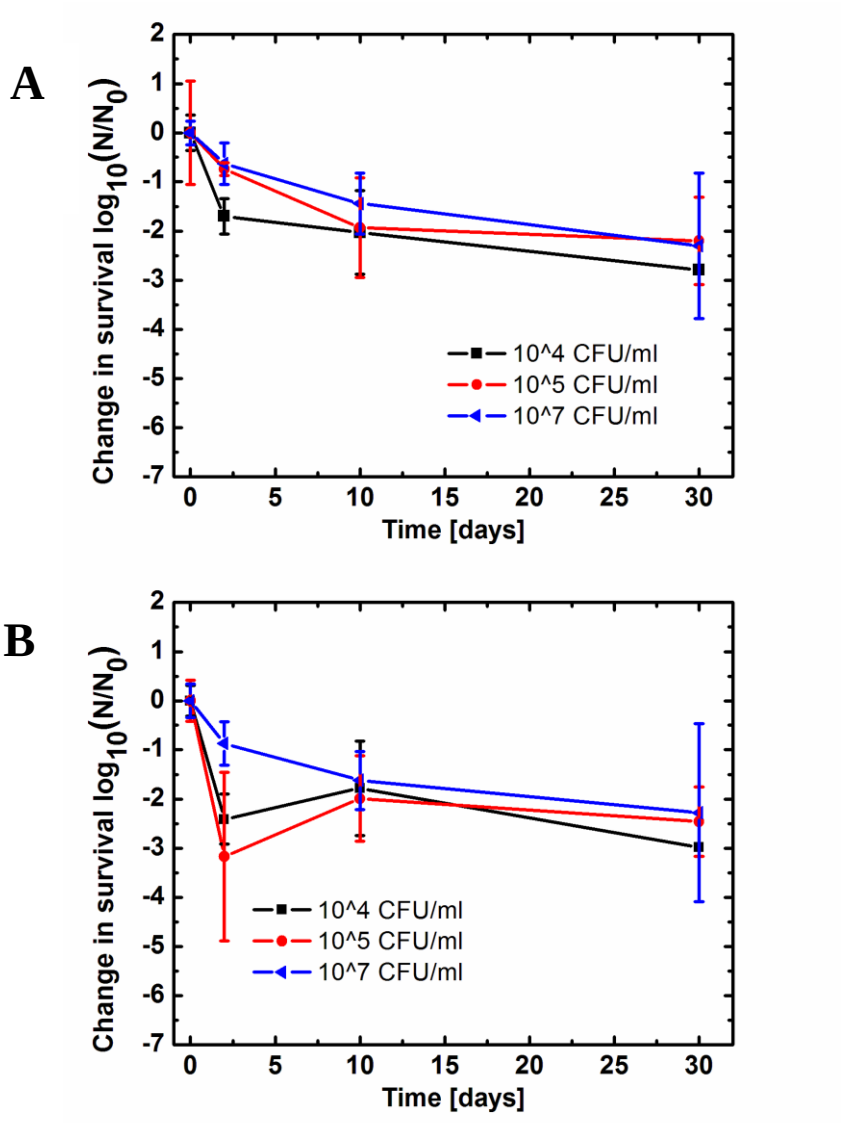
The average log cfu $surface^{-1}$ for discs inoculated with a range of bacterial suspension levels and held under standard humidity and temperature conditions for up to 30 days is shown in Figure 4.2. The initial drying (80 minutes) resulted in a nearly 1 log cfu $surface^{-1}$ reduction for all of the inocula, therefore before the discs were placed in the controlled environment the average microorganism levels were ~10^6, ~10^4, ~10^3 cfu $surface^{-1}$, for the 10^7, 10^5 and 10^4 inocula, respectively. There was a relatively high rate of bacterial death at the beginning of the survival curves for all isolates and a decrease in the death rate after 2 days. This was also observed in the previous trials described in Chapter 3. For the samples inoculated with ~10^7 cfu ml^{-1} suspension, the level of bacteria reduction occurring during desiccation conditions for 30 days, was similar ($p=0.872$) for all 4 serotypes and it was approximately 2.23 log cfu $surface^{-1}$.

The surfaces inoculated with the ~10^5 cfu ml^{-1} suspension had average microbial reduction levels, after 30 days, ranging from 2.20 log cfu $surface^{-1}$ (*S.* Cubana 2) to 3.02 log cfu $surface^{-1}$ (*S.* Muenchen). There was no statistical difference between the four serotypes ($p=0.456$). The inocula of ~10^4 cfu ml^{-1} were reduced to levels below detection (<0.7 log cfu $surface^{-1}$) for all serotypes. Overall, there was a similar reduction level for all inocula used in the experiment ($p>0.526$).

The temperature dependent survival of four *Salmonella* strains kept at 33 % RH for 30 days is shown in Figure 4.3. Overall there was no difference in the

survival rate between serotypes, regardless of the temperature that they were held at: 15°C±1°C (p=0.906), 25°C±1°C (p=0.872) and 37°C±1°C (p=0.416). However, differences were observed between samples held at different temperatures (p<0.01).

The lowest reduction after 30 days was observed for strains held at 15°C±1°C and ranged from 1.51 log cfu surface^{-1} (*S.* Typhimurium 'HR') to 1.98 log cfu surface^{-1} (*S.* Muenchen). The results gained from testing discs held at 25°C±1°C were not statistically different from those held at 15°C±1°C and they ranged from 1.76 log cfu surface^{-1} (*S.* Mbandaka 1) to 2.3 log cfu surface^{-1} for (*S.* Cubana 2). The samples kept at 37°C±1°C had a statistically significant difference from the rest of the samples (p<0.01), regardless of the serotype. The microorganism reduction ranged from 3.62 log cfu surface^{-1} to 4.16 log cfu surface^{-1} for *S.* Cubana '2' and *S.* Mbandaka '1', respectively.



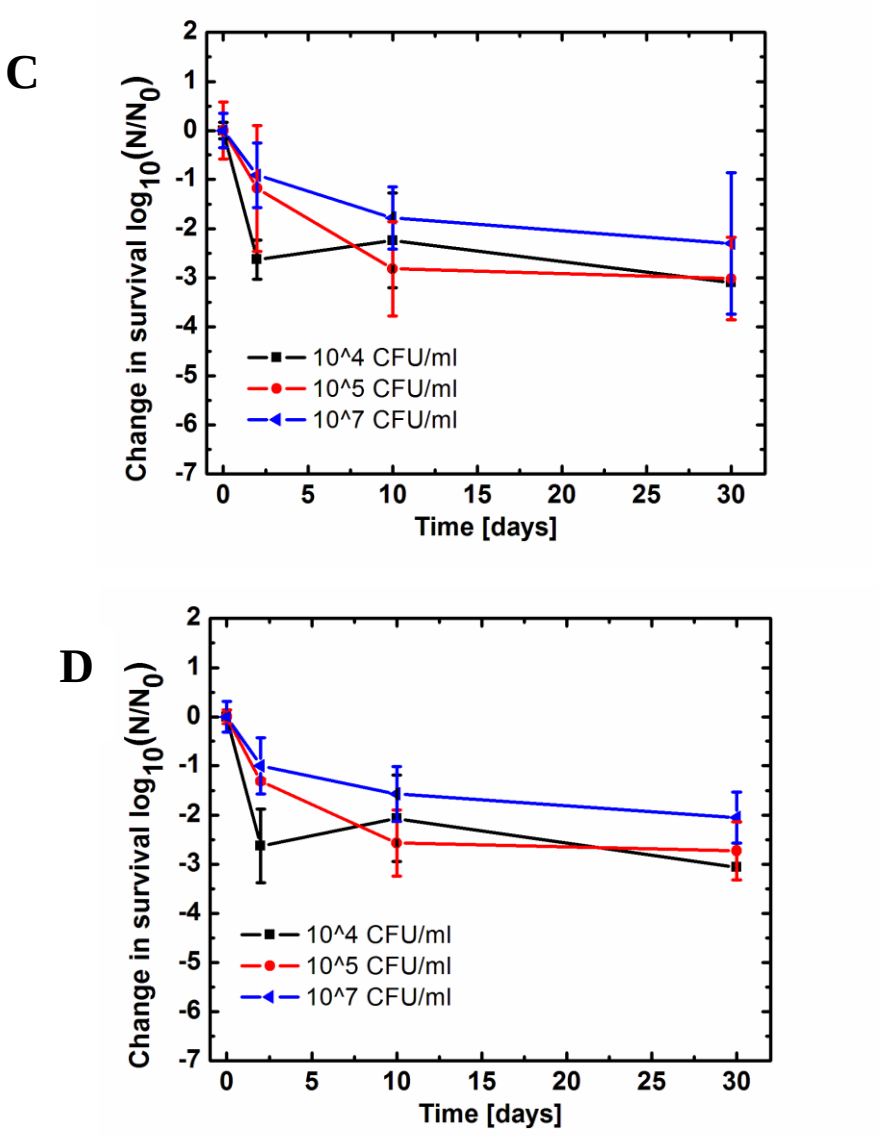
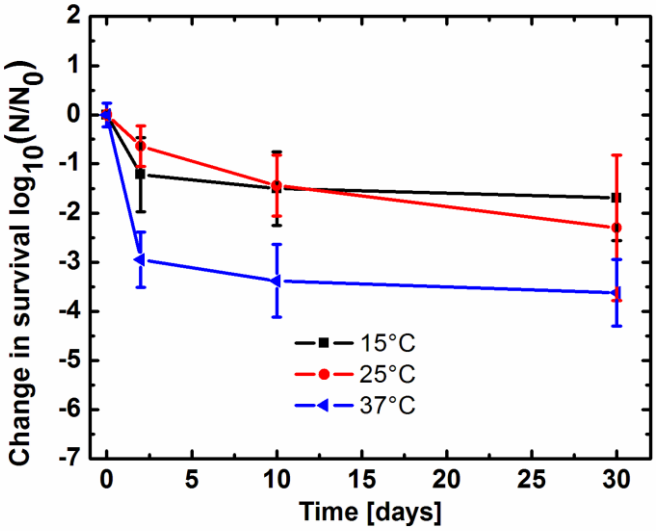
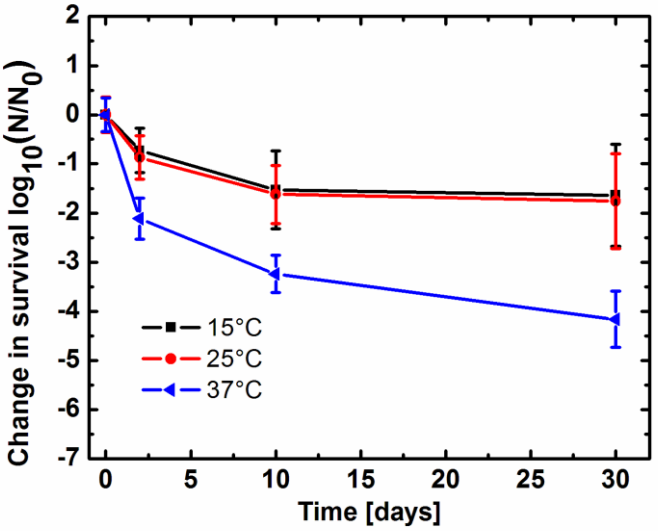


Figure 4.2 Survival of *Salmonella* suspended in MRD, inoculated onto stainless steel discs with a range of inocula levels (~10^4, ~10^5, ~10^7cfu ml^{-1}), dried and kept at 33 % RH and 25ºC±1°C for up to 30 days. Bars represent one standard deviation A) S. Cubana 2 (cereals environment); B) S. Mbandaka 1 (cereals environment); C) S. Muenchen; D) S. Typhimurium 'HR' NCTC 12023

A


Change in survival log10(N/N0)
Time [days]
15°C
25°C
37°C

B


Change in survival log10(N/N0)
Time [days]
15°C
25°C
37°C

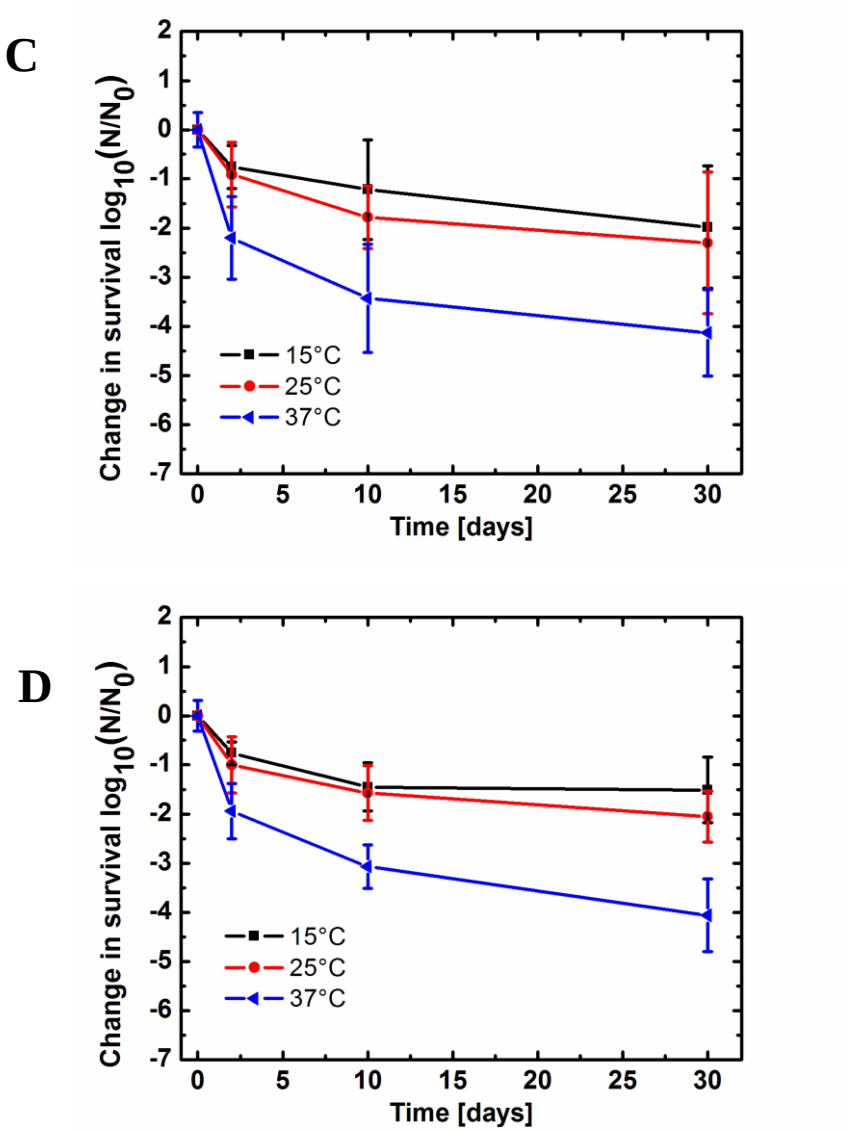
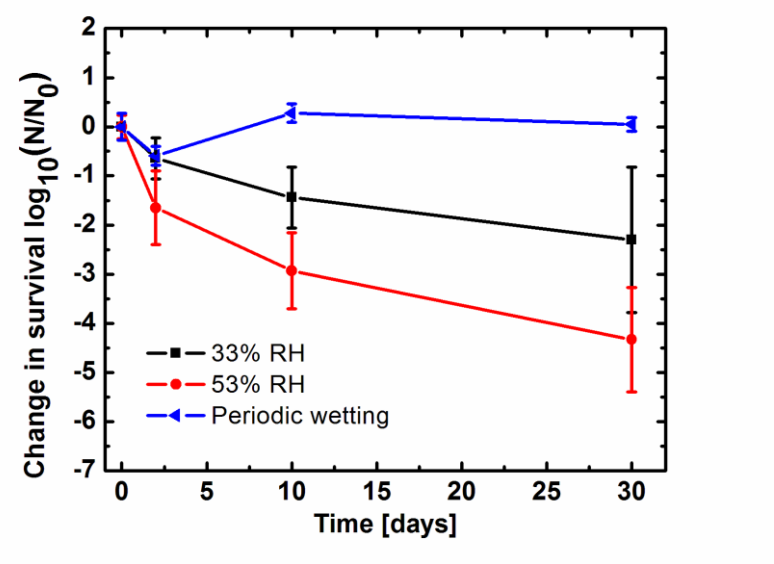
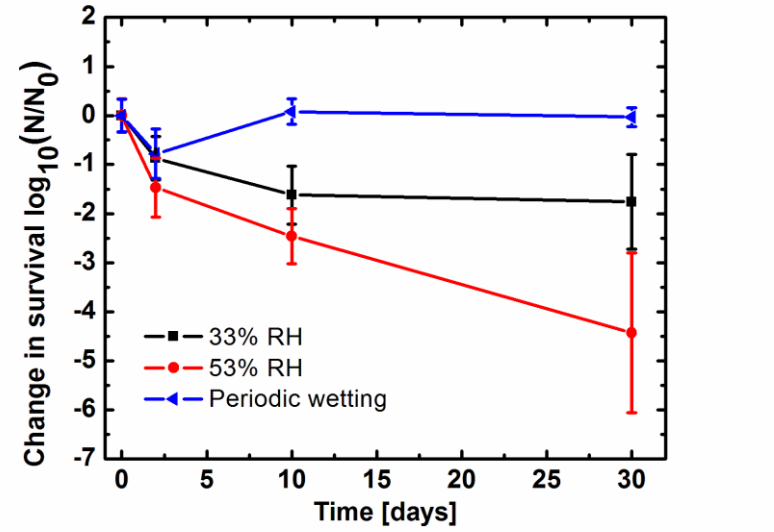


Figure 4.3. Survival of 4 strains of *Salmonella* suspended in MRD, inoculated (~10^7 cfu ml^{-1}) onto stainless steel discs, dried and kept at 33 % RH and range of temperatures: 15ºC±1°C, 25ºC±1°C and 37ºC±1°C for up to 30 days. Bars represent one standard deviation. A) S. Cubana 2 (cereals environment); B) S. Mbandaka 1 (cereals environment); C) S. Muenchen; D) S. Typhimurium 'HR' NCTC 12023

A


Change in survival $\log_{10}(N/N_0)$
Time [days]
33% RH
53% RH
Periodic wetting

B


Change in survival $\log_{10}(N/N_0)$
Time [days]
33% RH
53% RH
Periodic wetting

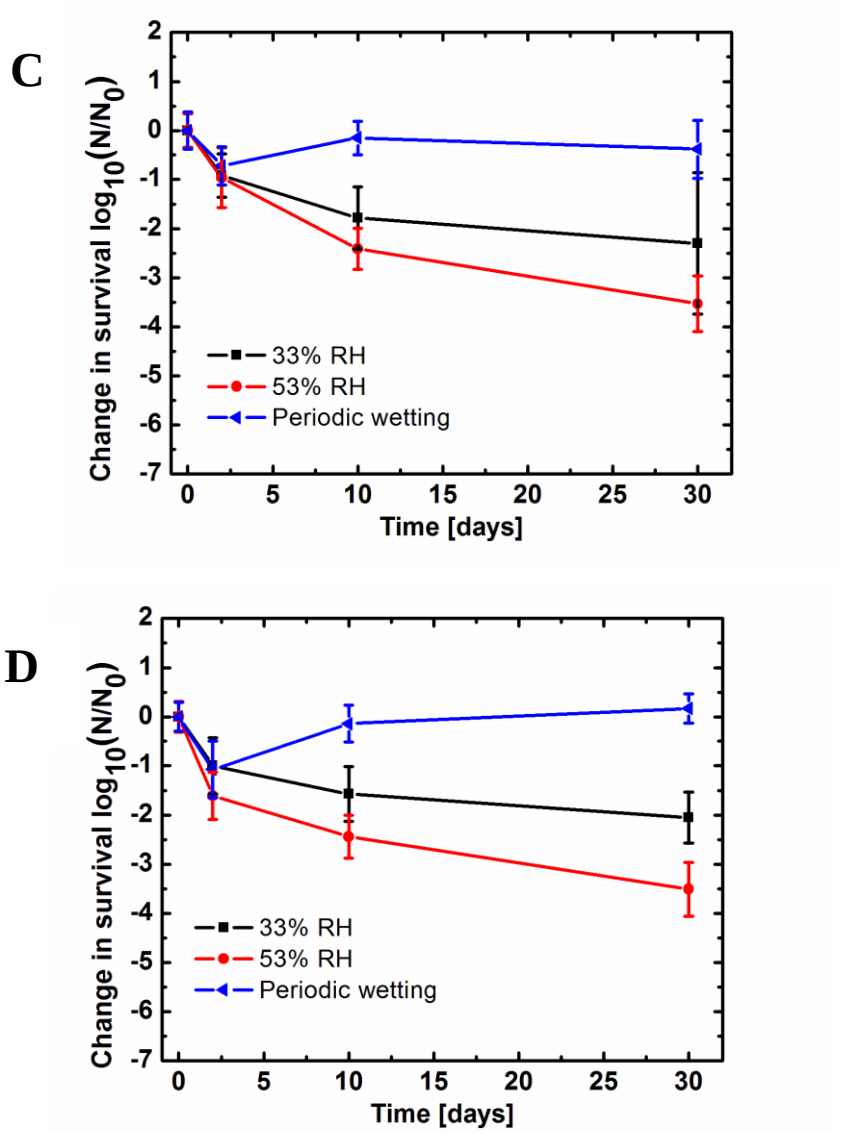
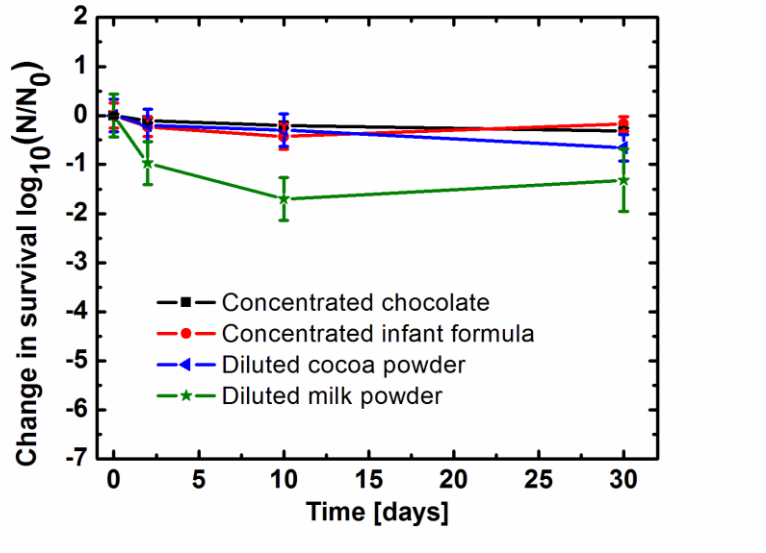
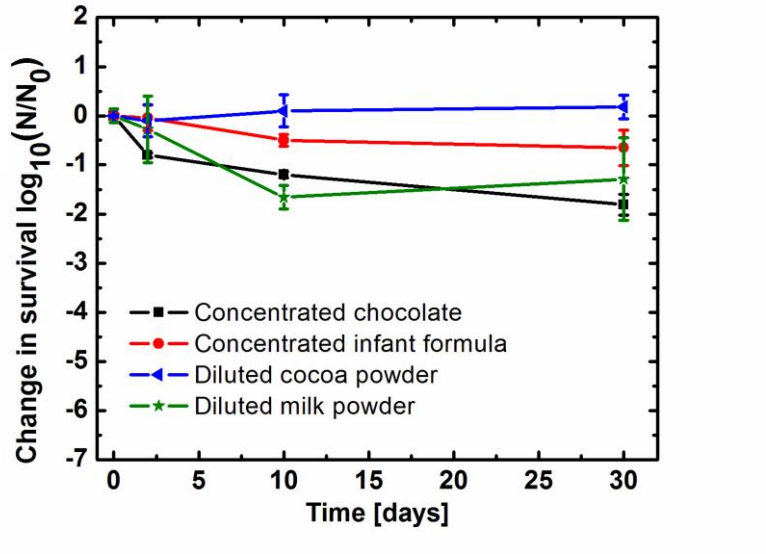


Figure 4.4. Survival of 4 strains of *Salmonella* suspended in MRD, inoculated (~10^7cfu ml^{-1}) onto stainless steel discs, dried and kept at a range of humidity levels: 33 % (with and without periodic wetting) and 53 % and a temperature of 25ºC±1°C for up to 30 days. Bars represent one standard deviation. A) *S.* Cubana 2 (cereals environment); B) *S.* Mbandaka 1 (cereals environment); C) *S.* Muenchen; D) *S.* Typhimurium 'HR' NCTC 12023

A


Change in survival $\log_{10}(N/N_0)$
Time [days]
Concentrated chocolate
Concentrated infant formula
Diluted cocoa powder
Diluted milk powder

B


Change in survival $\log_{10}(N/N_0)$
Time [days]
Concentrated chocolate
Concentrated infant formula
Diluted cocoa powder
Diluted milk powder

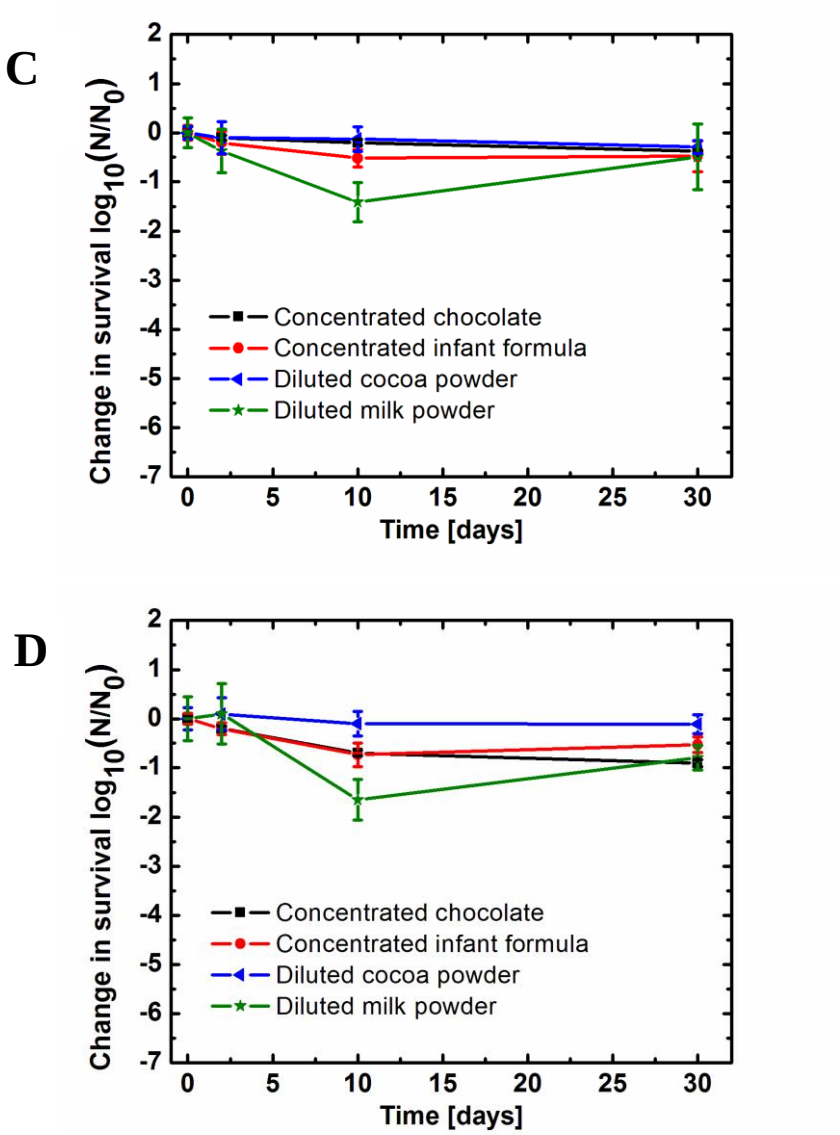


Figure 4.5 Survival of *Salmonella* suspended in MRD with addition of: concentrated chocolate powder, concentrated infant formula powder, diluted cocoa powder and diluted milk powder, inoculated (~10^7cfu ml^{-1}) onto stainless steel, dried and kept at 33 % RH and 25ºC±1°C for 30 days. Bars represent one standard deviation. A) S. Cubana2 (cereals environment); B) *S*. Mbandaka1 (cereals environment); C) *S*. Muenchen; D) *S*. Typhimurium 'HR' NCTC 12023

The effect of humidity on the survival of the four *Salmonella* strains is shown in Figure 4.4. Two different humidity levels were used: 33 % and 53 %. Additionally, some discs kept at 33 % humidity were wetted every 7 days, starting from the seventh day after inoculation. Overall there was no difference in the survival rate between all serotypes under each humidity condition: 33 % ($p=0.872$), 53 % ($p=0.119$) and periodic wetting ($p=0.341$). However, there were differences observed between survival of all bacterial strains held in different humidity conditions ($p<0.001$ for all strains). The highest survival rate was associated with strains which were frequently wetted and the numbers ranged from being 0.17 log cfu surface^{-1} higher than inoculum (*S.* Typhimurium 'HR'); to 0.38 log cfu surface^{-1} lower than the inoculum level (*S.* Muenchen).

The next level of survival was represented by samples held at 33 % humidity and the reduction ranged from 1.76 log cfu surface^{-1} (*S.* Mbandaka 1) to 2.30 log cfu surface^{-1} (*S.* Cubana 2). As humidity increased to 53 %, the reduction rate increased. It ranged from 3.51 log cfu surface^{-1} to 4.43 log cfu surface^{-1} with minimum and maximum values obtained from *S.* Typhimurium HR and *S.* Mbandaka 1 analysis, respectively.

Figure 4.5 displays *Salmonella* levels on surfaces kept for 30 days with the addition of different, powdered food debris. When performing ANOVA analysis between serotypes with each food type addition, the results for diluted milk powder and concentrated infant formula showed no difference between serotypes ($p=0.247$ and $p=0.608$, respectively). The survival of *Salmonella* with the addition of diluted cocoa powder was significantly higher ($p<0.001$) for *S.* Mbandaka 1, compared to the rest of the serotypes. There was also a significant difference between serotypes survival when the concentrated chocolate powder was added to the suspensions ($p<0.001$).

The results for *S.* Muenchen desiccated cells, after 30 days, displayed the best survival rate with the addition of diluted cocoa powder then with concentrated chocolate powder, concentrated infant formula, and diluted milk powder, with log cfu surface^{-1} reductions of 0.29, 0.37, 0.48 and 0.49, respectively. There was no difference in the survival rate between both concentrated products ($p=0.267$) and both diluted products ($p=0.834$). *S.* Cubana 2 cells survived the best when concentrated infant formula powder was added (0.17 log cfu surface^{-1} reduction).

The addition of diluted milk powder had a significantly lower effect (1.32 log cfu surface^{-1} reduction).

When analysing the survival rate of *S.* Mbandaka 1 cells, it can be concluded that the results differ from the results achieved with the other serotypes, when the diluted cocoa powder was added. This food product enhanced the survival rate and, after 30 days, the level of microorganisms was 0.18 log cfu surface^{-1} higher. A slight reduction was observed when concentrated infant formula was added (0.65 log cfu surface^{-1}), a higher reduction observed when diluted milk powder (1.29 log cfu surface^{-1} reduction) and concentrated chocolate powder (1.81 log cfu surface^{-1} reduction) was used.

S. Typhimurium HR had the lowest reduction rate, after 30 days, when diluted cocoa powder was added to the bacterial suspension (0.11 log cfu surface^{-1}). Addition of concentrated infant formula powder also gave low log cfu surface^{-1} reduction, which on average was 0.53.

4.2.4 SEM Analysis

The same four cultures were used as described in Section 4.2.3 to conduct SEM analysis of cells. Desiccated cells were observed under the Scanning Electron Microscope. Two types of cells were examined: cells freshly dried on the surfaces and cells desiccated for 30 days under 25ºC±1°C temperature and 33 % RH condition.

The bacteria were grown and test suspensions were prepared as described in Section 2.2.2. The discs were inoculated as previously described and dried in a 37°C±1°C incubator for 80 minutes. Some of the discs were examined directly using SEM (Section 2.4.4), whilst the others were left for 30 days in desiccating conditions and then examined by SEM. Ten images were taken of each strain's cells at single condition. The images were chosen randomly.

Figures 4.6 - 4.9 show the imagines obtained from each examined isolate. Stainless steel surfaces without inoculum are also shown to demonstrate there was no surface coverage before inoculation was conducted (Figure 4.10). All strains seem to have more additional substances covering the cells after 30 days of desiccation as compared with cells dried only for 80 minutes. The cells also appear to be more rounded and smaller when they are exposed for longer time to the dry conditions. The length of the cells on the first day of desiccation is approximately 1.5 µm and after 30 days of desiccation approximately 0.7 – 1 µm.

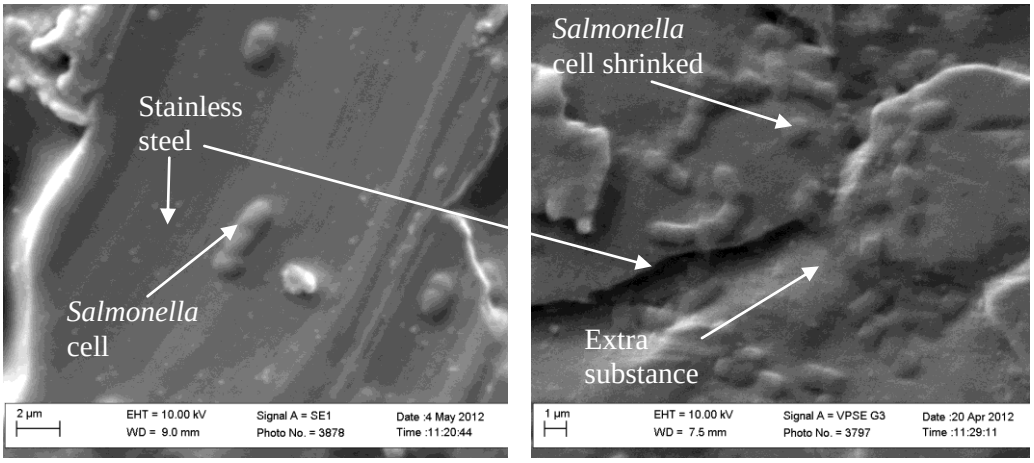


Figure 4.6 SEM of *S*. Cubana '2' suspended in MRD, inoculated (~10^7cfu ml^{-1}) onto stainless steel, dried and kept at 33 % RH and 25ºC±1°C for 30 days, A) immediately after drying; B) 30 days after drying. Discs were silver coated.

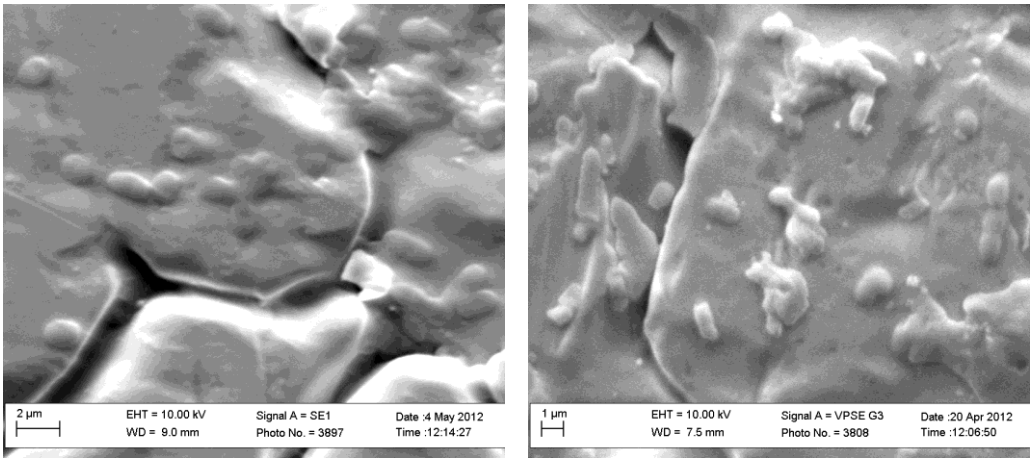


Figure 4.7 SEM of *S*. Muenchen suspended in MRD, inoculated (~10^7cfu ml^{-1}) onto stainless steel, dried and kept at 33 % RH and 25ºC±1°C for 30 days, A) immediately after drying; B) 30 days after drying. Discs were silver coated.

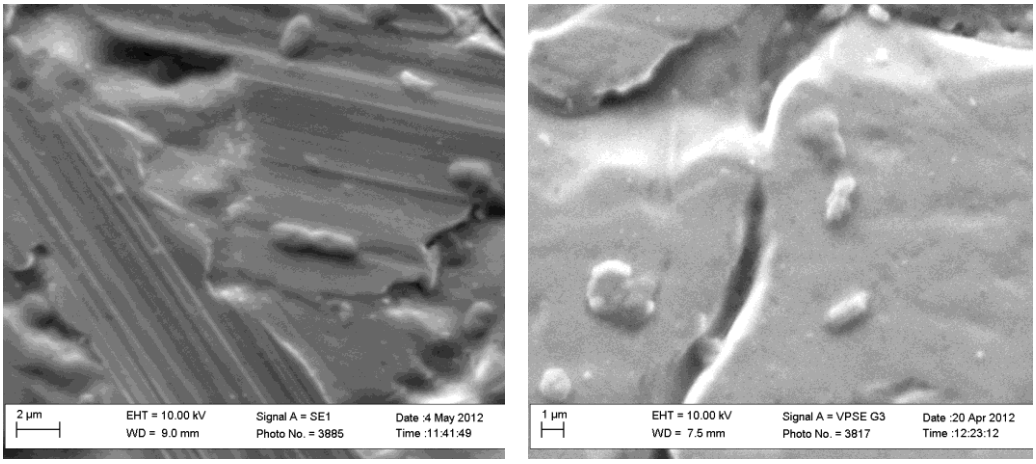


Figure 4.8 SEM of *S*. Mbandaka '1' suspended in MRD, inoculated (~10^7cfu ml^{-1}) onto stainless steel, dried and kept at 33 % RH and 25ºC±1°C for 30 days, A) immediately after drying; B) 30 days after drying. Discs were silver coated.

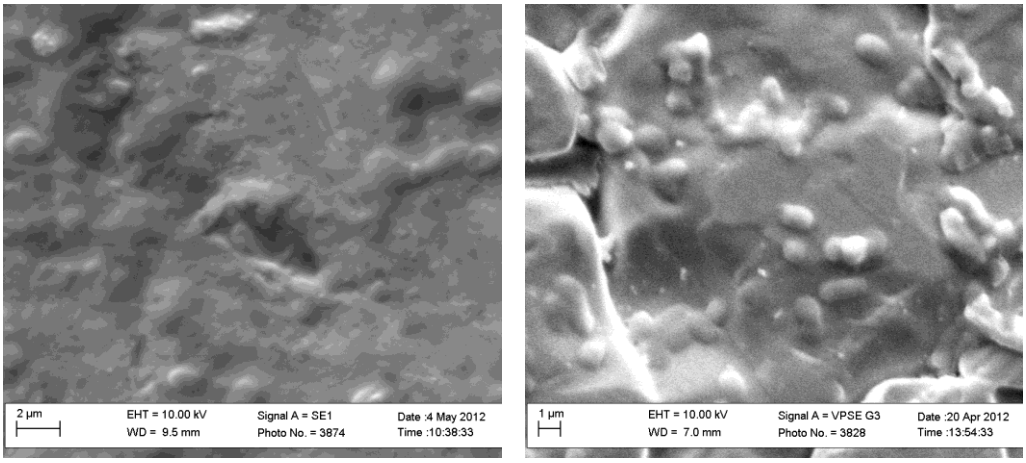


Figure 4.9. SEM of *S*. Typhimurium NCTC 12023 suspended in MRD, inoculated (~10^7cfu ml^{-1}) onto stainless steel, dried and kept at 33 % RH and 25ºC±1°C for 30 days, A) immediately after drying; B) 30 days after drying. Discs were silver coated.

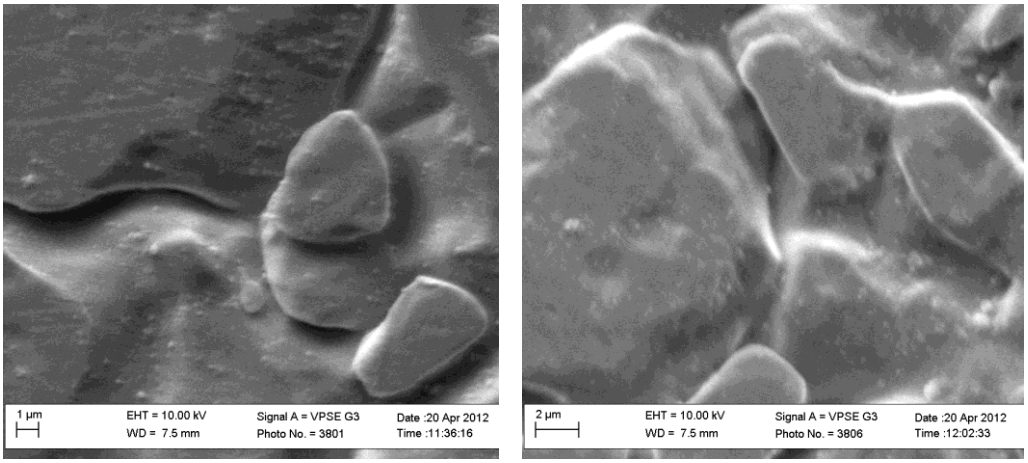


Figure 4.10 SEM of stainless steel surfaces without inoculation

4.3 DISCUSSION

4.3.1 Droplet Drying Mechanism

The results indicated that the cells present in a suspension are drawn to the edges of the droplets during drying. This confirms literature findings where one of the droplet evaporation mechanisms is described as having a larger evaporation at the edge which causes a capillary flow resulting in depositing particles in a ring structure (Deegan *et al*., 2000b). Nagayama (1995) describes this flow towards the boundary in liquid films as a convective flow. This phenomenon is called the coffee ring effect and it accrues sessile droplets pinned to the solid surface (see Figure 4.11, Mampallil, 2014). In this mechanism it is also important that the solvent meets the

surface at a non-zero contact angle and the solvent evaporates (Deegan *et al*., 1997; Huber, 2011).

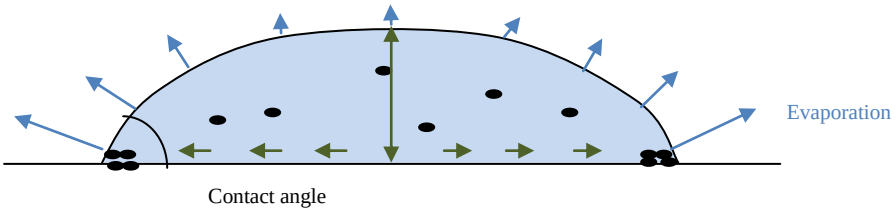


Figure 4.11 Sessile droplet drying mechanism

The other drying option could be a non-pinned contact line where the particles are deposited randomly during drying, however this was not observed during the experiments. Another reason why the cells were traveling towards the edge of the droplet could be oxygentaxis, where the cells move towards oxygen source (Tuval *et al*., 2005), therefore to the edge of the drying droplet. The movement of the particles towards the edge of the droplet could potentially cause a multilayer of microbial cells; particles which are on the outer droplet layer could protect the cells which they covered. This could potentially cause higher survival rates of bacteria in suspensions with higher numbers of cells (high concentration), compared to suspensions with lower numbers of cells, where the protective effect is not that high. A similar protective effect was observed by Butler *et al*. (2009), where more concentrated cell suspensions had higher resistance to antibiotics. In the current study experiments with a range of cell concentrations levels, confirmed however that the cell inoculum level did not have an effect on the reduction levels, indicating that there may not be a protective effect of an outer layer. Similar observation was made by Hingston *et al*. (2013) where desiccation inactivation kinetics were independent of initial contamination levels of *Listeria monocytogenes*. Unfortunately viability staining was unable to determine the presence of viable cells on surfaces and thus suggest either that viable cells were found predominantly around the droplet edge or where randomly distributed throughout the droplet. However there was an effect of food products added to the suspension which clearly enhanced cell survival. This may be due to a higher number of particles, forming a more solid layer around the bacterial cells. Additionally, in studies conducted by Butler *et al*. (2009) motile cells had added advantage, which could be in addition to a

capillary flow causing faster formation of a multilayer. All of the selected strains had good swim abilities as was shown in Chapter 3, and all of the strains had similar responses to different cell concentration levels.

4.3.2 Resistance of Desiccated Cells to Different Environmental Conditions

The environmental conditions were chosen according to the factory environment observations made, which was also within a range reported by Mettler and Carpentier (1998) where the humidity of pastry industry premises was between 50 and 65 %. Overall results indicate that lower temperatures, lower humidity, presence of food debris, and periodic wetting favour the survival of all four *Salmonella* strains.

Enhanced survival could be due to a reduced water loss caused by protective factors or the switching on of resistance genes. If reduced water loss was the biggest factor, then survival at lower temperatures and higher moisture levels would be expected as this would reduce water loss. Particle protection would also, clearly, reduce water loss. However greater survival was seen at lower humidity where desiccation pressure would be greater. Therefore potentially, two survival mechanisms could be present physical protection and genetic response. It could be beneficial to see if the cells protected by particles had the same genes switched on as the cells at lower temperature and humidity.

Good survival of cells inoculated onto surfaces with food particles could be potentially caused by either nutrient availability to the desiccated cells or food particles protecting the microbial cells by forming a layer around them. The low $\log_{10}$ cfu surface^{-1} reductions after 30 days must be due to survival of the inoculum as there is no water present that could lead to growth. As the level of the surviving cells is similar to the level of the inoculums, the protective effect is also apparent throughout the 30 days of the trial, including within the first 100 hours when desiccation has been shown to be prevalent. Food debris may have a protective effect due to the physical presence of particles surrounding the cell and providing generic desiccation resistance or particular nutrients within the food debris enabling a specific physiological protective mechanism, e.g. maintaining membrane rigidity (see Chapter 6). It is also known that some of the food compounds such as trehalose or sucrose may replace the water in the membranes of drying cells and preserve the structure of proteins, therefore increasing resistance to desiccation (Billi *et al*., 2000; Crowe *et al*., 1987; Leslie *et al*., 1995).

In studies conducted by Kusumaningrum *et al*. (2003) the effect of food residues on the survival of cells on stanless steel surfaces was also investigated. The experiments showed that food residues (saline solution, milk, minced chicken fillet) improved the survival of the pathogen, probably by forming a layer that might protect the cells on stainless steel surfaces. Similar observations were made by Louis *et al*. (1994) with addition of trehalose and sucrose (Hiramatsu *et al*., 2005) to the surviving *E. coli* cells. Results obtained by Kim *et al*. (2008) also indicated that infant formula provides protection for attached cells, as well as cells in biofilm, against lethality on exposure to desiccation.

In the experiments conducted during this thesis, as the temperature and humidity were reduced, the survival of *Salmonella* increased. It is possible that this triggers a cold stress and osmotic shock stress response which aids survival. Survival therefore could be greater at lower rather than higher temperatures and relative humidity, due to the protective effects of the physiological stress response. The influence of storage temperature on the survival of *Salmonella* in low water activity environments has previously been investigated in a number of studies. *Salmonella* inoculated into food products such as pecan halves (Beuchat and Heaton, 1975) and peanut butter products (Burnett *et al*., 2000) demonstrated significantly higher survival at 5°C compared with 21°C. The importance of storage temperature on desiccated cells was also examined by Hiramatsu *et al*. (2005) and it was demonstrated that decreasing storage temperature from 25ºC to 4ºC increased the survival of 18 *Salmonella* strains on paper discs from 70 days to 24 months.

The effect of temperature, relative humidity and suspending menstrua on the resistance of *Listeria monocytogenes* to drying was investigated by Palumbo and Williams (1990). The microorganism is Gram-positive, therefore it differs from *Salmonella* significantly, however, it showed similar results. *L. monocytogenes* survived longer on the surfaces of glass coverslips at lower temperatures, lower relative humidity and with the addition of food products. The same conclusions in respect to temperature, humidity and food debris were made by Kim *et al*., (2008) in relation to *Cronobacter sakazaki* on stainless steel.

Bae *et al*. (2012) also investigated the survival of *Salmonella* Typhimurium attached to stainless steel surfaces and kept in a range of humidity levels for 5 days. The results for cells kept in 100 % humidity, indicated an increase in microbial level by 1 log cfu surface^{-1}. Similar observation was made in these studies, where after

addition of water onto the surfaces, the level of *Salmonella* increased by approximately 1 log cfu surface^{-1}. The same conclusion can be made after analysing the results of Bae *et al*. (2012) from cells kept at 23 % and 43 % relative humidity. After 5 days the *Salmonella* levels reduced by approximately 2.5 log cfu surface^{-1} for both humidity levels. In these studies, the same period of time at 33 % and 53 % humidity caused approximately 2 log cfu surface^{-1} reduction.

Muller *et al*. (2012) investigated survival of surface attached *Salmonella* isolates after prolonged exposure to high humidity (82 %) and low temperature (10°C), and to low humidity (49 %) and high temperature (25°C). Generally, after 30 days, *Salmonella* survival was greater at high humidity and low temperature (1-2 log cfu ml^{-1} reduction) than low humidity and high temperature (3-7 log cfu ml^{-1} reduction). *Salmonella* response to lower temperatures corresponds to these studies, however increased humidity did not lower the survival. This is perhaps due to the stronger protective effect of temperature, rather than the negative effect of high humidity, which was also shown by Helke and Wong (1994).

Habimana *et al*. (2014) showed a difference between survival of *S.* Agona at two different humidity levels, with lower humidity favouring the survival. In other study with *E. coli*, over a 6 log reduction was achieved after 21 and 9 days at 35 and 85 % RH respectively. Similarly to these studies, no reduction was observed in 98 % humidity. Addition of 1 % glucose or 2 % peptone increased the survival. Survival was not affected by the number of cells inoculated on the surfaces, which also was observed in these studies (Moretro *et al*., 2010).

Development of cross-tolerance to diverse environmental stresses, following exposure to a single stress is very common in bacteria (Abee and Wouters, 1999; Kultz, 2005, Vorob'eva, 2005). It is hypothesised therefore that low temperatures and low humidity cause a stress response which is triggered before desiccation is started. The incubation of bacterial suspension was undertaken at 37ºC and for desiccation experiments it was moved to 37ºC, 25ºC or 15ºC. The cells which were moved to two last temperatures were subjected to considerable lower temperatures (>13ºC lower) when they were still in a droplet form and they, most likely, experienced a cold shock. The stress responses which are triggered during a cold stress are potentially similar to the ones triggered during osmotic stress. First of all changes in the fatty acid profile occur to maintain membrane fluidity (Suutari and Laakso 1994; Chattopadhyay and Jagannadham 2001; Kumar *et al*. 2002;

Chattopadhyay, 2006) which could also be necessary during desiccation stress to maintain turgor pressure. Heat shock and cold shock proteins are also induced at low temperatures which facilitate transcription and translation (Rey *et al*., 1994; Porankiewicz and Clarke 1997; Chow and Tung 1998). A similar reaction was also observed during desiccation (Gruzdev *et al*., 2012; Li *et al*., 2012). Additionally, cryoprotectants are accumulated which could be the same substances which protect the cells from the osmotic (desiccation) stress e.g. sugars or glycine betaine (Chattopadhyay, 2006 and Ko *et al*., 1994). This could be a reason why cells in lower temperatures survived better the desiccation process.

The reason why in higher humidity the survival was lower could be because, after drying of the droplet, there was still some water available from the air, therefore stress responses were not triggered early enough, before cells went into a dormant state (Deng *et al*., 2012).

When water was inoculated mimicking periodic wetting, *Salmonella* survived even better. It is hypothesised that the higher numbers on discs were a result of better resuscitation which could suggest that the level of survival is the same, just that the cells are recovered better. The other hypothesis is that when water is available, viable cells start a slow growth over time (approximately 2 days), taking up nutrients from dead cells. This will be further investigated in the next chapter.

4.3.3 SEM Analysis

Desiccated cells appeared to be covered with a substance which potentially could give a protective effect for microbial cells. The biggest difference in appearance of surface attached cells was seen in *S.* Typhimurium 'HR' and *S.* Cubana 2 which is aligned with the results obtained from Chapter 3 where the rugose phenotype was investigated. These two cells had the darkest and most wrinkled colonies. The other two strains however also showed signs of producing extracellular substances. Habimana *et al*. (2014) also looked at the SEM imagines of desiccated *Salmonella* Agona cells. They observed that after 2 months of desiccation at 35 % RH the cells appeared to be covered with a film-like substance as compared with cells desiccated for 1 hour, however the cells' morphology, was unaffected. In contrast, cells subjected to 2 months exposure to 85 % RH were covered with a granular-like substance and were connected with long, tubular structures, which possibly could be desiccated EPS strands. These observed physical attributes associated with desiccation could indicate the presence of both cell-to-cell

communication and adaptive response to environmental stress conditions (Habimana *et al*., 2014).

De Goffau *et al*. (2009) investigated cell morphology of Gram-negative and Gram-positive microorganisms under desiccation stress. Gram-negative rods displayed wave-like patterns, forming larger waves as they became increasingly filamentous at low humidity. In contrast, cells of *Bacillus subtilis* became shorter, curved, and eventually almost coccoid. This is a Gram-positive microorganism but followed a similar response as the observed *Salmonella* cells. Creation of filaments was not seen for desiccated *Salmonella* cells.

4.4 CONCLUSIONS

The factory visits gave a good understanding of the environmental conditions at dry food manufacturing sites. This helped to establish experimental parameters which made the trials more relevant to real manufacturing situations. When varying these parameters, survival was observed to be enhanced by low temperatures and humidity, the presence of particulate material and available water. It is proposed that enhanced survival was due to both a protective effect of any surrounding particulate material or the adoption of a stress response. Surface adhered cells also exhibited morphology changes with attachment time resulting in cell shrinkage and the presence of extra cellular structures, possibly due to EPS production. Such survival mechanisms need to be further investigated.

The aim of this thesis is not only to understand potential *Salmonella* survival mechanisms but also to help the food industry to control this pathogen. The results indicate that to reduce the probability of *Salmonella* survival, dry food factories should operate under high temperature or high moisture conditions. This may, however, increase energy costs (higher temperatures) and have negative effects on the product and its cleanability due to product agglomeration (high humidity). The presence of any water in the environment should be strictly prohibited as this would enhance *Salmonella* survival. Dry food manufacturers should undertake daily audits of the plant to ensure that there are no leaks in water pipe-work or the building fabric. Methods should also be developed to conduct effective dry cleaning and disinfection as the presence of food residues has a great effect on protecting living *Salmonella* cells.

The introduction of *Salmonella* to the factory environment could happen via many different routes, however water leaks from roofs are often associated with these incidents. This was mimicked in the experiments undertaken by inoculation of *Salmonella* solutions onto disc surfaces via droplet depositing and the implications of this method on *Salmonella* survival rates was investigated. Microbial cells were found to accumulate on the edge of the drying droplet, however higher concentrations of cell suspension did not influence survival rates.

CHAPTER 5
RESISTANCE OF DESICCATED CELLS TO WET DISINFECTION

5.1 INTRODUCTION

Following the results described in Chapter Three, it was evident that *Salmonella* cells are resistant to long-term desiccation. It is also known that exposure to a single stress condition e.g heat (Humphrey *et al*., 1995) or acid (Leyer and Johnson, 1993) is commonly associated with the development of cross-tolerance to other stress conditions (Abee and Wouters, 1999). Therefore the work described in this chapter focused on the investigation of the potential cross-tolerance of *Salmonella* to disinfectants, following desiccation. Additionally, the influence of water addition during wet disinfection or any other water introduction event, on bacteria survival was also investigated.

It is commonly known that bacteria attached to surfaces are less sensitive to disinfection than bacteria in suspension (Behnke *et al.,* 2011; Moretro *et al.,* 2012), therefore a suspension test using selected cells was not carried out, and surface-attached cells were tested instead. Disinfectant testing is a common way to approve the use of specific disinfectants in the food industry (Gutiérrez-Martín *et al*., 2011; Holah, 2012). There are European standards developed to test the efficacy of disinfectants against bacteria, yeasts, moulds and spores under a range of conditions (Anon., 2008; Anon., 2009). The use of these standards is now mandatory in Europe for the approval of disinfectants for use in the food and allied industries (Regulation No 528/2012, www.eur-lex.europa.eu). A number of reports and publications exist which contain findings from testing of different disinfectants, contact times or interfering substances (Beltrame *et al*., 2012), few publications, however, focus on the resistance of desiccated cells to disinfectants. Beltrame *et al.* (2012) investigated the influence of different sanitizers on food contaminant bacteria, however they highlighted that it is important to conduct disinfectant testing with microorganisms attached to surfaces (factory based trials). The significant factor for any challenge testing, such as disinfection, is to take any relevant pre-challenge exposure into consideration. That is why in this work the cells pre-exposed to desiccation were tested, as this is likely to happen within low water activity environments.

Contamination could be potentially dried out on the surfaces for long periods of time before any disinfection process. Humphrey (2004) talked about the design of laboratory trials and the necessity to reflect potential conditions which could induce a stress response. Therefore, if the *Salmonella* strains are likely to be exposed to dry conditions in the factory environment, disinfectants should be also tested against desiccated cells.

Studies described in Chapters 3 and 4 gave evidence that *Salmonella* could survive on factory surfaces for prolonged periods of time, regardless of the environmental conditions. It is also apparent that cells which are resistant to one particular stress may become resistant to other stresses (Chapter 1). It is known from factory visits undertaken during this studies, that dry processing plants are primarily dry cleaned only or wet cleaned periodically, therefore if the cells are introduced to the factory environment, they are desiccated for long periods of time and potentially could become resistant to disinfection procedures. For example, in the dry milk industry, the typical production time is approximately 30 days before wet cleaning of spray drying and associated equipment is undertaken (factory visits). Therefore, experiments were conducted to investigate if *Salmonella* cells which have been attached to surfaces for 30 days had the same resistance to disinfectants as cells freshly prepared and dried on to the surfaces for 80 minutes.

Dry cleaning by physical abrasive methods and vacuuming, followed by decontamination with alcohol (ethanol or propanol), is the preferred cleaning and disinfection method in dry processing environments. However, it is not entirely effective and may not remove all organic and inorganic residues, including microorganisms. To control some hazards, wet cleaning and disinfection are preferred, e.g.:-

- Wet cleaning is undertaken approximately every 12 weeks in the chocolate industry to break the life-cycle of the chocolate moth.
- Wet cleaning is undertaken every 30 days to control *Salmonella* and *Cronobacter* in infant formula manufacture.
- Wet cleaning may be undertaken after every batch of allergenic material in the dry ingredients processing sector, which may be daily to weekly.

Wet cleaning and disinfection clearly introduces water into the dry processing environment and the effect of such water on any *Salmonella* cells

attached to surfaces in a desiccated state prior to wetting, is largely unknown. This investigation was undertaken, therefore, to analyse, the influence of any water presence on the survival and potential growth of desiccated *Salmonella* cells.

5.2 RESULTS

5.2.1 Resistance of Desiccated Cells to Sodium Hypochlorite and Propanol

Following the studies described in Chapter 3, four *Salmonella* strains were selected for further investigation. Two of the strains were those showing one of the highest survival rates: *S.* Typhimurium NCTC 12023 (high heat resistant; HR) and *S.* Muenchen isolated from a cocoa bean environment. The rationale for choosing these strains was to check if the same strains which are resistant to desiccation are also resistant to various disinfectants. The other two strains which were chosen were showing medium resistance to desiccation: *S.* Cubana (2) and *S.* Mbandaka (1), and they were both isolated from cereal production environments.

The disinfectants which were tested were sodium hypochlorite and propan-2-ol (§2.1.8). These disinfectants were chosen because sodium hypochlorite is one of the commonly used disinfectants in the food industry to control pathogens and propan-2-ol is most likely to be used routinely in dry processing factories. The methods related to bacterial suspension preparation, relative humidity chambers preparation and enumeration of microbial counts are described in Sections 2.2.2 – 2.2.5.

50 µl of each suspension was deposited onto stainless steel discs and after drying samples were either subjected directly to a standard disinfectant test (5 minutes contact time) or placed in air tight plastic boxes which maintained an RH of 33% ± 1% and a temperature of 25 ±2 ºC and, after 30 days, were subjected to a standard disinfectant test. The disinfectant test used for the experiment was closely based on the BS EN 13697:2001 surface disinfectant test (§2.5). The only differences were the choice of disinfectant test strain, the method of inoculum preparation prior to testing and the omission of interfering substances. Following the protocol of EN 13697:2001, chemical products causing a 4 log reduction of test strains, in 5 minutes and under the defined conditions of the test, can be marketed in Europe as surface disinfectants for use in the food and allied industries.

The methodology was verified using a number of controls (§2.5.3-§2.5.5) which included neutraliser control (checks toxicity of neutraliser), water control

(checks toxicity of water) and neutraliser test (checks effectiveness of neutraliser). All the controls had similar microbial levels as the inoculum levels, indicating that the water and neutralisers were not toxic to *Salmonella* strains, and that the neutralisers inactivated both disinfectants sufficiently (see Tables 5.1 and 5.2).

The starting level (just before the disinfectant test) of *Salmonella* serotypes on discs was 0.80-2.00 log cfu surface^{-1} lower for the cells dried for 80 minutes and kept for 30 days in desiccating condition than for those just dried for 80 minutes. Therefore the disinfectant test results shown in this chapter, had higher reduction levels for freshly dried cells rather than desiccated cells, however this was associated with different starting levels, not the difference in resistance to disinfection. For the test to be considered as a 'pass' result, more than a 4 log cfu surface^{-1} reduction must be reached.

Table 5.1. Control results for sodium hypochlorite disinfectant testing.

Serotype	Desiccated (days)	Neutraliser control (log_{10} cfu surface^{-1})	Neutraliser test (log_{10} cfu surface^{-1})	Water control (log_{10} cfu surface^{-1})
S. Cubana 2	0	5.04 ±0.18	5.93±0.43	5.56±0.17
	30	4.82±0.56	5.04±0.00	5.65±0.46
S. Mbandaka 1	0	6.83±0.25	6.48±0.04	4.92±0.73
	30	6.05±0.12	5.66±0.15	5.98±0.11
S. Muenchen	0	5.15±0.09	5.25±0.20	5.80±0.11
	30	5.19±0.43	4.45±1.07	4.06±0.96
S. Typhimurium	0	5.87±0.01	5.31±0.02	6.02±0.11
	30	5.14±0.51	5.40±0.18	5.42±0.26

Table 5.2. Control results for propan-2-ol disinfectant testing.

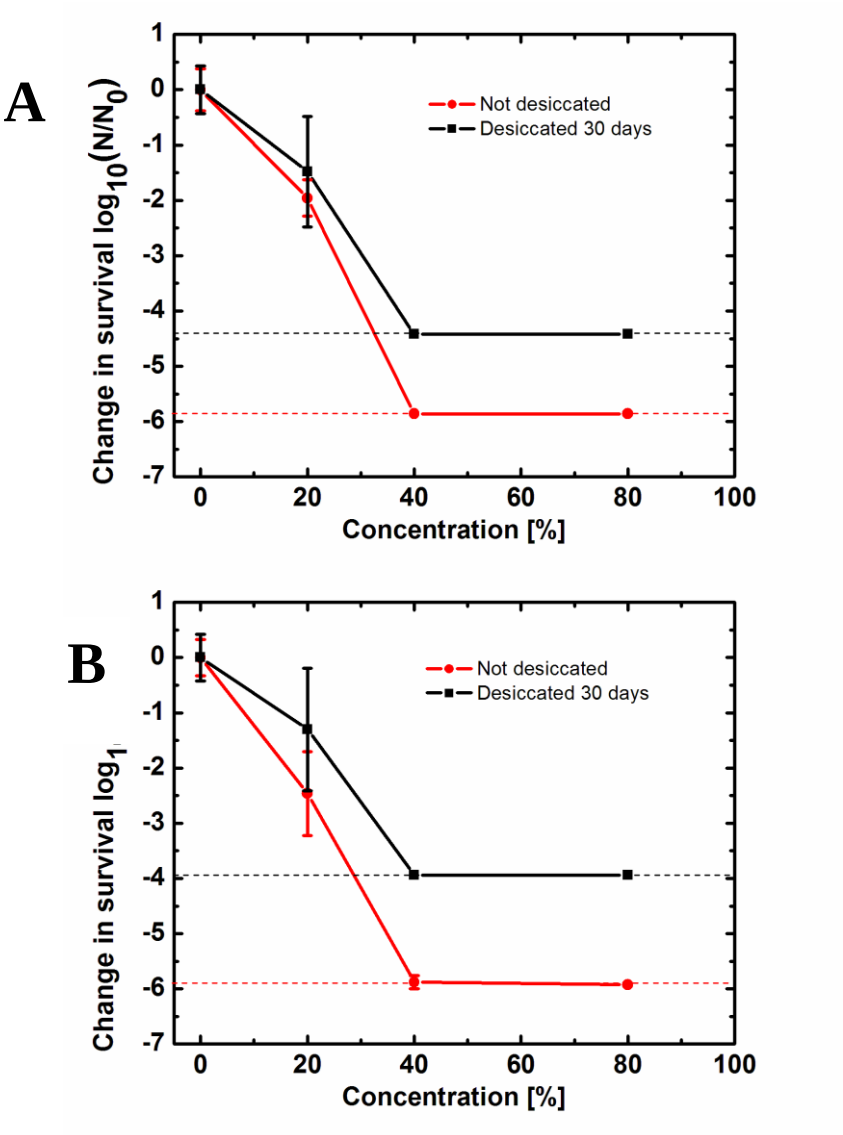
Serotype	Desiccated (days)	Neutraliser control (log_{10} cfu surface^{-1})	Neutraliser test (log_{10} cfu surface^{-1})	Water control (log_{10} cfu surface^{-1})
S. Cubana 2	0	5.82 ±0.20	6.04±0.07	6.56±0.38
	30	5.64±0.56	5.93±0.20	5.12±0.44
S. Mbandaka 1	0	6.35±0.25	5.72±0.20	6.62±0.32
	30	5.83±0.17	5.12±0.10	4.64±1.43
S. Muenchen	0	6.15±0.10	6.27±0.25	6.59±0.44
	30	5.20±0.45	4.50±0.27	4.97±1.34
S. Typhimurium	0	5.90±0.08	5.21±0.10	6.41±0.07
	30	4.14±0.51	4.40±0.20	5.30±0.27

The surface disinfectant test was carried out using three different concentrations of propan-2-ol; 20%, 40% and 80% (see Figure 5.1). 70-80%

propanol is the typical in-use strength for disinfectant products in the food industry. The 20% concentration did not have a sufficient effect (<4 log cfu surface^{-1} reduction) on *Salmonella* cells subjected to the test on the day of inoculation (dried cells) and 30 days after inoculation (dried and desiccated cells). The reduction levels of the cells treated just after 80 minutes drying, for all serotypes, ranged from 1.42 log cfu surface^{-1} to 2.46 log cfu surface^{-1} and the minimum and maximum results were associated with *S.* Muenchen and *S.* Mbandaka '1', respectively. There was a similar reduction range for the cells desiccated for 30 days; from 1.31 log cfu surface^{-1} to 2.16 log cfu surface^{-1} and minimum and maximum results were achieved for *S.* Mbandaka '1' and *S.* Typhimurium 'HR', respectively.

There was a greater effect on the cells treated with the 40% concentration of propan-2-ol. All of the samples treated on the day of inoculation reduced in microbial level to below the detection limit. Therefore, *S.* Typhimurium 'HR', *S.* Muenchen, *S.* Cubana '2' and *S.* Mbandaka '1' had a reduction level greater then 5.71, 5.89, 5.86 and 5.88 log cfu surface^{-1}, respectively. For the surfaces which were kept under controlled temperature and humidity conditions for 30 days, the level of reduction was at least 1.15 log cfu surface^{-1} lower.

The lowest reduction level was associated with *S.* Mbandaka '1', followed by *S.* Muenchen, *S.* Cubana '2' and *S.* Typhimurium 'HR', with average reduction levels >3.94, 4.20, 4.42 and >4.56 log cfu surface^{-1}, respectively. The difference was largely associated with lower starting levels after 30 days of desiccation. Despite the fact reduction in two *Salmonella* strains desiccated for 30 days was lower, in all cases the reduction was sufficient to pass the surface disinfectant test (minimum 4 log cfu surface $^{-1}$ reduction). For the 80% concentration, all of the serotypes showed a reduction in microbial count to below detectable levels. The minimum level of reduction for cells dried for 80 minutes was greater than 5.71 log cfu surface^{-1} and for cells attached for 30 days to the surfaces it was greater than 3.94 log cfu surface^{-1}. These reduction levels were associated with *S.* Typhimurium 'HR' and *S.* Mbandaka (1), respectively. For most of the strains and concentrations, there was no statistically significant difference between survival of freshly dried cells and desiccated cells (minimum p=0.28), except *S.* Mbandaka (1) at 20% concentration, where desiccated cells had a significantly lower reduction level (p=0.05).


A
Change in survival log10(N/N0)
Not desiccated
Desiccated 30 days
Concentration [%]
B
Change in survival log1
Not desiccated
Desiccated 30 days
Concentration [%]

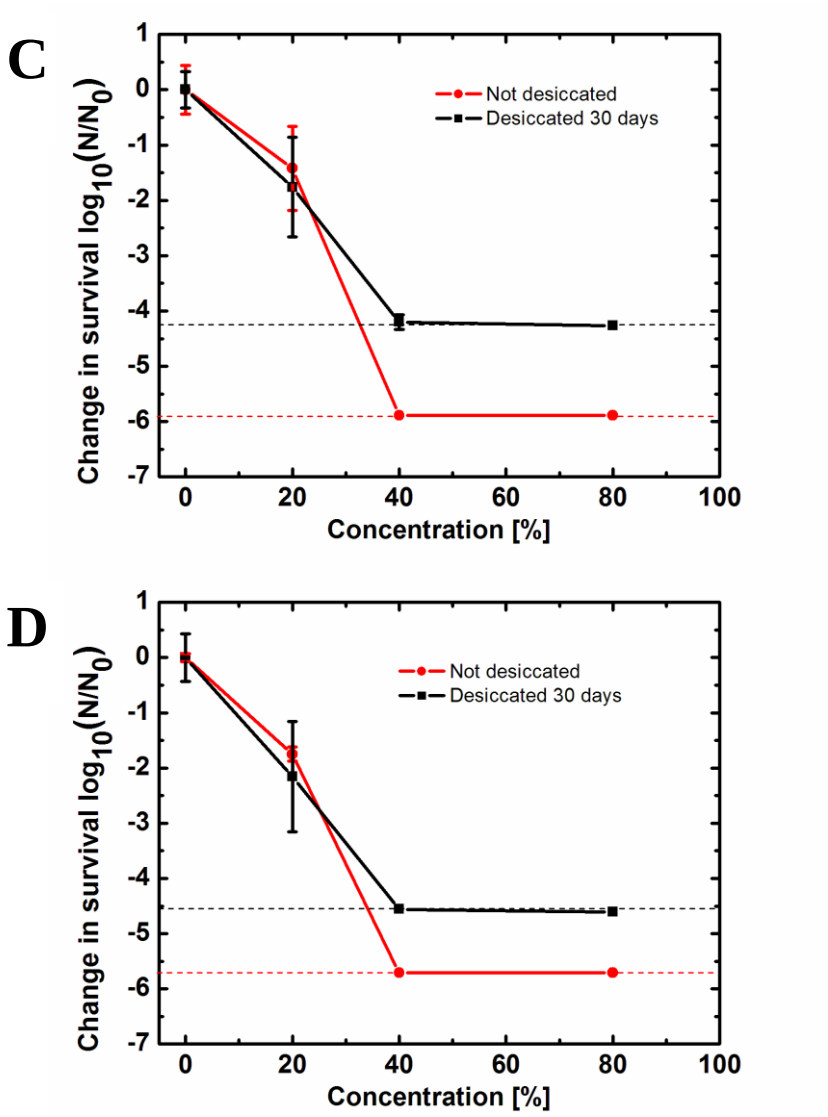
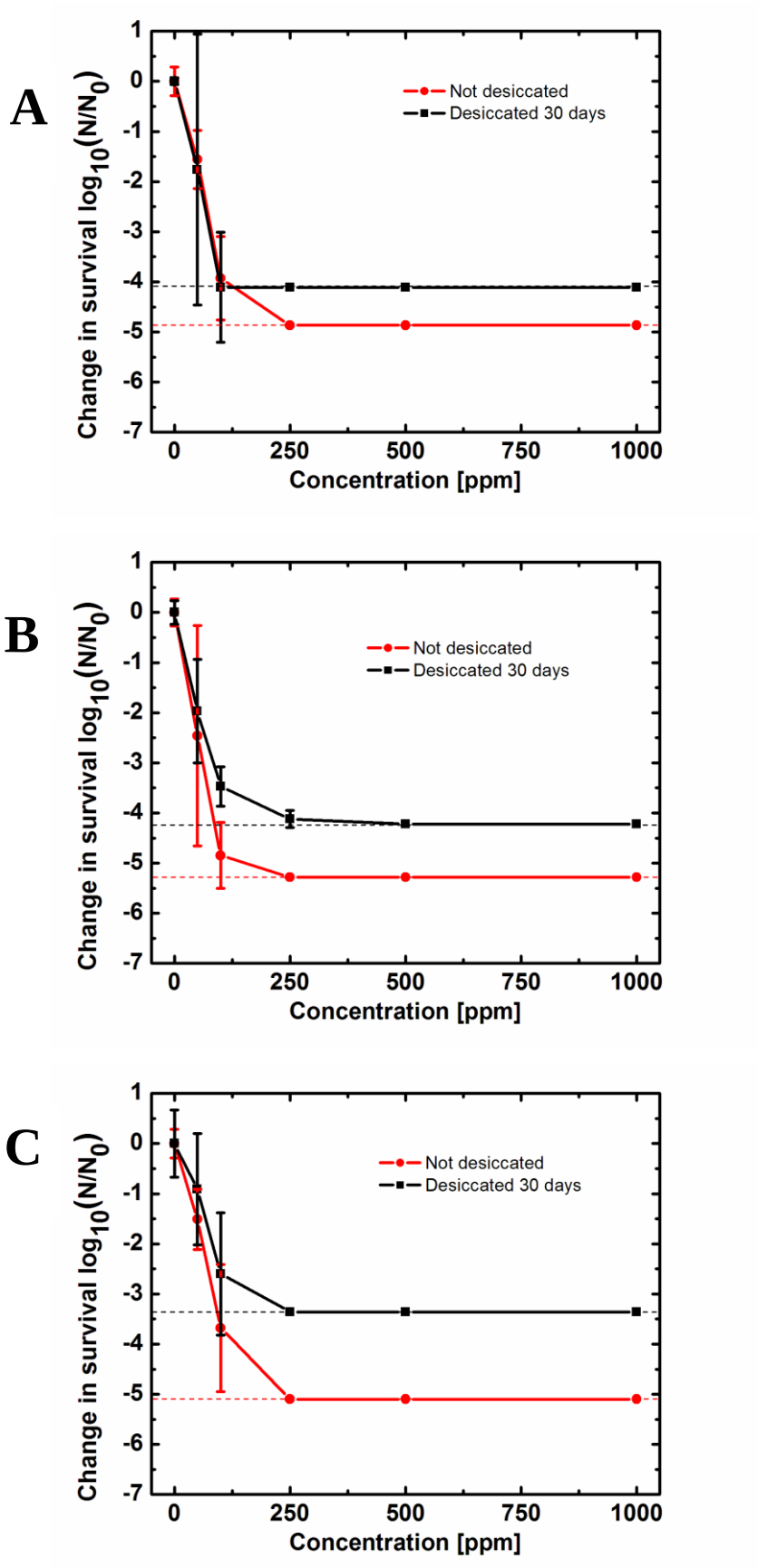


Figure 5.1. Propanol-2-ol (20, 40 and 80%) testing on 4 strains of *Salmonella* suspended in MRD, inoculated (~10^7cfu ml^{-1}) onto stainless steel discs, dried and directly subjected to the disinfectant for 5 minutes (not desiccated) or kept at 33% RH and 25ºC for up to 30 days and then subjected to the disinfectant for 5 minutes (desiccated 30 days). Bars represent one standard deviation. A) *S.* Cubana (2), B) *S.* Mbandaka (1), C) *S.* Muenchen, D) *S.* Typhimurium 'HR' NCTC 12023. Dotted lines represent reduction limit with detection limit of 5 cfu $surface^{-1}$ (0.7 log cfu $surface^{-1}$), when reduction reaches dotted lines this indicates viable count were not detected.


A
B
C
Not desiccated
Desiccated 30 days
Change in survival log10(N/N0)
Concentration [ppm]
1
0
-1
-2
-3
-4
-5
-6
-7
0
250
500
750
1000

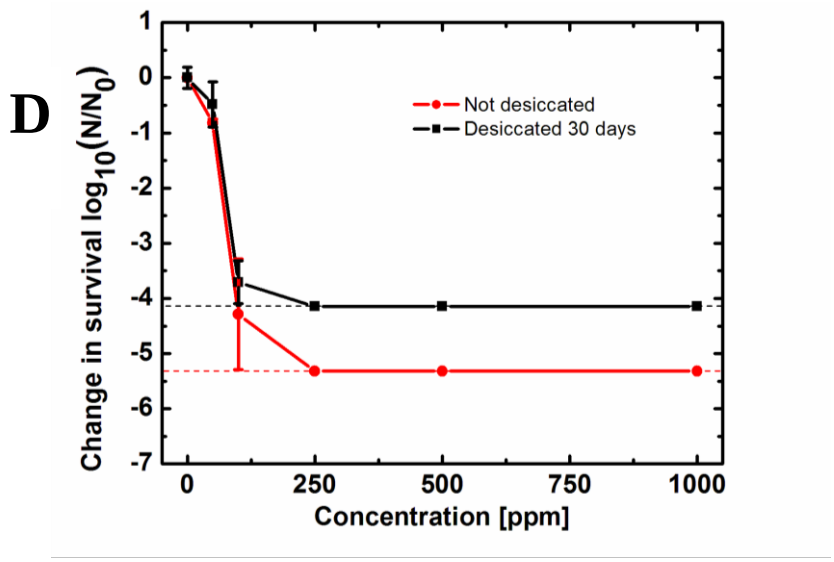


Figure 5.2. Sodium hypochlorite (0, 50, 100, 250, 500 and 1000 ppm) testing of 4 *Salmonella* strains suspended in MRD, inoculated (~10^7cfu ml^{-1}) onto stainless steel discs, dried and directly subjected to disinfectant test (not desiccated) or kept at 33% RH and 25ºC for up to 30 days and then subjected to the test (desiccated 30 days). Bars represent one standard deviation. A) *S*. Cubana (2), B) *S*. Mbandaka (1), C) *S*. Muenchen, D) *S*. Typhimurium 'HR' NCTC 12023. Dotted lines represent reduction limit with detection limit of 5 cfu $surface^{-1}$ (0.7 log cfu $surface^{-1}$), when reduction reaches dotted lines this indicates viable count were not detected.

The results of the surface disinfectant test with sodium hypochlorite are shown in Figure 5.2. Five concentrations of disinfectant were used: 50, 100, 250, 500 and 1000 ppm. 500-1000ppm sodium hypochlorite is the typical in-use strength for disinfectant products in the food industry.

The starting level on surfaces (just before disinfectant test) of *Salmonella* serotypes was approximately 1 log cfu $surface^{-1}$ lower for the cells dried for 80 minutes and kept for 30 days in desiccating condition than for those just dried for 80 minutes. The 500 and 1000 ppm concentrations caused a reduction in microbial levels to below detectable counts, for all serotypes. Therefore, the log cfu $surface^{-1}$ reductions were at least >3.36 for cells attached to the surfaces for 30 days and at least >4.87 for cells only dried for 80 minutes.

The 50 ppm concentration gave the lowest reduction in microbial levels for both types of cells: attached for 30 days and dried for 80 minutes. This concentration caused a reduction ranging from 0.8 log cfu $surface^{-1}$ to 2.46 log cfu $surface^{-1}$ for cells tested on the day of inoculation and these minimum and maximum values were associated with *S*. Typhimurium ´HR´ and *S*. Mbandaka (1), respectively. Slightly

lower, but not significant (minimum p=0.23), reduction levels were related to cells attached for 30 days to the surfaces, and they ranged from 0.48 log cfu surface^{-1} reduction to 1.97 log cfu surface^{-1} reduction. They were linked to *S.* Typhimurium `HR` and *S.* Mbandaka '1', respectively.

Increasing the concentration to 100 ppm resulted in larger reduction levels for all serotypes. The cells tested on the day of inoculation showed average reduction levels between 3.68 and 4.85 log cfu surface^{-1}, and they were linked with *S.* Muenchen and *S.* Mbandaka '1', respectively. The microorganisms which were kept in desiccation conditions for 30 days showed a slightly lower, but not significant (minimum p=0.20), range of reduction levels between 2.60 and 4.11 log cfu surface^{-1} for *S.* Muenchen and *S.* Cubana '2', respectively with only the *S.* Cubana '2)'serotype passing the test. *S.* Typhimurium `HR', *S.* Muenchen and *S.* Cubana '2' exhibited a drop in microbial levels to below detection, after treatment with 250 ppm of sodium hypochlorite for both attachment conditions. *S.* Mbandaka '1' cells were also reduced to below detectable levels, however only for the cells treated on the day of inoculation. The cells attached to the surfaces for 30 days showed a 4.12 log cfu surface^{-1} reduction.

5.2.2 Periodic Wetting

Following the studies described in Chapter 4 (§4.2.2) which showed high survival level of *Salmonella* with water addition, additional studies were conducted using only the *S.* Typhimurium NCTC 12023 (high heat resistant; 'HR') strain and they were aimed at investigating the reason for the high number of microorganisms present on the surfaces which were periodically wetted. Periodic wetting was conducted the same way as previously undertaken (§6.2.2). The discs were sampled immediately after initial drying (time 0) and on days 7 (before wetting), 8, 14 (before wetting), 15, 21 (before wetting) and 22 after initial inoculation with the bacterial suspension. Microscopic observation of discs was also conducted after acridine orange staining (§2.4.5). The differences in colour of the stained cells are most probably due to a different exposure of the samples to light (personal experience) and not likely due to the difference in viability.

The numbers of microorganisms at different time points are shown in Figure 5.3. The microbial level after initial drying had an average value of 6.41 log cfu surface^{-1} which was reduced after 7 days by 0.84 log cfu surface^{-1} but increased up to 6.96 log cfu surface^{-1} after water was inoculated. A similar pattern was observed

when water was introduced a second and third time. The numbers increased each time water was introduced and then declined after 7 days of desiccation.

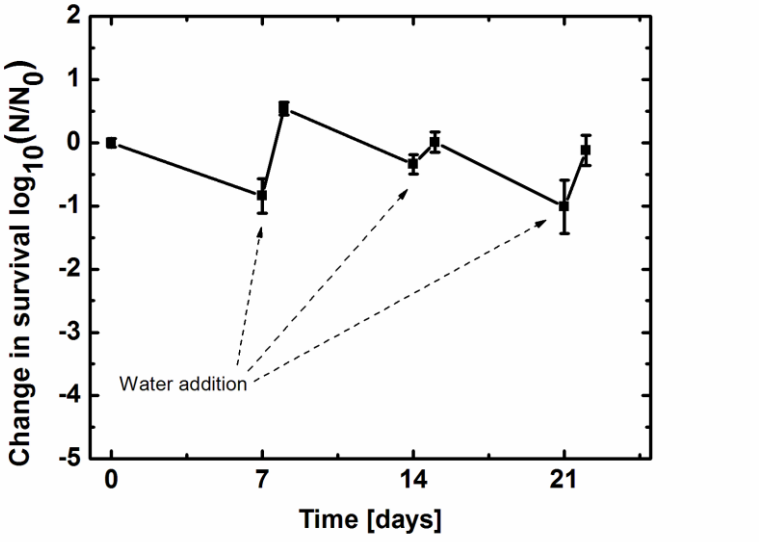


Figure 5.3. Survival of *S.* Typhimurium 'HR' suspended in MRD, inoculated onto stainless steel discs (~10^7cfu ml^{-1}), dried and kept at 33% RH, 25ºC for up to 30 days and inoculated with water on day 7, 14 and 21. Bars represent one standard deviation.

The findings obtained from enumeration of bacteria on agar plates were confirmed by microscopic images of the disc surfaces. Before any water was introduced, the number of bacteria on the surfaces was the lowest (Figure 5.4A). Twenty four hours after water was inoculated the number of cells visibly increased (Figure 5.4B). After wetting, the surfaces were wet for approximately 2 days before they completely dried, therefore presumably microorganisms continued to grow and on day 14, the image shows even higher cell levels (Figure 5.4C). This is most likely a mixture of viable cells and dead cells, because the results from culturing the cells showed that the counts were actually lower on day 14 than on day 8. The second wetting was performed on day 14 and samples taken one day after appear to have higher microbial counts (Figure 5.4D). After the third inoculation with water it emerged that cells formed a multilayer film and were surrounded with thick material which was clearly seen from life image, however it is not well recognisable on the photographs (Figure 5.4E). Further studies were conducted to investigate whether water inoculation after longer periods of time had a similar effect on bacterial numbers, as after only 7 days, and also if wetting increased survival of a strain less resistant to desiccation.

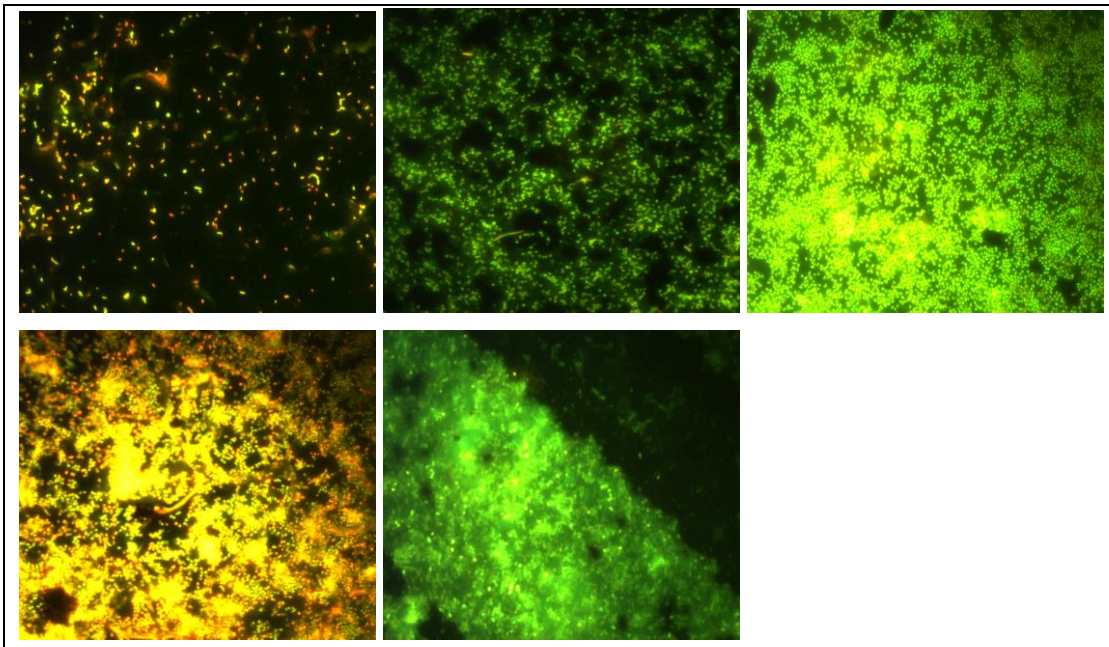


Figure 5.4. *S*. Typhimurium cells attached to stainless steel surfaces, stained with acridine orange and observed under 1000 fold magnification with UV light for: A) 7 days; B) 8 days and inoculated with water on day 7; C) 15 days and inoculated with water on day 7; D) 16 days and inoculated with water on day 7 and 14; E) 22 days and inoculated with water on day 7, 14 and 21. Difference in color is due to the time difference to light exposure.

Two strains were chosen: desiccation resistant *S*. Typhimurium NCTC 12023 (high heat resistant; HR) and desiccation sensitive *S*. Typhimurium (isolated from whey powder). Periodic wetting was conducted using the same method as previously undertaken (§5.2.2), and for samples inoculated with water approximately every 14 days, the days of wetting were as follows: 14, 28, 50, 63, 87 after initial inoculation with bacterial suspension. For samples inoculated with water approx. every 28 days, the exact days of wetting were as follows: 28, 56 and 87 after initial inoculation with bacterial suspension. Discs were sampled immediately after initial drying (time 0) and on each day of water addition (before adding water), and one and two days after each water addition.

Figure 5.5 shows the viable cells number at different time points of drying and water addition. Dotted lines on the graphs show the detection limit. *S.* Typhimurium (whey powder) cells declined to below detectable levels after 14 days (Figure 5.5A), however every time the water was added, at least one sample showed countable results. The standard deviation is high, because the rest of the samples usually did not show any growth. S. Typhimurium 'HR' strain showed similar

patterns as when it was wetted every 7 days (Figure 5.5B). The drop in viable counts in-between wetting was however higher because the time period was longer.

The microscopic images of *S.* Typhimurium (whey powder isolate) show some growth during the 87 days time period (Figure 5.6).The increase of cells was especially observed after the second wetting. The growth was however much higher for S. Typhimurium 'HR' cells (Figure 5.7), which had fewer cells at the beginning of the experiment and much higher concentration of cells in the end of the trial.

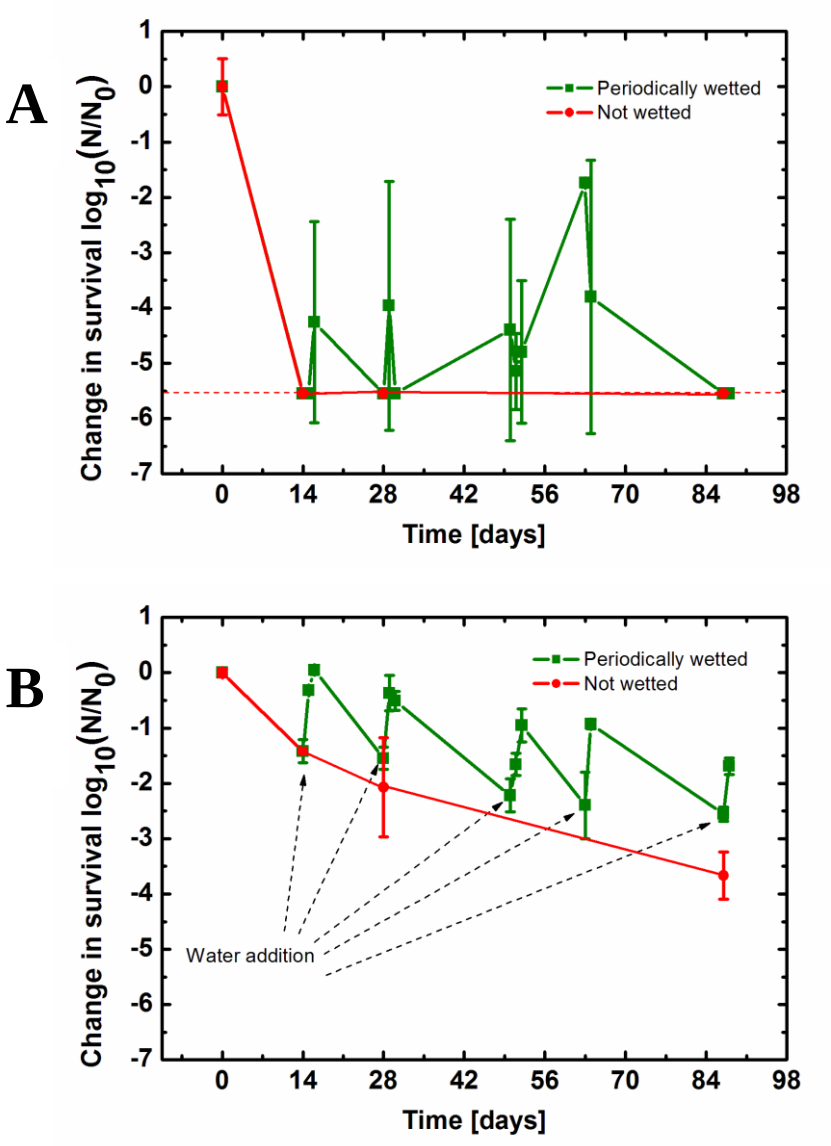


Figure 5.5 Survival of *Salmonella* suspended in MRD, inoculated onto stainless steel discs (~10^7cfu ml^{-1}), dried and kept at 33% RH, 25°C for up to 30 days and inoculated with water on day 14, 28, 50, 63 and 87. Bars represent one standard deviation. A) S. Typhimurium (whey powder isolate) and B) *S*. Typhimurium 'HR'.

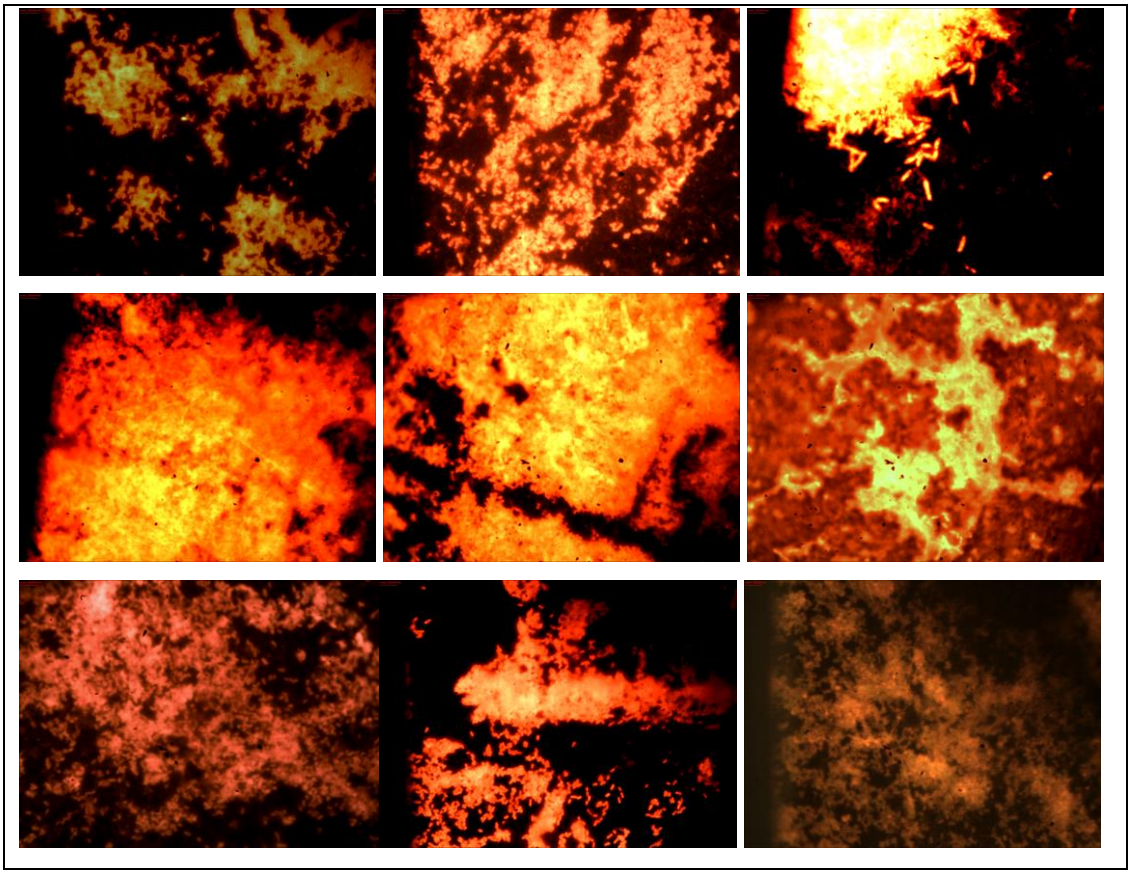


Figure 5.6 Microscopic imagines of *S*. Typhimurium (whey powder strain) inoculated onto stainless steel discs, kept under 25°C and 33% RH and wetted with sterile water at a range of time points, stained with acridine orange and observed under 1000 fold magnification with UV light: A) sample on day 0, not wetted, B) sampled on day 15, wetted on day 14, C) sampled on day 28, wetted on day 14; D) sampled on day 29, wetted on day 14 and 28; E) sampled on day 30, wetted on day 14 and 28; F) sampled on day 50, wetted on day 14 and 28; G) sampled on day 51, wetted on day 14, 28 and 50; H) sampled on day 90, wetted on day 14, 28, 50, 63 and 87; I) sampled on day 90, not wetted. Difference in colour is due to the time difference to light exposure.

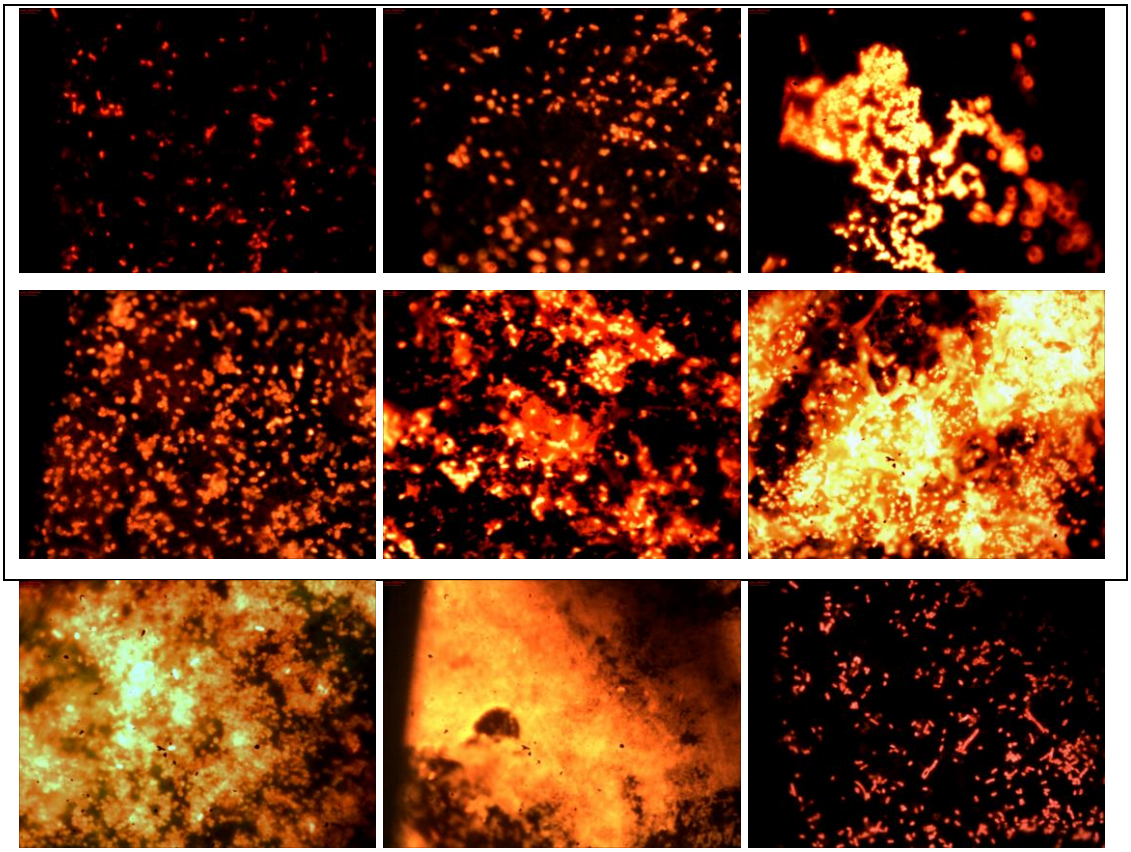


Figure 5.7 Microscopic imagines of *S*. Typhimurium NCTC12222 (HR strain) inoculated onto stainless steel discs, kept under 25°C and 33% RH and wetted with sterile water in a range of time points, stained with acridine orange and observed under 1000 fold magnification with UV light: A) sample on day 0, not wetted, B) sampled on day 15, wetted on day 14, C) sampled on day 28, wetted on day 14; D) sampled on day 29, wetted on day 14 and 28; E) sampled on day 30, wetted on day 14 and 28; F) sampled on day 50, wetted on day 14 and 28; G) sampled on day 51, wetted on day 14, 28 and 50; H) sampled on day 90, wetted on day 14, 28, 50, 63 and 87; I) sampled on day 90, not wetted. Difference in colour is due to the time difference to light exposure.

Wetting conducted every 28 days did not have any effect on the survival of *S.* Typhimurium (whey powder isolate). Numbers had fallen below the detection limit and recovery was also not possible after water was added to the dry cells (Figure 5.8A). There was also no clearly visible cell number increase observed on the microscopic images taken during the experiments (Figure 5.9). *S.* Typhimurium 'HR' showed growth every time water was added, to the levels close to the initial ones (Figure 5.8B). The images taken during the period of the experiment showed a slight increase in cell numbers after first (Figure 5.10D) and second (Figure 5.10E) water addition. The analysis of the images was conducted in qualitative, not quantitative way. The comparison was based on visual observations.

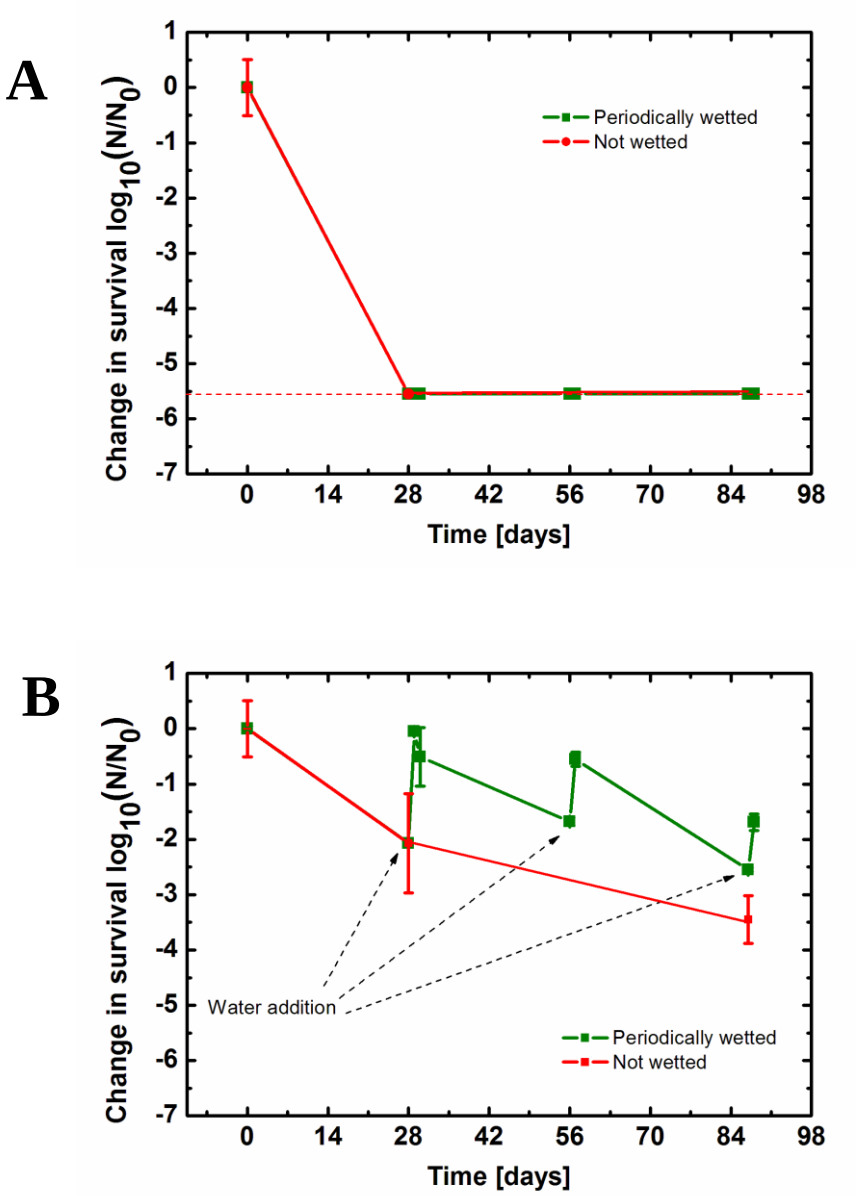


Figure 5.8 Survival of *Salmonella* suspended in MRD, inoculated onto stainless steel discs (~10^7cfu ml^{-1}), dried and kept at 33% RH, 25ºC for up to 30 days and inoculated with water on day 28 and 60. Bars represent one standard deviation. A) *S*. Typhimurium (whey powder isolate) and B) *S*. Typhimurium 'HR'

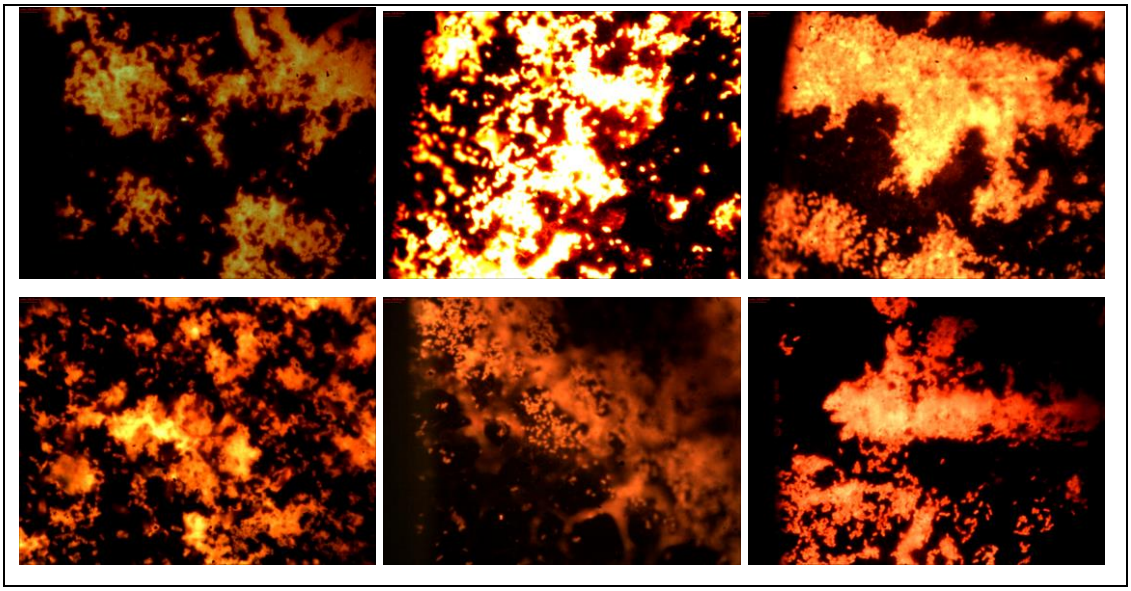


Figure 5.9 Microscopic imagines of *S*. Typhimurium (whey powder strain) inoculated onto stainless steel discs, kept under 25°C and 33% RH and wetted with sterile water in a range of time points, stained with acridine orange and observed under 1000 fold magnification with UV light: A) sample on day 0, not wetted, B) sampled on day 28, not wetted, C) sampled on day 29, wetted on day 28; D) sampled on day 30, wetted on day 28; E) sampled on day 90, wetted on day 28 and 60; F) sampled on day 90, not wetted. Difference in colour is due to the time difference to light exposure.

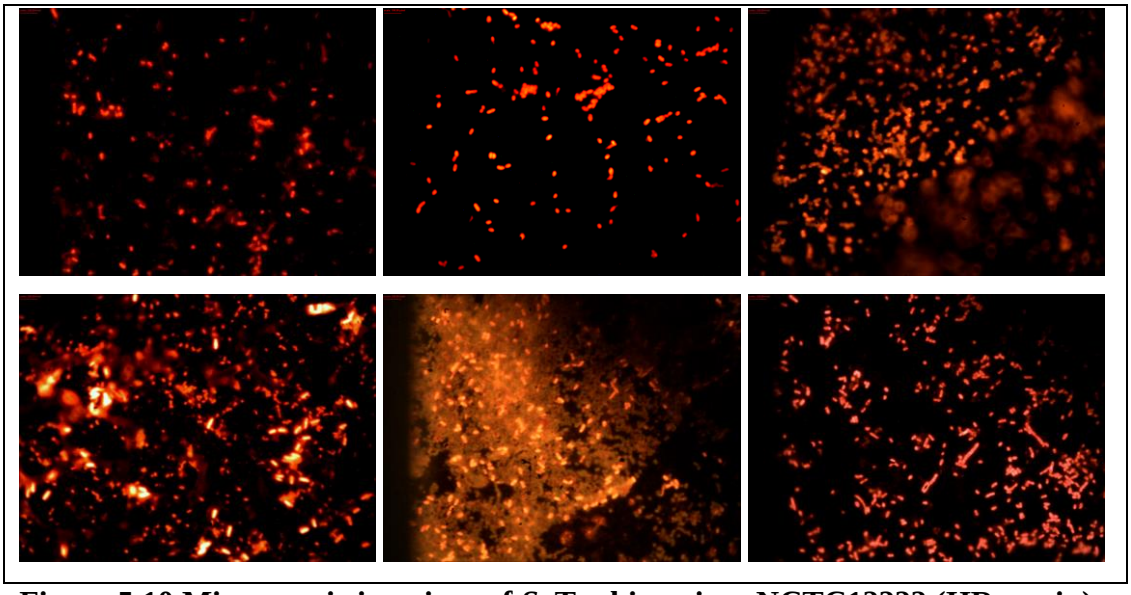


Figure 5.10 Microscopic imagines of *S*. Typhimurium NCTC12222 (HR strain) inoculated onto stainless steel discs, kept under 25°C and 33% RH and wetted with sterile water in a range of time points, stained with acridine orange and observed under 1000 fold magnification with UV light: A) sample on day 0, not wetted, B) sampled on day 28, not wetted, C) sampled on day 29, wetted on day 28; D) sampled on day 30, wetted on day 28; E) sampled on day 90, wetted on day 28 and 60; F) sampled on day 90, not wetted

5.3 DISCUSSION

5.3.1 Resistance of Desiccated Cells to Sodium Hypochlorite and Propanol

A range of propanol and sodium hypochlorite disinfectant concentrations was chosen for 3 reasons. Firstly, concentrations were chosen below the in-use concentration of the disinfectant to help elicit observation of any resistance changes between desiccated and recently dried cells. Secondly, in-use concentrations were chosen to ascertain if any established resistance patterns of desiccated cells rendered such in-use concentrations unsuitable for routine use in the food industry. Thirdly, it is also common in EU standard disinfectant tests to choose at least one concentration which will fail the test and one concentration which will pass it. This is done as a control to ensure that the selected strains are robust enough for the disinfectant test but not too robust as not to show any apparent disinfectant effect. This effect was achieved in the results, i.e. pass and fail results, for both disinfectants.

In addition, all test controls were also positive by the test standards which indicates the test conditions were appropriate and the decline of *Salmonella* cells during disinfection was caused by the disinfectants only and no other toxic materials within the test protocols.

The results of this chapter indicate that there is no evidence that cells adhered to stainless steel for 30 days develop resistance to disinfectants, such that in-use concentrations of disinfectants (e.g. 500 ppm hypochlorite acid and in excess of 1000 ppm if the presence of pathogens is expected) are unable to provide effective surface decontamination. This is only correct of course, if the disinfectant has direct contact with the cells, is left for the required contact time and the cells are not protected with food debris or harboured in equipment features. There was no difference between the selected strains which, in general, had high resistance to desiccation in the first place. The strains which were sensitive to desiccation were not chosen as after 30 days they would not have had high enough surviving numbers to perform disinfection studies.

The cells which were attached to the surfaces for 30 days had slightly lower reduction levels for the 40% concentration of propan-2-ol and 100 ppm and 250 ppm concentrations of sodium hypochlorite. However, the 40% concentration of propan-2-ol still passed the requirements of the test which is 4 log cfu surface^{-1} reduction.

Studies conducted by Lindsay and Holy (1999) showed that sessile cells of *B. subtilis* and *P. fluorescens* were significantly more resistant to disinfection than their

planktonic counterparts. This was also confirmed by Raffellini *et al*. (2011) with *E. coli* attached to the stainless steel surfaces. This was the prime reason why surface disinfectant tests were developed in the first place (Anon, 2009), why they are currently used in European disinfectant approval and also why they were used in this study.

Gruzdev *et al*. (2011) conducted similar studies, when they exposed desiccated *Salmonella* cells to a range of disinfectants. The microorganisms were exposed to desiccation for a shorter time than in these studies, which was 22 hours. The 5 minute treatment of *S.* Typhimurium cells with 50 ppm and 100 ppm of sodium hypochlorite, gave ~4.0 and 4.3 log cfu well^{-1} reduction, respectively. In these studies the reduction levels for *S.* Typhimurium were much lower for cells attached for 30 days: 0.48 and 3.17 log cfu surface^{-1} for the 50 ppm and 100 ppm concentrations, respectively. The cells which were dried for only 80 minutes showed very similar log reductions to that described by Gruzdev *et al.* (2011) which was 4.29 log cfu surface^{-1} reduction with 100 ppm sodium hypochlorite. The cells which were dried for 80 minutes in this work were more similar to the cells dried for 22 hours by Gruzdev *et al.* (2011) than the cells desiccated for 30 days. Taking into consideration the results from both studies, it can be concluded that there could possibly be a higher resistance to disinfectants in desiccated cells; however this is only noticeable at very low disinfectant concentrations, which are not used for surface disinfection in food factory environments.

Salmonella Typhimurium cells attached to stainless steel surfaces for 5 days were subjected to chlorine and alcohol (58.8%) based disinfectants (Bae *et al*., 2012). After 5 minutes exposure, 4.95 and >5.20 log count reductions were achieved, respectively. This was confirmed in work described in this chapter, where *S.* Typhimurium cells treated with 250 ppm sodium hypochlorite and 40% propan-2-ol achieved a >4.11 and 4.56 log cfu surface^{-1} reduction, respectively. Condell *et al*., (2012) also showed that working concentrations of 7 different disinfectants were effective against dried onto 96-well plate *Salmonella*.

Studies conducted by Stocki *et al*. (2007) are also comparable with these studies and they demonstrated that treatment of egg conveyor belts with disinfectants at the manufacturer's recommended concentrations should eliminate *Salmonella* contamination. However the investigation in one of the stock poultry farms indicated that a *S.* Senftenberg clone persisted for more than 2 years, despite cleaning and

disinfection, desiccation and depopulation, and subsequently infected *Salmonella* free layers (Broennum Pederson, 2008). This highlights that the presence of additional organic matter could potentially deteriorate the efficacy of disinfection. The disinfection process is also strongly influenced by the type of surface. This should also be considered in dry food environments and materials should be chosen carefully to reduce this potential contamination problem. It must also be ensured that any organic materials such as powders, dust, and food debris are removed before the disinfection process.

5.3.2 Periodic Wetting

The trials, with periodic wetting, were conducted to investigate whether the wetting caused microbial growth or only better recovery of microorganisms after the cells were rehydrated for a longer period of time. The results obtained by microbiological analysis and microscopic observation indicated that there was a growth of microorganisms each time water was inoculated, which however does not eliminate the theory that cells were also better recovered. Most of the cells appeared orange after staining which usually indicates more metabolically active bacteria compared with cells which stain green (Rapposch *et al*., 2000). This is partially confirmed in these studies as when the time period of desiccation increases, the cells are becoming more yellow and green (Figures 5.6-5.10). However, in a case of *S.* Typhimurium isolated from whey powder, even when the counts indicate all cells are not viable, the colour is still orange. This tried to be investigated further with the Live/Dead stain, however the method was not effective with desiccated cells (results not shown). It brings again the question about presence of viable but not culturable cells (VBNC). The recovery method however was validated and additionally an agar incorporation method was used to pick up low cell numbers. McFeters et al., (1991) states that staining with acridine orange might be helpful with investigation of physiological activity of bacteria, but it has to be done in defined conditions, staining procedure, environmental factors etc. That could be a reason why the stained samples in this work are not consistent with this interpretation. The effect of water addition was significant regardless of whether it was added after 7, 14 or 28 days of desiccation for *S.* Typhimurium 'HR'. This suggests that this particular strain of *Salmonella* can survive desiccation and any time water is available, can start growing again, using nutrients derived from the drying process and any cell death: no nutrients were added in the applied water. The medium which was used for re-

suspending the inoculum contained only 0.1% of peptone, therefore the rest of the nutrients to allow growth could potentially be taken from the dead cells.

In the case of *S.* Typhimurium isolated from whey powder, water addition after 28 days did not have any effect on the growth and survival of the cells. Cells were already not viable when the water was added, therefore it was not possible to recover the cells. When, for the same strain, water was added every 14 days, again most of the samples had the cells which were lethally damaged during desiccation; however one or two samples out of nine discs tested at each time point, were showing microbial growth. For this reason the results showed a large standard deviation. A time period of 14 days was probably the borderline for the ability of the cells to survive, therefore most cells were dead; however a minority of samples still had some viable cells. This shows that, in the absence of water, the cells would not survive, however when water was added every 2 weeks, there was a chance for recovery of cells and further survival.

The ability to recover bacteria from a dried state is used in preserving cultures by freeze drying and subsequent rehydration (Valdez *et al*., 1983; Valdez *et al*., 1985). Bacteria can also be recovered from dried food after long storage (Wang *et al*., 2004; Sani *et al*., 2013; Yang *et al*., 2015), however the ability to recover *Salmonella* from a dry state and growth without nutrients and osmoprotectants has not been broadly studied. This is particularly important when wet disinfection is used, which gives the possibility to introduce water to the dry bacterial cells. Moretro (2010) tested disinfectants against STEC on surfaces, and showed a desired effect, however it was also highlighted that the environment should be dried completely after disinfection as humid conditions will promote the survival of STEC. Therefore, if water is used the hygienic design of the equipment has to enable sufficient cleaning and disinfection procedures, be rapidly drainable and any dead spaces which could harbour microorganisms, protect them from sanitation or retain water, must be avoided.

5.4 CONCLUSIONS

The studies described in this chapter addressed the concern associated with disinfection of *Salmonella* in dry environments. It was evident from Chapter 3 and 4 that this pathogen can survive on stainless steel surfaces under different environmental condition; therefore it is crucial for the food industry to know if it is

possible to decontaminate the surfaces with commonly-used disinfectants. The results indicate that working concentrations of disinfectants such as 250 ppm of sodium hypochlorite or >40% of propanol are able to deliver the required 4 log reduction in viability. Responses of the cells which were only dried vs. cells which were dried and desiccated for 30 days were not significantly different for all of the strains tested. This finding confirms that dried *Salmonella* could be reduced during wet or dry chemical disinfection, however only if the disinfectant has direct contact with the cells and there is no food debris present. To gather more evidence on whether 30 day adhered cells develop enhanced disinfectant resistance, which could be correlated with enhanced desiccation resistance, further work is required in which tests are repeated with lower disinfectant concentrations.

Wet cleaning and disinfection, however brings water to the dry environment which could remain after cleaning e.g. when equipment is not hygienically designed or drying is not effective. Any addition of such water droplets could lead to resuscitation of dried cells and immediate growth, even if the nutrients are not present. Additionally, strains which are not highly resistant to desiccation, could survive for much longer periods of time when water is introduced periodically. Therefore, if wet disinfection is used, the environment should be capable of drying out immediately.

Moreover, it could be considered to test disinfectants used in low water activity food factories with outbreak strains which have been desiccated previously to recreate real factory conditions. This would ensure that the worst case scenario is taken to consideration and that disinfectants are effective at the conditions which they are going to be used under.

CHAPTER 6
DESICCATION STRESS RESPONSES

6.1 INTRODUCTION

The mechanisms of *Salmonella* survival in dry environments are still not completely understood. This is surprising as the responses of bacteria to other stress conditions such as: osmotic (Booth and Louis, 1999), oxidative (Pomposiello and Demple, 2000), nutrient (Hengge-Aronis, 2000), pH (Audia, Webb and Foster, 2001) thermal (Moat *et al*., 2002) etc. have been widely studied. The perception to date is that the same genes which are responsible for the osmotic resistance are also responsible for desiccation resistance (Chapter 1; Moat *et al.,* 2002). However several recent studies looking at changes in gene expression following desiccation stress have started to bring a new perspective to underlying desiccation adaptation mechanisms (Deng *et al*., 2012; Gruzdev *et al*., 2012a; Li *et al*., 2012; Finn *et al*., 2013). An initial comparison of these findings was therefore carried out and Figure 6.1 shows the list of genes up-regulated during desiccation, which were found to be in common between these studies. Whilst there were upregulated genes that were common to two studies, none of the up-regulated genes were common for all three studies.

In the study conducted by Li *et al.* (2012), transcriptomic profiles of *Salmonella* Tennessee, a desiccation tolerant strain, and *Salmonella* Typhimurium LT2, a less tolerant strain, were compared following 2 h air-drying at 11% relative humidity. *S.* Typhimurium exhibited high expression levels of protein biosynthesis genes, while *S*. Tennessee demonstrated greater induction and expression of genes associated with stress response, envelope modification, trehalose biosynthesis and biodegradation pathways. Furthermore, accumulation of trehalose was significantly induced in S. Tennessee, but not in LT2. Up-regulation of ribosomal protein genes was also observed in air-dried *S*. Typhimurium SL1344 on a polystyrene surface (Gruzdev *et al*., 2012a). In addition, they also reported on the induction of genes involved in amino acid metabolism, stress response, energy production and ion transport.

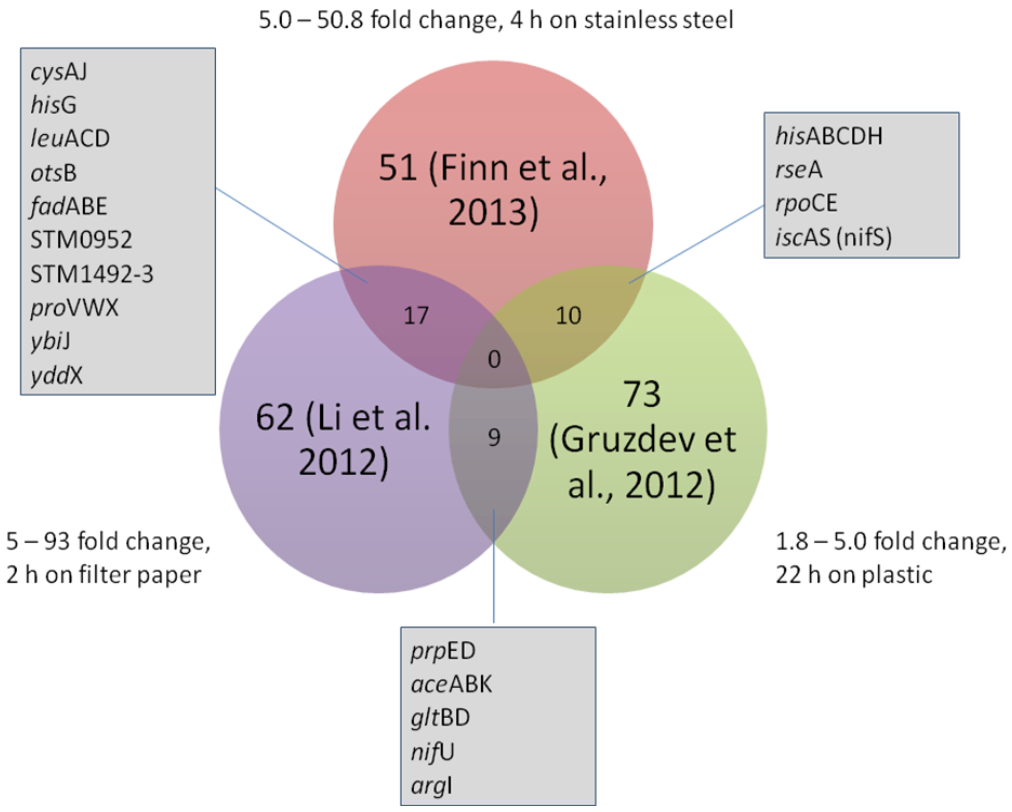


Figure 6.1 Comparison of genes identified as up-regulated during desiccation as reported by 3 different studies.

Some similar transcriptomic profiles were also reported in a *S.* Typhimurium ST4/74 strain that was dehydrated on stainless steel (Finn *et al.*, 2013a). This later study demonstrated down-regulation of 187 and up-regulation of 79 genes involved in transport of osmoprotectants, trehalose biosynthesis, formation of FE-S clusters, histidine metabolism and oxidative stress response (Finn *et al.*, 2013a). Gruzdev et al. (2012) investigated the involvement of several of the upregulated genes in desiccation tolerance by site-directed mutagenesis. These included genes encoding isocitrate lyase *aceA*, the lipid A biosynthesis palmitoleoyl acyltransferase *ddg*, modular iron-sulfur (Fe-S) cluster scaffolding protein *nifU*, the global regulator gene *fnr*, alternative sigma factor *rpoE*, and several transporters, *proP*, *proU*, *osmU* and *kdpA*.

Expression of genes obtained by three studies described in Figure 6.1 was compared to the results of transcriptomic studies during osmotic stress (Deng *et al.*, 2013). Only four genes were in common with one of the other studies: *dnaK* (heat shock protein), *rseA* (sigma-E factor regulatory protein), *rpoH* (RNA polymerase sigma factor RpoH) and *ibpA* (small heat shock protein). In the Deng *et al.* (2013)

study, gene expression analysis of *S.* Enteritidis in a high osmotic environment (peanut oil) and LB broth, demonstrated a massive repression of most *Salmonella* genes in the peanut oil compared to the broth. The authors reported the induction of only 12 genes, mostly encoding heat- and cold-shock proteins at 216 h compared to 72 h.

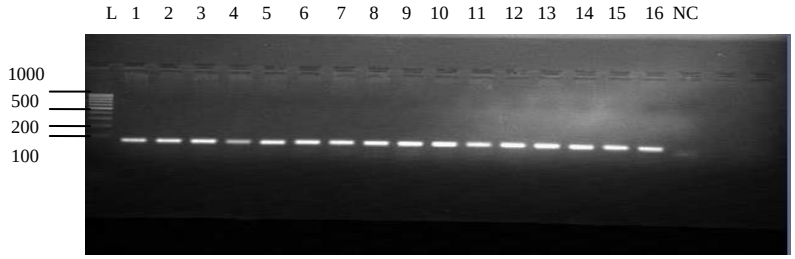
Although transcriptomic studies have shed new light on the genes that take part in how *Salmonella* responds to low a_w environments, the specific role of each gene in the bacterial stress response, remains to be further understood. Additionally, some of the findings are not consistent; therefore in this chapter of the thesis the focus was on confirming which genes are responsible for desiccation resistance of *Salmonella* cells attached to stainless steel. Moreover, the mechanisms of survival during desiccation can be studied not only by gene expression analysis, but by looking at the behaviour of the cells in different conditions such as: pre-stressing the cells by desiccation, comparing physical desiccation response of cells to the osmotic stress response, investigating the presence of key genes in strains which have different responses to desiccation or investigating better conditions for resuscitation. All of these approaches were tried in this work to better understand the underlying mechanisms of desiccation resistance of *Salmonella* which could potentially be used by food manufacturers to develop better strategies for control of this pathogen in the food processing environment.

6.2 RESULTS

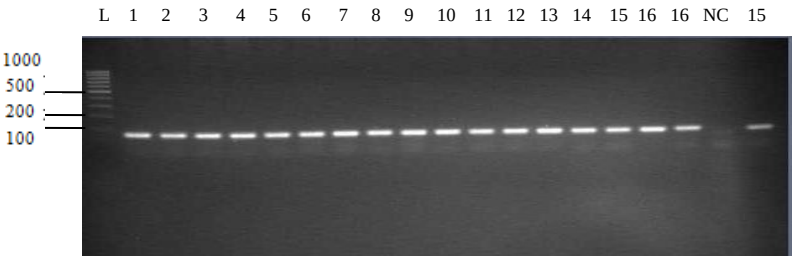
6.2.1 PCR of Selected Genes

At the time that initial gene expression studies were undertaken in this work, only a study published by Gruzdev *et al*., 2012 was of a similar nature (first global trancriptomics of desiccated *Salmonella* Typhimurium L2). Following the results of this paper, seven genes regulating different functions of the cells were selected for this work and *Salmonella* isolates described in Chapter 2 were screened for the presence of these genes. Additionally, *Salmonella* Typhimurium L2 was studied as a positive control. Six upregulated genes from different functional groups (*ddg*, *glpK*, *hisD*, *aceA*, *nifU*, and *kdpA*), displaying the highest fold change in the microarray study of Gruzdev *et al.* (2012) were selected. If these genes were mainly responsible for the survival of desiccated cells, potentially, the sensitive strains could lack some of the genes.

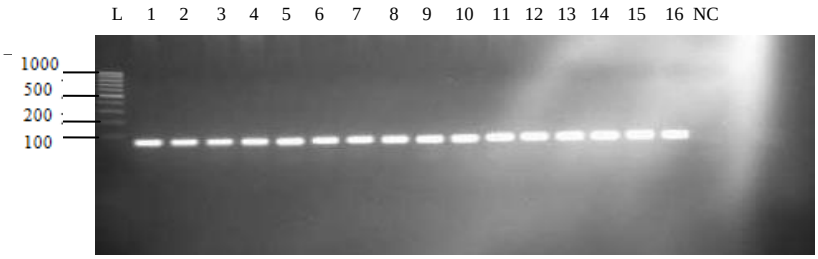
A


L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 NC
1000
500
200
100

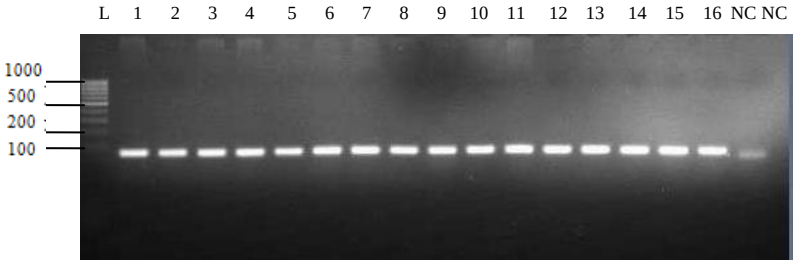
B


L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 16 NC 15
1000
500
200
100

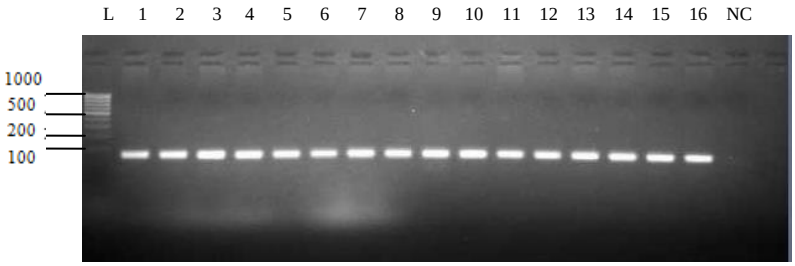
C


L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 NC
1000
500
200
100

D


L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 NC NC
1000
500
200
100

E


L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 NC
1000
500
200
100

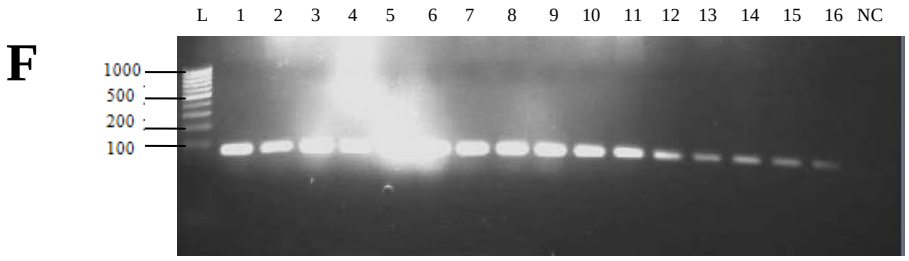


Figure 6.2. PCR results of DNA templates extracted from 16 *Salmonella* strains specific for 6 genes A) *nifU*, B) *kdpA*, C) *hisD*, D) *glpK*, E) *ddg*, F) *aceA*. 1- *S*. Agona; 2 – *S*. Napoli, 3 – *S*. Muenchen; 4 - *S*. Enteritidis; 5 – *S*. Tennesee; 6 – *S*. Typhimurium (whey powder); 7 – *S*. Typhimurium DT104; 8 – *S*. Typhimurium NCTC 12023; 9 – *S*. Typhimurium NCTC, NCOOO 74-17; 10 – *S*. Lomita (1); 11 – *S*. Lomita (2); 12 – *S*. Cubana (1); 13 - *S*. Cubana (2); 14 – *S*. Mbandaka (2); 15 – *S*. Mbandaka (1); 16 – *S*. Typhimurium L2; L – 1000 bp lambda DNA ladder, NC – negative control. Gel running conditions: 1 x TAE, 85 V, 2 h, 2 % agarose

Strains were grown and DNA was extracted according to the method in Section 2.6.2. The concentrations of DNA samples were measured using a Nanodrop spectrophotometer (Section 2.6.3). The PCR was then conducted, followed by gel electrophoresis (Sections 2.6.4, 2.6.14 and 2.6.15). Figure 6.2 shows the PCR results for the 16 *Salmonella* strains studied.

The bands were present in the expected positions (~90 bp) for the six selected genes in all strains studied, which indicates these genes may still be responsible for desiccation survival, however their involvement could be regulated by their expression rather than presence. The other possibility is that the genes are not intact as only small fragments of the whole genes are amplified during PCR and, from this data, it is not possible to assess what is the condition of the rest of the genes fragments. This experiments however, validate the suitability of the primers used for further qRT-PCR reactions.

6.2.2 Investigating the Desiccation Resistance of Cells Pre-exposed to Desiccation

As the earlier survival kinetics studies (Chapter 3) had shown that there were two sub-populations present for each strain, a more desiccation-sensitive one and a more desiccation-resistant one, studies were conducted to investigate if cells which had been previously desiccated had developed resistance to subsequent desiccation. Suspensions of *S.* Typhimurium NCTC 12023 (heat resistant; 'HR') and *S.*

Typhimurium (whey powder isolate) were prepared and inoculated on the stainless steel surfaces as described in Sections 2.2.2-2.2.3. Discs (n=3) were kept under controlled environment conditions (33% RH and 25°C). Surviving cells were subsequently recovered after 46 or 50 days (different biological replicates) by placing discs in 10 ml of MRD and vortexing for 1 minute. A loopful of suspension was streaked on TSA plates which were incubated for 24 h at 37°C. The colonies were identified as *Salmonella* Typhimurium using tests described in Sections 2.4.2-2.4.3. Three biological replicates were analysed.

Single colonies grown on TSA were transferred to 50 ml of TSB which was then incubated at 37°C for 20 hours in a shaking incubator. The same procedure was followed for *Salmonella* cultures stored on cryobeads at -80°C. The fresh inocula were prepared as described in Section 2.2.2 and transferred onto disc surfaces (Section 2.2.3). Discs were dried and kept in desiccating conditions for up to 50 days. The number of bacteria surviving on discs was assessed (Section 2.2.4) at different time periods and the log reduction from time 0 after drying to each sampling point was calculated (see Figure 6.3).

The log reductions in viable counts for *S.* Typhimurium (whey powder isolate) ranged from 2.2 to 2.8 log cfu surface^{-1} after 10 days. After 21 days, the cells were not detected for all studied conditions. There was no statistical difference between cells prepared from frozen culture and cells which were from cultures which had previously survived desiccation (both were not detected after 21 days). A similar observation was made with *S.* Typhimurium 'HR' (p-value=0.44) where, after 50 days of desiccation on stainless steel discs, the reduction levels were 2.5, 2.8 and 2.8 log cfu surface^{-1} for cells which had not been desiccated before, and cells which were desiccated for 50 and 46 days, respectively.

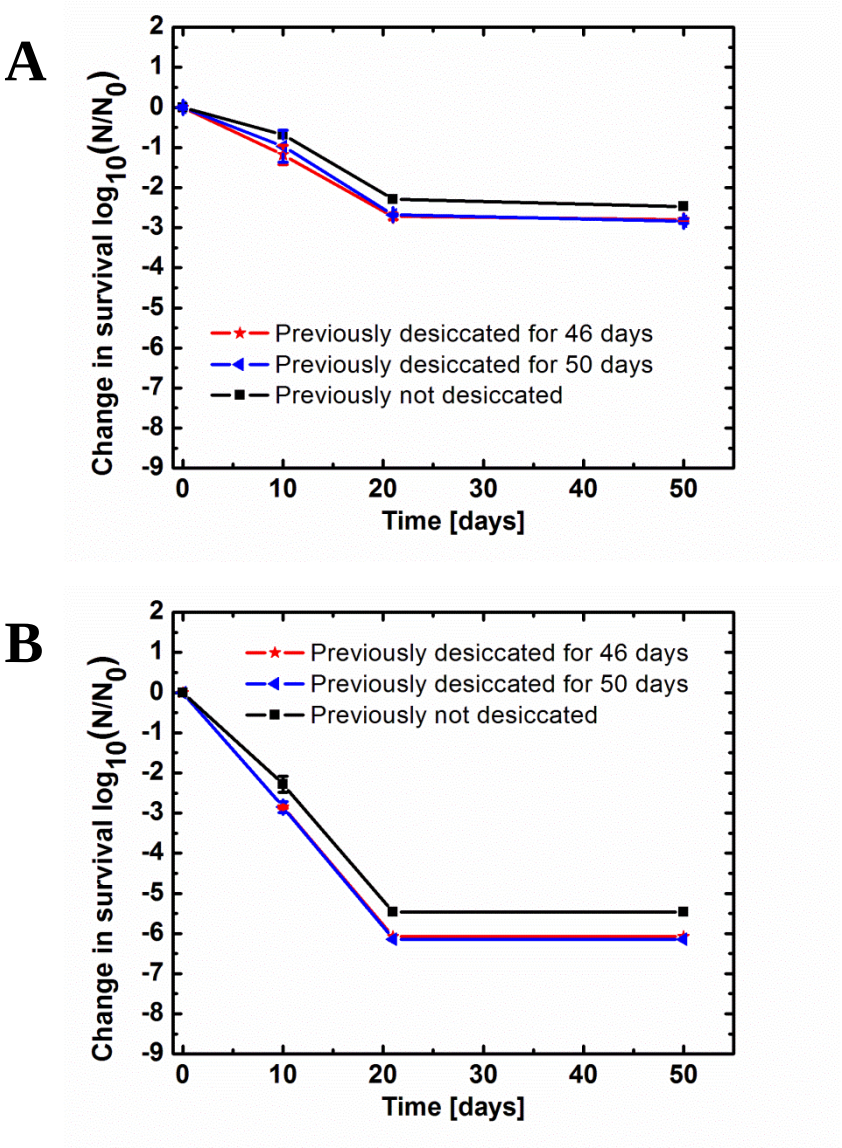


Figure 6.3 Reduction of $\log_{10}$ cfu surface^{-1} counts of two *Salmonella* strains A) *S.* Typhimurium 'HR' and B) *S.* Typhimurium (whey powder), Lines represent: red – cells previously desiccated for 46 days, recovered and subjected to desiccation for another 50 days; blue - cells previously desiccated for 50 days, recovered and subjected to desiccation for another 50 days; black - cells not desiccated previously and subjected to desiccation for 50 days. Bars represent one standard deviation.

6.2.3 Comparison of *Salmonella* Responses to Hyperosmotic Shock and Desiccation

The literature search revealed that some of the genes responsible for the osmotic shock response may be also responsible for desiccation resistance. Further experiments were conducted to compare responses of three *Salmonella* strains: *S.* Typhimurium (whey powder; sensitive to desiccation), *S.* Typhimurium 'HR' NCTC12023 (resistant to desiccation) and *S.* Napoli (originally isolated from

chocolate, therefore presumably resistant to high osmolality). The bacterial suspensions were prepared as described in Section 2.2.2. They were used directly to inoculate discs and also they were added to humectants' solutions for the osmotic responses studies.

The final concentrations of inoculated humectants were as follows:

- Sodium chloride 4.8 M (calculated a_w=0.86, measured a_w= 0.80 at 22.4°C)
- Sodium chloride 1.3 M (calculated a_w=0.96, measured a_w= 0.96 at 22.5°C)
- Sucrose 5.8 M (calculated a_w=0.86, measured a_w= 0.84 at 22.8°C)
- Glycerol 100% (measured a_w=0.08 at 23.2°C)

The discs were inoculated with 50 µl of suspension. The humectants were diluted to their final concentrations with MRD and bacterial suspension. Each humectant was added to bacterial suspensions gradually (10 ml every 10 - 20 minutes during an 80 minute period) to simulate a gradual drying process (also 80 minutes). The concentration of bacteria per disc and per ml of each humectant was designed to be the same which was approx. $1x10^{-7}$ cfu ml^{-1} or cfu $disc^{-1}$. The suspension for discs was not directly diluted, however by inoculating only 50 µl, it was diluted 12 fold. The suspension for humectants was also diluted 12 fold (2.5 ml suspension added to 47.5 ml humectant-MRD mixture).

Figure 6.4 shows the reduction of *Salmonella* counts for all three strains under the presence of three different humectants and under desiccation conditions over the 48 days period. For all strains studied, the presence of 100% glycerol caused a very rapid decrease in colony count, with numbers falling below detectable levels (0.5 cfu ml^{-1} or 5 cfu $disc^{-1}$) after 3 days. Similar results were observed for high concentrations of sodium chloride 4.8 M (a_w=0.86, only undertaken for *S.* Typhimurium 'HR' (NCTC 12023) and *S.* Typhimurium (whey powder), however the decline in viable counts was faster for *S.* Typhimurium isolated from whey powder (after 5 days counts dropped to below detectable levels; Fig 6.4C), as opposed to *S.* Typhimurium 'HR' (after 7 days counts dropped to below detectable levels; Fig 6.4B). Sodium chloride in concentration of 85% and glycerol in 100% concentration are often used as antimicrobials (Saegeman *et al.*, 2008) , therefore the results were as should be expected. The low concentration of sodium chloride seemed to have no large effect on *S.* Napoli and *S.* Typhimurium 'HR' (NCTC 12023) with no significant reduction after 48 days.

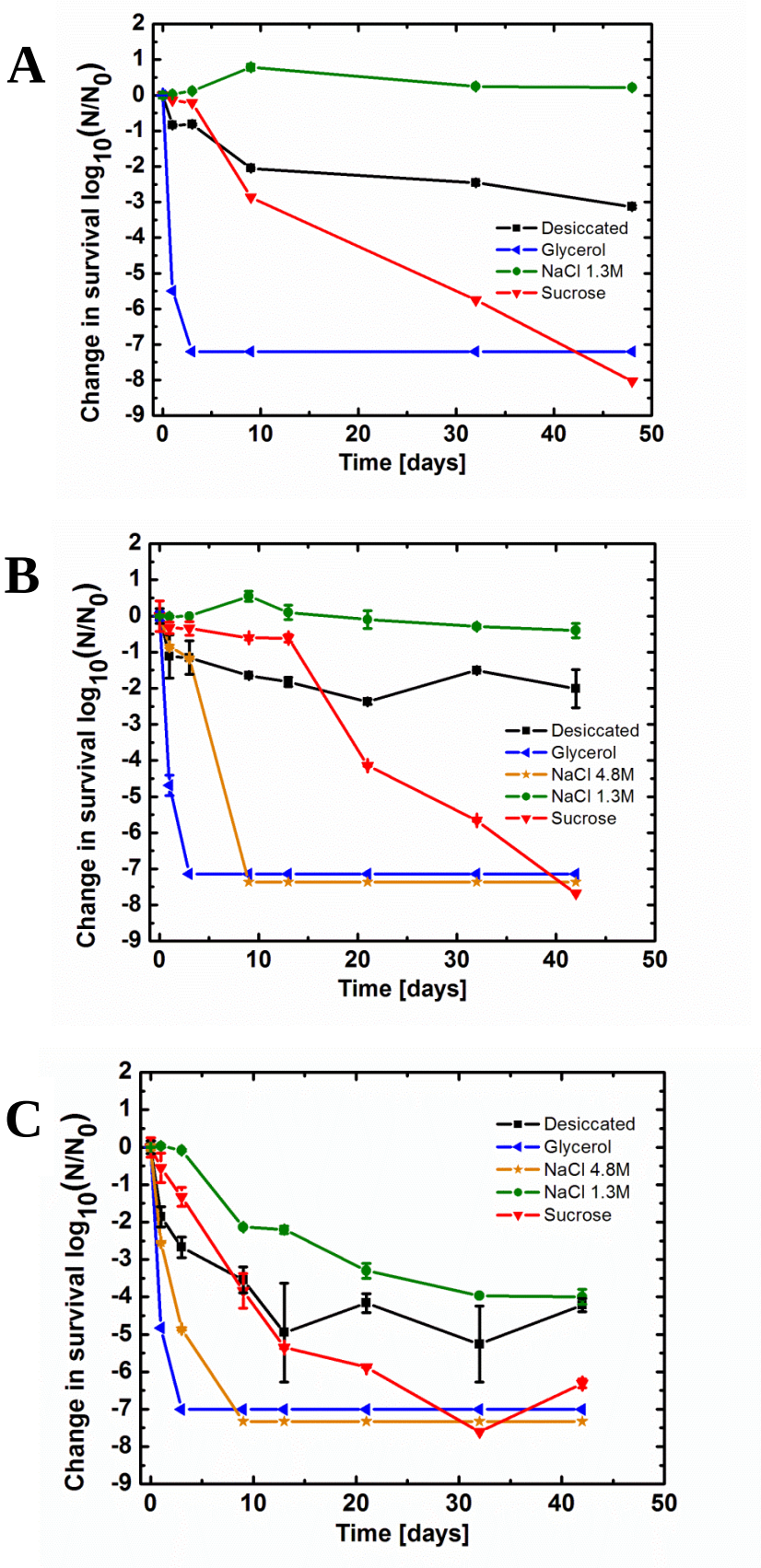


Figure 6.4. Resistance of three *Salmonella* strains to desiccation and osmotic stress A) *S*. Napoli, B) *S*. Typhimurium 'HR' (NCTC 12023) and C) *S*. Typhimurium (whey powder)

However, a significant effect was observed on *S.* Typhimurium isolated from whey powder (4.0 log_{10} cfu ml^{-1} reduction).

There was some correlation between cells immersed in sucrose solution and cells desiccated on stainless steel discs for the first 13 days of the trial for all strains. After 13 days, cells immersed in sucrose solution continued to die off and the numbers of cells inoculated onto stainless steel remained constant.

6.2.4 Recovery of Desiccated Cells with Betaine Solution

One of the known response mechanisms of *Salmonella* to osmotic stress is the accumulation of compatible solutes such as betaine. *S.* Typhimurium (whey powder) and *S.* Typhimurium NCTC 12023 'HR' were used to study the influence of the presence of betaine in a recovery medium for desiccated cells. Discs were inoculated as described in Sections 2.2.2-2.2.3 and were kept under desiccation conditions for 290 days.

The recovery of bacteria from a sub-set of the discs (n=3) was performed with 10 ml PBS solution, according to the standard procedure described in Section 2.2.4. The remainder of the discs were recovered using 10 ml of 1 mM betaine diluted in PBS. The number of bacteria surviving on discs and recovered with the two different solutions was assessed and the average log reduction from time 0 to 290 days after drying was calculated (Table 6.1). The experiments were repeated three times.

Table 6.1 Recovery of *Salmonella* strains after 290 days under desiccating conditions with or without betaine in the recovery medium

	S. Typhimurium (whey powder)		*S.* Typhimurium 'HR' (NCTC 12023)	
	Log_{10} cfu $surface^{-1}$	Log_{10} cfu $surface^{-1}$ reduction	Log_{10} cfu $surface^{-1}$	Log_{10} cfu $surface^{-1}$ reduction
Inoculum	6.25±0.36	0.00±0.36	6.68±0.50	0.00±0.50
Recovery without betaine	<0.7±0.00	>5.55±0.00	2.19±0.13	4.50±0.13
Recovery with betaine	<0.7±0.00	>5.55±0.00	1.06±0.51	5.62±0.51

S. Typhimurium (whey powder) cells were not recovered after 290 days under desiccation conditions despite the recovery solution used. *S.* Typhimurium 'HR' cells were recovered from both solutions, however, the recovery without betaine was significantly higher than recovery with betaine (p-value=0.04).

6.2.5 Gene Expression Studies

6.2.5.1 Method Summary

Two isolates were chosen for further gene expression studies, one of which is more resistant: *S.* Typhimurium 'HR' (NCTC 12023) and one more sensitive to desiccation: *S.* Typhimurium (whey powder). The bacterial overnight suspensions were prepared as described in Section 2.2.2. For both strains, cells in suspension and cells dried on stainless steel discs were analysed for expression of selected genes. The RNA was extracted at different time points as described in Figure 6.5. The time points were selected based on the survival kinetics generated in Chapter 3.

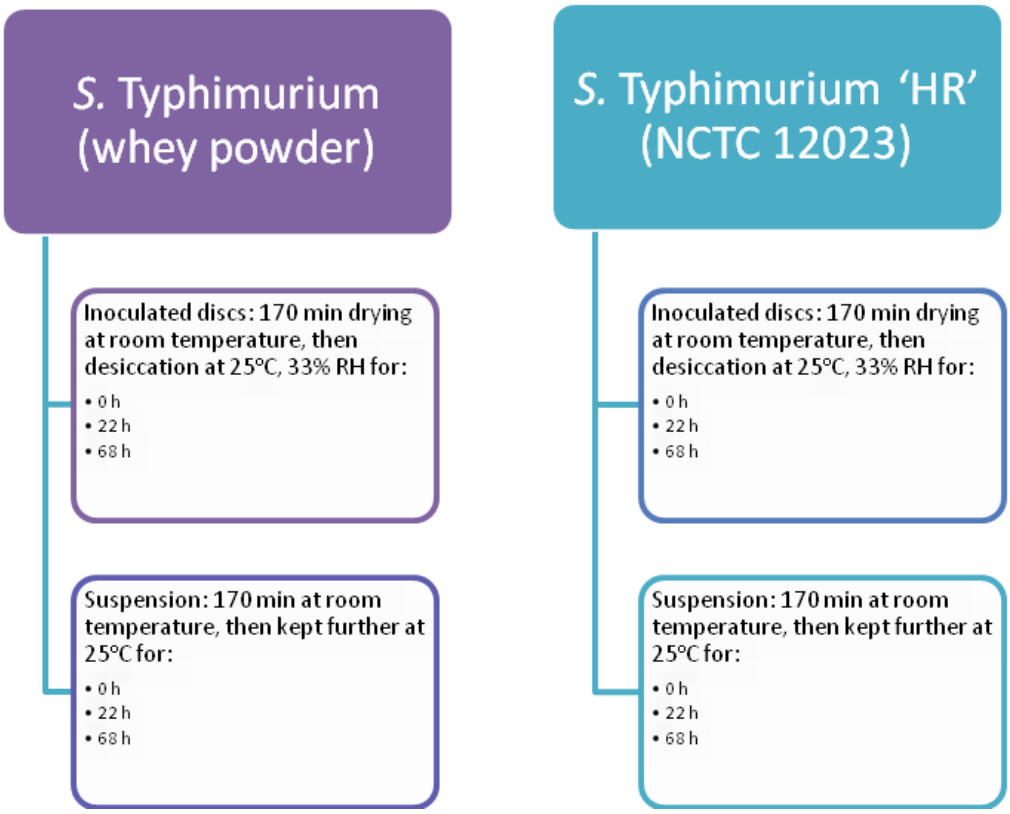


Figure 6.5 Experimental design for gene expression studies

Before real-time RT-PCR was undertaken, a range of protocols were used to extract RNA with the aim of achieving good quality, quantity and purity of RNA (§2.6.6-2.6.8). After RNA extraction, based on NanoDrop spectrophotometer readings (§2.6.3), a subsample of each RNA sample was made and if the concentration was high enough, it was diluted with RNAase-free water so all samples had the same concentration and then were stored at -80^{o}C.

All samples underwent the reverse transcription procedure. cDNA was generated from an RNA population using a Transcriptor First Stand cDNA Synthesis kit (Section 2.6.10). Random hexamers instead of oligo (dT) primers were used to convert RNA to cDNA because bacteria do not have stable poly (A)+ tails associated with eukaryotic RNA. The stock solutions of cDNA samples were stored frozen (-20^{o}C) and were used to make standard curves and analyze gene expression of bacterial cells. Two control samples were performed during reverse transcription. The first one was with use of template RNA supplied with the kit and the second was with isolated RNA samples but without use of transcriptase.

Each individual cDNA was stained with OliGreen reagent together with a water control (Section 2.6.11). A cDNA standard pool was made using each cDNA sample. It was then diluted 1:10 or 1:2 (10 further dilutions) and run with all primers. Based on the readings obtained, each cDNA was diluted so it fell within the standard curve.

SYBR Green 1 nonspecific fluorescent dye was used for monitoring real-time amplification and melting point analysis was performed to analyse if only one amplicon was present (Section 2.6.16). To ensure that generated real-time RT-PCR data were of high quality, several quality control steps including negative and positive controls were undertaken. Two negative controls were run: a no-template control and a no-reverse-transcriptase control. The first control was to confirm that no contamination was present in the PCR reagents and the second control was to ensure there was no contaminating DNA in the RNA samples.

In addition, each sample underwent real-time RT-PCR reaction in triplicate together with diluted pooled samples to determine amplification efficiencies. If the difference in efficiency of different samples is not considered, there can be systematic errors in calculating the amount of target sequence present in the sample.

The Pfaffl method described in Section 2.6.17 was used to calculate the fold change of genes expressed during desiccation as compared with non-desiccated samples at the same time point. The fold change was than adjusted according to the changes of the housekeeping gene. Figure 6.6 presents the steps taken before and during real-time RT-PCR analysis of selected genes.

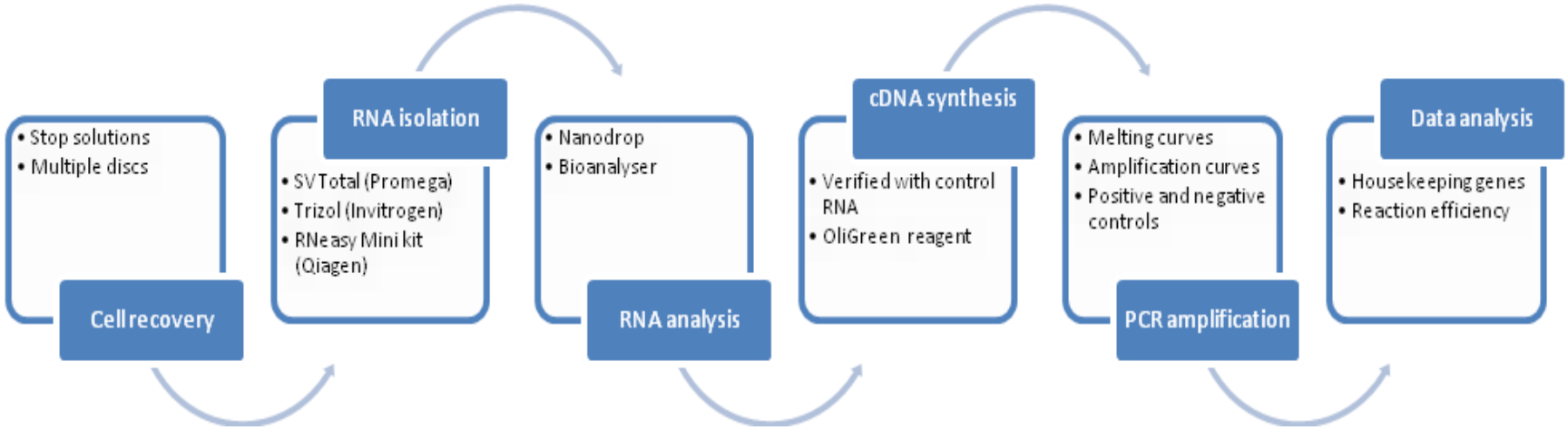


Figure 6.6. Real-time RT-PCR schematic including controls and validation steps

6.2.5.2 Selection of Target Genes

The literature search revealed that some of the genes responsible for the osmotic shock response may also be responsible for desiccation resistance. The results of the experiments in Section 6.2.3 of this chapter also indicated that at least at the beginning of the desiccation and osmotic stress process, the responses might be the same. That is why some of the genes responsible for the osmotic stress responses were investigated in this study. The reasoning for the selection and the additional genes selected are described in Table 6.2.

Table 6.2 Genes selected for real-time RT-PCR studies. Housekeeping genes are highlighted in grey.

No.	Gene (LT2 ortholog)	Function	Reason
1	*dnaK* (STM0012)	Heat shock protein	Expression increased significantly in 2 studies (Gruzdev *et al*., 2012 and Deng *et al*., 2012)
2	*kdpA* (STM0706)	K+ uptake	Osmotic shock gene, induced in Gruzdev *et al.*, 2012, mutant had lower survival in long term desiccation studies
3	*otsB* (STM1929)	Catalyzes the formation of trehalose, dependent on *rpoS* protein	Aviles *at al*. 2013, induced in 2 studies (Li *at al*., 2012 and Finn *et al.*, 2013)
4	*aceA* (STM4184)	Isocitrate lyase, carbon flow in the cell, energy production and conversion	Potentially released during osmotic stress, induced in 2 studies (Gruzdev *et al*., 2012 and Li *et al*., 2012)
5	*gltB* (STM3330)	Glutamate regulation, glutamate synthase large subunit	Glutamate accumulates after osmotic shock (Moat *et al*., 2002), Induced in 2 studies (Gruzdev *et al.*, and Li *et al.*, 2012)
6	*prpE* (STM0371)	Growth on propionate	Induced in 2 studies (Gruzdev *et al*., 2012 and Li *et al*., 2012), responsible for fatty acids metabolism
7	*nifU* (STM2542)	Involved in Fe-S cluster assembly	Mutant decreased resistance to desiccation in Gruzdev *et al.*, 2012
8	*ddg* (STM2401)	Lipid A biosynthesis lauryl acyltransferase	Outer membrane fluidity control. In Gruzdev *et al*., 2012 mutant decreased resistance to desiccation

No.	Gene (LT2 ortholog)	Function	Reason
9	*proU* (STM2809-2811)	ProXVW - involved in proline metabolism	ProU – high affinity betaine transporter and low affinity proline transporter (both are known osmoprotectants). Induced in Li *et al.*, 2012 and Finn *et al*., 2013
10	*proP* (STM4290)	Hypothetical amino acid permease	Amino-acids transport and metabolism. Induced in Finn *et al*., 2013
11	*osmU* (STM 1491-1494)	Synthesis of a periplasmic trehalose	Operon consisting of 4 genes responsible for transport of osmoprotectants (Frossard *et al*., 2012, Finn *et al*., 2013)
12	*rsu*A (STM2222)	Coding for 16S rRNA pseudouridylate synthase A	Did not exhibit differential expression during desiccation in Gruzdev *et al*., 2012, Deng *et al*., 2013 and Aviles *et al*. 2013
13	*gap*A (STM1290)	Coding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase	Was found not to change expression in Finn *et al*., 2013
14	*agp* (STM1117)	Glucose-1-phosphatase	Housekeeping gene in Li *et al*., 2012
15	*ffh* (STM2677)	signal recognition particle protein	-

6.2.5.3 Gene expression analysis with the SV Total RNA Isolation System (Promega) used for RNA extraction

Discs were inoculated with one drop of 50 µl of bacterial suspension prepared as described in 2.6.5 and were placed in a safety cabinet for 170 minutes to dry. The remaining bacterial suspension in a closed container was also placed in a safety cabinet. Immediately after this, extraction using the SV Total RNA Isolation System was undertaken with 3 different recovery methods (Section 2.6.6):

a) Recovery with stop solution, lysozyme and lysis buffer
b) Recovery with lysozyme and lysis buffer
c) Recovery with lysis buffer

After addition of lysis buffer the instructions of the SV Total RNA Isolation System technical Manual were followed (Section 2.6.6). The isolated RNA was then analysed using the NanoDrop spectrophotometer (Section 2.6.3) and the results of

this analysis are shown in Table 6.3. The concentration of RNA and A260/A280 and A260/A230 ratios are shown as these reflect the quantity and quality of obtained RNA.

The three methods gave very similar results (Table 6.3), therefore it was decided to further develop the method A, due to the use of stop solution, which is important to protect RNA from further changes. The A260/A280 values were in most cases higher than 1.80, which means that samples where free of contaminants which absorb at or near 280 nm. However the 260/230 values were low (0.76-1.82) which indicates the presence of contaminants which absorb at 230 nm. The low yield (maximum of 33.49 ng μl^{-1}) was also observed.

Table 6.3 RNA concentrations and quality obtained using SV Total RNA isolation kit with three different recovery methods, A) with stop solution, lysozyme and lysis buffer, B) with lysozyme and lysis buffer and C) with lysis buffer. S – suspension, D – cells desiccated on discs

Method	Sample ID	Type	ng μl^{-1}	260/280	260/230
A	S. Typhimurium	S	33.49	1.97	1.49
A	S. Typhimurium 'HR'	S	7.98	1.64	0.76
A	S. Typhimurium	D	34.48	2.05	1.82
A	S. Typhimurium 'HR'	D	15.29	1.99	1.44
B	S. Typhimurium 'HR'	S	23.08	2.01	1.55
B	S. Typhimurium 'HR'	D	18.36	1.97	1.55
C	S. Typhimurium 'HR'	S	18.28	1.85	1.16
C	S. Typhimurium 'HR'	D	13.57	1.82	1.33

The amount of starting material was increased to raise the RNA concentration. In further experiments 3 drops of 50 µl bacterial suspension were used for disc inoculation and suspension testing. A full trial with RNA extraction at all time-points (0, 22 and 68 h) was conducted. The yield increased for *S.* Typhimurium samples and the concentration ranged from 51.00 to 1835.12 ng μl^{-1}, however for most of the samples it stayed in the lower range. The concentrations of RNA isolated from *S.* Typhimurium 'HR' samples were lower, and ranged from 19.4 to 405.1 ng μl^{-1}, additionally the quality of RNA stayed low (260/230 values of 0.2 – 1.5).

Two samples were precipitated by adding NaCl and ethanol (Section 2.6.9) to improve the quality of the RNA, and the results obtained are shown in Table 6.4.

Table 6.4 RNA concentrations and quality obtained using SV Total RNA isolation kit with stop solution, lysozyme and lysis buffer and 3 drops of 50 µl bacterial suspension for each sample, purified with NaCl and ethanol.

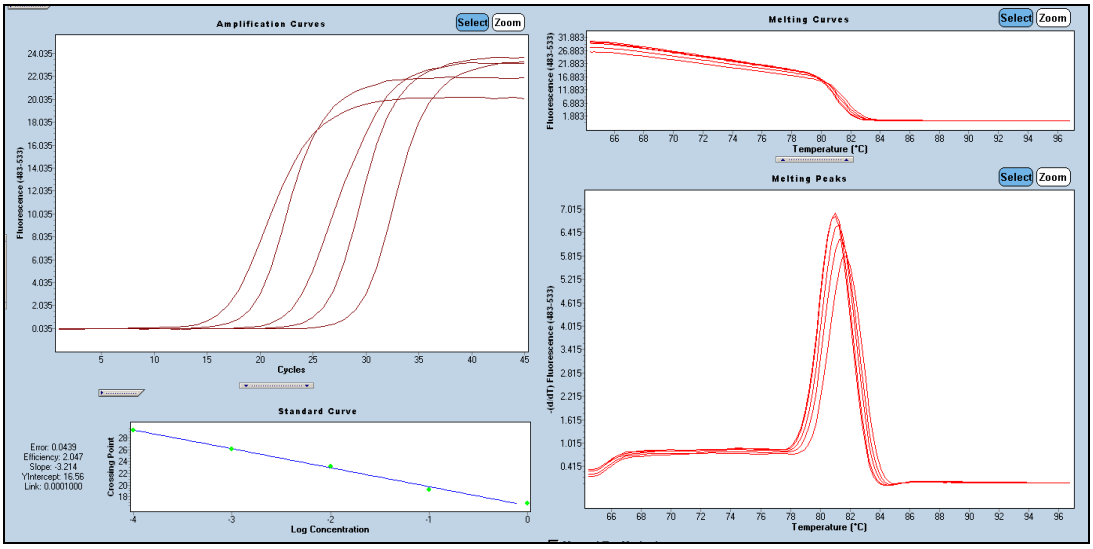
Purification	Time (h)	Strain	Type	ng μl^{-1}	260/280	260/ 230
Before	0	*S.* Typhimurium	S1	124.89	1.9	0.82
	0	*S.* Typhimurium 'HR'	D2	43.82	1.76	0.51
After	0	*S.* Typhimurium	S1	24.1	1.64	0.56
	0	*S.* Typhimurium 'HR'	D2	81.7	1.81	1.08

The *S.* Typhimurium sample had lower concentrations and quality after precipitation than before treatment (Table 6.4). The *S.* Typhimurium 'HR' sample showed a slight improvement in 260/230 values, however they were still low (1.08). The rest of the samples were not treated with NaCl and ethanol.

All RNA samples were reverse transcribed using the Roche kit and the cDNA was analysed using Oligreen reagent. The obtained values of Oligreen run were higher for *S.* Typhimurium (whey powder) than for *S.* Typhimurium 'HR' with an average of 17.11 and 3.67, respectively. This indicates that that RT reaction efficiency was higher for *S.* Typhimurium (whey powder) RNA samples as the starting concentration of RNA samples was the same for both isolates.

Equal volumes of all cDNA samples for each strain were pooled together to create a standard pool which was used to create the standard curve. The samples were run on a LC480 light cycler (Section 2.6.16) to check linearity/efficiency and hence suitability. This was done for 7 genes: *rsuA* (housekeeping gene), *kdpA, aceA, gltB, prpE, nifU, ddg*. Examples of standard curves and melting points are shown in Figure 6.7. The standard curves covered the exponential phase between 15 and 30 cycles which gave an indication of how single RNA templates should be diluted to fall between 20 and 25 cycles. The melting points showed only one temperature peak for all the replicates indicating there are no contaminants with different melting points. An aliquot of each cDNA sample was taken and diluted appropriately so as to fall within the standard curve – this was the 'working' cDNA for each sample.

A



B

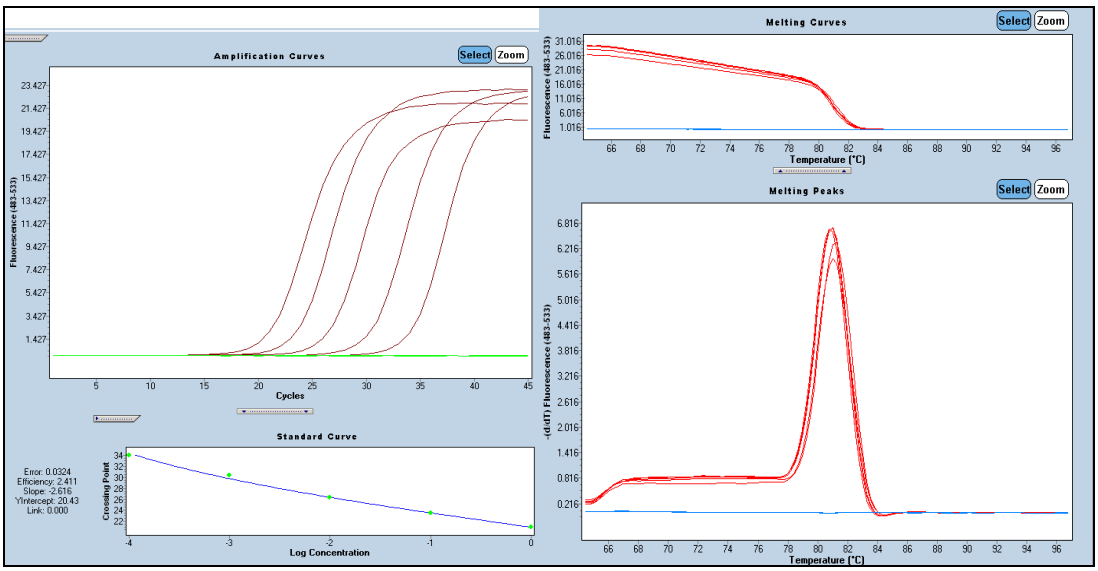


Figure 6.7 Examples of standard curve and melting points for *aceA* gene A) *S.* Typhimurium standard pool, B) *S.* Typhimurium 'HR' standard pool

The real-time RT-PCR was performed for four genes: *rsuA* (housekeeping), *kdpA*, *aceA* and *gltB*. The amplification efficiency (Ep) was close to 2 for all genes studied and for both strains (Table 6.5). The details of the calculations are described in Section 2.6.17. The fold change (FC) was very low and similar for all genes studied (Table 6.5) with the rsuA gene which was chosen as a housekeeping gene having the highest fold change of expression (3.56 fold change for S. Typhimurium 'HR' at 22h). For this reason the fold change normalised against housekeeping gene was not calculated.

This indicated that the quality and quantity of RNA are not sufficient for real-time RT-PCR, therefore a different RNA extraction method was investigated.

Table 6.5 Results of real-time RT-PCR

Gene	Time (h)	S. Typhimurium (whey powder)					S. Typhimurium 'HR' (NCTC 12023)				
		Ep	C_T susp	C_T disc	ΔC_T (s-d)	FC	Ep	C_T susp	C_T disc	ΔC_T (s-d)	FC
rsuA	0	2.05	16.39	17.73	-1.33	0.38	1.58	14.84	13.88	0.95	1.54
	22		16.08	16.70	-0.63	0.64		16.69	13.89	2.79	3.56
	68		16.59	17.75	-1.15	0.44		14.96	13.5	1.46	1.94
kdpA	0	1.92	24.99	26.54	-1.55	0.36	1.89	24.63	25.78	-1.14	0.48
	22		24.86	25.30	-0.44	0.75		26.14	24.84	1.30	2.28
	68		25.54	26.24	-0.70	0.63		24.82	25.31	-0.49	0.73
aceA	0	1.91	24.96	26.08	-1.12	0.48	1.97	24.81	25.75	-0.94	0.53
	22		24.79	25.32	-0.53	0.71		26.47	25.16	1.31	2.44
	68		25.53	25.76	-0.23	0.86		25.33	25.86	-0.54	0.69
gltB	0	1.92	26.25	26.81	-0.55	0.70	1.98	25.42	26.74	-1.32	0.41
	22		25.58	27.10	-1.52	0.37		26.99	25.81	1.17	2.23
	68		25.54	26.63	-1.09	0.49		26.21	26.23	-0.02	0.99

(Section 2.6.17 for calculation methods), Ep – amplification efficiency; CT – crossing point; FC – fold change; FCN – fold change normalised against housekeeping gene, HK – housekeeping gene; ΔC_T (s-d) - the difference between average C_T of samples recovered from suspension and samples dried on discs, at the same time point (§2.6.17).

6.2.5.4 Gene Expression Analysis with the Trizol (Invitrogen) and RNeasy Mini kit (Qiagen) Used for RNA Extraction and Purification

Bacterial suspensions were grown as described in Section 2.6.5, however for validation of the housekeeping genes, the RNA was also extracted after 16 hours, 20 hours and 24 hours of incubation of a working culture. The bacterial suspension used for further gene expression studies was incubated for 20 h. The RNAprotect (Qiagen) was used as a stop solution, Trizol (Invitrogen) was utilized to extract RNA and the RNeasy Mini kit (Qiagen) was used to clean extracted RNA (Section 2.6.7).

During the first experiment 3 drops of 50 µl bacterial suspension were used for disc inoculation and suspension testing. The concentrations of RNA were very low (8-100 ng μl^{-1}, results not shown) and, additionally, they had to be further diluted to obtain the same final concentration for all RNA templates. The final concentration was approximately 10 ng μl^{-1} and A260/A230 ratios were in most cases below 1. The 260/280 ration were between 2.5 and 1.5 which could be acceptable (results not shown).

Very low yields of RNA were obtained, which also resulted in very low quantities of cDNA as was seen after analysis with Oligreen reagent (maximum value 2.32, results not shown). Real-time RT-PCR was performed on standard pools of cDNA templates for both strains. The genes which were analysed, were the selected housekeeping genes: *gapA, agp, rsuA* (1^{st} primer set), *ffh, rsuA* (2^{nd} primer set) and one target gene: *kdpA.* Standard pools were diluted and standard curves were created. The efficiency for all genes ranged from 2.00 to 2.39 however the concentrations were so low that in some cases (e.g. *kdp, ffh*) there was no amplicon detected on dilutions -3 and -4 of the standard pools (results not shown).

The gene expression of the selected housekeeping genes *rsuA, agp, gapA* and one target gene *prpE* in the individual cDNA samples was also analysed. Figure 6.8 shows the amplification curves for individual cDNA samples, standard pools and melting points for the *gapA* gene for both *Salmonella* strains.

The amount of cDNA was very low, and in many cases the Ct was above 35 cycles (results not shown). This is not ideal and often the standard curve had to be extrapolated to obtain the Ct values for individual samples.

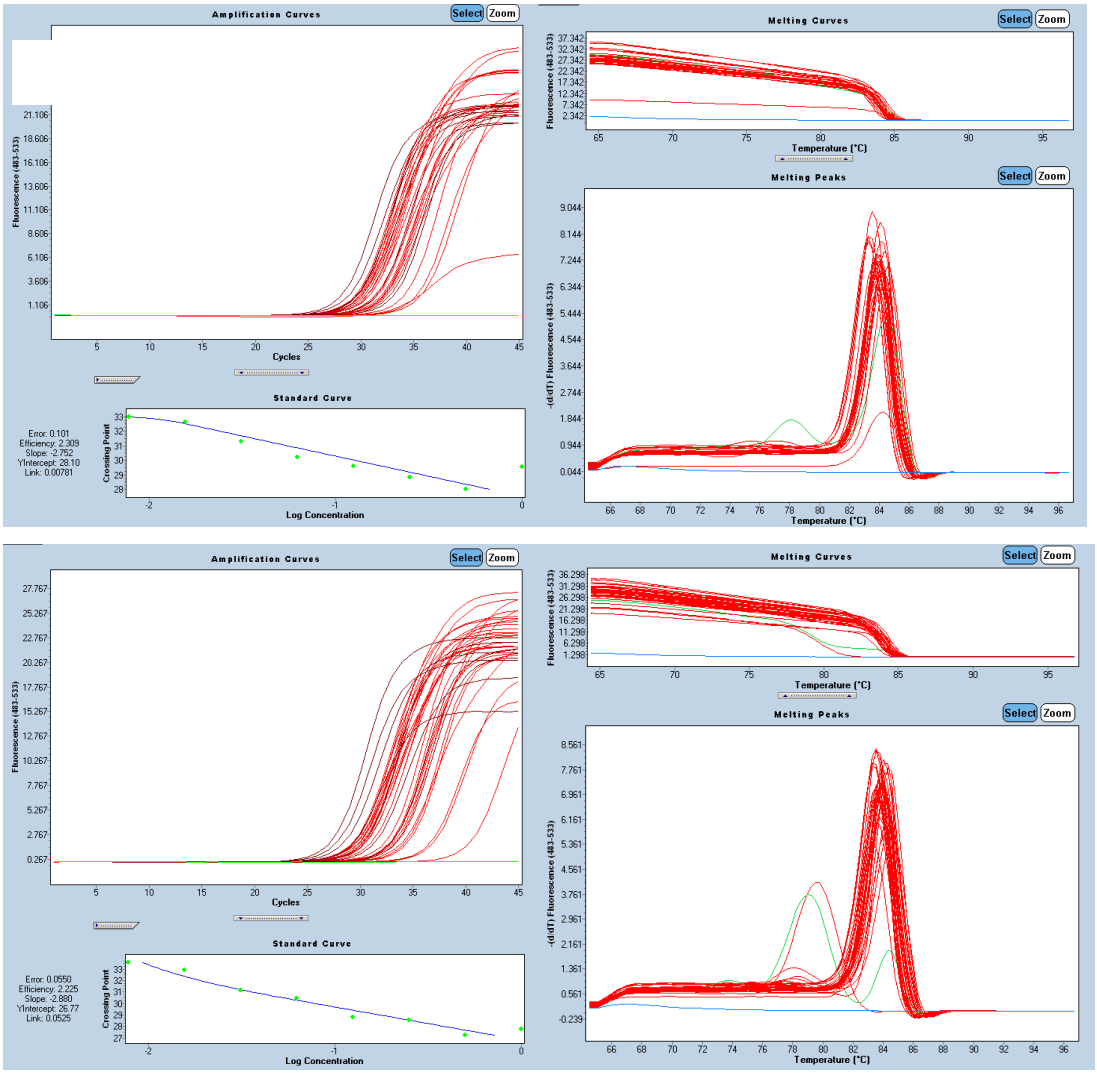


Figure 6.8 Examples of standard curve, individual samples curves and melting points for *gapA* gene A) *S.* Typhimurium, B) *S.* Typhimurium 'HR'

The results obtained with such small quantities of cDNA are not reliable and even though the results for some housekeeping genes studied did not indicate large changes in expression (e.g. rsuA: 0.1-2.9 fold change), this work had to be repeated to obtain higher cDNA levels and therefore lower Ct values. Some of the negative controls had small amounts of cDNA, however with different melting points, which indicates that this did not interfere with the results (Figure 6.8).

A further experiment of RNA extraction was conducted to investigate the suitability of housekeeping genes and to increase the amount of RNA extracted. *S.* Typhimurium 'HR' was only used for this trial. The RNA was extracted as described above, although this time RNA samples were diluted to 25 ng μl^{-1} so there was a higher amount available for the RT reaction. The samples were extracted during different growth times of the *Salmonella*, after 16, 20 and 24 hours of growth. If

housekeeping genes are suitable, there should be no changes in their expression during different growth times.

After undertaking NanoDrop readings, the RNA samples were also analysed using a Bioanalyser (see Figure 6.9). The NanoDrop readings showed low 260/230 values (0.6 - 0.8). The Bioanalyser results also indicated poor quality and integrity of the RNA from both trials: with RNA diluted to 10 ng μl^{-1} and to 25 ng μl^{-1} as there was neither 18S or 28S ribosomal band present and a shift towards shorter fragment sizes was observed. The RNA integrity numbers (RIN) for all the samples ranged from 1.8 to 2.7 (results not shown). The examples are shown in Figure 6.9.

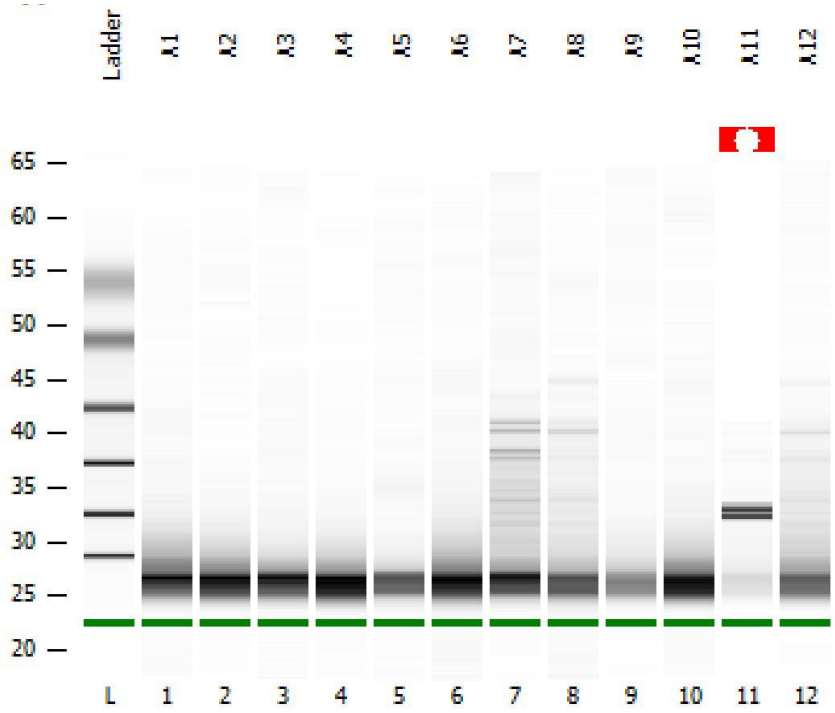


Figure 6.9 Bioanalyser results of RNA extracted using Trizol.
A1 – S. Typhimurium 'HR', inoculum grown for 16 hours
A2 – S. Typhimurium 'HR', inoculum grown for 20 hours
A3 – S. Typhimurium (whey powder), inoculum grown for 20 hours
A4 – S. Typhimurium 'HR', inoculum grown for 24 hours
A5 – S. Typhimurium (whey powder), suspension at 0 h time point
A6 – S. Typhimurium 'HR', suspension at 0 h time point
A7 – S. Typhimurium (whey powder), cells desiccated on disc at 0 h time point
A8 – S. Typhimurium 'HR', cells desiccated on disc at 0 h time point
A9 – S. Typhimurium (whey powder), suspension at 22 h time point
A10 – S. Typhimurium 'HR', suspension at 22 h time point
A11 – S. Typhimurium (whey powder), cells desiccated on disc at 22 h time point
A12 – S. Typhimurium 'HR', cells desiccated on disc at 22 h time point
For good quality of RNA bands at 40 and 45 positions should be present

Despite the quality of RNA the real-time RT-PCR was also run to check if amplification was present. The genes which were investigated were: *rsuA, gapA, agp* and *prpE*. The Ct values were very high (above 30) which suggests the amount of initial cDNA was very low (results not shown).

Following the above experiments, the next trial was conducted to check if an increased amount of bacterial culture would give better results in terms of quality and quantity of RNA. Two drops of 50 µl bacterial suspension were inoculated onto each disc. After initial drying of the discs (Section 2.6.5), 5 discs of each isolate were placed in 10 ml of Bacteria RNAprotect and 10 drops of bacterial suspension were transferred to 9.5 ml of RNA protect. The results were better in terms of concentration (diluted to approximately 60 ng μl^{-1}, results not shown), however the quality remained poor (260/230 ratio 0.7 – 0.9). The pool of the samples was analysed by real-time RT-PCR to access if any amplification was present. The Ct values were in some cases still above 30 cycles and the fold change values were similar for each gene (results not shown).

The RNA concentrations were increased to 60 ng μl^{-1} therefore to give higher cDNA quantity and better amplification curves. This however did not happen; therefore a troubleshooting for the RT reaction was undertaken. The controls were run to check the efficacy of the RT process. The control RNA template (50 ng μl^{-1}) from a Roche kit was used. It was reverse transcribed and later used at the initial concentration but also diluted 10^{-1} and 10^{-2}. These products were then amplified using standard PCR with primers supplied by an RNeasy Mini kit manufacturer (§ 2.6.14 and 2.6.15). The PCR was followed by gel electrophoresis (Figure 6.10) which did not show any bands on the gel indicating, therefore, that there was no cDNA present.

Additionally real-time PCR was run with samples where RNA was reverse transcribed with RT polymerase and also where RNA was put through the same conditions, however without RT polymerase. This was done to check if the RNA itself had any DNA contamination. Only four templates were chosen to perform this control analysis (results not shown). Two genes were selected: *hisD* and *gapA* and tested on both strains. There was similar amplification (results not shown) from cDNA samples and from samples which were put through the RT reaction but without reverse transcriptase (RNA samples). This indicates that cDNA was not

transcribed and contains the same contamination which was present in the original RNA samples.

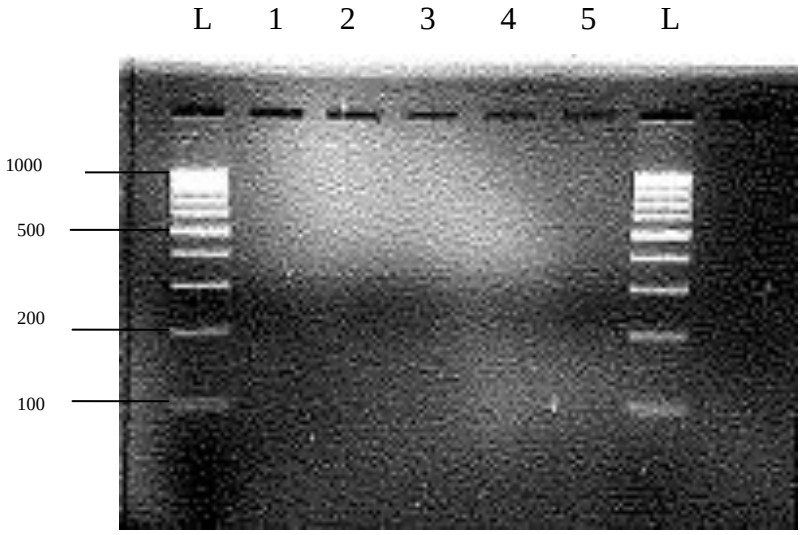


Figure 6.10 PCR results of reverse transcribed control RNA, 1 – RNA 1:1, 2 – RNA 1:10, 3 – RNA 1:100, RNA without RT polymerase, 5 – water control, L – 1000 bp ladder. Gel running conditions: 1 x TAE, 85 V, 2 h, 2 % agarose

The final trial was conducted to investigate if the reagents were contaminated with DNase/Rnase which could affect the integrity of the RNA. An *E. coli* strain was also used to check if the *Salmonella* strains used were particularly difficult to extract RNA from. An additional method described in the Rneasy Mini kit handbook (2.6.8) was also tried. All extractions of RNA were performed with new reagents and tubes. All methods resulted in low concentrations and low quality of RNA as indicated by the 260/230 ratios (Table 6.6).

6.2.5.5 Gene expression analysis with the RNeasy Mini Kit (Qiagen) used for RNA extraction

Initial trials were performed to establish if the method was effective in extracting good quality RNA from desiccated cells, if the kit was not contaminated and cannot negatively affect the quality of RNA (therefore new and old kits were used) and also to investigate if the strains were specifically challenging in terms of RNA extraction. Apart from both *Salmonella* strains, *E. coli* K12 MG1655 was also used. One *S.* Typhimurium 'HR' sample was directly isolated from the LB broth. Suspensions were prepared as described in Section 2.6.5, and a portion was used to inoculate stainless steel discs which were dried for 170 minutes. Each disc was inoculated with 3 droplets of 50 µl.

Table 6.6 NanoDrop readings of selected RNA samples and concentration of bacteria at studied time points

Time	Strain	Type	Method	Cfu ml^{-1}	NanoDrop after extraction ng μl^{-1}	260/280	260/230
0	*S.* Typhimurium	D	2.6.7	$2.55x10^{8}$	97.2	2.07	0.86
0	*S.* Typhimurium 'HR'	D	2.6.7	$2.02x10^{9}$	235.3	2.13	1.02
0	*S.* Typhimurium 'HR'	S	Rneasy Handbook	$1.43x10^{7}$	157.1	2.01	0.96
0	*E. coli* K12 HG 1655	S	2.6.7	$3.17x10^{7}$	83.3	1.62	0.57

The RNA was extracted from both, surface attached cells (5 discs for each sample) and from suspensions (15 times 50 µl) directly after drying the inoculum (time 0) using RNeasy Mini Kit (Qiagen) as described in Section 2.6.8.

The results improved significantly when compared with previous methods (Table 6.7). The yield was much higher and the 260/280 and 260/230 values in an acceptable range. The *E. coli* strain had much better RNA quality and quantity than both *Salmonella* strains.

Table 6.7 NanoDrop readings of selected RNA samples

Strain	Condition	RNA kit	Nanodrop after extraction ng μl^{-1}	260/280	260/230
S. Typhimurium	D	new	988.15	1.95	2.11
S. Typhimurium 'HR'	D	old	791.09	1.74	2.10
S. Typhimurium 'HR'	S	new	629.88	1.59	2.10
S. Typhimurium 'HR'	LB	new	581.99	1.27	2.06
E. coli	S	new	2508.8	1.90	2.10
E. coli	D	new	1307.9	2.02	2.14

Following a successful preliminary trail, the RNA extraction was conducted following the same protocol. The amount of bacterial suspension used for disc inoculation and for RNA isolation was decreased to 10 times 50 µl. After specified time periods, discs and bacterial suspensions were transferred to a stop solution (5:1 RNAprotect reagent to PBS).

Table 6.8 shows the results of RNA quantity and quality from 3 different biological replicates. All samples were diluted to a concentration of 100 ng $\mu l^{-1.}$

RNA integrity was also analysed using a Bioanalyser and examples of results are given in Figure 6.11. Samples 1-4 show the results of selected *S.* Typhimurium RNA and samples 5-6 shows the results of *E. coli* isolated RNA. The difference in integrity is very large. The *E. coli* samples show an RNA integrity number (RIN) above 8.0 (results not shown). All *Salmonella* samples show an average integrity of 4.0 RIN (results not shown). As many of the isolation methods were already tried and some of the literature suggests that good real-time RT-PCR results can also be obtained with lower RNA quality, the RT and real-time PCR were undertaken (Fleige and Pfaffl, 2006; Gingrich, 2006).

RT was undertaken as described in Section 2.6.10. To check the effectiveness of the process, a control RNA sample was also reverse transcribed. The cDNA was then amplified using the PCR reaction with supplied primers and the product of PCR

was run on an agarose gel using gel-electrophoresis (Section 2.6.15). The bands were visualised using UV. The presence of amplified cDNA amplimers confirmed that the RT process was successful (Figure 6.12). Additionally, cDNA samples were analysed using OliGreen reagent. All samples showed high values after the analysis, indicating the presence of cDNA (see Table 6.8).

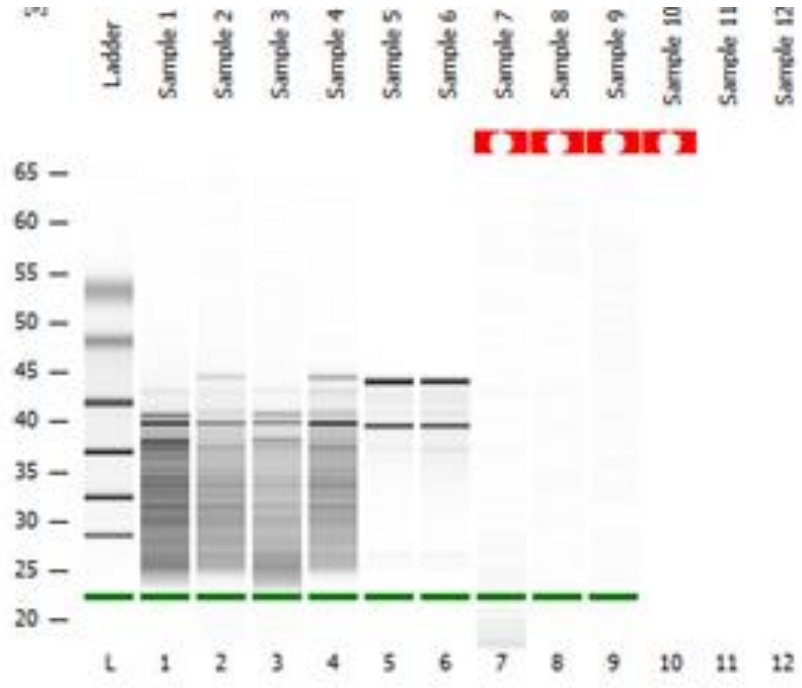


Figure 6.11 Bioanalyser results of RNA templates, 1, 3, 4 – different biological replicas of *S*. Typhimurium (whey powder); 2 – *S*. Typhimurium 'HR' inocula; 5 - *E. coli* K12 MG1655 suspension; 6 – *E. coli* K12 MG1655 desiccated on disc

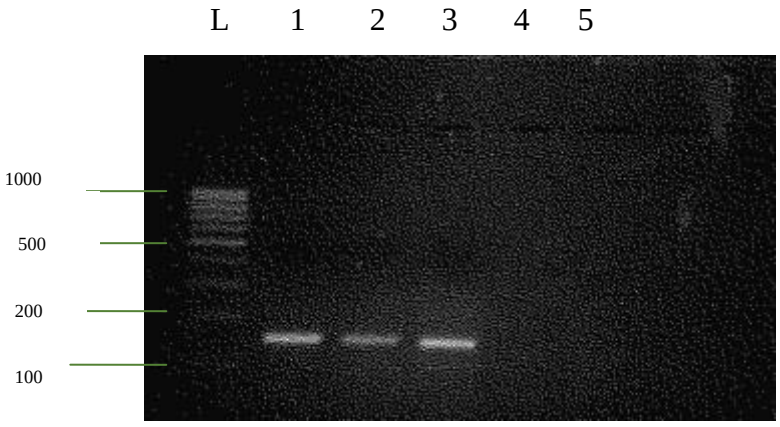


Figure 6.12 PCR results of reverse transcribed control RNA, 1 – RNA 1:1, 2 – RNA 1:10, 3 – RNA 1:100, 4 - RNA without RT polymerase, 5 – water control, L – 1000 bp ladder. Gel running conditions: 1 x TAE, 85 V, 2 h, 2 % agarose

Table 6.8 Average NanoDrop spectrophotometer results from three biological replicates.

Time (h)	Strain	Condition	Cfu ml^{-1}	Nanodrop after extraction			Nanodrop diluted to 100 ng μl^{-1}			Oligreen
				Ng μl^{-1}	260/280	260/230	Ng μl^{-1}	260/280	260/230	
Inoc	S. Typhimurium	PBS	$1.73x10^{9}$	505.7	2.08	1.60	74.9	2.07	1.70	34.28
Inoc	S. Typhimurium 'HR'	PBS	$1.50x10^{8}$	672.8	2.13	1.99	164.0	2.10	1.99	26.99
0	S. Typhimurium	S	$2.00x10^{8}$	503.2	2.11	1.40	121.5	2.11	1.40	28.21
0	S. Typhimurium 'HR'	S	$1.15x10^{8}$	584.3	2.09	1.89	130.6	2.08	1.89	29.87
0	S. Typhimurium	D	$9.83x10^{6}$	595.8	2.09	1.89	130.8	2.09	1.86	36.23
0	S. Typhimurium 'HR'	D	$1.08x10^{7}$	635.1	2.10	1.87	146.2	2.11	1.87	27.26
20	S. Typhimurium	S	$1.58x10^{8}$	455.9	2.10	1.64	118.9	2.10	1.67	35.5
20	S. Typhimurium 'HR'	S	$5.33x10^{8}$	557.8	2.03	1.39	120.2	2.02	1.40	21.78
20	S. Typhimurium	D	$2.33x10^{6}$	702.8	2.10	1.99	131.5	2.10	1.96	14.27
20	S. Typhimurium 'HR'	D	$1.65x10^{7}$	726.2	2.12	1.89	133.9	2.11	1.89	16.92
70	S. Typhimurium	S	$4.07x10^{8}$	695.2	2.08	1.92	123.1	2.08	1.92	16.07
70	S. Typhimurium 'HR'	S	$2.83x10^{8}$	666.7	2.11	1.90	131.9	2.11	1.90	22.57
70	S. Typhimurium	D	$3.10x10^{5}$	680.8	2.11	1.85	140.5	2.10	1.83	25.34
70	S. Typhimurium 'HR'	D	$3.20x10^{6}$	580.9	2.11	1.79	143.3	2.11	1.79	22.71
-	Control RNA cDNA	S	-	-	-	-	-	-	-	18.41
-	Water	S	-	-	-	-	-	-	-	5.105
-	Oligreen control	S	-	-	-	-	-	-	-	122.26
-	No-transcriptase	S	-	-	-	-	-	-	-	41.97

All *S*. Typhimurium (whey powder) templates were pooled together from all replicates, the same was done for *S.* Typhimurium 'HR'. The preliminary real-time RT-PCR was performed to find out which dilutions could be used for further experiments. All 12 genes were tested with templates at their original concentration and they were also diluted 1000 fold. Negative controls were performed with no cDNA template and with RNA which had gone through RT without polymerase. The average C_T was 24-25 for both isolates and above 40 for negative controls samples (results not shown). Following this experiment, real-time RT-PCR was performed with individual templates (diluted 100 fold) from each replica experiment, however the C_T values were very low and there was an amplification present in the negative control samples (results not shown). Therefore, a decision was made to perform a real-time RT-PCR on pooled samples from all three trials. Samples which were representing the same conditions were added together, therefore the amount of suspension was large enough to dilute it only two fold.

Two housekeeping genes were used: *gapA* and *ffh*, however *gapA* was chosen for all the calculations because it had better stability at all conditions (Table 6.9). All 'no template' controls showed no evidence of amplification or had C_T values above 35 cycles. Most of the RNA controls had C_T values above 35 cycles however three had values below 30 cycles. The calculations are based on average C_T values from 3 technical replicates. ΔC_T (s-d) is the difference between the average C_T of samples recovered from suspension and samples dried on discs, at the same time point. The details of the calculations are described in Section 2.6.17. The Ep values (reaction efficiency) ranged from 1.49 to 2.46, however most of them where close to 2.

The results suggest that the genes in *S*. Typhimurium (whey powder) seem to be induced more during desiccation. However a major reason for this may be that the expression of genes in suspension is decreasing and this is causing a difference between expression in suspension and expression during desiccation.

Table 6.9. Results of real-time RT-PCR of samples extracted with RNeasy Mini Kit

Gene	Time	*S.* Typhimurium (whey powder)						*S.* Typhimurium (NCTC 12023, 'HR')					
	(h)	Ep	C_T susp	C_T disc	ΔC_T (s-d)	FC	FCN	Ep	C_T susp	C_T disc	ΔC_T (s-d)	FC	FCN
gapA	Inoc	1.99	19.53	-	-	-	-	2.05	20.36				-
	0		22.56	22.77	-0.21	0.86			22.69	22.04	0.66	1.60	
	22		24.04	22.75	1.29	2.43			22.10	22.60	-0.49	0.70	
	68		25.08	23.04	2.04	4.07			23.57	22.53	1.04	2.11	
ffh	Inoc	1.85	19.61	-	-	-		1.88	23.56	-	-	-	
	0		24.62	22.35	2.27	4.06			24.49	23.35	1.14	2.04	
	22		26.07	23.00	3.07	6.64			22.04	24.39	-2.35	0.23	
	68		27.47	23.84	3.63	9.38			25.74	25.53	0.21	1.14	
kdpA	Inoc	2.03	19.71					2.04	25.15				
	0		25.50	23.02	2.48	5.82			25.47	25.59	-0.12	0.92	
	22		28.13	23.38	4.75	29.0			22.07	26.68	-4.61	0.04	
	68		29.10	24.94	4.15	19.0			25.50	29.55	-4.05	0.07	
hisD	Inoc	1.49	22.24					2.01	24.60				
	0		26.63	23.56	3.07	3.40			24.86	24.38	0.48	1.40	
	22		27.56	24.33	3.22	3.61			21.89	25.22	-3.32	0.10	
	68		29.73	26.08	3.65	4.28			26.00	26.40	-0.40	0.76	
otsB	Inoc	2.10	21.25					2.14	23.34				
	0		23.40	22.80	0.60	1.56			23.11	21.46	1.64	3.49	
	22		25.84	22.87	2.97	9.01			23.30	21.64	1.66	3.53	
	68		27.49	22.74	4.75	33.6			26.29	23.38	2.90	9.09	
aceA	Inoc	2.00	21.59					1.90	24.51				
	0		23.08	20.81	2.27	4.79			24.93	21.02	3.91	12.4	
	22		25.81	21.83	3.98	15.7			22.97	20.81	2.17	4.03	
	68		27.52	21.50	6.01	63.8			26.26	21.27	4.99	24.8	
gltB	Inoc	1.77	23.10					1.81	24.84				
	0		30.13	27.85	2.28	3.66			25.38	24.98	0.40	1.27	
	22		31.23	27.50	3.73	8.33			22.64	24.86	-2.22	0.27	
	68		31.74	38.35	3.39	6.89			25.72	26.60	-0.88	0.59	

Gene	Time (h)	*S.* Typhimurium (whey powder)						*S.* Typhimurium (NCTC 12023, 'HR')					
		Ep	C_T susp	C_T disc	ΔC_T (s-d)	FC	FCN	Ep	C_T susp	C_T disc	ΔC_T (s-d)	FC	FCN
prpE	Inoc	2.03	26.19					1.86	25.89				
	0		30.10	27.23	2.87	7.58			26.73	26.14	0.60	1.45	
	22		32.36	28.05	4.31	21.0			23.68	26.96	-3.28	0.13	
	68		34.91	30.28	4.63	26.3			27.87	27.98	-0.11	0.92	
nifU	Inoc	1.90	19.38					1.77	22.02				
	0		23.08	-	-	-			24.13	21.20	2.92	5.34	
	22		24.04	20.71	3.33	8.50			21.62	21.33	0.28	1.18	
	68		25.54	21.24	4.30	15.8			24.87	22.46	2.42	3.99	
ddg	Inoc	2.29	20.86					1.58	25.18				
	0		24.80	23.19	1.61	3.80			25.05	24.25	0.80	1.44	
	22		26.76	24.19	2.57	8.37			22.85	25.06	-2.21	0.37	
	68		27.10	24.85	2.25	6.45			26.05	26.59	-0.54	0.78	
proU	Inoc	1.87	20.22					1.75	27.16				
	0		26.45	23.49	2.96	6.43			26.90	27.06	-0.16	0.91	
	22		28.74	24.16	4.58	17.8			24.49	27.80	-3.31	0.16	
	68		28.74	25.46	3.28	7.86			28.45	29.03	-0.58	0.72	
proP	Inoc	2.46	20.71					1.85	23.81				
	0		24.78	22.93	1.85	5.30			24.23	24.56	-0.33	0.82	
	22		27.43	23.98	3.45	22.3			21.77	25.49	-3.72	0.10	
	68		28.47	25.47	3.01	15.0			25.28	27.45	-2.17	0.26	
osmU	Inoc	1.94	22.88					1.60	27.88				
	0		28.62	26.25	2.37	4.79			27.96	28.11	-0.14	0.94	
	22		31.17	27.39	3.81	12.5			25.18	29.49	-4.32	0.13	
	68		32.09	28.50	3.58	10.7			29.23	31.67	-2.44	0.32	

Note. (Section 2.6.17 for calculation methods), Ep – amplification efficiency; C_T – crossing point; FC – fold change; FCN – fold change normalised against housekeeping gene, ΔCT (s-d) is the difference between average C_T of samples recovered from suspension and samples dried on discs, at the same time point

6.3 DISCUSSION

6.3.1 PCR of Selected Genes

The first published studies investigating gene expression of desiccated *Salmonella* indicated several genes which could be involved in desiccation resistance (Gruzdev *et al*., 2012), therefore at the start of this work trials were undertaken to check if all 15 *Salmonella* isolates had these selected genes, especially the ones which were sensitive to desiccation. Six upregulated genes from different functional groups (*ddg*, *glpK*, *hisD*, *aceA*, *nifU*, and *kdpA*), displaying the highest fold change in the published microarray study, were selected. The PCR results showed the presence of all the genes in the 15 *Salmonella* isolates therefore greater sensitivity to desiccation is not a result of gene loss. However their involvement could be regulated by their expression rather than presence and so their expression was further studied with real-time RT-PCR as these genes may still be responsible for desiccation. The other possibility is that the rest of the genes fragments are not intact which cannot be seen in the PCR reaction. The experiments however validated the primers used for further analysis.

6.3.2 Investigating Desiccation Pre-exposed Cells for the Development of Desiccation Resistance

The survival of cells which had been previously desiccated was compared with the survival of unchallenged cells. There was no significant difference between these two types of cells in response to desiccation. This was also seen in Chapter 5 where addition of water was studied. The cells were dried for up to a month, then water was added and they were desiccated again. Increased survival following each desiccation and recovery process was not observed.

Developing resistance to a particular stress by subjecting microorganisms to this stress is commonly observed with antibiotic resistance. With time, bacteria start to develop mechanisms of resistance to antibiotics which include e.g. alteration/eradication of target binding sites, alteration of porin channels etc. (Pan *et al*., 2015; Oliphant and Eroschenko, 2015; Domenech *et al*., 2015). Similarly, thermal adaptation can enhance heat resistance. Ferrando *et al*. (2015) showed that selected thermal adaptation improved by 2 log orders the thermal resistance of two *Lactobacillus plantarum* strains, but cell growth in the presence of NaCl was enhanced only in one strain. Responses of bacteria to cold stress can be enhanced

with pre exposure to low temperatures. For example, a *Lactobacillus plantarum* L67 that went through cold stress showed improved survival rates under freeze-thawing conditions and long-term storage (Song *et al*., 2014). Also, previous osmoadaptation by exposure to fat (20–60%) or salt (5%) increased desiccation resistance significantly in *Listeria monocytogenes* cells (Hingston *et al*., 2013). In this study however, improved survival was not observed after one cycle of drying and rehydrating and neither with repeated cycles (Chapter 5).

6.3.3 Comparison of *Salmonella* Responses to Hyperosmotic Shock and Desiccation

A number of studies suggest that similar responses in bacteria are triggered by desiccation and osmotic stress (Yousef and Courtney, 2003; Moat, 2002). Gene expression studies were carried out to look into the specific genes responsible for these responses, however, first the physical responses of selected *Salmonella* strains were analysed to compare their survival during drying and hyperosmotic stress. One strain was originally isolated from chocolate which caused an outbreak (*S.* Napoli) and could presumably be more resistant to high osmolarity. The other two strains were associated with low and high desiccation resistance. The studies showed that all strains did not survive high concentrations of NaCl salt (nonglass-forming, ionic solute) and glycerol (glass-forming, membrane permeable). This could be expected as concentrations used for the experiments are known to have antimicriobial effect Saegeman *et al.,* 2008). However the presence of sucrose (glass-forming, non-membrane permeable; Stewart *et al*., 2005) had a similar effect as desiccation during the first thirteen days. Later however, desiccated cells did not show any further decline in viability whilst the cells immersed in sucrose solution declined to undetectable levels. The response therefore, could be similar at the beginning of the stress conditions, whilst other mechanisms could be present later. Another option is that sucrose, as a membrane impermeable solute, slows down the molecular mobility of the system (the capacity of the molecules present to move), therefore the lag time before death is longer (Stewart *et al*., 2005).

In studies conducted by Hiramatsu (2005), sucrose and NaCl were added to bacterial suspensions which were then dried on paper discs for 24 hours. The addition of sucrose increased survival, however the addition of NaCl decreased the number of bacteria.

Table 6.10 Genes responsible for osmoresistance

Gene	Function	References
kdpABC	K^+ uptake	Gruzdev, Moat, 2002; Asha and Gowrishankar, 1993; Frymier *et al*., 1997 Booth and Higgins, 1990.
trk (supG)	K^+ regulation, not transcriptionally regulated	Moat, 2002; Balaji *et al*., 2005 Booth and Higgins, 1990.
Kup	K^+ uptake	Moat, 2002; Balaji *et al*., 2005, Booth and Higgins, 1990.
speE	Used to transform putrescine to spermidine	Moat, 2002
potE	Protein excretes putrescin	Moat, 2002
Glt	Glutamate regulation- it accumulates after osmotic shock	Gruzdev *et al*., 2012, Li *et al*., 2012
Gdh	Protein excretes putrescin	Moat, 2002
otsAB	Synthesise trehalose, dependent on RpoS protein	Li *et al*., 2012, Aviles et al., 2013
tre/osm	Synthesis of a periplasmic trehalase	Moat, 2002
proP/proU	Mediate uptake of osmoprotectants	Li *et al*., 2012, Finn *et al*., 2013, Culham *et al*., 2003; Sutherland *et al*., 1986
envZ/*ompR*	2 component regulatory system encoded by *ompB* locus, osmotic control of gene expression, *omp*R gene is responsible for synthesis regulation of the Vi capsular polysaccharide	Moat, 2002, Chowdhury *et al*., 1996.
ompF-ompC	Encode the osmoregulated porins	Moat, Balaji *et al*., 2005
invA	Invasion genes	Chowdhury, 1996, Chowdhury, 2012; Aviles *et al*., 2013; Asakura *et al*., 2012
opuBB	*opuE* (in *B. subtilis*) is encoding a proline transporter and *opuD* encodes a glycine betaine transporter	Hecker and Volker, 2001; Li *et al*., 2012
STM1493	Putative periplasmic component	Li *et al*., 2012
ddg	Outer membrane fluidity control	Gruzdev *et al*., 2012
*rpo*S	Stress responses, transcription, responsible for desiccation resistance and osmotic stress in *E.coli*	Stasic *et al*., 2012
*rec*A	Involved with homologous genetic recombination and recombinational repair	Aranda *et al*., 2011; Asada, *et al*., 1979

Survival was also significantly higher in beef powder containing 0.5 or 3% sodium chloride compared to beef powder containing 20% salt (Ryu *et al*., 1999). A number of studies suggest that the same RH, achieved with different humectants and means, has a different influence on bacterial survival which is dependent on molecular mobility and ionic stress (Gould 1985; Townsend and Wilkinson 1992; Vijaranakul *et al*. 1995, 1997). Some nonglass-forming ionic solutes such as NaCl may have a toxic effect itself as opposed to glass-forming solutes such as sucrose (Stewart *et al*., 2005).

Table 6.10 contains a range of genes which have been proven to be responsible for or involved with osmotic and desiccation stress responses, therefore suggesting similar survivability under these two stresses. To investigate further the differences in osmotic and desiccation stress, real-time RT-PCR was performed on selected genes (highlighted in Table 6.10). Not all of the genes responsible for desiccation were studied by real–time RT-PCR. Some of them are not transcriptionally regulated e.g. *trk* or *rpoS*, or are only induced slightly during osmotic stress e.g. *kup*. Also, the *recA* gene was not studied as most of the stress conditions would cause DNA damage and its expression is not strictly associated with desiccation resistance (Asada *et al*., 1979; Setlow and Setlow, 1996; Pitcher *et al*., 2007).

6.3.4 Gene Expression Studies

6.3.4.1 Method Selection

Real–time RT-PCR offers several advantages over other methods which can be used for gene expression studies and it was used in this work to study gene expression during desiccation. Methods such as northern blot analysis or RNase protection assays are often time consuming and sometimes can show difficulties in reliably generating appropriate levels of gene expression.

There are several protocols for isolating RNA from bacteria. For most purposes it is advisable to work with total RNA, rather than mRNA, since the purification is simpler and provides sufficient RNA for real-time PCR (Fraga *et al*., 2008). Investigation of the gene expression of surface attached cells is a challenging procedure. First of all it must be ensured that the tested RNA is truly representing cells in a desiccation state. As soon as the discs are placed in liquid, the gene expression can be dramatically changed and can give a false indication of genes

involved in desiccation resistance. That is why several stop solutions were used in this study.

SYBR Green 1 nonspecific fluorescent dye was used for monitoring real-time amplification. These types of dyes do not distinguish between different dsDNA products therefore melting point analysis was performed to analyse if only one amplicon was present (Fraga *et al*., 2008). In general, the selection of housekeeping genes and the method used was based on the literature which describes studies also focused on desiccated *Salmonella* gene expression (Table 6.11). The housekeeping genes should not change their expression at different water availability levels; however during the experiments it was evident they were expressed differently depending on conditions. It is not clear why the difference occurred and it is further analysed in 6.3.4.2 section. The recovery of desiccated cells and RNA extraction could be very challenging; therefore the selection of an appropriate method was extremely important.

Table 6.11 Comparison of the published methods used for studying gene expression in desiccated *Salmonella*

	Finn *et al*., 2013	Li *et al*., 2012	Gruzdev *et al*., 2012	Deng *et al*., 2012
Growth media	LB	TSB	LB/ double deionised water	LB
Housekeeping gene	*gapA*	*agp* – glucose 1phosphatase	*rsuA* (16s rRNA)	*rsuA* (16s rRNA)
Stop solution	RNA*later* Stabilization solution (Ambion)	-80°C	Phenol/ ethanol solution	RNAprotect Bacteria Reagent (Qiagen)
RNA extraction	Trizol (Invitrogen)	UltraClean Microbial RNA Isolation Kit (Mo bio)	SV Total RNA Isolation System (Promega)	RiboPure RNA purification kit (Ambion)
RNA purification	Dnase, Rnase inhibitor	UltraClean Microbial RNA Isolation Kit (Mo bio)	SV Total RNA Isolation System (Promega)	RiboPure RNA purification kit (Ambion)
RT	QuantiTect SYBR Green RT-PCR Kit (Qiagen)	QuantiFast SYBR Green PCR kit (Qiagen)	QuantiTect RT kit (Qiagen)	Transcriptor First Strand cDNA Synthesis Kit (Roche)
Real-time PCR			Fast Plus EvaGreen qPCR Master mix (Biotium)	Not known
Replicates	3 biological	3 biological	2 biological	2 biological

The medium chosen to grow *Salmonella* cultures was LB. All of the housekeeping genes listed in Table 6.20 were analysed for shifts in expression. Several stop solutions, RNA isolation and purification kits were investigated and Roche kits (Transcriptor First Strand cDNA Synthesis Kit and LightCycler 480 SYBR Green I Master) were chosen for reverse transcription and real-time RT-PCR.

All methods highlighted in blue in Table 6.11 were used in this work. The methods therefore were already established in similar studies which suggested they should be suitable. The results however indicate that the RNA obtained with most of the methods and upstream processes was not of the highest possible quality. One of the quality problems was low A 260/A 230 ratios which are typically due to guanidine thiocyanate contamination or other impurities e.g. some reagents used for RNA purification (Trizol) which can absorb at 230 nm. However, when the samples where precipitated by adding NaCl and ethanol, an improvement was not seen. The method which gave the best results for RNA was with the use of the RNeasy Mini kit (Qiagen) for extraction and purification. The quality and quantity were of a high standard, however the integrity of the RNA, which uses an algorithm assigning integrity values to RNA measurements, still remained low (approximately 4 RIN). This is very important for studying gene responses during desiccation as this could affect the final results. Differences in the recovery of RNA may be a reason why all four studies which investigated desiccation mechanisms give different results (see 6.1; Deng *et al*., 2012; Gruzdev *et al*., 2012a; Li *et al*., 2012; Finn *et al.,* 2013). The reason for poor recovery might also be specific to the *Salmonella* strains which were selected, as RNA isolated from an *E. coli* strain had very good yield, quality and integrity. Further studies would have to be conducted to investigate the reasons for the difficulties with RNA extraction.

6.3.4.2 Gene Expression

The selection of genes for expression studies was based on the published literature in which at least one of the previous studies had shown their involvement in desiccation or osmotic shock (see §6.2.5). The housekeeping genes were also based on literature and according to the previous studies, they should not change expression at different water activity conditions. However, regardless the RNA extraction methods there have been always an upregulation or downregulation of four selected housekeeping genes. The fold change of expression as compared with

cells in suspension ranged from 0.2 to 9. For this reason, the expression of genes which were studied to investigate desiccation resistance mechanisms of *Salmonella* was not normalised against housekeeping genes (see as an example Table 6.9). The results where however analysed just based on fold change of expression during desiccation when compared to genes in the suspension at the same time point. This would have to be further confirmed by conducting more studies, with suitable housekeeping gene. The obtained results just give an indication of the changes during desiccation and highlight difficulties associated with experimental conditions and influence of desiccation on genes which supposed to stay unchanged.

Overall, the results of the studies indicated that the selected genes were induced during desiccation when compared to genes in the suspension at the same time point; however this was observed mostly for the *S.* Typhimurium isolated from whey powder which is more sensitive to desiccation. The highest fold change in expression was associated with *kdpA, aceA, prpE, proP* and *proU*. Half of the studied genes were showing the highest increase in fold change at 68 hours time point and half after 22 hours. *kdpA* gene had the highest fold change after 22 h of desiccation. This could be reflecting what is seen in osmotic shock mechanism, in that the different gene systems are expressed in succession. K^+ ion influx is a rapid initial response. Following this in Gram negative bacteria glutamate is synthesized by two enzymes: glutamate dehydrogenase (GDH) and glutamate synthase (GS) and accumulates quickly; this synthesis is dependent on the K^+ ion as an initiator for synthesis. Following this, trehalose is synthesized by the otsAB operon under RpoS control.

The desiccation resistant strain (*S.* Typhimurium 'HR' NCTC 12023) showed an increase in fold change only for three genes: *aceA*, *nifU* and *otsB*. *aceA*, similarly as for the desiccation sensitive strain showed the highest fold change after 68 hours. The rest of the genes were showing fold change below or close to 1 which indicates that there was the higher amount of RNA extracted from the samples in suspension than from desiccated cells. A potential reason for this could be the Lurini-b Broth (LB) media used for cultivation of *Salmonella*. It contained 0.5% of NaCl which could potentially cause stress responses to occur during growth; therefore any later changes were not seen.

It seems like *S.* Typhimurium (whey powder isolate) had higher amount of RNA corresponding to studied gens than *S.* Typhimurium 'HR'. The reason for this is not known. One possibility is that the inoculum was produced by removing all the nutrients and taking the cells from 37°C to approximately 25°C, which could be associated with starvation and cold shock. The sensitive strain had possibly the highest expression of the genes only directly after this process was undertaken and the resistant strain possibly had these genes at a similar or even higher level throughout the experimental time period. However, the fold change analysis was undertaken based on a comparison of genes in suspended and desiccated cells at the same time point and the results are interpreted based on this comparison. The reason why the Ct values of genes studied during desiccation in resistant strain as compared with the sensitive strain where higher, could also be because of the ability of the resistant strain to go into a dormant state, where most genes are repressed (Deng *et al*., 2012). It is possible also that the fact these genes are expressed at a higher level initially in the resistant strain makes it more preadapted to desiccation when they are placed on a disc. This could explain why the whey powder isolate shows a much greater initial die off unlike the `HR` strain which is resistant over the initial period.

A gene with one of highest fold change was *prpE* which belongs to the functional group of fatty acid metabolism. In studies conducted by Li *et al*. (2012) this was the biggest group of genes induced during desiccation (up to 51%). The authors also investigated *prpE* and its expression after 2 hours of desiccation was much higher than in cells in suspension, especially in a resistant strain (*S.* Tennessee, 40-fold) as compared with a sensitive strain (*S.* Typhimurium LT2, 28-fold). The induction of this gene was not observed in the other two studies focused also on transcriptomic analysis of desiccated *Salmonella*, however with different strains (Gruzdev *et al*., 2012 and Finn *et al*., 2013). The increase of genes responsible for fatty acid metabolism is however understandable as starved, desiccated cells are in need of energy, and oxidation of fatty acids generates more ATPs per carbon than oxidation of glucose (Li *et al*., 1999). Additionally, osmotic pressure creates a need for better membrane rigidity which can be regulated by fatty acids (Li *et al*., 2012).

The group of genes responsible for osmoprotectants synthesis and transport: *osmU* operon (osmotic induced lipoprotein precursor), *otsB* (trehalose biosynthesis), *proU* and *proP* showed increased fold change during desiccation of the sensitive

strain (*S*. Typhimurium isolated from whey powder) and only the *otsB* gene showed lower Ct values in desiccation resistant *S.* Typhimurium 'HR' (NCTC 12023) strain. Again, in studies conducted by Li *et al*. (2012), the situation was opposite as higher expression was seen in a resistant strain with the exception of *proU* which was induced 12.5 fold in the resistant strain and 14.9 in a sensitive strain. In studies conducted by Finn *et al*. (2013) *proP, proU, osm* and *otsB* were all up-regulated, especially the first 3. The authors concluded that these were the genes mainly responsible for desiccation as they regulate the osmotic pressure of the cell by accumulation of osmoprotectants; expression of *proU, kdp, otsB* and *proP* is therefore increased to maintain osmotic stability of the cells. *proU* and *osmU* systems are known ABC transporters and the *proP* system belongs to the major facilitator superfamily of permeases. All three transporters have been shown to promote survival under osmotic stress caused by NaCl (Finn *et al*., 2013). OtsB is important in the production of trehalose, which binds water that would otherwise be lost from the cell during drying and dry environment exposure (Aviles *et al*., 2013).

In studies with *S*. Typhimurium in 0.3 M NaCl solution it was evident that genes are induced in three stages after osmotic shock: in 4-5 minutes *proU, rpoS* and *proP* were induced, in 8-9 minutes *kdpA* and in 10-12 *ompC* and *otsB*. After 30 minutes genes came back to their original state (Balaji *et al*., 2005). It is difficult to relate this work as the gene expression analysis of desiccated cells is much more challenging than cells immersed in hyperosmotic solution. First of all, it is difficult to establish when the process of desiccation is starting, then it is not clear when and if it is ending. Drying of droplets takes 2 hours therefore some cells could already have gone through the response mechanisms. Some analogy can be seen however, as *proU, prop* and *kdp* seem to be more induced at the middle time points and *otsB* at the last time point (after 68 hours).

Genes responsible for protein biosynthesis and transport: *hisD* and *gltB* showed higher fold change as compared with cells in suspension in *S*. Typhimurium isolated from whey powder and lower fold change in the resistant *S*. Typhimurium 'HR' (NCTC 12023) strain. A similar pattern was also observed by Li *et al*. (2012), where genes responsible for protein biosynthesis were much more induced in a desiccation sensitive strain. This could be due to the fact that proteins are synthesised with a high energy input and strains which are not focusing on these

processes may have more energy for other processes, and therefore are more resistant to desiccation (Li *et al.*, 2012). These two genes were also induced by 2.4 fold in a desiccated *S.* Typhimurium SL1344 which was relatively resistant in studies conducted by Gruzdev *et al*. (2012) and could indicate the additional role of histidine and glutamate in the adaptation to desiccation.

A group of genes belonging to the energy production functional group included: *aceA* and *nifU* genes. *aceA* is one of the glyoxylate shunt pathway genes which can be induced under glucose starvation (Fischer and Sauer, 2003). The gene could potentially help release free radicals causing oxidative stress (Gruzdev *et al*., 2012) which is known to accrue during desiccation (Paul *et al*., 2004). In this work, it showed higher fold change in both strains, especially after 68 hours of desiccation, where the oxidative stress is probably greatest. It was also induced after 2 hours in studies conducted by Li *et al*. (2012), however more in a sensitive strain. It was highly expressed (3.3 fold increase) in desiccated *Salmonella* on plastic surfaces (Gruzdev *et al*., 2012). This gene can be particularly involved in adapting cells to a desiccation stress. Additionally, the concept of an oxidative stress presence during desiccation is supported by increased expression of the NifU-like protein gene by up to 4 fold for both strains, 3.34 in Gruzdev *et al*. (2012) and up to 5.3 for a resistant strain in Li *et al*. (2012). This gene is particularly involved with iron-sulfur cluster assembly, having the role of a cluster precursors destined for nitrogenase maturation (Johnson *et al*., 2005). However the cellular processes which are utilising the clusters are very complex, therefore it is difficult to allocate it to a specific one.

The gene which is also responsible for transport, however of the inorganic ion K^+, is *kdpA* (potassium transporting ATPase subunit A). In the current study, the cDNA corresponding to this gene was detected slower in the resistant strain and faster in the sensitive strain. Its increased expression was not observed in the Li *et al*. (2012) inoculated on paper discs, however it was the highest up-regulated gene in the Gruzdev *et al*. (2012) work. Desiccation causes loss of turgor pressure and increase of intracellular ion strength which may trigger *kdpA* induction (Epstein, 2003; Ballal *et al*., 2007). After turgor is restored the *kdp* level returns to the pre-stress value, which might be a reason why its expression is increased in some studies, but not in the others, because of the different time periods studied. Increase

of the K^+ ion concentration in the cytoplasm could potentially trigger all of the desiccation responses (Balaji *et al*., 2005).

The *ddg* gene is responsible for lipid transport and metabolism (lipid A biosynthesis lauryl acyltransferase) and has been reported to be involved with a cold shock response in *E. coli* (Carty *et al*., 1999). It modifies lipid A composition which enables the cells to maintain membrane fluidity. This can be crucial during desiccation. Only the desiccation sensitive strain showed indication of an increase of this gene expression in this work. It was also induced in studies conducted by Gruzdev, however the increase in expression was not noted when *Salmonella* were dried on paper discs for two hours (Li *et al*., 2012).

The same genes' responses are not the same at the same water activity obtained with different humectants (Balaji *et al*., 2005), which indicates that drying could have different response mechanisms than osmotic shock. Some studies also suggest induction of σ^{32}, σ^{S} and σ^{E} during osmotic shock (Aldsworth et al., 1998; Bianchi and Baneyx, 1999), however only some genes which have a regulon controlled by these sigma factors were studied e.g *otsA*. Differences in the expression of genes in all the analysed studies may be associated with recovery methods used to resuscitate damaged cells (Aviles *et al*., 2013). It could also be caused because of other experimental conditions such as different media used for culturing (especially use of Lurini-b Broth) and suspension, surface types, time periods, amount of dry suspension used etc. To obtain a satisfactory understanding of the desiccation responses, it would be necessary to correlate the transcription of desiccation regulated genes over time with a variety of relevant parameters, such as turgor pressure (Asha and Gowrishankar, 1993), membrane tension, water content, cytoplasmic volume, internal solute concentration, or ionic strength (Higgins *et al*., 1987). To confirm the involvement of the genes studied in the desiccation process, proper housekeeping genes would have to be found and knock out mutants of the selected genes could be obtained and subjected to desiccation.

6.4 CONCLUSIONS

A number of approaches were undertaken to better understand the mechanisms of desiccation resistance of *Salmonella*. It was evident from the studies conducted in this chapter and from water inoculation studies that cells are not

becoming more resistant as they are subjected to multiple desiccation times. Perhaps more drying and re-suspending cycles are needed to observe any resistance increase. Secondly, the increase of viable cell numbers after desiccation and subsequent recovery with betaine solution was not observed, which suggests that recovery itself is not affecting the achieved survivability results. It was hypothesised that response to osmotic shock could be similar to responses to desiccation. This hypothesis was then analysed by investigating the survivability of *Salmonella* desiccated on stainless steel surfaces and in the presence of different humectants. Whilst the responses were similar for cells on surfaces and in sucrose solution for up to 13 days of desiccation, after this time the pathogen level declined to below detectable levels in sucrose although the cells dried on stainless steel stayed at a high level. This suggests the sucrose continues to dehydrate the cells as against the desiccation process which comes to equilibrium. Another hypothesis is that there is other desiccation protection mechanism than just that responsible for osmotic shock, which was further investigated by gene expression analysis. The isolation of high quality RNA and upstream analysis was challenging, however the best method was ultimately selected. The biggest difficulty, was however selection of appropriate housekeeping gene. All four genes showed changes during desiccation therefore fold change of studied genes could not be normalised against any housekeeping gene. Overall qRT-PCR results were still analysed as they can show at least an indication into possible gene expression in the desiccated cells. Genes in the sensitive strain showed higher fold change during desiccation as compared with cells in suspension, than the genes of the resistant strain. The genes which had highest fold change in *S.* Typhimurium 'HR' (NCTC 12023) strain were *otsB* (the late stage osmotic shock response), *aceA* and *nifU* (both in a group of energy production genes) which might be crucial for desiccation survival. In *S.* Typhimurium (whey powder isolate) all the selected genes were showing higher fold change and the highest was associated with genes responsible for transport of osmoprotectants, energy production and fatty acid metabolism. Further studies must be conducted to investigate genetic mechanisms responsible for desiccation resistance of *Salmonella* and most suitable housekeeping genes. This work gave a good understanding of methodology importance, selected time periods, media, control samples and identified the need to consider additional aspects during any desiccation studies.

CHAPTER 7
CONTROL OF MICROBIAL CROSS-CONTAMINATION FROM THE FACTORY ENVIRONMENT TO THE PRODUCT

7.1 INTRODUCTION

The results from the previous chapters indicate that there is a high probability that *Salmonella* could survive in factory environments after it is introduced there from the external environment. Wet cleaning and disinfection procedures could remove the pathogen, however these methods are not often used in dry processing factories, additionally the infrastructure of the factory and equipment often makes wet sanitation procedures ineffective (observations from factory visits). A root-cause analysis of foodborne outbreaks of *Salmonella* listed in Table 1.5 (Carrasco, 2012; Smith *et al*., 2004) shows that microbiological cross-contamination has been a contributing factor to several well-documented outbreaks of foodborne illness. In total there were at least 39 of such outbreaks (51 including other pathogens; Beuchat *et al*. 2013) with the numbers significantly growing since 1980s. Thirteen outbreaks were caused potentially by cross-contamination from the factory environment (equipment, dust, people or pests). Fifteen food products which caused the outbreaks were not thermally treated or the heat treatment was not sufficient to destroy potential contamination therefore it is difficult to say if the pathogen came from the raw material or was transferred to the product during the production process. The source of contamination is not known for 11 of the selected outbreaks however, 8 of these foods had undergone validated thermal treatment and Hazard Analysis and Critical Control Points (HACCP) records did not show any failures of these processes. It can be assumed, therefore, that the contamination came from the factory environment. There are therefore two most common reasons of contamination of dry products: 1) cross-contamination from the environment after the bacterial reduction step or 2) lack of a decontamination step or a thermal step which was not designed to control potentially present pathogens (historically, many thermal treatments such as roasting were optimised for organoleptic product quality). The food processing steps where thermal treatment is used as a Critical Control Point (CCP) are usually well controlled by the HACCP system, therefore it is not likely that the cause of any outbreak is a failure of the processing step because this would be monitored and

corrected by HACCP corrective actions. These problems need rapid solutions. At the moment, there are technologies being developed to decontaminate low water activity foods (Bonvehi and Isal, 2000; Bari *et al*., 2010; Mtenga *et al.*, 2013, Ban and Kang, 2014) and existing processes are being validated (GMA, 2010). The most challenging issue is, however, to control cross – contamination after the last product decontamination critical control point.

In most HACCP or other hazard analysis-based food safety systems, cross-contamination is controlled and managed predominately by prerequisite programmes (PRPs; Anon., 2011a) which are generic in nature (Anon., 2003; Bas, 2006, Anon, 2009, Anon., 2011, see Figure 7.1). Establishments must ensure that their prerequisite programmes reflect the current work environment and operational practices within their establishment and comply with specific commodity policies, manuals, procedures and associated regulations (Anon., 2014), however there is no guideline on how to select the prerequisites which are critical to control cross-contamination.



Figure 7.1 Schematic of current Food Safety Management systems in food manufacturing factories

The challenge however is to identify the prerequisites or activities which are more significant to control food safety or are associated with higher hazard.

The concept of a ranking system for PRPs has been addressed by ISO 22000 (Anon., 2005), which differentiates operational PRPs (OPRPs) from PRPs. An OPRP is defined as a PRP *identified by the hazard analysis as essential in order to control the likelihood of introducing food safety hazards to and/or the contamination or proliferation of food safety hazards in the product(s) or processing environment.*

In other words, ISO 22000 suggests that a hazard analysis may identify some routes of cross-contamination that are so important to the safety of the food product that their control is essential (however they are not CCPs as the environment is not part of the process flow) and are thus elevated to a higher classification of PRP, that is, an OPRP. No guidance has been provided, however, as to the hazard analysis steps to be undertaken to determine OPRPs from PRPs.

There are two important factors which must be determined in a factory to enable better control of cross-contamination and to establish OPRPs. First of all it must be established where the microorganisms are coming from (sources) and how and when they are transferred within the factory processing environment and to the product (vectors and line interventions). The concept of sources, vectors and line interventions is shown schematically in Figure 7.2.

General knowledge and literature shows that pathogenic microorganisms can enter food processing areas from several main routes (Kornacki and Doyle, 2010): the external environment (Abdelzaher *et al*., 2010; Holvoet *et al*., 2014), raw materials (Alwakee and Nasser, *et al*., 2011; Lima *et al*., 2011; Batool *et al*., 2012; Berghofer *et al*., 2013), people (Guzewich and Ross, 1999a; Olsen *et al*., 2000; Greig *et al*., 2007; Todd *et al*., 2007), equipment (FSM, 2013; www.ehedg.org) and in-plant microbiology laboratories (Holah, 2013). Once inside, pathogens may persist for long periods as was shown in chapter 3 and 4. When pathogens persist in the environment, they generally survive in harbourage sites, for example, poor hygienic design features of processing equipment or damaged areas of the plant's building structure (Carpentier, 2004; Codex Alimentarius, 2005; Carpentier, 2007; Holah, 2010; Carpentier and Cerf, 2011). When a harbourage site also provides an environment suitable for growth, it can be considered a growth niche (Kornacki, 2006; Kornacki and Doyle, 2010; Holah, 2013). Both harbourage sites and growth niches are potential sources of contamination within the processing environment and they should be identified within the processing plant. For a pathogen to move from a source within the processing environment to other locations (and perhaps even into product), a vector is required. It should be noted that cross-contamination usually occurs as an event (line intervention) in which a number of vectors may be involved.

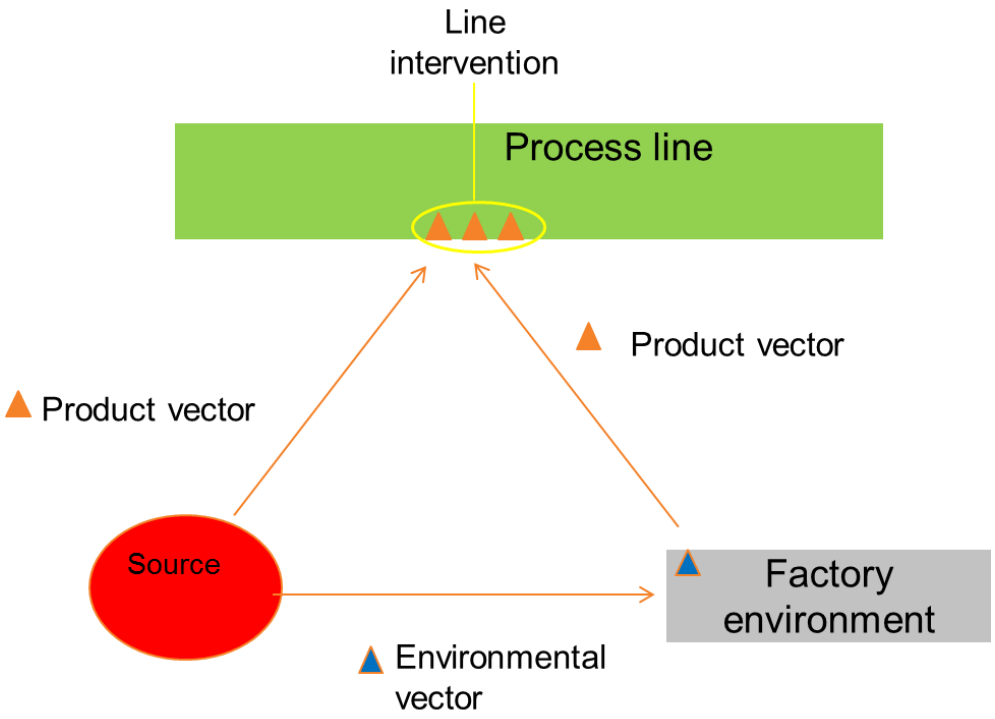


Figure 7.2. Schematic of a concept of sources, vectors and line intervention events.

Obtaining detailed knowledge about each of these events in a food factory, the vectors associated with them and the sources which the vectors could come into contact with, could better help control cross-contamination incidents.

It was shown that *Salmonella* can survive for prolonged periods of time on surfaces and it can be challenging to remove it from a dry food factory environment. Additional control mechanisms are therefore needed to avoid food contamination incidents. This chapter gives a systematic approach to create an OPRP management programme and potentially use OPRPs to control and manage cross-contamination of the product. It proposes incorporation of an OPRP programme into an already well-established HACCP system to create a food safety plan. Two critical concepts of microbial cross-contamination: sources within factory environment and vectors (pathogen transfer possibilities during line interventions) are explored. This chapter also presents a method for identifying and risk-ranking sources, vectors of contamination and line intervention events. This is important for the production of all food commodities, however in this chapter the main focus is on dry food processing. The concept of controlling cross-contamination in dry food processing

was developed and then implemented in a factory producing infant formula milk which is shown as a case study.

7.2 DEVELOPMENT OF AN OPERATIONAL PREREQUSITE MANAGEMENT PROGRAMME

7.2.1 Initiation of the Programme

7.2.1.1 Concept

Food manufacturers are now very familiar with HACCP as a food safety management tool and the development of an operational prerequisite management programme should follow these principles as closely as possible. This is to ensure that factory food safety policies do not become overcomplicated and that the learning of this new proposed food safety management technique can be more easily incorporated into a factory's existing systems. Figure 7.3 shows a typical HACCP process flow and the additional steps which have to be undertaken to create an operational prerequisite programme. HACCP and OPRPs plans, together, create a comprehensive food safety plan.

The programme should be developed in parallel with the HACCP plan, therefore the preliminary HACCP steps such as management commitment, defining scope of the studies and other information about the product should be already implemented within the HACCP study. The process flow is composed in the same way as for HACCP studies, however, additional, different hygiene zones should be drawn around specific steps. The line interventions and sources should also be placed next to the specific process steps. Identification of potential sources, vectors and line intervention events should be done by a cross-functional team by walking the line and examining the processing equipment and environment. The hazards should already have been identified by the HACCP analysis, however the sources and vectors of these hazards will be determined and analyzed during the OPRP assessment (see Figure 7.3, principle 1). The system described in these studies focuses only on biological hazards and specifically *Salmonella* and *Cronobacter* (due to the case studies), however it could potentially be extrapolated to control other hazards. When observing and identifying potential contamination sources and vectors, any current measures for controlling such observed sources and vectors should be recorded as shown in Appendices H and I.

Food Safety Plan

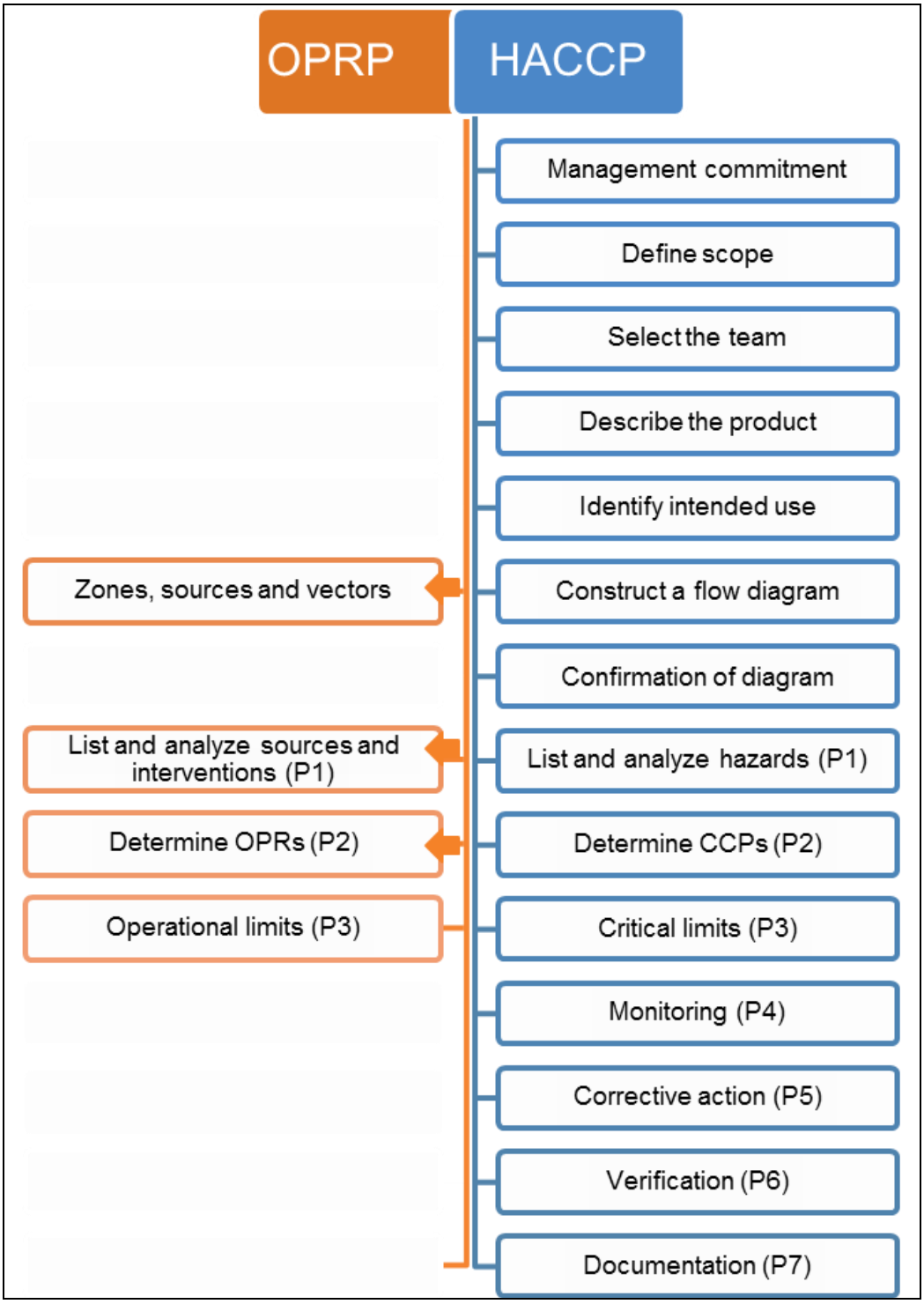


Figure 7.3 Combining HACCP and OPRP plans

P=principle

The largest difference in this approach from the HACCP study is the analysis of sources and vectors, and determination of OPRPs (principles 1 and 2) which are done in addition to analysis of hazards and determination of CCPs (principles 1 and 2 from the HACCP system). After CCPs are determined, the OPRPs studies will focus on process interventions after the last decontamination process CCP and also the identification of sources in areas surrounding these processing steps. A specific risk ranking tool has been developed to access the risk associated with the hazard sources and vectors identified. Principle 3 is focused on determining operational limits for the OPRPs as the OPRPs are not likely to have critical limits as is the case of CCPs. The OPRPs will have to be further monitored, corrected if out of control, verified and recorded (as in a HACCP plan).

7.2.1.2 Case Studies

The development of the concept is described on an example of a case study undertaken in one dry milk processing plant, however it was also implemented in more milk processing facilities owned by different food manufacturers (Holah, 2010a,b). The primary work was focused on developing the idea and the final case studies were chosen to show the whole concept. The factory studies focused on identifying potential sources of microorganisms and routes of cross-contamination to the product. The hazards which the exercise was focused on included *Salmonella* and *Cronobacter* spp. which were identified during the HACCP assessment.

The part of the factory which was investigated contained four milk processing lines (L1, L2, L3 and MW). The factory is located in a 4 story building with ingredients being delivered on the ground floor and then transported to the 4th floor via an elevator. The processing starts on the top floor by dosing the ingredients to the processing lines. There is no heat step or microbial inactivation step during processing, all ingredients are decontaminated at other establishments, and thus have already passed the last decontamination CCP related to microbial hazards. The flow charts of the infant formula milk powder processing lines are shown in Figure 7.4.

The factory has two hygiene zones: low and high; however the high hygiene zone can be separated further into two hygiene levels: one where transport is undertaken and another where production is taking place.

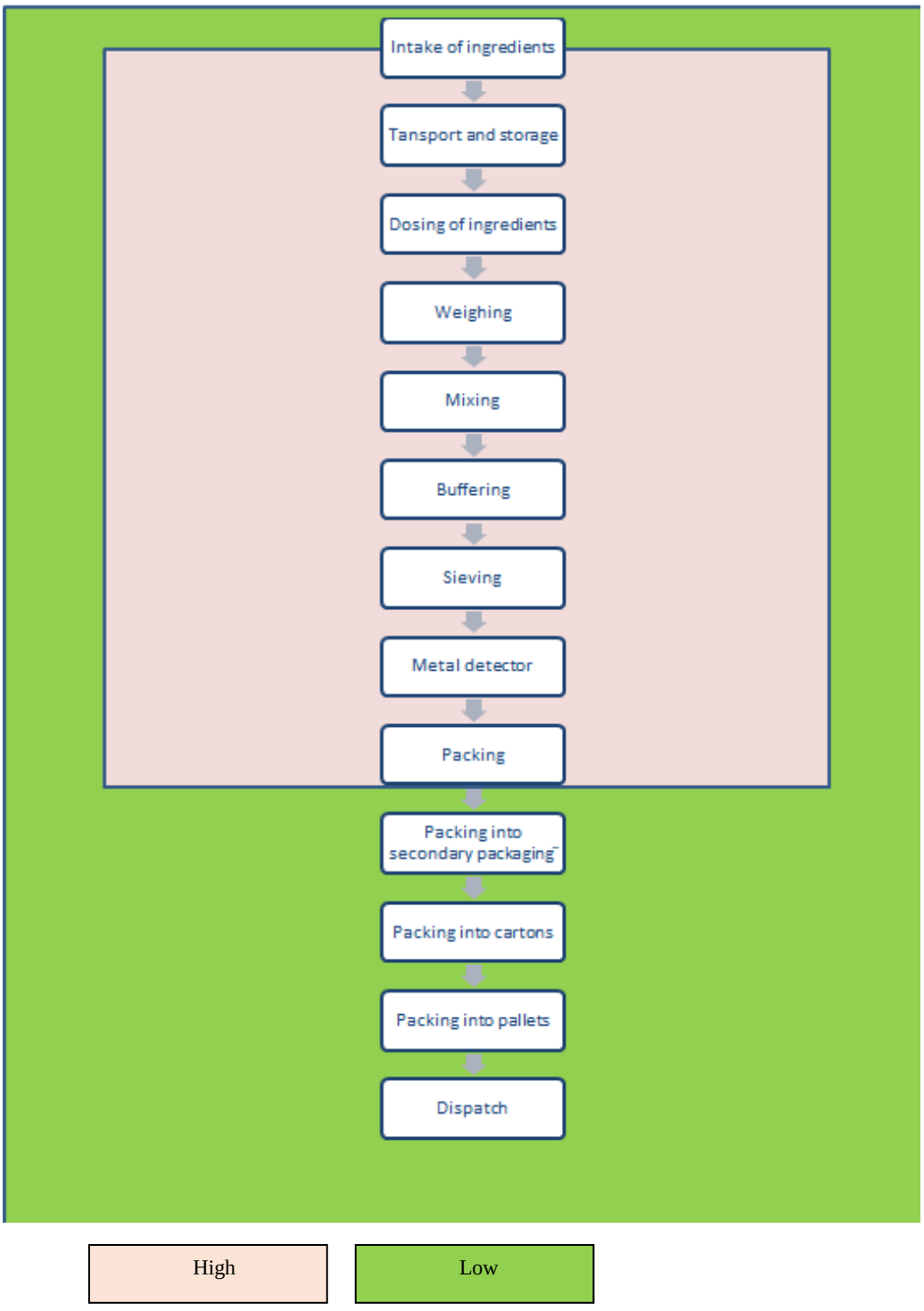


Figure 7.4 Process steps of the infant formula milk powder processing line including hygiene zones where the steps are located

The high hygiene zone starts at the dock where the ingredients are delivered by the company's own trucks (there is no physical barrier between the basic hygiene environment and the high hygiene zone – just a line on the floor). The zone then includes elevators and the corridors where the materials are transported; it then extends to the production areas. The production areas, however, are physically separated from the corridors, and operatives' footwear has to be changed on the entrance to these areas. There are also separate forklift trucks in the production and transport areas, however they have a common charging station. Occasionally, product is stored in corridors next to the elevators or in corridors next to the processing areas.

The low hygiene area starts after the product is packed in its primary packaging and finishes when the product is dispatched from the building.

The hygienic zones were marked on the process flow (Figure 7.5) and Figures in Sections 7.2.2.2 and 7.2.3.2 also contain potential sources and interventions to the processing line.

7.3 RISK RANKING OF SOURCES (PRINCIPLE 1)

7.3.1.1 Concept

Potential sources of microorganisms in food factories are associated with harbourage sites and growth niches.

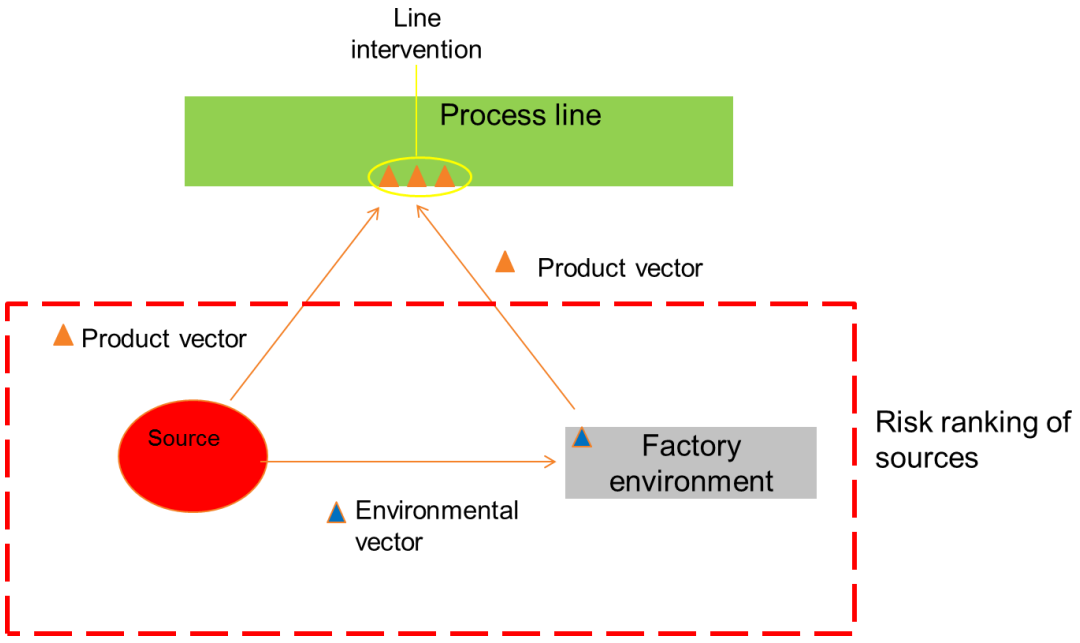


Figure 7.5 Points taken into consideration when risk ranking sources

Harbourage sites can be defined as physical areas in which pathogens can lodge and be protected from cleaning and disinfection actions, for example, poor hygienic design features of processing equipment or damaged areas of the plant's building structure. When a harbourage site also provides an environment suitable for growth, it can be considered a growth niche.

Potential sources can be determined by numerous means, including dismantling equipment to identify potential harbourage sites and niches, as well as an inspection of the environment and building structures. A review of microbiological data collected as part of an environmental monitoring programme may help identify potential sources in the production environment. However, a history of negative results for a particular pathogen on an environmental site does not indicate that the site is not a potential source for other pathogens, or that the site could not become a source in the future, especially when the construction of the site is not consistent with accepted hygienic design principles.

The factory should be inspected for the potential hazards and sources defined in Section 7.1; each potential source should be listed and risk ranked. Risk ranking should be done with the current identified control measures in place and again with potential control measures which could be implemented to lower the risk. When risk ranking the sources, the possibility of the area being a source and the possibility for microorganisms to spread from this source to the product and other areas via vectors, is considered (Tables 7.1 and 7.2)

The assessment of the probability of a structure to act as a source is a three point scale as, in terms of control, it is usually a repair (e.g. a cracked tile is replaced). A source is thus either present or it may be or it is not (Table 7.1). The assessment of the probability for the spread of microorganisms (Table 7.2) has a larger scale, because this is most likely going to be changed by any control measures applied, and it gives the possibility to show different levels of control in a food factory.

During the development of this approach, different scales were tried. For example, 3 point scales were tried for both ranked points, however this could not show much of a difference between the different control measures applied, which is very important for recognizing operational prerequisites and further improvement of control measures.

Table 7.1 Probability of the area of various sizes becoming a source of contamination (PS)

Rating	Meaning	Status
A	Low (virtually impossible due to absence of harborage sites and microbial niches and no known occurrences of pathogens in this area)	No source
B	Medium (relatively few harborage sites and/or microbial niches and no known or single occurrences of pathogens in this area)	Potential source
C	High (presence of multiple harborage sites and/or microbial niches and/or frequent occurrences of pathogens in this area)	Source

Table 7.2 Probability of spreading microorganisms from the source to the product and environment (PV)

Rating	Meaning
1	Extremely Unlikely (virtually impossible to spread to the product and other areas)
2	Remote (relatively few possibilities to transfer microorganisms to other areas and no possibility to transfer directly to the product)
3	Occasional (occasional possibilities to transfer microorganisms to other areas and/or relatively few possibilities to transfer directly to the product)
4	Reasonably Possible (frequent possibilities to transfer microorganisms to other areas and/or occasional possibilities to transfer directly to the product)
5	Frequent (frequent contact with vectors to the product and to other areas)

Also, a five point scale was designed for both ranked points, however this became too difficult to manage and would not be practical to use in a food factory. Additionally quadrant graphs and decision trees were also considered as a tool; however the chosen approach was a compromise between the best approaches for simplicity and effectiveness of the method.

7.3.1.2 Case Studies

The infant formula milk factory was assessed for potential sources and the available environmental monitoring data were analysed, however detailed hygienic design assessment of the processing equipment was not performed.

The investigated factory had a history of detecting *Cronobacter* spp. in the factory environment. Samples for *Cronobacter* spp. analysis are taken in the high hygiene zone on all stories of the building, the positive results are mainly attained from the floors (e.g. where high traffic of people is observed) and vacuum cleaners' samples. *Salmonella* spp. have never been detected in the factory environment or in the final product.

The identified sources and vectors associated with them were scored against the criteria described in section 7.2.2.1 and they are listed in Appendix H, however

two examples are also given in Table 7.3. The identified sources are also placed on the processing flow chart (Figure 7.6). The potential sources were scored with the current controls which are applied. In a normal HACCP study risks are ranked with and without controls. In this approach, the score without controls at all is not done, however it was considered during development of the concept. It always resulted in unacceptable levels of risk associated with each source, because if there were no factory walls, no hand washing procedures, or no cleaning etc. there would always be a possibility of pathogens contaminating the product from the outside environment.

The approach to scoring the sources could be explained for the example of source number 3: roof. It is scored C for the probability of the area becoming a source. This is because pathogens could be introduced there by birds, they could grow there (moisture, temperature, time, nutrients) or could be harbored because of presence of protective features. With the current controls such as hygienic building design and controlled access with additional protective clothing, the score for probability to spread the microorganisms is 3. This still did not receive the lowest score because there could be better controls implemented such as e. g. the entrance to the roof could be locked or access to the roof could be moved from the high hygiene zone (e.g. access to the roof from outside the building or from low risk is preferred). The hazards for all the identified sources are the same: presence and/or growth of pathogenic bacteria (e.g. *Salmonella* and *Cronobacter*) in harborage sites and environmental niches and their spread via environmental and product vectors.

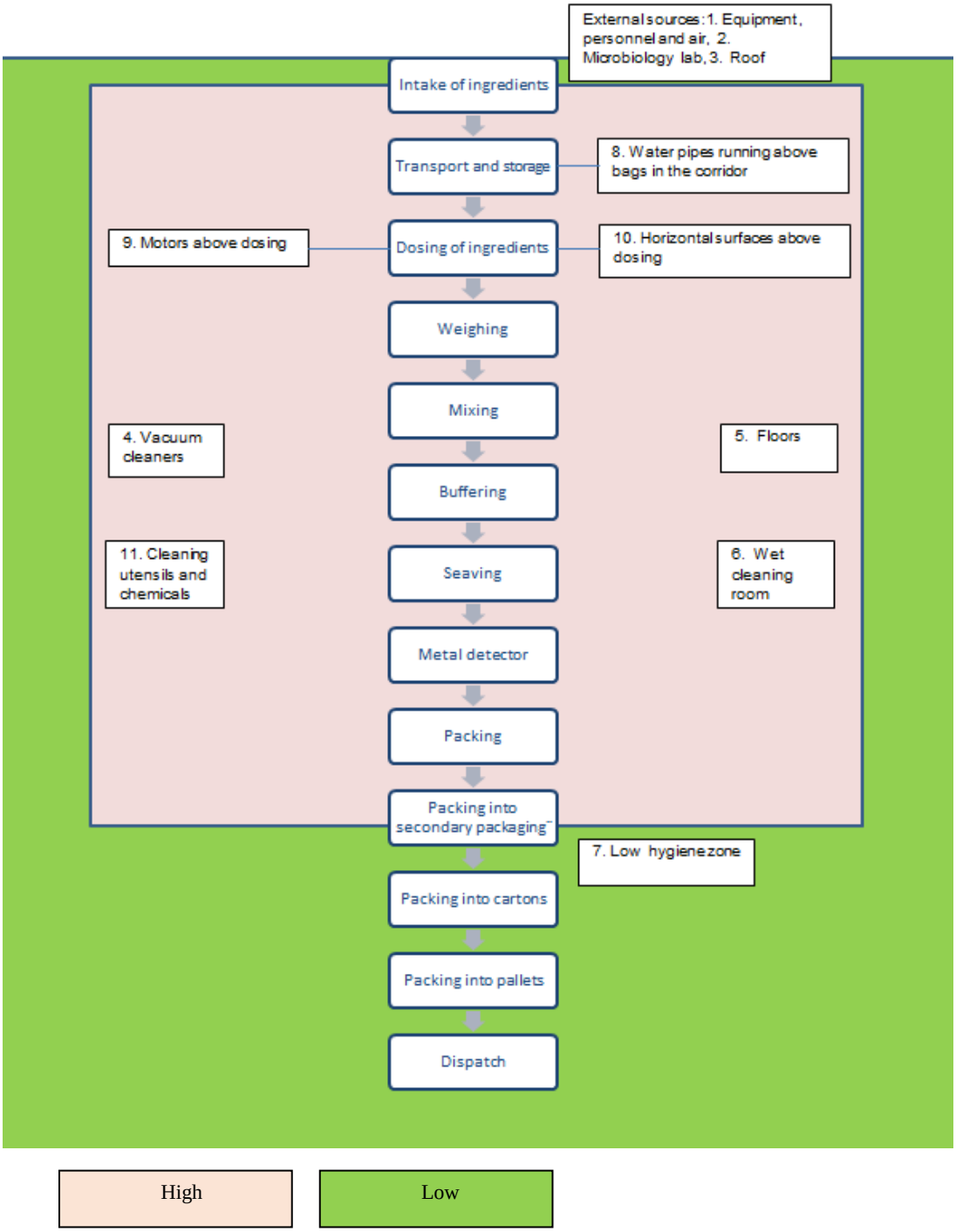


Figure 7.6 Process steps of the infant formula milk powder processing line including hygiene zones where the steps are placed and potential pathogen sources

Table 7.3 Examples of potential sources of pathogens and their current control measures

No.	Source	Vectors	Current control measures	PS	PV
1	Roof	People Clothing Equipment	Roof is designed and maintained to avoid leakage. The entry to the roof is protected from unauthorized movement by a chain. Personnel who enter the roof must inform a super-visor and wear shoe covers and additional overalls.	C	3
2	Vacuum cleaners – have a history of positive isolations and contain a number of harborage sites where microorgan-isms could be protected from cleaning	Vector itself People Equip-mint Floors	Cleaning: all vacuums are labeled, they are all cleaned at the same time in the same area, they are placed on plastic sheets in the corridor, outside the door to the processing area. The doors are frequently opened during cleaning (there could be aerosols in the air). The plastic sheet is placed directly on the floor therefore people walk on it and the cleaned parts are also placed on it. Personnel who clean the vacuums are often entering production in the same clothes. All shoes worn in the high hygiene zone are hanging next to the place where the vacuums are cleaned. There is no schedule for cleaning the hoses which are all cleaned together (product contact/non product contact). There is insufficient drainage on the drying wall. HEPA filters are not used in the vacuum cleaners.	C	4

7.3.2 Risk Ranking of Vectors Associated with Line Interventions (Principle 1)

7.3.2.1 Concept

A vector can be defined as anything (air and other gases, water and other liquids, physical objects or people) that carries or transfers a pathogen from one place to another. Vectors may be further described as those that carry a pathogen from a source to another location within the processing environment, that is, an environmental vector, or those that carry a pathogen from a source to the product or product ingredients, that is, a product vector (Figure 7.7). It should be noted that cross-contamination to food products manufactured in pipework and vessels usually occurs as an event (line intervention) when the line is opened and in which a number

of vectors may be involved. For example, collecting a product sample from an enclosed process line for quality control analysis by inserting a sampling bag into the product stream by hand may introduce potential product vectors of the operator's hand (or glove), the operator's sleeve, the sampling bag and the air.

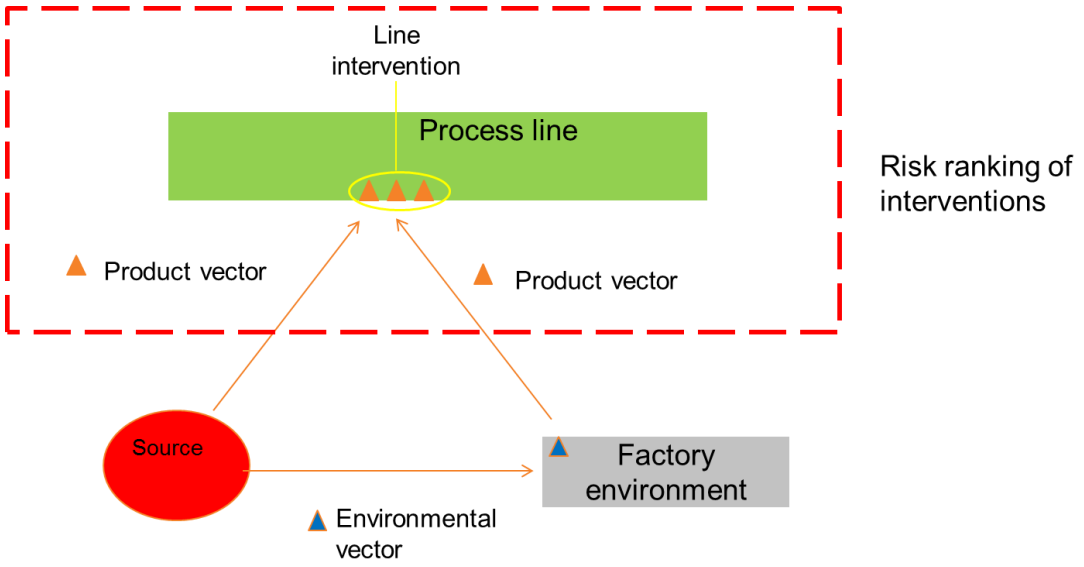


Figure 7.7 Points taken into consideration when risk ranking process interventions

The same cross-functional team should perform a comprehensive review of the process and environment for potential product/product contact surfaces cross-contamination vectors during line interventions. The process and processing environment should be observed during all shifts, when all types of products are produced and when infrequent procedures are performed. In some cases, all personnel may not perform the same task in the same manner. Therefore, interviews of line operators, maintenance staff and quality and sanitation personnel may also help determine potential cross-contamination vectors. Line interventions may occur at both fixed and defined time intervals, or randomly. Observations for potential cross-contamination vectors should be made independently of known or likely pathogen sources, as contamination could arise from temporary sites and be transferred to other sites and/or the product stream. It is unlikely that microbiological sampling of vectors would be helpful, as the likelihood of observing a pathogen on a potential vector would be very small.

Since the cross-contamination usually occurs as an event (line intervention) in which a number of vectors may be involved, in the following concept, single vectors are not listed. Instead, all intervention events on the processing line, packed product or packaging itself, and all vectors associated with them are listed. These intervention events are ranked by the possibility of vectors to carry microorganisms and the extent these vectors are in contact with the product/product contact surfaces (Table 7.4 and Table 7.5).

The severity of the hazard to the consumer of the product is not included in the risk assessment because the severity of the hazards should already have been established and assessed as part of the HACCP study (Principle 1).

Table 7.4 Probability of caring contamination by the group of product vectors during an intervention (PC)

Rating	Meaning
1	Extremely Unlikely (unlikely contact of all vectors with any potential source or source directly before intervention*)
2	Remote (relatively few possibilities of vectors to contact potential source, no direct contact of vectors with a source directly before intervention*)
3	Occasional (repeated possibilities of vectors to contact potential sources, no contact with a source or relatively low possibility to contact a source directly before intervention*)
4	Reasonably Possible (repeated contact with potential sources and occasional contact with sources directly before intervention*)
5	Frequent (frequent contact of multiple vectors with sources, potential sources directly before intervention* or one of the vectors is a source itself)

*Directly before intervention means there was no effective disinfection/decontamination in-between contact with a source/potential source and touching the product.

Table 7.5 Probability of contaminating the product during intervention (frequency and complexity of action, PP)

Rating	Meaning
A	Low (relatively few possibilities of direct contact with the product or product contact surfaces, no direct vectors)
B	Moderate (occasional contact with small proportion of product or product contact surfaces using few possible vectors)
C	High (direct contact with large amount of product and/or multiple contact with small proportion of product and product contact surfaces during production using multiple vectors)

Overall the approach takes into consideration: frequency of the vector coming in contact with food, frequency of a vector coming in contact with probable sources in the facility, presence of confirmed sources in the same area, history in the plant, environmental sampling, the amount of product or the area size of the product contact surface which comes in contact with the vectors and the number of vectors.

7.3.2.2 Case Studies

The author performed a comprehensive review of the process and environment for potential product cross-contamination vectors during line interventions. For this particular risk assessment, environmental vectors were not considered as due to the nature of the enclosed processed line, the product could not be contaminated other than via line interventions. The observations were made during a single shift only however sufficient information was collected for the case studies. Figure 7.8 shows all processing steps and the line interventions (product and product contact surfaces interventions) associated with these steps during or outside of production time. Table 7.6 shows one example of line intervention with its current control measures, the rest of the identified interventions are listed in Appendix I.

The hazard was already established during the HACCP study and it is the introduction of pathogenic bacteria (e.g. *Salmonella* or *Cronobacter*) from the pathogens' sources, via product vectors during line interventions that is assessed here.

The interventions are ranked with the controls currently implemented. For example, the intervention shown in Table 7.6: operators making holes with a knife into the big bag to enable powder removal is scored 3C. Score 3 is reflecting the contamination level of the vectors (hands, air, knife, clothing) which have direct contact with the product. The score is quite high because the knife does not have any cleaning schedule and it is kept in a holder on the wall. There is a possibility that this knife will come into contact with a potential source during this time and there is no cleaning and disinfection step before using it to pierce the bags. The score is not higher than three because the knife is hygienically designed, therefore it is probably not a source itself, operators wash their hands on entry to the processing area and the air is filtered. The C score is reflecting the fact that the knife comes in direct contact with a small amount of product, however on a frequent basis (each bag).

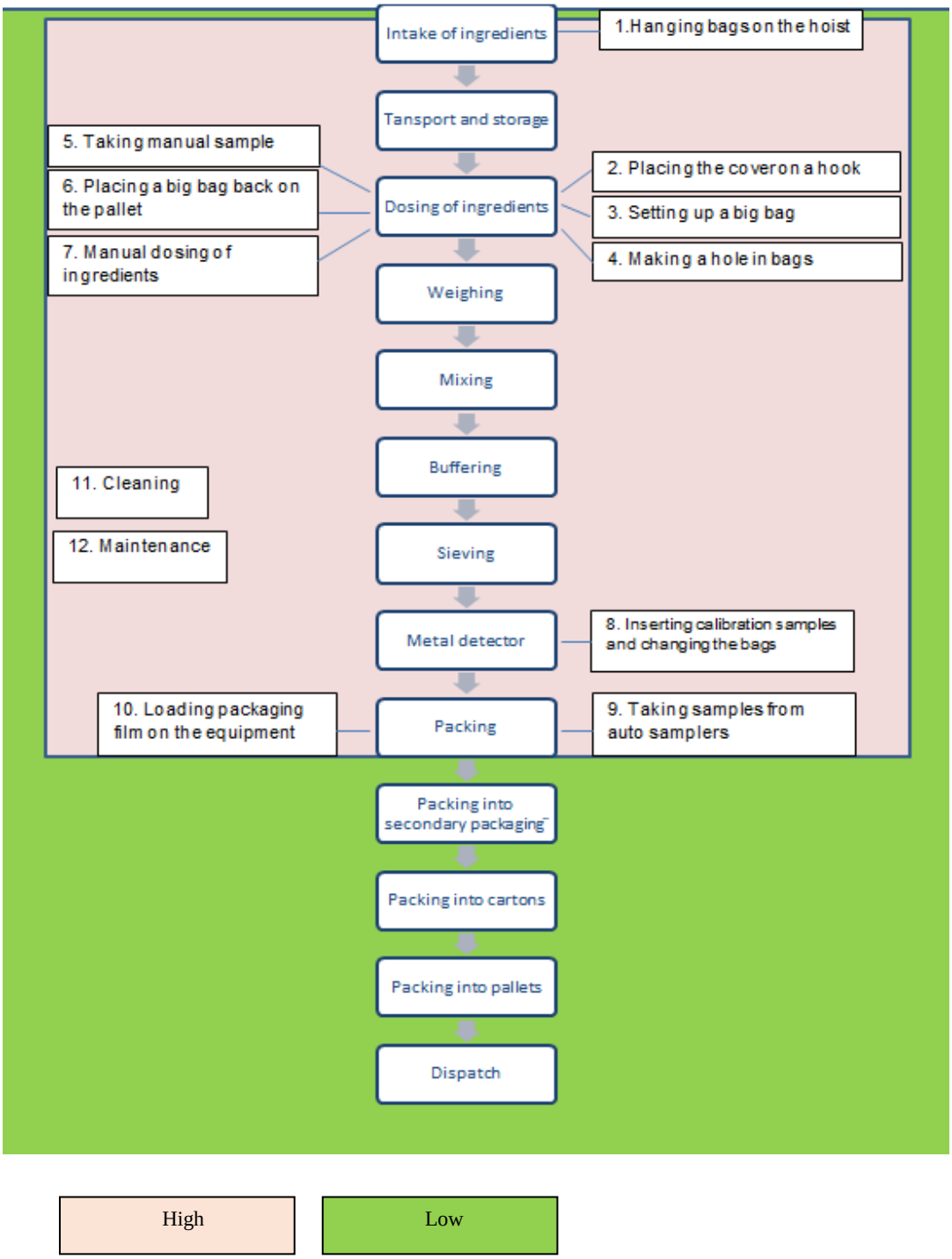


Figure 7.8 Process steps of the infant formula milk powder processing line including hygiene zones where the steps are placed and line interventions

Table 7.6 Examples of line interventions and their current controls

No	Process step	Intervention and hazard	Vectors	Current control measures	PC	PP
1	Dosing	Operators make holes with a knife into the big bag to enable powder removal.	Air	Positive pressure is maintained in the production rooms. Air is filtered.	3	C
			Hands	Hands of operators are not disinfected before use however they are cleaned at the entrance to the high risk area.		
			Clothing	Factory clothes are washed in a laundry which is certified, they are changed daily.		
			Knife	The knife is stainless steel but not cleaned and disinfected. The knife is kept in a holder attached to a wall (also not cleaned).		

7.3.3 Determination of OPRPs (Principle 2)

7.3.3.1 Concept

Although many potential sources and cross-contamination vectors may be identified during the assessment of a processing environment, the degree of control necessary for each source and line intervention has to be determined using the risk analysis tools described in Sections 7.2.2 and 7.2.3. After risk-ranking sources and interventions, both scores obtained for sources and both scores obtained for interventions are combined. Table 7.7 below is used to assess if the analysed source or intervention brings unacceptable, high, moderate or low risk of product contamination.

Table 7.7 Risk ranking - final score interpretation

Probability PS/PP	Probability PV/PC ⇩	A	B	C
1		Low	Low	Low
2		Low	Moderate	Moderate
3		Low	Moderate	High
4		Low	High	Unacceptable
5		Moderate	Unacceptable	Unacceptable

PS - Probability of the area becoming a source of contamination
PV - Probability of spreading microorganisms from the source to the product and environment
PP - Probability of contaminating the product during intervention (frequency and complexity of action)
PC - Probability of carrying contamination by the group of product vectors during an intervention

If the risk of product/processing line contamination is unacceptable (or high) the source or intervention should be controlled via an OPRP and additional controls should be applied to bring the risk to an acceptable level.

OPRPs are generally used to control activities that present a risk of direct product contamination in the post-lethality area of production, such as the management of key intervention steps into the process and sources in the processing areas.

Risk ranking of sources and vectors should be recorded as illustrated in the sections above. Undertaking a risk analysis with current and improved controls can help identify whether current or intended controls are sufficient to reduce either the risk of the source or the contamination event to an acceptable level. As a minimum, this allows consideration of the adoption of controls for the uncontrolled sources and vectors identified, which may have an immediate impact on improved food safety. If current controls are not sufficient to adequately control the hazard risk, additional controls must be undertaken.

Higher risk scores for sources may be used to help prioritize resource allocation, identify where additional control measures are needed and/or to justify capital expenditure. Likewise, higher risk scores for vectors may help prioritize actions taken to reduce the frequency of the vector (e.g. by reducing line interventions via installation of external devices for checks, such as ultrasound or see-through equipment walls), identify where additional control measures can lower the risk associated with the vector and/or to eliminate the vector altogether.

In summary, based on the hazard assessment, an appropriate combination of control measures should be selected which is capable of preventing, eliminating or reducing food safety hazards to defined acceptance criteria. The existing control measures should be reviewed for their effectiveness. The control measures selected should be categorized to allow management through operational PRPs or by the HACCP plan. Methodology and parameters used for this categorization shall be described in the Operational Prerequisite Management Programme documents and results recorded.

Table 7.8 Sources and interventions and their current and proposed controls

No	Source/ Intervention	Vectors	Current control measures	PS/PP	PV/PC	Proposed control measures	PS/PP	PV/PC
1	Vacuum cleaners – they have a history of positive isolations and contain a number of harborage sites were microorganisms could be protected from cleaning	Vector itself, People, Equipment Floors	Cleaning: all vacuums are labeled, they are all cleaned at the same time in the same area, they are placed on plastic sheets in the corridor, outside the door to the processing area. The doors are frequently opened during cleaning (there could be aerosols in the air). The plastic sheet is placed directly on the floor therefore people walk on it and the cleaned parts are also placed on it. Personnel who clean the vacuums are often entering production in the same clothes. All shoes worn in the high hygiene zone are hanging next to the place where the vacuums are cleaned. No schedule for cleaning the hoses which are all cleaned together (product contact/ non product contact). There is insufficient drainage on the drying wall. HEPA filters are not used in the vacuum cleaners.	C	4	The vacuum cleaners which have tested positive for *Cronobacter* or *Salmonella* should be disposed of. More hygienically designed vacuums should be used or low cost vacuums could be used for short periods of time e.g. 3 months and then they could be disposed of. HEPA filters should be used in the vacuums. Cleaning and changing of bags should be performed in a dedicated area, separated from the high hygiene zone, with dedicated people, utensils and clothing. All vacuums should be dedicated to specific areas, including spare ones (environment or product contact surfaces). Hoses should be dedicated to a specific vacuum cleaner and should have the same cleaning schedule as the vacuum cleaners.	B	3
2	Operators make holes with a knife into the big bag to enable powder removal	Air Hands Clothing	Positive pressure is maintained in the production rooms. Air is filtered. Hands of operators are not disinfected before use however they are cleaned at the entrance to the high risk area. Factory clothes are washed in a laundry	3	C	Personnel should disinfect their hands and wear gloves before performing this activity. The knife should also be disinfected each time before use.	2	C

		which is certified and has separation between dirty and clean areas, they are changed daily.			
	Knife	The knife is stainless steel but not cleaned and disinfected. The knife is kept in a holder attached to a wall (also not cleaned).			

7.3.3.2 Case studies

To illustrate this approach, the example of the vacuum cleaners as a source can be used. The current controls applied such as labeling and cleaning of vacuums are not sufficient to control the hazard (Table 7.8) this source scores 4C which is unacceptable. It might be necessary to implement better cleaning procedures, control personnel clothing and make the use of vacuums dedicated or purchase vacuums of a more hygienic design (see Table 7.8). After proposing more comprehensive controls, the sources are scored again and it is evident that it is possible to lower the risk level to moderate (3B, Table 7.8). The same is illustrated for the example of interventions: making holes in a big bag to enable powder removal (see Table 7.8). The final score for this line intervention is 'high', however this score could be reduced by implementing more controls (e.g disinfecting the knife and hands before use). Before new controls are implemented the sources and interventions which score high or unacceptable must be controlled via OPRPs.

7.3.4 Establishing Operational Limits, Monitoring, Corrective Actions, Verification and Record Keeping Procedures (Principles 2-7)

7.3.4.1 Concept

As the control of product interventions and sources which score high or unacceptable in the risk ranking section, are especially important to product safety, these controls could be described as OPRPs. An OPRP requires the establishment of operating limits (or control limits), monitoring activities, corrective actions for when a control limit is not met, verification activities and record-keeping procedures. Sources and interventions should be analysed separately for each single hazard.

In the same manner as CCP records, the records of an OPRP should be incorporated into a food safety plan to ensure all essential conditions, which are frequently monitored, were met during the manufacture of a product and when the factory is in out-of-production mode. The OPRPs cannot usually be continuously monitored, therefore the frequency of checks must be established. If a deviation in an OPRP were to occur, then the affected product should be placed on hold while a cross-functional team is assembled to review the associated risk and make a decision on product disposition.

The management strategy described above for OPRPs is essentially the same as for CCPs as defined under HACCP. However the difference between a CCP and

an OPRP is that CCPs are generally described for specific steps in the manufacturing process to eliminate or reduce a significant hazard to an acceptable level, for example, pasteurizing milk or running a liquid product through a screen of defined particle size. On the other hand, OPRPs are generally described for procedures or programmes that address some aspect of the processing environment or the interaction of the processing environment with the process, for example, the manual piercing of an ingredient bag with a knife during a production run or taking a manual sample from the product stream etc.

The effectiveness of OPRP plans designed to control hazards must be validated (e.g. cleaning could be validated to ensure visible cleanliness and target ATP or microbiological values etc.)

7.3.4.2 Case Studies

The case studies have described sources and interventions associated with the presence and spread of pathogenic bacteria within a factory and directly to the product. Table 7.9 describes the control measures, operational limits, monitoring, corrective actions and records associated with the vacuum cleaner which was identified by risk assessment to be one of the OPRPs. The hazard is *Salmonella* or *Cronobacte*r which could be transferred onto the equipment product contact surfaces during cleaning. The vacuum cleaners have often tested positive for *Cronobacter*, therefore they could be a potential source of pathogens.

Current control measures are not sufficient to control the hazard, however in the future, controls could include disposing of the vacuum cleaners which have tested positive for *Cronobacter* or *Salmonella* or using more hygienically designed vacuums with HEPA filters. Cleaning and changing of bags should be performed in a dedicated area, separated from the high hygiene zone, with dedicated people, utensils and clothing. All vacuums should be dedicated to specific areas, including spare ones (environment or product contact surfaces). Hoses should be dedicated to a specific vacuum cleaner and should have the same cleaning schedule as the vacuum cleaners.

Table 7.9. OPRPs for controlling presence and spread of pathogen within factory environment and to the product

No.	Source/ Interven-tion	Hazard	Control measures	Operational limit	Monitoring and verification	Corrective action	Records
1	Vacuum cleaners used for product contact surfaces	Post-process microbial contamination of product contact surfaces	Disposal of vacuum cleaners which have tested positive for *Cronobacter* or *Salmonella.* Purchase of hygienically designed vacuums with HEPA filters	All vacuums tested negative for *Salmonella* and *Cronobacter*	Check micro-biological results once a day	Put on hold any potentially contaminated product Determine the need for equipment to be decontaminated	Laboratory results log book
			Cleaning and changing of bags should be performed in a dedicated area , separated from the high hygiene zone, with dedicated people, utensils and clothing according to the designed schedule. Hoses should be dedicated to a specific vacuum cleaner and should have the same cleaning schedule as the vacuum cleaners. All vacuums should be dedicated to specific areas, including spare ones.	Concentra-ton of isopropanol 70% (target 75%)	Check con-centration of isopropanol before scheduled cleaning	Investigate the cause of the problem and take appropriate actions Staff retraining	Vacuum cleaners sanitation log
				ATP after cleaning <150	ATP measurement after every clean and before use	Review of cleaning procedures	Post-decontaminat ion ATP sampling records
				All vacuums are labeled	Check labels on vacuums before use and before clean		Vacuum cleaners registry log

The monitoring activities could include ATP testing prior to use of vacuums which could apply an operational limit to an assessment of the cleanliness of the vacuum cleaners. Verification of cleanliness could be periodically undertaken by microbiological sampling. Also, prior to using the vacuums, checks should be made on the last microbiological results and the labels. Before cleaning of vacuums, the concentration of disinfectant should be checked, and during cleaning, observations could be made to ensure the procedure was being done correctly and that there were no extrinsic factors that could act as additional cross-contamination vectors.

Records should be kept of all sanitation activities of vacuum cleaners, whether cleaning procedures had been correctly followed, ATP and microbiological counts, concentration of disinfectant and vacuum numbers. Corrective actions would review the training of the staff against cleaning equipment decontamination programmes.

The whole OPRP plan would have to be further validated, verified and reviewed which can be done together with the same HACCP activities. The validation activities should cover the whole Food Safety System (HACCP and OPRP) and the specific Operational Prerequisites. Validation could for example include the effectiveness checks of control measures, such as validation of cleaning and disinfection efficacy of vacuum cleaners. The activity would also include document review of the OPRP plan. Verification activities should include e.g. auditing, microbiological testing, trending etc. If any change e.g. to the factory environment, practices, key personnel, equipment or process itself are implemented, the review of the programme should be undertaken.

7.4 DISCUSSION

As was explained in Section 7.1, the most common food safety problems do not include failure of the HACCP CCPs. Instead, the microbiological safety is usually affected by prerequisites such as inefficient employee hygiene practices, niche environments, plant renovations, lack of hygienic equipment design, post-processing contamination, ineffective application of sanitation practices etc. (CDC, 2014; RASFF, 2014). There is also an increase in new and antibiotic resistant infectious foodborne agents such as *Campylobacter jejuni* (Wardak *et al*., 2006) or *Salmonella* Typhimurium DT104 (Briggs and Fratamico, 1999). The evolution of

these new agents, new vectors transmitting known pathogens and resistance to harsh environmental conditions and biofilm resistance to disinfectants (Bridier *et al*., 2011; Campanac *et al.*, 2002) makes prevention of food contamination a moving target for those in charge of ensuring food safety. Also, the aging population is at a higher risk of developing illness from contaminated food (Lund and O'Brian, 2011). These and other changes over time significantly increase the risk of contracting a foodborne illness, which is demonstrated by an increased number of outbreaks associated with dry foods which previously were considered as safe without the necessity of implementing stringent control measures (Table 1.1). All of these factors bring a need to take a new look at food Good Manufacturing Practices (GMPs) and food safety management systems.

The requirement to further focus on prerequisites has already been highlighted in many food industry guidelines such as the Food Safety and Inspection Service (FSIS) document (Anon., 2014) which talks about preventing *Listeria monocytogenes* contamination in the post-lethality processing environment through Sanitation Standard Operating Procedures (SSOP) or other prerequisite programmes. Also the Food Standards Agency in the UK produced a guideline on controlling cross-contamination of *E. coli* 0157 in ready-to-eat food (Anon, 2013). All these guidelines however, do not give a systematic approach for selecting OPRPs, they just emphasise the importance of prerequisites in controlling cross-contamination.

Some of these concerns were also confirmed in this thesis. It was shown that *Salmonella* can survive on stainless steel surfaces for up to a year and presumably much longer. The presence of water, food debris, lower temperatures and humidity further increased the survival rate. Dry cleaning methods currently used are not sufficient to remove *Salmonella* and wet cleaning is both not compatible with factory and equipment design and introduces an additional risk with respect to potential microbial growth. It must be assumed that a pathogen such as *Salmonella* will find its way to the factory environment and as soon as it is introduced, it can establish itself within production areas. Therefore, it is extremely important to control cross-contamination with pathogens from the factory environment to the final product.

The assessment, management and ultimately control of cross-contamination sources and vectors will likely include both the HACCP plan and its foundational PRPs (Ropkins and Beck, 2000). Therefore, this approach is consistent with

established HACCP models, in which hazards that may be introduced at a process step should be considered in the process hazard analysis (Khandke and Mayes, 1998).

The approach gives a freedom to the method taken to establish sources of microorganisms and their vectors. Potential pathogen sources and cross-contamination vectors in a processing plant can be determined by a physical examination of the processing environment and may include microbiological sampling. Sources and vectors may be associated with a specific process step or may affect the processing line in general. Regardless of how the identification and assessment of cross-contamination sources and vectors are incorporated into a food safety plan, efforts to control them should include reducing the number of possible sources and vectors within a processing environment and developing specific measures to reduce the risk associated with those that remain or which are intrinsic to the food production process. For example, the usage of water in certain processing environments may be significantly reduced or eliminated in an effort to control the establishment, growth and movement of pathogens (sources and vectors). It is also not that significant how the risk analysis of vectors and sources is going to be undertaken. The approach proposed in this chapter uses a specific scoring system which fits very well with the purpose, however any other approach could be used, such as quadrant graphs, logic tables, Codex Alimentarius (2009) risk assessment grid (Low/Medium/High) or decision trees (Campden BRI, 2009). The HACCP team should set the numerical combined score value that they consider to be significant.

With this approach OPRPs are used to control activities that present a risk of direct product contamination in the post-lethality area of production.

Figure 7.9 suggests the new concept of food safety management in food factories. HACCP and CCPs are focused on the most critical parts of the production process and are most important in controlling food safety. Operational Prerequisites are designed to control most vital activities and features of the factory environment and are also very important but not as critical as CCPs. On the bottom of the pyramid is the whole prerequisite program which is more general and controls broad aspects of good manufacturing practices which, in case of failure to control, are not posing a direct and significant hazard to people’s health.

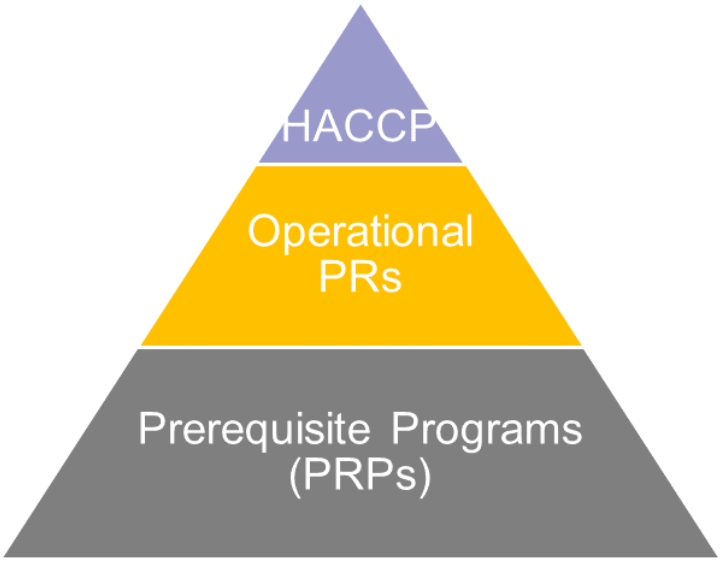


Figure 7.9 Schematic of proposed Food Safety Management system in food manufacturing factories

7.5 CONCLUSIONS

There are some viewpoints suggesting that everything managed outside of the CCPs in the HACCP plan (e.g. general hygiene issues) will be forgotten or not taken very seriously. On the other hand, including every aspect of risk in the CCPs makes the management of the system very complicated and resource demanding (Wallace and Williams, 2001). It is crucial to formalise the approach to both HACCP and prerequisites programs to control all food safety issues within the factory and the approach described in this chapter provides an effective way to do it.

Elevating the control of sources and vectors to the level of OPRPs and managing them in a similar way to CCPs focuses attention on the control of what is thought to be the highest risk of cross-contamination from the processing environment to the product. It may provide a means to demonstrate increased confidence in product safety should a pathogen be found in the manufacturing environment.

The concept of identifying sources and vectors of cross-contamination, assessing their risk and managing their risk through OPRPs in a fashion similar to CCPs is a developing study and will have to be further verified in more factory scenarios. Taken beyond microbiological hazards, the same source-and-vector approach may be used to evaluate and control non-microbiological hazards, such as allergens or foreign material.

CHAPTER 8
GENERAL CONCLUSIONS

The objectives of these studies were to obtain more insights into the survival mechanisms of *Salmonella* attached to stainless steel surfaces under dry conditions and thus propose ways to control this pathogen in factory environments. These studies highlight the unique characteristics of low-moisture foods environments and *Salmonella's* responses to them. The work was strongly based on real dry food manufacturing problems and gaps in scientific knowledge, therefore each step of the studies revealed more insights about the pathogen behaviour and possible control methods. Knowing that *Salmonella* was the main food safety hazard for dry foods, because it could survive extensive time periods within the food matrix, it was investigated as to whether it could also survive on food processing surfaces. When it was apparent that *Salmonella* was very resistant when attached to surfaces under a range of environmental conditions, the physiological characteristics and genetic responses were investigated to better understand the mechanisms of its endurance. Moreover, the current control measures used in the food industry were tested, and additionally new management tools were proposed for better control. The thesis therefore, followed a logical sequence which included both, scientific understanding and industrial applicability. Even though the thesis brings more understanding and knowledge about *Salmonella* behaviour in low moisture foods, more studies are needed to have complete and confirmed data.

8.1 *SALMONELLA* – A PATHOGEN OF CONCERN

Salmonella was chosen for this study as it is the main pathogen of concern for low water activity foods (a_w<0.85) causing approximately 77% of reported outbreaks associated with this type of food (Beuchat *et al.*, 2012). Over half of these outbreaks were caused by cross-contamination from the environment to the product, which emphasise the importance of controlling this pathogen in the factory environment. For example, in 2014, there were 214 alerts associated with *Salmonella* in low moisture feed and food in Europe (RASFF database, 2015). An additional concern is associated with its extremely low infectious dose, which, interpreted from some outbreaks data, could be as low as <10 cfu (D'Aoust *et al.*,

1975). The survival and cross-contamination of just single *Salmonella* cells in the food processing environment is thus potentially critical for food safety. Also, as foods become less moist, the heat resistance of *Salmonella* increases, which brings additional challenges to the food manufacturers and their established processing methods.

8.2 *SALMONELLA* SURVIVAL ON ENVIRONMENTAL SURFACES

The first question which had to be asked concerned the ability of *Salmonella* to survive on food processing area environmental surfaces. An important factor to help answer this question was the selection of a range of appropriate strains. Typically, scientific work is based on use of culture collection strains isolated from human or animals which have no history of surviving in dry conditions. In these studies, 15 isolates of *Salmonella* were used and they were isolated from low a_w foods or their environments. Strains associated with outbreaks were also selected, together with strains used for thermal validation of processes and disinfectant testing. Most of the selected strains survived well during 30 days of attachment under desiccating conditions, however there were a few strains such as: *S.* Typhimurium NCTC12023, *S.* Typhimurium DT104 or *S.* Muenchen with particularly high survival rates, which could be used for further desiccation studies. The importance of choosing appropriate strains was highlighted by the fact that the same *Salmonella* serovars (e.g. *S.* Typhimurium) had strains with the highest and lowest survival rates. Therefore for any future desiccation studies, it is not only important to choose appropriate serovars but also to know the origin of the particular strain.

The microbiological method selection can also be challenging for this type of investigation. The cells are desiccated and attached to surfaces, therefore recovery of them for enumeration purposes could pose a concern. A number of recovery methods were tested and the one selected, with its use of a short soaking, diluent and additional agar incorporation method, was proven to work with these types of cells. The results obtained, therefore, are associated with actual *Salmonella* death rather than poor recovery. Because the work conducted was strongly influenced by industry practises and needs, it was also crucial to compare the chosen recovery method to the existing industry ISO standard enumeration method. The results obtained by using both methods were comparable in terms of identifying the absence or presence of

microorganisms; the industry method, however was not used, as it did not give quantitative results. It is not suggested to change the existing standard to the method proposed in this study, because in the food industry, the number of detected *Salmonella* cells is not important. In general, this pathogen should not be present in any food or food processing environment, therefore it is sufficient to obtain 'presence' or 'absence' results. Especially when food products are tested, it is important to be able to analyse large quantities in one sample, as often single colonies could be present and distribution is usually not homogeneous. The same approach is relevant to surfaces, as areas as large as possible are swabbed and the whole volume of the recovery medium can then be incubated to check for growth. In the case of these studies, the numbers of survivors were high, therefore it was not necessary to have more sensitive methods. For valid results it was also important to show a comparison of the survival of cells in suspension with attached cells and it was proven that cells in suspension have greater survival abilities than desiccated cells attached to surfaces.

One of the most important findings of the studies is the proof that *Salmonella* can survive on a surface for over a year without nutrients and water, which emphasises the fact that the first and important factor to control it, is prevention of entry of this microorganism to the factory environment. This is fairly possible if the pathogen is carried by people, equipment, pests etc., however if it is carried within a raw material it is not possible to stop its entry into the processing environment. Therefore once this pathogen is introduced to the factory it is likely to survive there for years as time was not a significant factor influencing its decline. The mathematical model of *Salmonella* survival on surfaces, which was established in these studies, can help to understand its survival kinetics and, therefore help to develop control mechanisms. It was evident that the *Salmonella* populations can be divided to two subpopulations: sensitive and resistant to desiccation. As the sensitive subpopulation declined during the first days of desiccation, the resistant subpopulation remained viable for long periods of time, without signs of further declining. This suggests that up until 3 days of desiccation, some cells are undergoing changes and after this time period, the cells are in a stable state. This can be used for studying genetic responses and the time periods selected for gene expression investigations were based on that model. This information can also be

used for any other studies focused on desiccated *Salmonella* cells e.g. studying disinfection properties or cross-contamination potential, as it is not necessary to wait longer than 3 days because the physical responses of the cells after this time do not change significantly.

8.3 THE EFFECT OF FACTORY ENVIRONMENTAL CONDITIONS ON *SALMONELLA* SURVIVAL

As it was proven that *Salmonella* can survive on environmental surfaces, it was then important to know how survival could be affected by the factory environment. The vital aspect of the study was, therefore, a choice of the experimental conditions. These were again based on real industrial settings which were selected during a number of factory visits. This makes the study much more applicable to the food industry as the findings could be directly related to processing conditions. First of all, the surface material selected was stainless steel, which is the most common material used in food factories. All international standards and guidelines (3A, EHEDG, ISO) recommend the use of stainless steel for food contact surfaces. Some of the published studies which investigated the responses of *Salmonella* during desiccation, used paper discs (Li *et al.*, 2012) or polypropylene (Gruzdev *et al.*, 2012) which does not reflect real processing scenarios. Other environmental conditions, such as temperature, humidity, the presence of food debris or the frequency of water use, were also based on food factory settings. The results of the studies suggested that lower temperatures and humidity, the presence of food debris and access to water, favoured the survival of *Salmonella* on stainless steel. The number of inoculated cells on the surface however, did not affect reduction levels. Therefore a significant contamination event is not necessary to allow survival, and finally, just few cells can cause illness.

The observation of the effect of water on *Salmonella* survival was especially important for the studies and their significance to the food industry. It is known worldwide that dry food processing environments should be kept dry as introducing any water will increase the risk of *Salmonella* growth. This GMP advice is ostensibly followed, however manufacturers still undertake wet cleaning to remove allergenic ingredients, to control known pathogen sources or as a definitive batch separation process when running continuous production. It has been perceived that if

water introduction is not conducted on a daily basis, the risk is much lower. However these studies showed that the addition of water caused growth of resistant strains, regardless of whether it was added after a week or a month and regardless of whether nutrients were present. Additionally, the strains which were sensitive to desiccation survived considerably longer periods of time, when water was added periodically. As some of the strains were also highly motile, there may be a higher possibility of pathogen transfer and further cross-contamination to the product or product contact-surfaces under wet conditions. This also raises concerns of whether food industry practices are robust enough to adequately control *Salmonella* and consequently, reduce foodborne outbreaks and recalls in dry food production.

8.4 POTENTIAL PHYSIOLOGICAL SURVIVAL MECHANISMS

The proven ability of *Salmonella* to survive over a year on stainless steel without nutrients and water raised further questions about the mechanisms responsible for such survival. It is commonly suggested that cells undergo similar responses during osmotic shock as during desiccation, as in both scenarios, water availability is higher inside the cell than in the surrounding environment (Moat *et al*., 2003). *Salmonella* strains were tested in a number of trials to verify this hypothesis. First of all, isolates were screened for selected properties such as: RDAR phenotype, filament formation, the presence of extracellular material (analysed by SEM), auxotrophic mutation and swim and swarm abilities which were suggested to be related to osmotic stress resistance. There was no common pattern which could suggest that strains resistant to desiccation always had the properties which are known to support osmoresistance. These properties were present only in some strains resistant to desiccation. Secondly, when the survival of selected strains in a dry state was compared to the survival of cells immersed in different humectants, it was evident that the responses were different. The most similar osmotic stress survival pattern was associated with sucrose as a humectant, however after 10 days, cells attached to surfaces demonstrated constant survival levels for a further 50 days whilst cells immersed in sucrose declined to undetectable levels. It could be argued, that recovering the cells brought them into highly hypoosmotic conditions which could damage them and give a false security that attached cells were dead whereas in fact, they may have died during recovery. This was, however tested with the addition

of betaine and the results showed that this osmoprotectant did not improve cell recovery.

The influence of the surface inoculum droplet drying mechanism was also considered in these studies. It was shown that the cells accumulated on the edge of the droplet; therefore a possible multilayer was created which might influence the obtained results. An experiment with different inoculum levels was therefore undertaken, which demonstrated that the reduction rate was always the same, regardless of cell number. This suggested that cell accumulation during desiccation was not a major survival factor. Another concern regarding cell behaviour was associated with water addition onto dry, attached cells with minimal amounts of nutrient present. It was not clear if the water caused better recovery or microorganism growth. With the use of vitality stains applied to cells attached to the surfaces, it was shown microscopically that cell numbers increased when water was present, suggesting, therefore, that growth had occurred. It was also demonstrated that known heat and antibiotic resistant cells survived desiccation very well, however, cells which were previously subjected to desiccation stress, did not show any enhanced resistance to subsequent desiccation, disinfection or osmotic shock. This suggests that mechanisms responsible for heat and antibiotic resistance are also playing an important role in desiccation stress responses.

8.5 POTENTIAL GENETIC SURVIVAL MECHANISMS

In this type of investigation it is also crucial to understand what is happening within the cell at the molecular level. The selection of methods was not that straight forward when assessing the expression of genes. RNA extraction proved to be very challenging and commonly used methods were not very successful. All three RNA extraction methods which were tested were based on published methods used previously for the transcriptomics of *Salmonella* in dry conditions (Gruzdev *et al.,* 2012, Li *et al.,* 2012, Finn *et al.,* 2013). However, information about the quality and integrity of the extracted RNA was not reported. These studies compared the three commonly used RNA extraction methods and showed that the MiniPrep kit (Qiagen) proved to give the highest yield, quality and integrity of RNA for the *Salmonella* strains studied. The integrity however was still not excellent, as compared with *E.*

coli cells used for control purposes. An investigation therefore is required to be undertaken to find out the reasons for this difference.

The expression of genes responsible for osmotic shock, fatty acid metabolism, protein synthesis and transport were studied during desiccation. Gene expression was analysed for desiccation resistant and sensitive *Salmonella* strains, and also for cells in suspension and dried on stainless steel. Cell types were analysed at different time points as suggested by earlier survival kinetics. The largest challenge was associated with suitability of housekeeping genes selected. All four genes showed changes in expression during desiccation, therefore they could not be used for normalisation of studied genes. The qRT-PCR results were still analysed, however without normalisation against a housekeeping gene. When the desiccation resistant strain was tested, only some of the genes showed higher fold change (*aceA, nifU* and *otsB*, responsible for osmotic stress relieve and trehalose production), most genes however were having higher Ct values during desiccation as compared with cells in suspension. The sensitive strain showed a higher level of fold change for all selected genes during desiccation, as compared to cells in suspension, and most of the genes had their greatest change after 68 hours of desiccation. As the published literature to date reports different mechanisms involved during desiccation, these studies provide additional knowledge to better understand processes inside the cell and methods to study these processes. However, additional work is still needed.

8.6 FACTORY BASED CONTROL MECHANISMS

The results of these studies suggested that *Salmonella* persistence could be reduced if dry processing environments were kept dry, operated at a high temperature and high humidity with frequent cleaning to remove dust and food particles. The question was then asked if the currently used wet disinfection methods are effective and necessary to control desiccated *Salmonella*. From the trials conducted, there was no evidence that dried *Salmonella* cells were gaining disinfectant resistance which would be sufficient enough to survive treatment with working concentrations of propanol or sodium hypochlorite. This however could be different when food debris is present and/or the hygienic design of the equipment prevents full access of the disinfectant to the bacterial cells. If cells are subjected to lower disinfectant concentrations, long-term desiccated cells might be more resistant

than freshly dried ones and this could cause a food safety issue. Even though both alcohol and sodium hypochlorite, used at their specified concentrations, are effective in terms of *Salmonella* decontamination, the use of wet disinfection is forcing the factories to use water in the processing environment which, if uncontrolled, could possibly cause more risk (related to potential cell growth) than bring actual benefits.

The food industry should re-evaluate current food processing protocols, and cleaning and disinfection procedures in the production environments of low-water activity foods in order to assure food safety. The food manufacture should limit wet cleaning or introduce quick, validated drying directly after cleaning, as well as regularly checking for any leaks (e.g. roof and pipes). There is also an additional challenge for chemical and decontamination methods providers to develop methods which can effectively remove heavy soils, allergens or pathogens without the use of water. Moreover, microbiological laboratories and food safety experts should then develop methods to validate and verify these novel decontamination techniques. These studies have provided an excellent background and base for such future work, as it has proposed *Salmonella* strains, recovery methods, time scales and processing conditions which are appropriate for future testing.

8.7 ENHANCED FOOD SAFETY MANAGEMENT TOOLS

Practical advice to control *Salmonella* in dry environments is not sufficient alone and the implementation of a management tool to manage the risk of *Salmonella* is also a priority. Based on the findings of these studies, published literature and factory observation, a control system was proposed to reduce the possibility of cross-contamination of *Salmonella* from factory surfaces to the final product. This control system can be represented as a schematic circle as shown in Figure 8.1. The basic philosophy of this method is to put in place barriers to prevent *Salmonella* entry into the processing environment, prevent the harbourage and growth of any cells that enter the building and equipment fabric, reduce the vectors which could transfer *Salmonella* to the product or product contact surfaces and finally, provide effective cleaning and disinfection methods.

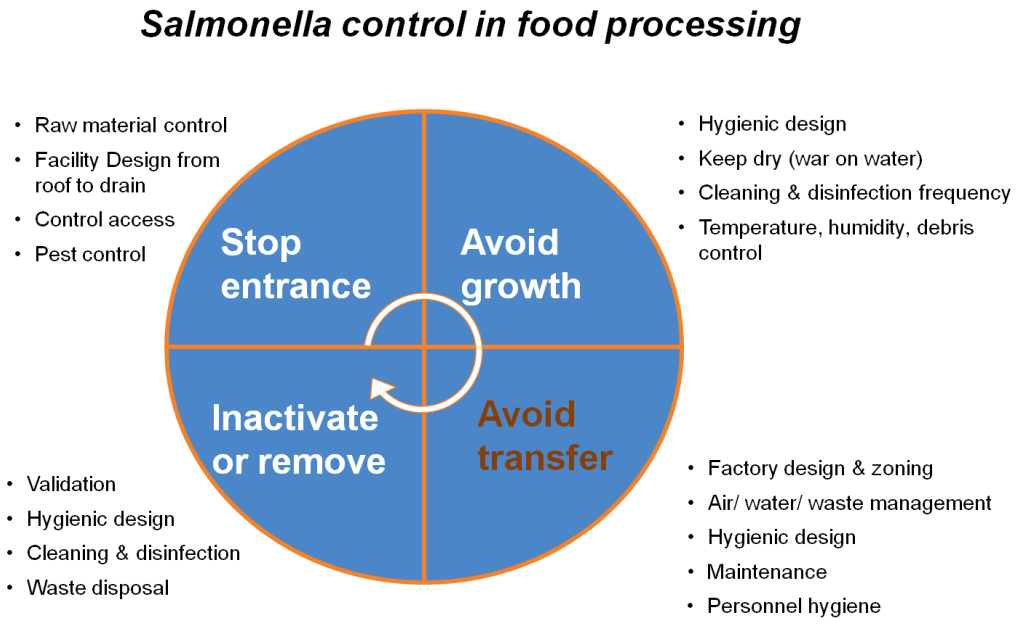


Figure 8.1. Basic approach to control *Salmonella* and other hazards in the food processing environment, adapted from Warren, 2012

This proposed method was further developed by building on the commonly used food safety approach known as the Hazard Analysis and Critical Control Points (HACCP) system. The HACCP system functions well for process control, however it does not control environmental cross-contamination routes, leading to product contamination following the last thermal Critical Control Point (CCP), which are responsible for most *Salmonella* outbreaks associated with dry foods. The approach is based on identifying any possible routes of product contamination during and outside of the processing window (which are usually associated in dry food manufacturing with line interventions); and any established and potential sources of *Salmonella* in the factory environment. These are then risk-ranked to establish the most critical factors to maintain food safety, and their control measures, monitoring practices etc. are further improved and controlled. The line interventions and sources which have the highest likelihood to introduce a hazard to the product are then designated as Operational Prerequisites and their control is elevated, specific limits are applied and corrective actions are defined in case anything is out of target. Based on this approach, most of the crucial aspects of cross-contamination control are covered: controlling *Salmonella* cross-contamination vectors controls its transfer, and controlling its sources controls its entry, growth and presence (Figure 8.1). The whole system would need regular verification and appropriate record keeping.

The GMPs which are listed in Figure 8.1 are well established in the food industry, the point which was lacking, was to have a systematic approach to classify which activities are the most important to control *Salmonella* in each individual factory. The approach is also applicable to other pathogenic microorganisms, e.g. *Cronobacter sakazaki* in dry processing environments, and *E. coli* 0157, *Listeria* or *S. aureus* in general food processing. Furthermore, this proposed prerequisite management programme can also be adjusted to control other hazards, which could be: allergens, chemical, physical or biological hazards. However, even if the food manufacturer effectively prevents the entry of *Salmonella* to the factory building, its transfer to the product and between zones, its growth via reduction of environmental niches and harbourage sites; and has the ability to kill or remove any microorganisms present, there are still some bottlenecks in the dry food industry, which are making implementation of these controls difficult. Firstly, poor agriculture practices for raw material production (seeds, nuts, spices) may lead to high levels of pathogens entering dry food processing facilities. Secondly, many existing dry food factories were not designed for appropriate zoning, therefore it is difficult to apply appropriate segregation. Thirdly, the design of equipment to process low a_w food is very often not designed for wet cleaning. Lastly, there is a lack of dry disinfection methods and appropriate validation techniques.

Therefore, novel dry surface disinfection methods such as cold plasma, pulsed light, ozone, UV, and the heating of production areas to elevated temperatures may show promise in the future (Moat *et al*., 2009, Rutala *et al*., 2010, Levya *et al*., 2012, Niemira and Boyd, 2014). Additionally, intelligent software could be used to collect data from *Salmonella* contamination events worldwide and predict the possibility of incident occurrence.

8.8 FURTHER STUDIES

The results of these studies give an insight into *Salmonella's* survival under conditions in dry processing environments, suggesting that once this pathogen is introduced to the factory environment, there is a serious risk of its persistence.

Academically, more knowledge and work is needed to develop appropriate methods to study desiccation resistance. Gene expression during desiccation would have to be further investigated as published literature to date reports different

mechanisms involved during desiccation. Extraction of RNA has to be improved, however most importantly common understanding of desiccation time and conditions must be developed. It is crucial to know when the desiccation process starts and if all tested cells have initialised the responses or are still in 'wet' conditions. Verification of the results is also needed, which can be done by the creation of knock-out mutants of genes indicated to be involved in desiccation. The resistance of these mutants to drying as compared with wild type strains would then have to be analysed.

From the food industry perspective, it is important to investigate if *Salmonella* stress responses can be correlated to practical factory controls. This could lead, in the future, to selecting more effective intervention measures if they were based on an improved understanding of *Salmonella*'s underlying cell biology and molecular responses. However this would have to be further studied as current knowledge is still not consistent. It would also be valuable to compare the physical and molecular responses of cells attached to different surface materials and their properties. This could indicate the types of surfaces which favour survival. The potential resistance of desiccated *Salmonella* to lower concentrations of disinfectants as compared with cells freshly dried would also have to be further investigated to understand better if there is an increased risk of survival. A very important factor which could further influence common factory practices is the effect of water and drying on *Salmonella* survival. It would be extremely beneficial to establish a time window in which surfaces have to be dried to avoid the possibility of *Salmonella* growth and increased survival.

In general, the widespread occurrence of *Salmonella* in the natural environment and various sectors of the dry food industry, its resistance to dry conditions, its ability to recover and grow after long periods of time, the physiological and molecular adaptability of this organism to dry conditions, the low infectious dose, the continued global increase in outbreaks associated with low aw products and the enormous impact on the food industry, all identify a need to introduce more effective *Salmonella* control measures for the safe production of low a_w food products.

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APPENDIX A

RIBOPRINT PATTERNS FOR THE SELECTED *SALMONELLA* ISOLATES

Sample Number	Label	DuPont ID Label	RiboGroup Default Library: ECORI	RiboPrint™ Pattern 1 kbp 5 10 15 50
114-3344-S-1	FH 158sm			
114-3344-S-2	FH 159 sa	Salmonella ser. Agona	PVUII 114-3344-S-2	
114-3344-S-3	FH 160 ss	Salmonella ser. Paratyphi B var.L (+)	PVUII 114-3344-S-3	
114-3343-S-4	FH 161 sn	Salmonella ser. Lille	PVUII 114-3343-S-4	
114-3343-S-5	FH 162 se	Salmonella ser. Enteritidis	PVUII 114-2743-S-7	
114-3343-S-6	FH 163 st	Salmonella ser. Tennessee	PVUII 114-3343-S-6	
114-3343-S-7	FH 164 sm	Salmonella ser. Muenchen	PVUII 114-2895-S-4	
114-3343-S-8	FH 165 st	Salmonella ser. Typhimurium/ 4,[5],12:i:-	PVUII 114-3141-S-7	
114-3344-S-6	FH 166	Salmonella PvuII Group I #	PVUII 114-3344-S-6	
114-3344-S-7	FH 167	Salmonella ser. Typhimurium	PVUII 114-2889-S-1	
114-3344-S-8	FH 168	Salmonella ser. Typhimurium/ 4,[5],12:i:-	PVUII 114-2889-S-1	
114-3385-S-4	8020	Salmonella ser. Lomita	PVUII 114-3385-S-1	
114-3385-S-5	8021	Salmonella ser. Lomita	PVUII 114-3385-S-1	
114-3385-S-6	8022	Salmonella ser. Cubana	PVUII 114-3385-S-6	
114-3386-S-6	8162	Salmonella ser. Mbandaka	PVUII 114-2748-S-1	
114-3386-S-7	8167	Salmonella ser. Mbandaka	PVUII 114-3386-S-7	
114-3386-S-8	8215	Salmonella ser. Cubana	PVUII 114-3385-S-6	

APPENDIX B

SALTS HUMIDITY TABLE (NOVATRON, PERSONAL COMMUNICATION)

Salt humid-ity	Chemical name of salt	Colour	Humidity at temperatures 15°C - 30°C															
			15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
4%	Caesium Fluoride	White	---	---	---	---	---	---	3.8	3.8	3.7	3.6	3.6	---	---	---	---	---
6%	Lithium Bromide	White	6.9	6.9	6.8	6.8	6.7	6.6	6.6	6.5	6.5	6.4	6.4	6.4	6.3	6.3	6.2	6.2
11%	Lithium Chloride	White	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3
33%	Magnesium Chloride	Blue	33.3	33.3	33.2	33.2	33.1	33.1	33.0	33.0	32.9	32.8	32.8	32.7	32.6	32.5	32.4	32.4
53%	Magnesium Nitrate	Green	55.9	55.6	55.3	55.0	54.7	54.4	54.1	53.8	53.5	53.2	52.9	52.6	52.3	52.0	51.7	51.4
75%	Sodium Chloride	Violet	75.6	75.6	75.6	75.5	75.5	75.5	75.5	75.4	75.4	75.3	75.3	75.3	75.2	75.2	75.1	75.1
90%	Barium Chloride	White	90.9	90.8	90.7	90.7	90.6	90.5	90.4	90.3	90.3	90.2	90.1	90.1	90.0	90.0	89.9	89.9
97%	Potassium Sulphate	Yellow	97.9	97.9	97.8	97.7	97.6	97.6	97.6	97.5	97.4	97.3	97.3	97.3	97.2	97.1	97.0	97.0
98%	Potassium Dichromate	Orange	---	---	---	---	---	98.2	98.2	98.1	98.1	98.0	98.0	98.0	98.0	98.0	98.0	98.0

APPENDIX C

QUESTIONNAIRE TO ESTABLISH FACTORY CONDITIONS



The survival and control of *Salmonella* in low a_w environments

Of critical interest to this PhD/MSFR project study is an understanding of the food processing environment in which Salmonella attached to surfaces could survive. The factors that we believe are of importance include:-

- the environment temperature
- the environment humidity
- the material of construction of the environment surface (floor, walls - stainless steel will be used to model equipment)
- the degree of protection of the organism on the surface - is the surface absolutely clean, is it covered with a conditioning layer of e.g. carbohydrates, is it covered with visible dust, are there large amounts of product on the surface such that the organism is more likely to be product-bound or surface bound
- how often the surface is wet - is the surface completely dry 100% of the time, is there a wet clean once a month, once every 6 months etc.
- are there any operations that could foreseeably leak water onto an environmental surface and how often might this occur

We would wish to enlist the help of (company name) to understand what the range of these factors are (e.g. the minimum and maximum temperatures likely to be found in factories across the world) and what the 'typical' conditions might be. We will

use this information to model laboratory experiments in which we can hold Salmonella in defined conditions for long periods of time and which simulate as closely as possible realistic factory environments. To maximise the data that we could obtain, we would be very pleased if as many as possible individual (company name) manufacturing sites could supply us with the following information.

1. Information about the product
    a. Aw
2. What is the temperature in the production area? Could you give a range of temperatures through the year?
3. What is the relative humidity in the production area? Could you give a range of humidity through the year?
4. What types of food debris are present on the floor/surfaces? (sugar, nuts, finished product etc.)
5. Cleaning and disinfection process
    a. What type of cleaning is used (wet/dry)?
    b. If wet cleaning is used, how often it is performed?
    c. What chemicals are used for wet cleaning?
    d. Is there a drying process after wet cleaning?
6. Are there any other operations which use water which may come into contact with the environment? (accidental or intentional)
7. What are the materials of the surfaces which come in contact with the product?
8. Production process – what are the decontamination steps (roasting/cooking etc.)?

Whilst we will use this information to ensure the robustness and relevance of our laboratory work, no mention of (company name) or individual sites would appear in any internal or external document arising from this work and the data will remain absolutely confidential.

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APPENDIX D

VALUES OF THE COEFFICIENT OF DETERMINATION (R^2) FOR VARIOUS EQUATIONS.

Eleven equations were analyzed and only Weibull, nth-order, Churchill and Weibull two-populations shown the best R^2 values. The R^2 value was calculated for single strains and then an average was taken. The model with the best average and lowest standard deviation was chosen for further statistical analyses.

Serotype	Weibull two-populations	Weibull	nth-order	Churchill
S. Agona	0.9417	0.9279	0.9279	0.9751
S. Enteritidis	0.9452	0.9426	0.9426	0.9515
S. Muenchen	0.9392	0.8867	0.8867	0.6138
S. Napoli	0.9323	0.9316	0.9316	0.9251
S. Tennessee	0.9661	0.9661	0.9661	0.8653
S. Typhimurium	0.9815	0.9714	0.9714	0.9597
S. Typhimurium DT104	0.8785	0.8672	0.8672	0.9038
S. Typhimurium HR	0.9681	0.9117	0.9117	0.6017
S. Typhimurium DS	0.9417	0.9417	0.9417	0.8086
S. Cubana 1	0.9070	0.8948	0.8948	0.9581
S. Cubana 2	0.8547	0.8973	0.8973	0.6374
S. Lomita 1	0.9086	0.8877	0.8876	0.9436
S. Lomita 2	0.9482	0.9437	0.9437	0.9457
S. Mbandaka 1	0.8748	0.9015	0.9015	0.6177
S. Mbandaka 2	0.8865	0.8184	0.8184	0.6275
Average R^2	0.9249	0.9127	0.9127	0.8223
Standard deviation	0.0380	0.0401	0.0401	0.1542

Weibull

$$\log_{10}(S) = -\left(\frac{t}{\delta}\right)^{\alpha}$$

Weibull 2b

$$\log_{10}(S) = -\left(\frac{t}{\delta_1}\right)^{\alpha_1} + \left(\frac{t}{\delta_2}\right)^{\alpha_1}$$

Weibull b&d

$$\log_{10}(S) = -\left(\frac{t}{\delta_1}\right)^{\alpha_1} + \left(\frac{t}{\delta_2}\right)^{\alpha_2}$$

nth-order

$$\log_{10}(S) = -\delta \cdot t^{\alpha}$$

nth-order*2

$$\log_{10}(S) = -\delta \cdot t^{\alpha} - \delta_2 \cdot t^{\alpha} + \beta$$

Churchill

$$\log_{10}(S) = \beta_1 \cdot \exp(\delta_1 \cdot t) - \alpha - \beta_1 \cdot \exp(\delta_2 \cdot t)$$

Weibull two-populations

$$\log_{10}(S) = \beta_1 \cdot \left(\frac{t}{\delta_1}\right)^{\alpha_1} - (1 - \beta_1) \cdot \left(\frac{t}{\delta_2}\right)^{\alpha_2}$$

The subscripts 1 and 2 indicate the two different subpopulations (or the two different levels of resistance), S is the survival rate (cfu_t/cfu_0), β_1 is the fraction of subpopulation 1 in the total population, δ is the time needed for the first decimal reduction, α is a shape parameter and t is the time in hours.

APPENDIX E

TIME PLAN FOR SURFACE DISINFECTANT TEST

Equilibrate all the reagents and discs at room temperature.

Start clock

Min.	Action
0.00	100µl disinfectant concentration 1 onto disc 1, ensure dried inoculum is covered
0.15	100µl disinfectant 2 to disc 2
0.30	100µl disinfectant 3 to disc 3
0.45	100µl disinfectant 4 to disc 4
1.00	100µl disinfectant 5 to disc 5
1.15	100µl disinfectant 1 to disc 6
1.30	100µl WSH to disc W1
1.45	100µl WSH to disc W2
5.00	1 disc to 1 inactivation (inact) pot, placed upside down in inactivator and shake
5.15	2 disc to 2 inact pot
5.30	3 disc to 3 inact pot
5.45	4 disc to 4 inact pot
6.00	5 disc to 5 inact pot
6.15	6 disc to 6 inact pot
13.30	W1 DISC TO W1 INACT POT
6.45	W2 disc to W2 inact pot
7.00	Shake 4 inact pots at a time for 1 minute
8.15	Shake 4 inact pots at a time for 1 minute
10.00	Pipette 1ml 1 to 9 ml of diluent 1 -1
10.15	Pipette 1ml 2 to 9 ml of diluent 2 -1
10.30	Pipette 1ml 3 to 9 ml of diluent 3 -1
10.45	Pipette 1ml 4 to 9 ml of diluent 4 -1
11.00	Pipette 1ml 5 to 9 ml of diluent 5 -1
11.15	Pipette 1ml 6 to 9 ml of diluent 6 -1
11.30	Pipette 1ml W1 to 9 ml of diluent W1 -1

11.45 Pipette 1ml W2 to 9 ml of diluent W2 -1

13.15 100µL SDW TO C INACTIVATION POT

12.30 100µl SDW to C inactivation pot

13.00 100µl disinfectant 3 to Tc inactivation pot

13.15 100µl disinfectant 3 to Td inactivation pot

17.15 Cc clean disc to Cc inact pot

17.30 Cd dirty disc to Cd inact pot

18.15 Tc clean disc to Tc inact pot

18.15 Td dirty disc to Td inact pot

18.30 Shake 4 inact pots at a time for 1 minute

22.15 Pipette 1ml Cc to 9 ml of diluent Cc -1

22.30 Pipette 1ml Dc to 9 ml of diluent Dc -1

23.00 Pipette 1ml Tc to 9 ml of diluent Tc -1

23.15 Pipette 1ml Td to 9 ml of diluent Td -1

C1, c2, c3 = the disinfectant tests, clean conditions

D1, d2, d3 = disinfectant tests, dirty conditions

Disinfectant 1 – lowest concentration

Disinfectant 2 – middle concentration

Disinfectant 3 – highest concentration

W = the WSH controls

C = the neutralisation controls

T = the neutralisation tests

Test temperature of room between 18^{o}C +/- 1^{o}C and 25^{o}C +/- 1^{o}C

APPENDIX F

VIDEO – MOVEMENT OF PARTICLES AT THE START OF DRYING PROCESS

APPENDIX G

VIDEO – MOVEMENT OF PARTICLES AFTER 30 MINUTES OF THE START OF DRYING PROCESS

APPENDIX H

POTENTIAL SOURCES AND THEIR CURRENT AND PROPOSED CONTROL MEASURES IDENTIFIED IN A BABY MILK POWDER PROCESSING PLANT

No	Source	Vectors	Current control measures	Score		Proposed control measures	Score	
				PS	PV		PS	PV
1	External environment	Equip-ment surfaces etc. entering via docks	Packed ingredients enter the factory via docks. There is no physical barrier between the basic hygiene environment (outside environment at the dock) and the high hygiene zone – just a line on the floor. Therefore people and equipment could potentially move between zones without control. However the actual production area is further separated by physical walls and an obligatory change of footwear. There is no hand washing station. Different fork lift trucks (FLT) are used in the production zones and the corridors, however there is no physical barrier to stop them crossing over to the other side.	C	3	The dock area and the high hygiene area should be separated with an intermediate area where the secondary packaging of the ingredients could be removed and then the bags could be handled by designated personnel and equipment. The air pressure in the intermediate area should be maintained higher than the dock area to avoid the air movement from the outside environment to the high hygiene zone. The areas should be physically separated so the FLT could not cross between the areas. The doors should be interlocked so that when the intermediate area is accessed from the dock's side, it is impossible to access it from the high hygiene side.	C	2
		Personnel entering via changing room	All personnel when entering from outside must change their clothes (excluding underwear) and wash their hands before entering the high hygiene zones. The floor of the changing room is not physically divided between the high hygiene side and non-food production areas. Personnel are	C	1	The current controls reduce the possibility of personnel spreading contamination from the outside environment to the factory environment and directly to the product. The floor in a changing room could be physically separated.	C	1

			screened every 2 years for *Salmonella* carriage. A Personnel Hygiene policy is implemented.					
		Air	The air in production rooms is filtered	B	2	The current air filtration system is sufficient to stop contaminated air entering the processing areas	B	2
2	Microbiological laboratory	People, sampling equipment	The controls are described in point 4.1	B	1	The controls are sufficient to control contamination from the microbiological laboratories	B	1
3	Roof	People, clothing equipment	The roof is designed and maintained to avoid leakage. The entry to the roof is protected from unauthorized movement by a chain. Everyone who enters the roof must first inform a supervisor and wear shoe covers and additional overalls. Where are these disposed of on their return?	C	3	The entrance to the roof could be locked or it could be moved out from the high hygiene zone.	C	2/1
4	Vacuum cleaners – they have a history of positive isolations and contain a number of harborage sites were microorganisms could be protected	Vector itself, People, Equipment Floors	All vacuum cleaners are labeled, they are all cleaned at the same time in the same area, they are placed on plastic sheets in the corridor, just outside the door to the processing area. The doors are frequently opened during cleaning and there could be aerosols in the air. The plastic sheet is placed directly on the floor therefore people walk on it and the cleaned parts are also placed on it. Personnel who clean the vacuum cleaners are often subsequently entering production in the same clothes. All shoes worn in the high hygiene zone are hanging next to the place where the vacuum cleaners are cleaned.	C	4	The vacuum cleaners which have tested positive for *Cronobacter* or *Salmonella* should be disposed of. More hygienically designed vacuum cleaners should be used or low cost vacuum cleaners could be used for short periods of time e.g. 3 months and then they could be disposed of. HEPA filters should be used in the vacuums. Cleaning and changing of bags should be performed in a dedicated area, separated from the high hygiene zone, with dedicated people, utensils and clothing. All vacuum cleaners should be dedicated to specific areas, including spare ones (environment or product contact surfaces). Hoses should be dedicated to a specific	B	3

	from cleaning		The vacuum cleaners are cleaned once per week, however there is no schedule for cleaning the hoses which are all cleaned together (product contact/non product contact). HEPA filters are not used in the vacuum cleaners. There is also a washing room where the hoses are wet cleaned – soaked in a tank, however there is insufficient drainage on the drying wall.			vacuum cleaner and should have the same cleaning schedule as the vacuum cleaners.		
5	Floors have cracks, some places have additional metal plates on the top which can provide harborage sites underneath	Personnel, equipment Cleaning liquids	Floors are cleaned with damp cloths and disinfected with 70% alcohol once a week. Delladet (2%) is used to clean the floors, diluted the same way as Divosan. Concentrations are not checked. Openings in the floor are not protected so if there is any liquid on the upper floors, it leaks through to the lower levels.	B	4	The cracks should be repaired and the metal plate should be removed or sealed. The concentration of chemicals should be checked on a regular basis. Openings in the floor should have protection so if there is any liquid on the upper floors, it will leak go through to the lower levels.	A	4
6	Wet cleaning room	Personnel, equipment	The area is separated from the production environment by 2 doors. The personnel must change footwear when entering and leaving the area. Clothing is not changed. There is no effective segregation between clean and dirty equipment. Some of the cleaned	B	3	Additional overalls should be worn in the wet cleaning room. There should be an effective segregation between clean and dirty equipment and cleaned equipment should be dried in a different room than the washing room. All the shelves should be drainable.	A	3

			equipment is dried just next to the washing tank. Some shelves are not drainable.				
7	Low hygiene zone	Personnel, Equipment Samples Air	There is an airlock between the high and low hygiene areas on the ground floor, the packaging is transported via the airlock and the waste is disposed of in the same way. There is no inter-lock so if the door on the low hygiene side is open there is nothing to stop the door being opened from the high hygiene zone. Stairs go from the high hygiene zone to the low hygiene zone. There is no physical separation, only a chain which people could easily open and pass through. There are separate tools for use in low and high hygiene zones. The shoes which are used in the high hygiene zone are marked with an orange colour. Positive pressure is maintained in the production rooms. Some product samples are transferred from the low hygiene zone back to the high hygiene zone (via the air lock) to take some measurements	B	3	The airlock should have an interlock installed. There should be no other paths between low and high hygiene zones except via a changing room If the stairs are the fire escape route, there should be a fire door placed in-between low and high hygiene zones. The product should not come back from low to high hygiene, the measuring equipment should be placed in a low hygiene zone or two sets of the equipment should be purchased: one for high hygiene and the other for the low hygiene zone.	B	2
8	Water pipes in the corridor	Water	Ingredient bags are stored directly underneath the pipes, however the bags usually remain closed	B	4	The product should not be stored underneath the water pipes, they should be outside of the production areas.	B	3
9	Motors above dosing	Oil, dust	Above the openings where the content of the big bags is emptied there are drip trays so nothing should fall from the	B	3	Motors should not be mounted directly above product streams	B	2

	holes		motors or the ceilings to the product stream				
10	Horizontal surfaces above process lines	Dust	The service constructions above 2.5 meters are never cleaned. Some of those constructions are above the dosing equipment	B	3	The service constructions should not be horizontal, they should be angled or round to stop accumulation of dust. The surfaces should have a cleaning schedule	B 2

APPENDIX I

LINE INTERVENTIONS AND THEIR CURRENT AND PROPOSED CONTROL MEASURES IN A BABY MILK POWDER PROCESSING PLANT

No	Process step	Intervention	Product vectors	Current controls	Score		Proposed controls	Score	
					PC	PP		PC	PP
1	Intake of ingredients	Ingredients are delivered to the dock, secondary packaging is removed there and bags are hung by an operator on a hoist	Air Gloves	Air is not filtered Gloves are of the 'firm grip' types and are used to handle dirty equipment and surfaces. Operators are trained on how to handle the bags.	3	B	The area should have solutions described in Appendix 7.1 (source 1) to separate the external environment and high hygiene zone.	2	B
2	Dosing of ingredients	The covers for the dosing equipment (where the big bags are attached) hang on the side when the equipment is in use	Air Hands	The covers are frequently cleaned and disinfected. Positive pressure is maintained in the production rooms. Air is filtered. Personnel wear clean factory clothing and they wash their hands at the entrance to the high hygiene zone. Gloves are used when the covers are removed	2	B	The covers could be wiped with disinfectant when they are returned to the equipment.	1	B
3	Dosing of ingredients	Setting up a big bag on dosing equipment	Air Hands Gloves	Positive pressure is maintained in the production rooms. Air is filtered. Personnel wash their hands at the entrance to the high hygiene zone. Gloves are clean and used only for this activity. There is no hand disinfection before wearing gloves	2	B	More frequent training of the personnel could be performed to avoid mishandling of bags (driving over with them with a FLT, placing them directly on the floor)	1	B

			Clothing	Factory clothes are washed in a laundry which is certified and has separation between dirty and clean areas, they are changed daily.						
			Personnel	Personnel wear masks during this activity						
4	Dosing of ingredients	Operators make holes with a knife into the big bag to enable powder removal.	Air	Positive pressure is maintained in the production rooms. Air is filtered.	3	C	Personnel should disinfect their hands and wear gloves before performing this activity. The knife should also be disinfected each time before use.	2	C
			Hands	Hands of operators are not disinfected before use however they are cleaned at the entrance to the high risk area.						
			Clothing	Factory clothes are washed in a laundry which is certified and has separation between dirty and clean areas, they are changed daily.						
			Knife	The knife is stainless steel but not cleaned and disinfected. The knife is kept in a holder attached to a wall (also not cleaned).						
5	Dosing of ingredients	Small samples for analysis are taken directly from big bags using a stainless steel spoon	Air	Positive pressure is maintained in the production rooms. Air is filtered.	3	C	Personnel should disinfect their hands and wear gloves before performing the activity. The spoon should be disinfected each time before use.	2	C
			Clothing	Factory clothes are washed in a laundry which is certified and has separation between dirty and clean areas, they are changed daily.						
			Spoon	The spoon is kept on a table in the dosing area. The frequency of cleaning and disinfection of this spoon is unknown.						
6	Dosing of	If all of the powder	Air	Positive pressure is maintained in	4	B	Previously opened bags	4	B	

	ingredients	is not used from one big bag, then it is placed back on the pallet	Hands Equipment Pallet	the production rooms. Air is filtered. Hands of operators are not disinfected before intervention however they are cleaned on the entrance to the high risk area. The bag touches external parts of the equipment and occasionally the floor Pallet has a liner			could be used for a lower grade product.			
7	Dosing of ingredients	Manual dosing of ingredients	Air Hands Gloves External pack surface	Positive pressure is maintained in the production rooms. Air is filtered. Gloves are used	3	B	Personnel should disinfect their hands and wear gloves before performing the activity. Instructions should be provided on how to handle the bags to avoid cross contamination from outside, and how to remove secondary packaging.	2	B
8	Metal detector	Inserting calibration samples for the metal detector and changing the bags	Air Hands Gloves Calibration samples	The calibration samples for the metal detector are inserted every 4 hours. Positive pressure is maintained in the production rooms. Air is filtered. Personnel wear gloves and masks. Gloves are clean and used only for this activity. The samples are disinfected with alcohol after each use and left to dry in the designated place, next to the	3	C	The samples should be kept in higher hygienic conditions; the moisture should not be left on their surfaces for long periods of time.	2	C

				entrance to the filling area. The boxes where the samples are kept are cracked and parts of the plastic are missing. The drying is taking a considerable amount of time which may allow microorganisms to grow.					
9	Packing	Taking samples from auto samplers	Air Hands Gloves Plastic bags	Positive pressure is maintained in the production rooms. Air is filtered. Personnel wear gloves and masks. Gloves are put on directly before the activity. New gloves are used. The sample is collected into a plastic bag. The rest of the sampler is also covered with a plastic bag to avoid dispersing the powder. Bags are stored in a clean place and they are not stored in the laboratory prior to use.	2	B	Plastic bags should be issued from the raw material warehouse stores. Certified training in aseptic techniques could be provided to operators.	2	B
10	Packing	Transfer and set up of packaging film	 Air Hands Gloves Trolley Floor	Packaging is transported to the high hygiene area via an airlock. Positive pressure is maintained in the production rooms. Air is filtered. Hands are not disinfected before handling film Gloves are not used The trolley used to place the foil on the filling machine has no schedule of cleaning and disinfection. When the packing film is inserted onto the roll, some parts of it touch	3	B	The trolley should have a cleaning and disinfection schedule and it should be stored in a place to avoid accidental contamination. Packaging could be placed at a greater proximity from the floors, so direct contact with the floor could be avoided. All utensils used and rollers should	2	B

				the floor. However, the first part of the reel is then disposed of.			be disinfected.	
			Tape	The next roll of packing film is stuck to the previous one by double sided tape.				
			Scissors	Part of the film is cut off with scissors which are kept close to the equipment.				
			Rolls	It is not known how the rolls are cleaned and disinfected – some of them have direct contact with the internal part of the packaging.				
11	All	Cleaning of product contact surfaces		Equipment cleaning is undertaken once a week. During the cleaning procedures, all the openings to the processing lines are closed with polythene sheets and all remaining product is removed from the room. All areas are vacuumed, also deep inside the lines to remove as much residue as possible.	3	B	The cleaning chemicals could have their concentrations checked. The vacuum cleaners and the hoses should be managed as indicated in Table 5 (source 4). The uniforms used during cleaning cannot be used later during production activities.	2 B
			Air	Positive pressure is maintained in the production rooms. Air is filtered.				
			Hands					
			Gloves	Gloves are used				
			Cleaning utensils	Most of the cleaning equipment is not wet cleaned (except vacuum hoses); they are stored in an equipment store room in plastic bags. If anything is sent for wet cleaning it comes back dried and packed in a plastic bag. Vacuums				

				are known to be contaminated with *Cronobacter spp*.				
			Cleaning chemical	70% alcohol (ethanol) is used to disinfect some equipment; it is diluted in the office of the operative responsible for this task. There are appropriate instructions available, however dosing equipment is not provided. The volumes of alcohol and water are measured in dedicated containers and this is mixed together. The final concentration is not measured. Divosan (Johnson Diversely) is also used: it is diluted manually in a cleaning room, measuring cylinders are provided. TM90 (2%)+Divosan Extra (1%) One ingredient (30 kg) is used to flush clean lines in-between different products. The first batch of the new product is then rejected. The autosampler samples some of this cleaning flush, also a sample from the first pallet of the new product is taken.				
12	All	Maintenance	Air	Positive pressure is maintained in the production rooms. Air is filtered.	3	B	Hands should be disinfected before line intervention or gloves should be used	2	B
			Hands	Personnel wash their hands on entrance to the high hygiene zone.					
			Gloves	Gloves are not used					
			Tools	Tools are dedicated to each zone.					

	Tools are disinfected with 70% ethanol before use			

APPENDIX J

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Salmonella survival in low a_w environment

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ABSTRACT

Since the early 1970's a number of large *Salmonella* outbreaks have been associated with low a_w food products, including chocolate, cereal, peanut butter, spices, and powdered infant formula. This research is design to help the food industry to determine *Salmonella* survival in the production environment and thus understand the potential risk of cross contamination to the product. The studies sought to assess the survival of 9 isolates of *Salmonella* on stainless steel surfaces, the influence of different serotypes on survival and the comparison of the survival rate within the same serotype.

The following isolates were used for the experiments: *S.* Agona, *S.* Napoli, *S.* Enteritidis, *S.* Tennessee, *S.* Muenchen, *S.* Typhimurium (whey powder), *S.* Typhimurium DT104, *S.* Typhimurium NCTC 12023, *S.* Typhimurium, NCTC (PHLS), NC000 74-17. The stainless steel surfaces were inoculated with 10^7 cfu of each *Salmonella* isolate and placed in a controlled environment: relative humidity 33% and temperature 23°C. The number of microorganisms surviving on the stainless steel surfaces was assessed at various time points from 1 hour to 30 days.

Differential survival was observed for different serotypes of *Salmonella*. The highest survival rate was associated with *S.* Agona, *S.* Enteritidis, and *S.* Typhimurium DT104 where, after 30 days, the number of microorganisms on the surfaces ranged from 4.0 logs cfu to 4.5 logs cfu. There was 1 log cfu survival of *S.* Typhimurium NCTC (PHLS), NC000 74-17 after 10 days in experimental conditions, therefore differences within the same serotype were observed. The average number of *Salmonella* surviving after 30 days ranged from 2.5 logs cfu to 3.5 logs cfu.

The results of the studies could provide insight into potential growth and survival mechanisms for *Salmonella* in dry processing environments. These results will be incorporated into a Campden BRI guidance document on the survival of *Salmonella* in dry process environments with relation to strain, temperature, humidity, water and soiling, which would help the industry to control *Salmonella* endurance in the environment and thus in the final product.

Keywords: Salmonella; survival; water activity; dry; environment, surfaces

INTRODUCTION

For many years, low moisture foods, such as chocolate, were regarded as microbiologically safe due to their inherent product characteristics. The first outbreak of *Salmonella* that could be traced back to low moisture products was registered in the 1970's and since this time it has been the main pathogen of concern for these foods. Currently, there is growing concern for the potential presence and survival of *Salmonella* in low a_w products. The problem within the food industry is widespread with reports from the UK Food Standards Agency and Rapid Alert System for Foods and Feeds (RASFF) concerning such foods and ingredients as: whole egg powder, coriander, soy bean meal, Tahini, minced dehydrated white onions, sesame seeds, dried sage, peanut butter, black pepper, dried mushrooms, oat cereals, low a_w sausage, chocolate and flour being able to harbour *Salmonella.* In addition, the same *Salmonella* type has been isolated from products produced over a number of years (e.g. 10 years for Malt-O-Meal in the USA; [11]) from the same plant. The assumption has been that this particular *Salmonella* strain had survived in the plant for this time period and may have recontaminated products.

All of those facts underline the importance of understanding the mechanisms of *Salmonella* survival in low moisture environments, establishing the environmental factors likely to favour their long term survival and determining ways to control them.

Whilst there is some information on the survival of *Salmonella* in dried products, there is little information on the survival of *Salmonella* in the environment, both in terms of the length of survival and the possible factors contributing to such survival. It is also well known that as the water activity of foods becomes lower and the available moisture is decreased, the probability of growth is reduced; however the ability of organisms to survive is greatly increased.

The ability of *Salmonella* to survive for long periods in low a_w products e.g. halva [16] and peanut butter [5] has been documented. Uesugi *et al.* demonstrated potential for long-term environmental persistence of *Salmonella* in almond orchards. In his studies, 53 *Salmonella* isolates collected from the selected orchard during 5 years were identified as the same serovar: *Salmonella* Enteritidis PT 30 [24].

The approach of this study is to establish a survival model for *Salmonella* strains and compare the differences between each serotype and the same serotypes isolated from different products.. The environmental conditions which were used (RH=33% and T=23ºC) reflect the environment of low a_w food factories.

MATERIALS & METHODS

***Salmonella* strains.** All *Salmonella* isolates were obtained from Campden BRI culture collection. *S.* Agona was isolated from cotton seeds, *S.* Napoli from chocolate confectionary (1986 outbreak, Italy), *S.* Enteritidis was obtained from cereals, *S.* Tennessee from sesame seeds and *S.* Muenchen from cocoa bean environment. Four isolates of *S.* Typhimurium were used: multi-drug

resistant *S*. Typhimurium DT104, *S*. Typhimurium NCTC 12023 (high heat resistant; HR), *S*. Typhimurium, NCTC (PHLS), NC000 74-17 (used as a standard strain for disinfectant testing; DS) and *S*. Typhimurium isolated from whey powder. To ensure that the results would be as useful and applicable to the industry as possible, the same *Salmonella* strains, from factories manufacturing the same low a_w products as from the foods which caused recent *Salmonella* outbreaks, were used (Table 1).

Strain	Food	Year	Location	Number affected	References
S. Agona	Cereal	2008 1998	USA USA	28 209	[9] [7]
S. Napoli	Chocolate	1982	UK	245	[13]
S. Enteritidis	Almonds	2000 2005	USA, Canada Sweden	168 15	[14] [18]
S. Tennessee	Peanut butter Powdered infant formula	1996 1993	USA Canada, USA	628 3	[8] [6]
S. Typhimurium	Chocolate Peanut butter	1987 2008	Norway, Finland USA, Canada	361 684	[15] [10]
S. Typhimurium DT104	Halva	2001	Australia, Germany, Norway, Sweden, UK	>70	[1]

Table 1. Outbreaks of foodborne *Salmonella* infections associated with consumption of contaminated dry foods

Surfaces. Stainless steel discs (2 cm diameter, Grade 2 B 1.4301 (EN 10088-1), EN 10 088-2) were used. They were soaked in hot water with detergent for 60 minutes. Each disc was then cleaned with a sponge and rinsed with Sterile Distilled Water. They were then left to dry, wiped with alcohol wipes, and sterilised in the autoclave.

Test suspensions. The cultures were maintained at -80ºC in cryo vials containing glycerol. Working cultures of each serotype were sequentially prepared by adding a cryo bead to 150 ml Nutrient Broth (Oxoid) which was incubated for 24 h in a shaking incubator at 37ºC and 100 rpm. The broth was then centrifuged at 3000 g for 10 minutes. Pellets were resuspended in a 500 ml of Maximum Recovery Diluent (LabM; MRD) which formed 10^8 cfu/ml suspensions.

Preparation of controlled RH chamber. In this experiment, an RH of 33% ± 1% was established and maintained by the presence of saturated magnesium chloride salt. The sealed boxes were held at a temperature of 23±2ºC. For the measurement of temperature and RH, a calibrated, digital thermohygrometer was used (Rotronic; Art no Hygrolog-D).

Survival of *Salmonella* on stainless steel surfaces. 50µl of each suspension was deposited onto stainless steel discs to produce an inoculum of approximately 5 x 10^6 – 2 x 10^7 cfu/surface. Discs were placed in uncovered Petri Dishes and transferred to an incubator to dry at 30ºC for 1h 20 min. After drying, Petri Dishes were covered and placed in air tight transparent plastic boxes (32cm x 11cm x 21cm). The number of microorganisms surviving on the stainless steel surfaces was assessed at the following time points after drying: 0 h, 1 h, 3 h, 6 h, 24 h, 30 h, 2 days, 3 days, 7 days, 10 days, 15 days and 30 days.

Determination of viable counts of drying bacteria. The discs were aseptically transferred into sterile plastic universal containers (diameter 4-5cm) containing 10 ml MRD. The containers were vortexed for 1 minute to recover the remaining bacteria into suspension. Each sample was then serially diluted in MRD to 10^{-5} and plated out in duplicate using Tryptone Soya Agar (Oxoid; TSA).

To validate the bacterial recovery process, each disc was recovered from its container and then placed onto a prepoured TSA plate. A 0.1ml volume of sterile, distilled water was pipetted onto the disc and rubbed over the top and the bottom surface with a pipette tip for 30 seconds. The discs were then overpoured with TSA. All plates were incubated at 37°C for 48 hours. Plates were then enumerated and the colony forming units (cfu) per test surface calculated.

RESULTS & DISCUSSION

The serial dilution method used in this experiment was not sensitive enough to detect a viable cell concentration of less than 5 cfu/ml (0.7 log cfu). However, the agar incorporation method was capable of detecting any culturable colonies left on the surface after recovery. The initial bacterial level on the stainless steel discs ranged from 6.5 to 7.5 log cfu/surface. Strains had different sensitivity to drying which resulted with different starting levels when placing the discs in the boxes with controlled humidity. *S*. Typhimurium and *S*. Typhimurium DS were found to be most sensitive to drying (1.8 and 2.1 log cfu reduction respectively). *S*. Tennessee and *S*. Enteritidis were most resistant to drying (0.7 log cfu reduction). On the basis of the response to desiccation, the strains of *Salmonella* tested could be divided into two different groups: desiccation sensitive and desiccation resistant. The desiccation sensitive group included *S*. Typhimurium (isolated from whey powder) and *S*. Typhimurium DS. The rest of the strains belonged to desiccation resistant group: *S*. Agona, *S*. Typhimurium DT104, *S*. Enteritidis, *S*. Tennessee, *S*. Muenchen, *S*. Typhimurium and *S*. Napoli. Figure 1 shows representative survival curves for the 9 different isolates of *Salmonella*.

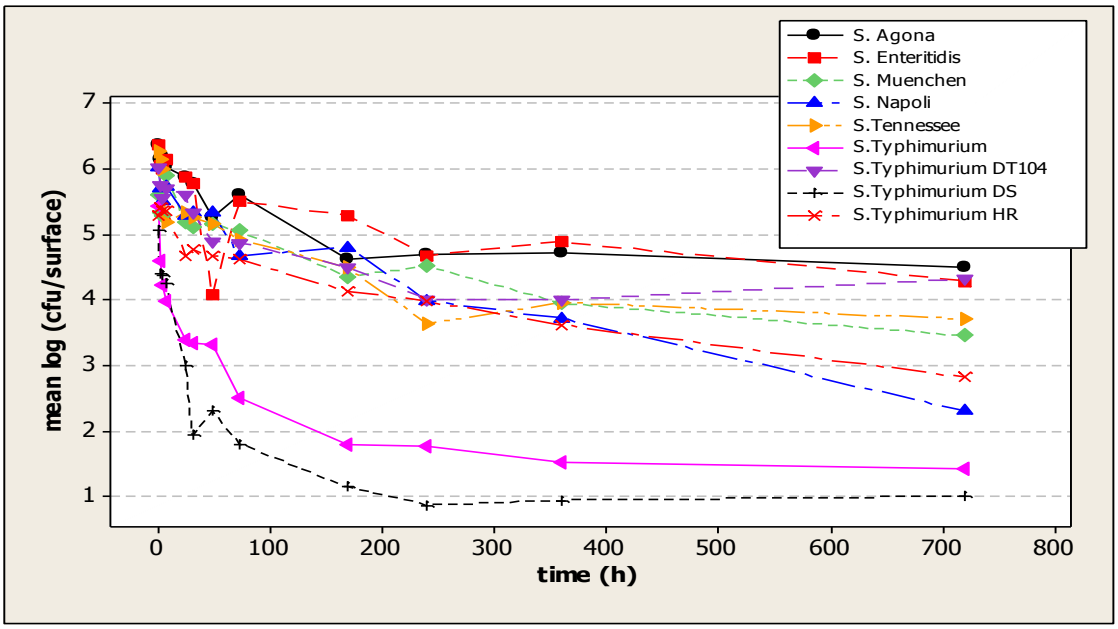


Figure 1. Survival of different strains of *Salmonella* suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33% RH and 23ºC.

There was a relatively high rate of bacterial death at the beginning of the survival curves for all isolates and a decrease in the death rate after 100 hours. For this reason, curves were split into two time periods: early survival (all results obtained from times: 0 h, 1 h, 3 h, 6 h, 24 h, 30 h, 2 days and 3 days) and later survival (all results obtained from times: 7 days, 10 days, 15 days and 30 days). Straight lines were fitted to each time period for each strain. Slopes were measured between time 0 h and 3 days and showed strong evidence of a reduction with time ($p < 0.001$) for each organism except *S*. Muenchen ($p = 0.09$). For the later sampling points (7 days and more), the slopes for the majority of strains showed no statistically significant evidence of decline, except for *S*. Napoli (strong, $p = 0.004$) and *S*. Typhimurium (weak, $p=0.049$). Most of the strains, after reaching a certain reduction level, showed no further, significant viability loss for 27 days (Figure 2.)

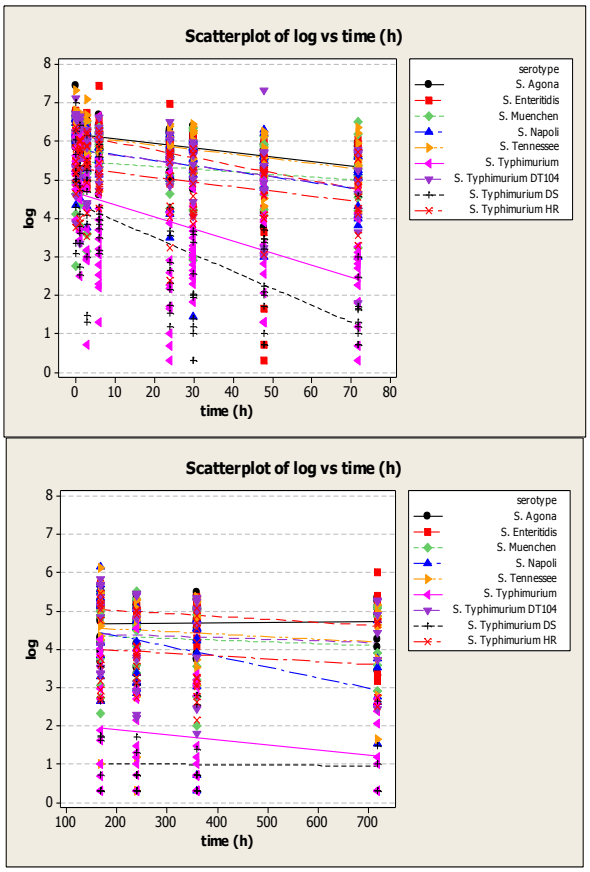


Figure 2. Survival rate of 9 *Salmonella* strains suspended in MRD, inoculated into stainless steel discs, dried and kept at 33% RH and 23ºC, time 0 h to 72 h (left) and time 168 h to 720 h (right).

The mean number of survivors for each *Salmonella* strain, after 30 days under the defined desiccating conditions, is shown in Figure 3. The highest log reduction was associated with *S.* Typhimurium and *S.* Typhimurium DS, where the level of bacteria dropped from 7.3 and 7.2 log cfu (inoculum) to 5.5 and 5.1 log cfu after drying and to 1.3 and 1 log cfu after 30 days, respectively.

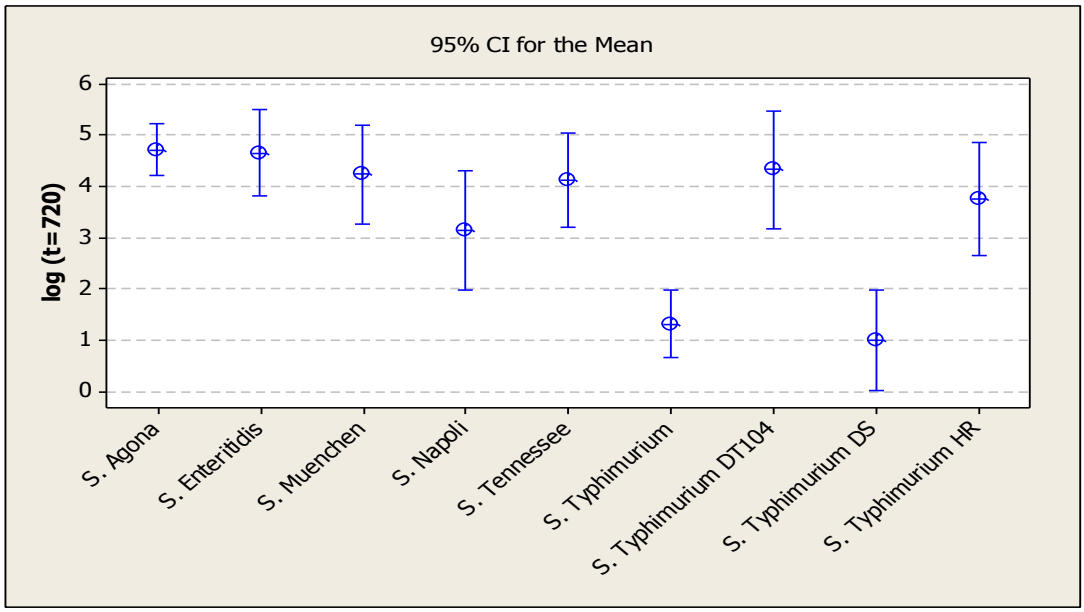


Figure 3. Overall reduction of *Salmonella* strains suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33% RH and 23ºC for 30 days.

The highest survival rate was associated with *S.* Agona, *S.* Typhimurium DT104 and *S.* Enteritidis, where the microorganism level dropped from 7.2, 7.1 and 7.0 log cfu to 6.4, 5.9 and 6.4 log cfu after drying and 4.5, 4.3 and 4.3 log cfu respectively, after 30 days on stainless steel surfaces. Differences in survival within the same serotype were noted, with *S.* Typhimurium DT104 and *S.* Typumurium HR surviving significantly better than *S.* Typhimurium and *S.* Typhimurium DS.

Kusumaningarum *et al.* reported already that *S.* Enteritidis survived for more than 96 h on stainless steel surfaces at 20 – 25ºC and 40 – 45% RH which was confirmed in these findings. Their experiments also showed that food residues (saline solution, milk, chicken fillet) improved the survival of the pathogen, probably by forming a layer that might protect the cells on surfaces [17].

It is not clear why some of the *Salmonella* strains survive better than others. The way by which *Salmonella* survives the inimical conditions of the environment may be via formation of filaments. It has been reported that when cells undergo stress conditions that are bacteriostatic, they elongate and produce filamentous cells. This has been demonstrated in low water activity conditions, extreme pH and refrigerated temperatures [19, 22]. However, based on the available literature, it is not clear whether *Salmonella* cells form filaments at an a_w less than 0.85. Anriany et al. showed that *S.* Typhimurium DT104 displays a rugose phenotype which has a unique function in allowing the cells to survive in the presence of acid and hydrogen peroxide and possibly other adverse conditions [4].

A number of researches have also investigated other mechanisms that may enhance *Salmonella* survival including accumulation of betaine via specific transporters [2], accumulation of proline [21] modification of the outer membrane [23] and the function of σ^E- and σ^S regulated genes [20, 3].

Evidence of *Salmonella* survival has been found in plant processing environments. An outbreak of *Salmonella* Agona associated with toasted oats cereal showed wide-spread low levels of the organism in the plant environment, including samples taken from the floor, production equipment and the exhaust system in the plant [12]. In a study involving contaminated chocolate by Craven *et al*. [11], investigators found that the cause of the contamination was un-controlled airborne spread of dust. Understanding the ability of *Salmonella* strains to survive in the environment is necessary to control this pathogen and prevent contamination of food being produced.

CONCLUSION

Some of the *Salmonella* strains studied survived at least 30 days and potentially could survive much longer. Strains also have different responses to desiccation. The results of this study will give an insight into potential growth and survival mechanisms for *Salmonella* in dry processing environments by providing strains that can be used to investigate physical and molecular changes which accrue within the cells under low a_w conditions. The relationship between *Salmonella* survival and environment is very complex. It is not easy to explain this relationship based only on one environmental factor which can be manipulated in the laboratory. Bacterial survival is affected by a complex of stresses in the environment, not by a single stress. For this reason, the strains which have the best survival level will be selected to perform further studies using a range of humidity levels, temperatures, variations in food debris components, water access or inoculation levels. All these conditions will reflect the genuine factory environment. Systematic studies will be directed to provide solutions for controlling *Salmonella* in dry foods and will help to determine equipment design, environmental conditions for reduced survival and novel methods/programs for plant decontamination. These results will be incorporated into a Campden BRI guidance document on the survival of *Salmonella* in dry process environments with relation to strain, temperature, humidity, water and soiling, which would help the industry to control *Salmonella* endurance in the environment and thus in the final product.

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APPENDIX K

CAMPDEN BRI OPEN DAY. 3 JUNE 2010. (POSTER)

Salmonella management in low a_w food products

Campden BRI
food and drink innovation

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Since the early 1970's a number of large *Salmonella* outbreaks have been associated with low a_w food products, including chocolate, cereals, peanut butter, spices, and powdered infant formula. This project is design to help the food industry to control *Salmonella* contamination in the production environment and in the low a_w products (particularly unvalidated, potential thermal processes) and thus produce safer food.





The effect of the thermal process will be assessed at different temperatures in a range of low aw products:

- low and high fat chocolate
- wheat
- flour
- sesame seeds
- pumpkin seeds
- hazelnuts, walnuts and peanuts
- mixed nuts

The effect of environmental conditions and product matrices on the long-term survival of surface adhered *Salmonella* serotypes will be studied. Experimental variables will include:

- A number of different serotypes at different inoculation levels
- Attachment to stainless steel, wall and floor materials
- Adjustment of humidity and temperature
- Variations in debris components
- Evaluations ranging from 1 hour to 1 year
- Physiological and molecular changes within the microbial cells

The regimes used to decontaminate the raw material and final product will be evaluated by challenge testing and validation techniques and protocols will be developed for future industrial use. Emphasis will be placed on the following:

- Format of the validation test for different scenarios
- Formulation of choice of marker organisms and test materials
- Verification procedure against *Salmonella*

The following decontamination regimes for dry processing environments will be evaluated:

- Alcohol or solvent based disinfectants
- Gaseous disinfectants e.g. ozone or vaporised hydrogen peroxide
- Wet chemical disinfection and rapid drying techniques

Proposed Publications:

- Guidance document on the decontamination of *Salmonella* in raw materials and final products
- Guidance document on the survival of *Salmonella* in dry process environments with relation to strain, temperature, humidity, water and soiling

Arrangement of formal Steering Group to:

- Gain information about thermal processing steps and factory environments in dry foods manufacture
- Acquire industrially relevant *Salmonella* strains for laboratory studies
- Conduct factory studies

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APPENDIX L

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Survival and death kinetics of *Salmonella* strains at low relative humidity, attached to stainless steel surfaces

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ABSTRACT

Salmonella is a major pathogen of concern for low water activity foods and understanding its persistence in dry food processing environments is important for producing safe food. The studies sought to assess the survival of 15 isolates of *Salmonella* on stainless steel surfaces. Additionally, the aim was to select a suitable model to describe and understand the strains' survival kinetics. *Salmonella* isolates were dried onto stainless steel surfaces, placed in controlled temperature (25 °C) and humidity (33%) conditions and their viability assessed at times from 1 h to 30 days. The highest survival rate was associated with *S.* Typhimurium DT104, *S.* Muenchen, and *S.* Typhimurium (NCTC 12023), where, after 30 days, the reduction ranged from 1.3 $\log_{10}$ cfu/surface to 1.6 $\log_{10}$ cfu/surface. The lowest survival was linked to a *S.* Typhimurium strain used in European Standard disinfectant approval tests and *S.* Typhimurium isolated from whey powder. For most of the strains, following an initial reduction in viability in the first hours (<72 h), no further reduction was seen over the 30 day period; therefore a 2-population Weibull model was fitted to model the survival kinetics.
The overall survival was neither serotype nor time related. All strains had two different subpopulations, one more resistant to desiccation than the other.
The results indicate the possibility of the long term survival of *Salmonella* on environmental surfaces (at least 30 days) and suggest the most suitable model to describe and predict survival kinetics. The results also identify strains that may be used to study stress response mechanisms and potential factory control measures in future studies.

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1. Introduction

Historically, low water activity foods ($a_w < 0.85$) were of little concern with regard to microbiological safety due to their product characteristics. However, during the last 40 years *Salmonella* outbreaks have been linked to a number of food products with low water activity including: cereal, seeds, nuts, chocolate, dried fruits and vegetables, halva, dried milk products, spices, salami, tahini and tea (www.cdc.gov/foodsafety/outbreaks/). These outbreaks are usually very specific and are characterised as widely geographically spread, affecting a large number of people and lasting for relatively long periods of time. Beuchat and Komitopoulou (2011) and the Centres for Disease Control and Prevention website (CDC, 2014) described at least 40 large, documented outbreaks of *Salmonella* associated with dry foods. There is, therefore, a growing concern for the potential presence of *Salmonella* in such products. As the manufacture of many of these foods contains a thermal processing step, post process environmental contamination is thought to be the major source for contamination. Indeed, the same *Salmonella* type has been isolated from products produced over a number of years (e.g. 10 years from cereals produced at the same plant; CDC, 2008). The assumption has been that the particular *Salmonella* strain isolated had survived in the plant for this time period and had contaminated products, presumably when Good Manufacturing Practices (GMPs) were out of control. Driven by these contamination incidents, guidelines on implementation of better controls have been published (Beuchat and Komitopoulou, 2011; Holah et al., 2011; Podolak et al., 2009; Scott et al., 2009). However, there are still a number of bottlenecks in the dry food industry which are making implementation of these controls difficult (poor agriculture practices, lack of segregation, hygienic design of production equipment or lack of dry decontamination methods).

Evidence of *Salmonella* survival has been found in food processing environments. A study focused on contaminated chocolate by Craven et al. (1975) showed that the cause of the contamination was the uncontrolled airborne spread of dust in the factory environment. Samples

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taken in an oil meal plant also detected *Salmonella* on the processing floor, in dust in the air and on equipment (Morita et al., 2006). In several studies *Salmonella* was isolated from filters, processing floors, mixers, air and brooms in feed mills or crushing plants over a long period of time, indicating *Salmonella*'s ability to survive in dry processing environments (Binter et al., 2011). It has been reported that once a production line is contaminated with *Salmonella* spp., the microorganism will establish itself on the equipment and facility surfaces. In studies conducted by Gounadaki et al. (2008) *Salmonella* species were recovered from 26.5% of the environmental and equipment samples from dry sausage processing facilities. It is evident, therefore, that *Salmonella* can be present in the food processing environment and potentially contaminate the product.

The ability of *Salmonella* to survive for long periods of time in low a_w products e.g. dried almond hulls (Uesugi and Harris, 2006), halva (Kotzekidou, 1998), peanut butter (Burnett et al., 2000), dry milk, cocoa powder and poultry feed (Juven et al., 1984) or even on walnut kernels (Blessington et al., 2012) has been documented. However, whilst there is some information on the survival of *Salmonella* in dried products there is limited information on the survival of *Salmonella* in the dry food processing environment, both in terms of the length of survival and the possible factors contributing to such survival. Uesugi et al. (2007) demonstrated the potential for long-term (5 years) environmental persistence of *Salmonella* in almond orchards. However, this was an open environment and may have been contaminated on an ongoing basis from an external source. Resistance of *Salmonella* strains to dry conditions was confirmed by Janning et al. (1994). In addition, Kusumaningrum et al. (2003) showed that *Salmonella* can remain viable on dry stainless steel surfaces and present the potential for contamination for considerable time periods.

The subspecies of most concern, in relation to food safety, is *Salmonella enterica* subsp. *enterica*. More than 99% of *Salmonella* isolated from humans belong to this subspecies (Old, 1992). The total number of *S. enterica* subsp. *enterica* serovars is approximately 2579 (Grimont and Weill, 2007), however the number of serovars associated with outbreaks in low a_w foods, is only ~30 (Beuchat and Komitopoulou, 2011; CDC, 2014). Therefore, it is important to assess if these strains can also survive in the low relative humidities of dry food processing factories and thus identify the need for appropriate control mechanisms.

Wet cleaning and disinfection is the preferred method in the food industry to control microbial contamination. Dry processing food factories are often dry cleaned only (which does not always remove all surface soiling and microorganisms) or wet cleaned periodically (e.g. following every batch or each time a different allergenic ingredient is processed). For example, in the dry milk industry, the typical production time is approximately 30 days before wet cleaning is undertaken. Therefore, due to the existing dry cleaning methods not being sufficient for microbial eradication it is important for the food industry to know if *Salmonella* can survive for prolonged periods of time on surfaces without water or until the next wet cleaning occurrence (e.g. for 30 days).

In order to ensure that the results would be as useful and applicable to the food industry as possible, the same *Salmonella* serovars as those from the foods which caused recent *Salmonella* outbreaks were used. Additionally, heat- and disinfectant-resistant (Anon, 2001) laboratory test strains were used to investigate if they were also resistant to desiccation. The studies aimed to (a) establish survival kinetics for 15 *Salmonella* strains, (b) assess any differences between and within serotype isolates, (c) show if *Salmonella* cells attached to stainless steel surfaces, with traceable amounts of nutrients, can survive for prolonged periods of time, and (d) choose the best kinetic model to describe the survival.

2. Materials and methods

2.1. Salmonella strains

A total of 15 *S. enterica* subsp. *enterica* serovars were obtained from the Campden BRI culture collection (Table 1). Identification and characterisation of the strains were carried out by ribotyping (Campden BRI, Dept. Food Microbiology) prior to experimental use. Four isolates of *S.* Typhimurium were used to compare differences in survival rate between the same serotype and six isolates from cereal manufacturing environments were also used to compare differences in survival of strains isolated from the same environment.

2.2. Surfaces

Stainless steel discs (2 cm diameter, Grade 2B, 1.4301 (EN 10088-1, EN 10 088-2)) were used (Resurgem Engineering Co Ltd, UK). They were soaked in hot water with detergent (Fairy liquid, Procter & Gamble) for 60 min. Each disc was then cleaned with a sponge and rinsed with sterile distilled water. They were then left to dry exposed to the air for 30 min, wiped with alcohol wipes (Izo-wipes; Johnson Diversey), and sterilised in an autoclave at 121 °C for 20 min.

2.3. Test suspensions

Cultures were maintained at −80 °C in cryo vials containing glycerol (Pro-Lab Diagnostics, Microbank). Working cultures of each serotype were sequentially prepared by adding a cryo bead to 150 ml Nutrient Broth (NB, Oxoid 0001) which was incubated for 24 h at 37 °C in a shaking incubator at 100 rpm. The broth was then centrifuged at 3000 *g* for 10 min (Camlab, ALC centrifuge, PK 120). The degree of cell washing necessary to remove any nutrients from the cell solution, which could have an impact on subsequent cell survivability, was assessed during initial experiments. The survival of *S.* Typhimurium 'HR' was accessed

Table 1
Salmonella isolates used for the experiments.

No.	Given code	Name	Source
1.	FH/Sa/159	*Salmonella* Agona	Cotton seeds
2.	FH/Sn/161	*Salmonella* Napoli	Chocolate confectionary, refrigerated 7 years (1986 outbreak Italy)
3.	FH/Sm/164	*Salmonella* Muenchen	Cocoa bean environment
4.	FH/Se/162	*Salmonella* Enteritidis	Cereal
5.	FH/St/163	*Salmonella* Tennessee	Sesame seeds
6.	FH/St/165	*Salmonella* Typhimurium	Whey powder
7.	FH/St/167	*Salmonella* Typhimurium DT104	HPA, Colindale
8.	FH/St/168	*Salmonella* Typhimurium	NCTC 12023, heat resistant "HR"
9.	FH/St/68	*Salmonella* Typhimurium	NCTC (PHILS), NC000 74-17, European standard disinfectant test strain "DS"
10.	FH/S1/172	*Salmonella* Lomita	Cereals environment "1"
11.	FH/S2/173	*Salmonella* Lomita	Cereals environment "2"
12.	FH/S3/174	*Salmonella* Cubana	Cereals environment "1"
13.	FH/S7/178	*Salmonella* Cubana	Cereals environment "2"
14.	FH/S5/176	*Salmonella* Mbandaka	Cereals environment "1"
15.	FH/S6/177	*Salmonella* Mbandaka	Cereals environment "2"

and compared between cells washed once or twice. There was no statistical significant difference in survival ($p > 0.05$). Following this, one wash was used for all the trials. After centrifugation, pellets were re-suspended in 500 ml of Maximum Recovery Diluent (MRD, LabM) which formed ~10^7–10^8 cfu/ml suspensions. These were directly used to achieve ~10^6–10^7 cfu inocula on discs.

2.4. Preparation of controlled RH chamber

A relative humidity (RH) of 33% ± 2.5% was used in all experiments, chosen to be reflective of dry food processing environments. The RH was established and maintained in sealed boxes (32 cm × 11 cm × 21 cm) by the presence of saturated magnesium chloride salt (Fisher Scientific, Laboratory reagent grade, $MgCl_2 \cdot 6H_2O$, Batch: 0936578). The sealed boxes were held in an incubator at the desired temperature: 25 ± 2 °C. A calibrated, digital thermohygrometer (Rotronic; HygroLog-D) was used daily for measurements of temperature and RH.

2.5. Inoculation of discs

A 50 μl volume of each test suspension was deposited onto the centre of stainless steel discs. Replicate discs ($n = 3$) were placed in an uncovered Petri dish and transferred to an incubator to dry at 30 °C for 80 min. After drying, Petri dishes were covered and placed in air-tight, humidity and temperature-controlled, transparent plastic boxes. The number of microorganisms surviving on the stainless steel surfaces was assessed at the following time points after drying: 0 h, 1 h, 3 h, 6 h, 24 h, 30 h, 2 days, 3 days, 7 days, 10 days, 15 days and 30 days.

2.6. Determination of viable counts of surface-inoculated bacteria

In order to assess the number of bacteria surviving on the discs, each disc was aseptically transferred into a sterile plastic universal container (diameter 4–5 cm) containing 10 ml MRD. Containers were left for 10 min at room temperature and vortexed for 1 min to recover the remaining bacteria into suspension. Each sample was then serially diluted in MRD and 1 ml volumes plated out in duplicate using Tryptone Soya Agar (Oxoid; TSA). This recovery method had been previously validated to maximise recovery from the surfaces. The method was not sensitive enough to detect a viable cell concentration of less than 5 cfu/surface (0.7 $\log_{10}$ cfu).

2.7. Agar incorporation method

To verify the bacterial recovery process, and to detect any cultivable colonies left on the surface after recovery, a modified method of EN 13697:2001 (Anon, 2001) was used. Each disc from which bacteria had been removed was recovered from its container, rinsed with 10 ml of sterile distilled water and then placed onto a pre-poured TSA plate. A 0.1 ml volume of sterile, distilled water was pipetted onto the disc and rubbed over the top and the bottom surface with a pipette tip for 30 s. The discs were then over-poured with approx. 10 ml TSA and plates were swirled to ensure even mixing. All plates were incubated at 37 °C for 48 h. Plates were then enumerated and the number of colony forming units (cfu) per test surface was calculated.

2.8. Statistical methods used

All experiments were statistically designed and the results gained were analysed using Minitab 16 statistical software. For statistical comparison the ANOVA test with 95% confidence was used. Results with a p value <0.05 were considered as statistically significant. Experiments were repeated at least 4 times using 3 replicates each time for each test condition and these data were used to develop a kinetic model. Trials involving the strains isolated from a cereal processing environment had three technical replicates for each strain at each time point. These strains were tested as a final part of the study and according to the sample power and size calculations, there was no need to conduct more replicates to be able to notice statistical differences between strains.

The results are expressed as the mean $\log_{10}$ cfu/surface reduction at each time point compared to time 0. The points on the graphs represent the mean of experimentally obtained values and the lines represent predicted kinetics gained by fitting the modified Weibull model (Eq. (1)).

2.9. Kinetic model

Several equations were analysed to build the most suitable empirical model (Weibull, nth-order, exponential, Churchill model, and modifications of them in order to account for both the weak and resistant populations). According to the best fitting parameters (data not shown), a modified Weibull equation considering two populations was selected:

$$\log_{10}(S) = \underbrace{\beta_1 \cdot \left(\frac{t}{\delta_1}\right)^{\alpha_1}}_{\text{Subpopulation 1}} \underbrace{-(1-\beta_1) \cdot \left(\frac{t}{\delta_2}\right)^{\alpha_2}}_{\text{Subpopulation 2}}. \quad (1)$$

The subscripts 1 and 2 indicate the two different subpopulations (or the two different levels of resistance; in the Results section, subpopulation 1 is resistant and subpopulation 2 is sensitive to desiccation), S is the survival rate (cfu_t/cfu_0), β_1 is the fraction of subpopulation 1 in the total population, δ is the time needed for the first decimal reduction, α is a shape parameter and t is the time in hours.

The goodness of fit for the obtained equations was analysed according to the coefficient of determination (R^2) and root mean square error (RMSE). In addition, the model performance was tested with data generated in this study, by means of the bias factor, (B_f) and the accuracy factor (A_f) (Álvarez et al., 2003; Klotz et al., 2007; Meneses et al., 2013).

The error between predicted and experimental *Salmonella* survival values was estimated by using the Root Mean Square Error (RMSE), which is a statistical measure of the differences between values predicted by a model and the values actually observed from experiments and can be expressed by an absolute value, in this case $\log_{10}$:

$$\text{RMSE} = \sqrt{\frac{\sum_{i}^{n} \left(S_{observed} - S_{predicted}\right)^2}{n}} \quad (2)$$

where, $S_{observed}$ is the measured population, $S_{predicted}$ is the predicted population by the empirical model and n is the total number of experimental measurements.

The bias factor is a measure of over-prediction ($B_f > 1$) or under-prediction ($B_f < 1$) of the predicted values by the empirical model in comparison to the experimental values. A bias factor of 1 indicates perfect agreement between observed and predicted generation times.

The accuracy factor indicates how the predictions differ from the observed data. An A_f of 1 indicates perfect agreement between all predicted and observed S values. An A_f value higher than 1 (e.g. 1.2) shows that the prediction is on average, either 20% smaller or 20% larger than the observed values (Ongeng et al., 2010). The A_f should thus always be analysed in combination with the B_f. It is worth mentioning that the deviation percentage to the observed value (A_f) is in reference to the values as "$\log_{10}$" and not to the number of colonies.

3. Results

Fifteen different *Salmonella* isolates were used to compare their survival rate and assess their resistance to desiccation. The initial bacterial level on the stainless steel discs ranged from 6.6 to 7.3 $\log_{10}$ cfu/surface. Strains had different sensitivities to initial drying (80 min at 30 °C) which resulted in different starting levels when placing the discs in the controlled humidity boxes *S.* Typhimurium "DS" and *S.* Typhimurium

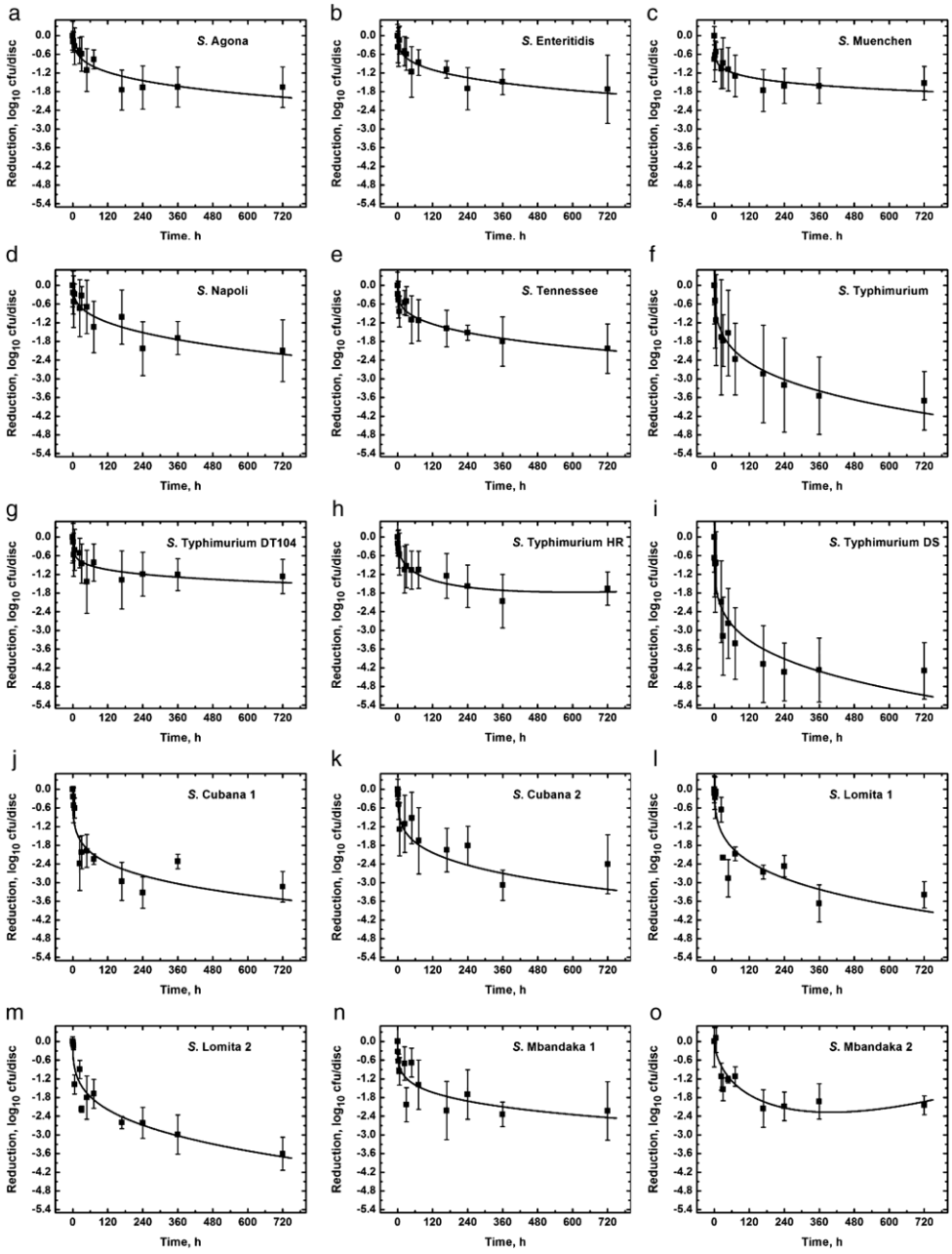


Fig. 1. Reduction kinetics of 15 strains of *Salmonella* suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33% RH and 25 °C for up to 720 h. Bars represent one standard deviation. Points represent mean experimental values.

strain FH/St/165 were found to be most sensitive to initial drying (a loss of viability of 2.08 ± 0.92 and 1.84 ± 0.57 $\log_{10}$ cfu/surface, respectively), and they were also most sensitive to the desiccating conditions at a controlled humidity and temperature (Fig. 1). The loss of viability after initial drying for the rest of the strains ranged from 0.65 ± 0.29 to 1.44 ± 0.82 $\log_{10}$ cfu/surface.

The highest $\log_{10}$ reduction was associated with *S.* Typhimurium strain FH/St/165 and *S.* Typhimurium "DS" where the level of bacteria, showed a 4.1 ± 0.9 and 4.3 ± 0.9 $\log_{10}$ cfu/surface reduction after 30 days, respectively. The lowest reduction rate, from the point after initial drying to 30 days, was associated with *S.* Muenchen, *S.* Typhimurium DT104 and *S.* Typhimurium "HR", where the microorganism levels dropped by 1.5 ± 0.50, 1.3 ± 0.60 and 1.6 ± 0.50 $\log_{10}$ cfu/surface, respectively. A similar reduction was also observed for both *S.* Agona and *S.* Enteritidis. The rest of the strains, after 30 days, showed reduction levels ranging from 2.1 to 3.6 $\log_{10}$ cfu/surface (Fig. 1).

With respect to the recovery of strains from the stainless steel discs, there was no indication, after trials with vital stains and epifluorescent microscopy, that microorganisms adhered to the stainless steel discs were in the viable but not culturable state (data not shown).

After obtaining broad experimental data, the most suitable model was selected and fitted to describe the mechanism of the reduction for each *Salmonella* strain. Table 2 shows the integrity of fit for the obtained equations. The coefficient of determination (R^2) is close to 1 for most of the strains, indicating that the suggested model fits relatively well to the experimental values. Nonetheless, the model may present deviations from the experimental data as it was not possible to fit the model to 100% of the experimental points. In order to find out whether this deviation created an under- or over-prediction, the B_f value was calculated. The B_f values are mostly above 1 indicating that the model is over-predicting the reduction rates for 13 out of 15 strains. This means that the real reduction is smaller than that plotted by the kinetic curve on the graphs and in some cases (S. Lomita "1") by as much as 67% (A_f 1.672942 ~67%). The reduction kinetics are under-predicted (i.e. the curve lies above the actual data points) only for *S.* Typhimurium (whey powder isolate) and *S.* Mbandaka "2". An over-predicted result is not desirable since a higher reduction level will be estimated compared to the real value. This must be considered when using the model to predict the survival rates of *Salmonella* strains and, depending on the A_f values, the appropriate percentage should be added or reduced ($1 > B_f$ or $1 < B_f$ respectively) from the predicted values.

The modified version of the Weibull model suggested the existence of two subpopulations within the population of each *Salmonella* strain. Alternatively, the model suggested two different levels of desiccation resistance. It can be seen that there was a relatively high rate of bacterial death at the beginning of the survival curves (subpopulation 2) for all isolates and a decrease in the death rate (except *S.* Typhimurium "DS" and *S.* Lomita "2") from up to 72 h (subpopulation 1) under desiccating conditions. The fastest decline rate was associated with *S.* Typhimurium strain FH/St/165 and *S.* Typhimurium "DS" where statistically significant differences could be seen between samples taken at time 0 and samples taken after 6 and 24 h in desiccating conditions, respectively. The slowest overall dying rate was shown for *S.* Muenchen and *S.* Typhimurium "HR". For these strains, a significant difference in microbial counts from time 0 could only be seen after 168 h under desiccating conditions. After the initial reduction in *Salmonella* count, the microorganism levels stayed constant. The strains which reached this state in the shortest time (after 24 h) were *S.* Muenchen, *S.* Typhimurium DT104 and *S.* Typhimurium "HR". Most of the strains, after reaching a certain reduction level, showed no further, significant viability loss for 27 days (Fig. 1), suggesting that time might not be a factor in prolonged survival. Significant differences were detected ($p < 0.005$) for all cases between the two sub-populations (or resistances).

Table 3 lists kinetic coefficients obtained using Eq. (1) and these describe the behaviour of the surviving subpopulation (parameters δ_1 and α_1) and the more sensitive subpopulation (parameters δ_2 and α_2; Table 3). δ_1 and δ_2 are expressed in hours and indicate the time needed for the first decimal reduction; β_1 indicates the fraction of subpopulation 1 (surviving population) in the total population. It is worth mentioning that this fraction is in relation to the survival when expressed as $\log_{10}$ cfu/surface and not to arithmetic values of the counts. α is a shape parameter and refers to the extent of the impact that time of exposure may have on the reduction rate. If α is lower, the reduction rate is less affected by time of exposure and thus the survival number of *Salmonella* is higher (however this value has to be analysed together with δ_1 and δ_2 to give an assessment of overall reduction rates). This can be seen for example with *S.* Agona. The first subpopulation (more resistant, surviving population is about 41% (β_1 fraction is 0.4148, which is equal to ~41%) of the whole population and is resistant to desiccation with numbers changing only slightly, between 120 and 720 h of desiccation (Fig. 1a). This behaviour is represented in the kinetic equation by the low value for α_1 (Table 3). In addition, the kinetic parameter δ_1 shows how slowly this population dies (2.6 h to achieve the first decimal reduction). A high value of δ_1 would mean that a longer time of exposure to an inimical process is necessary in order to achieve a significant cell reduction. In contrast the second more sensitive subpopulation of *S.* Agona has a δ_2 of 1.5 showing that the first decimal reduction of this subpopulation takes a shorter time and the higher value for α_2 in comparison to α_1 shows that this subpopulation is more sensitive to time of exposure. Considering *S.* Typhimurium (isolated from whey powder, FH/St/165) as an example of one of the most sensitive strains, it could be concluded that the resistant subpopulation, which is 71% of the population (line 6, Table 3), is less affected by exposure time than

Table 2
Values of the coefficient of determination (R^2), root mean square error (RMSE), bias factor, (B_f) an accuracy factor (A_f) for the obtained equation.

Serotype	R^2	RMSE ($\log_{10}$)	B_f	A_f
S. Agona	0.94173	0.22256	1.03933	1.29414
S. Enteritidis	0.94516	0.19514	1.042759	1.329144
S. Muenchen	0.93921	0.18180	1.01665	1.158471
S. Napoli	0.93234	0.26125	1.030013	1.322894
S. Tennessee	0.96606	0.16458	1.015739	1.178274
S. Typhimurium	0.98152	0.22091	0.992169	1.123399
S. Typhimurium DT104	0.87851	0.22872	1.085728	1.291501
S. Typhimurium HR	0.96805	0.15498	1.015047	1.749514
S. Typhimurium DS	0.94172	0.52380	1.111761	1.264831
S. Cubana 1	0.90704	0.48121	1.167163	1.399194
S. Cubana 2	0.85467	0.36153	1.337437	1.372376
S. Lomita 1	0.90857	0.56402	1.367244	1.672942
S. Lomita 2	0.94818	0.37543	1.319625	1.604673
S. Mbandaka 1	0.87479	0.39561	1.070405	1.300488
S. Mbandaka 2	0.88645	0.32754	0.825617	1.605119

Table 3
Kinetic coefficients for Eq. (1).

Serotype	β_1	δ_1	δ_2	α_1	α_2
S. Agona	0.4148	2.6468	1.5171	8.94E−7	0.2282
S. Enteritidis	0.3884	2.4615	1.5615	0.1041	0.2329
S. Muenchen	0.1839	2.2052	1.7739	2.21E−5	0.1466
S. Napoli	0.4805	2.3777	1.4872	0.2311	0.2966
S. Tennessee	0.3516	2.3822	1.4350	0.2892	0.2892
S. Typhimurium	0.7115	3.8797	0.00343	0.01544	0.2306
S. Typhimurium DT104	0.2940	2.4029	1.6910	1.57E−7	0.1503
S. Typhimurium HR	0.4689	2.5481	1.0244	0.5795	0.5003
S. Typhimurium DS	−0.1164	1.6939	1.8831	0.2377	0.2378
S. Cubana 1	0.6665	4.4996	0.0007	0.06119	0.1871
S. Cubana 2	0.1735	1.9410	1.8993	7.05E−7	0.2376
S. Lomita 1	0.7156	5.0027	0.0049	9.06E−5	0.2347
S. Lomita 2	0.2154	1.8388	1.9981	7.67E−9	0.2733
S. Mbandaka 1	0.2164	2.2764	1.7068	5.84E−7	0.2022
S. Mbandaka 2	0.3845	1.2024	1.5277	0.8149	0.7746

the sensitive population ($\alpha_1 < \alpha_2$) and more time is needed to reduce the number of cells by one decimal reduction ($\delta_1 > \delta_2$) compared with the second subpopulation. However, this *S.* Typhimurium strain is not very resistant to desiccation overall; this behaviour is dominated by the second subpopulation (Fig. 1f) where the value of δ_2 is 1000-fold lower than for the first subpopulation, reflected in a very fast reduction in microbial cell counts at the beginning of the desiccation process. Analysing other sensitive strains, *S.* Typhimurium "DS" seems to have a predominant sensitive subpopulation 2 (89%), *S.* Lomita "1" has a much lower δ_2 value than δ_1 and *S.* Lomita "2" has a high percentage of subpopulation 2 (79%) with considerably higher α_2 than α_1 value (ie with considerably less resistance). Therefore for each of these more desiccation sensitive strains it would suggest that a different factor is determining their survival kinetics.

In addition, the best surviving strains have different factors which are influencing their resistance to desiccation. For example, *S.* Muenchen and *S.* Typhimurium DT 104 have a small amount of the population which is more resistant (18% and 29%, respectively); however these subpopulations have a very low α_1 factor (very modest influence of time of exposure) and need slightly more time for one $\log_{10}$ decline in cell numbers ($\delta_1 > \delta_2$). For these strains the α_2 values are also relatively low showing that their sensitive populations are also less influenced by time of exposure. *S.* Typhimurium HR, however, has a much larger population which is resistant (47%) but the α and δ values for both subpopulations are not very different, thus producing an overall more resistant population.

An example of a moderately desiccation resistant strain is *S.* Cubana "1" (Fig. 1j, Table 3). The values for this strain indicate that most of the population (~66%) is resistant to harsh environmental conditions and no significant differences occur between 60 and 720 h. This resistant part of the population shows a very high value of δ_1 (4.5) in comparison to the sensitive population ($\delta_2 = 0.0007$). This means that a longer time is needed to achieve a significant reduction in microbial counts of this part of the population. The value of α_1 is smaller than the value of α_2, which means that the resistant population is not much affected by exposure time and no significant changes are expected as a function of time. The sensitive population, although a smaller part (~34%) of the population has a very low δ_2 and this is reflected in the very rapid reduction seen during the first 0–60 h on the graph (Fig. 1j), which is significantly different from the resistant population ($p = 0.005$).

Differences in survival kinetics and survival rates between different strains of the same serotype were noted, with *S.* Typhimurium DT104 and *S.* Typhimurium "HR" (heat resistant) being in the high resistance group, and *S.* Typhimurium (isolated from whey powder) and *S.* Typhimurium "DS" (a laboratory cultured strain used in European Standard disinfectant approval test methods) having low resistance to desiccation. In contrast there was no difference in the reduction levels between *S.* Lomita 1 and 2 (3.4 ± 0.4 and 3.6 ± 0.5 log cfu/surface reduction, respectively). However in contrast to the *S.* Typhimurium strains which were isolated from different environments, both *S.* Lomita strains were isolated from the same cereal environment, which may have selected specific desiccation tolerances in *Salmonella* strains harboured there.

4. Discussion

This study set out to examine the desiccation resistance of a range of *Salmonella* strains under industrially relevant conditions. The *Salmonella* strains chosen were associated with low a_w food outbreaks, were isolated from dry foods or their production environment or were known as resistant to other stresses such as heat or disinfection. Stainless steel was chosen as the most common surface used in the food industry (BSSA, 2013), and the temperature and humidity conditions were chosen to reflect dry food processing environments. It is known that the exposure to a single stress condition e.g. heat (Humphrey et al., 1995) or acid (Leyer and Johnson, 1993) is commonly associated with the development of cross-tolerance to other stress conditions (Abee and Wouters, 1999). Therefore the conditions used for the experiments were chosen to obtain an otherwise optimum growth environment for *Salmonella* cells (e.g. stationary phase, ambient temperature, and nutrients not limited), so, most likely, only desiccation resistance mechanisms were involved in the survival of serotypes. Stationary phase grown microorganisms were used rather than log-phase bacteria as it is known that they have greater resistance to multiple stresses (Aldsworth et al., 1998; Rychlik and Barrow, 2005) and this is thought to more closely reflect the growth phase cells are in in food environments.

The studies showed that the strain recognised as resistant to disinfectants (and so resistant to one stress factor) was not resistant to drying; in contrast the heat resistant strain of *S.* Typhimurium did show desiccation tolerance. Differences in the survival between different isolates of the same serotype (*S.* Typhimurium) were noticed. For most of the studied strains, an initial reduction in viability occurred in the first few days, with no or relatively little further reduction seen over the subsequent part of the 30 day period. This suggests that the length of the period of desiccation the strains are exposed to is not the most important factor in reducing the microbial population. During the first hours of exposure (<72 h), the cells are under stress and as they are only starting to switch on their response mechanisms, they may be more susceptible to desiccation. After 72 h, most of the serotypes seem to have developed a more resistant subpopulation possibly through fully expressed adaptive mechanisms; it is this subpopulation that can now persist for an extended period and shows a very slow death rate. This coincides with the findings from food borne outbreak investigations and factory sampling trials, which imply that some *Salmonella* strains have survived for years in factory environments.

The modified kinetic Weibull model fitted well with experimental data ($R^2 > 0.85$) and gave some insights into the survival behaviour for each *Salmonella* strain studied. The objective of analysing the model performance was to ensure a proper mathematical correlation between survival and time. It is recommended to analyse model performance with external data prior to universal applications on survival rate of *Salmonella* on stainless steel surfaces.

It is important that, for each strain, each parameter obtained by the model is analysed together with the other parameters obtained for that particular strain and it is not recommended that individual kinetic parameters are compared between strains since their meaning is strongly dependent on the rest of the values. This kind of comparison could only be possible if a 1-population Weibull model with the same α values for every strain (or the same β value for each strain) was used. The kinetic parameters obtained from the modified Weibull model, in combination with the graphs (Fig. 1), are considered to explain the kinetics of *Salmonella* survival for each single strain, but the kinetic parameters should not be used to range or group the strains in terms of most or least resistant. For these purposes, the graph for each strain can be used as a comparison reference. For most of the strains (except *S.* Typhimurium "DS" and *S.* Lomita "2") the second population was more susceptible to desiccation, which is reflected in the graphs by the values obtained from the early hours of desiccation. This population, therefore, would need less time for reduction in microbial counts ($\delta_2 < \delta_1$). Thus during the first hours of desiccation microorganism levels declined faster (2nd subpopulation) and during the late period of desiccation, microorganism levels stayed constant or declined slightly (1st subpopulation — surviving subpopulation). The added value of the model is the possibility to calculate exactly the percentage of subpopulations 1 and 2 and the rates that these subpopulations were declining. The kinetic parameters obtained suggested that each strain had a different response to desiccation in terms of the percentage of the population which were resistance, reduction levels, reduction rate and/or influence of time of exposure. Although extrapolation is not recommended, the model could also be helpful to predict reduction levels of each *Salmonella* strain at longer exposure times; this however, should only be used for indicative purposes.

In previous studies, Kusumaningrum et al. (2003) reported that *S.* Enteritidis survived for at least 96 h on stainless steel surfaces at 20–25 °C and 40–45% RH and Broennum Pedersen et al. (2008) found that *S.* Senftenberg attached to ELISA wells for 35 days at 40% humidity were reduced in microbial population by 3.45 $\log_{10}$ cfu/ml. This is a similar result to other serotypes investigated in this study. However, the reduction was much higher in the Kusumaningrum et al. (2003) studies. These differences could be caused because of different strains, different culturing methods prior to inoculation, different drying methods or different humidity levels. The difference in survival rate between isolates of the same *Salmonella* serotype seen in the current study was also observed by Humphrey et al. (1995).

Recent studies conducted by Gruzdev et al. (2012) also showed large variability in desiccation tolerance and long term persistence (up to 12 weeks) among different *Salmonella* serotypes as well as within a single serotype (Typhimurium). This confirms the findings of the present studies and highlights the importance of choosing an appropriate serotype/isolate when conducting any specific challenge testing. Muller et al. (2012) investigated the survival of 25 selected *Salmonella* strains attached to surfaces after prolonged exposure to controlled humidity and temperature. A strong decline trend was observed and only three *Salmonella* strains survived at desiccation conditions for up to 49 days. Variations in survival of all 25 isolates could be observed, including among several *S.* Typhimurium strains. The 25 strains were mainly porcine and clinical isolates, which may reflect their reduced resistance as compared with the 15 strains used in this study, primarily isolated from dry environments. The serotypes which are isolated more often from dry processing environments may have been selected to survive because they have acquired mutations or genetic information that enhances their desiccation tolerance (Binter et al., 2011). The number of 'mutators' among *Salmonella* is reportedly high (Jorgensen et al., 2000); therefore it can be argued that the more frequent the appearance of mutators, the better the ability of this organism to generate isolates better adapted to survive in different environments. As a consequence very closely related *Salmonella* serovars can have very different responses to stress (Jorgensen et al., 2000).

It is not yet clear from the present studies why some *Salmonella* strains survive better than others or what the differences are which make one subpopulation decline more rapidly at the beginning of the desiccation process and the other subpopulation reach a plateau phase. One way *Salmonella* survives severe environmental conditions may be because surface inoculated cells dry towards the outer ring of the inoculum (which has been observed by the authors under fluorescent microscopy, data not shown) thus forming a multilayer of cells on the edge of the ring. The outer layer may then die off faster, protecting the inner layers. This could be one way that the two subpopulations could arise. Additionally, initial decline of the population level, followed by no decline, could be explained by possible heterogeneity of the bacterial populations (differences in growth phases of cells; Booth, 2002). The single *Salmonella* inoculum may contain cells with different levels of expression of a particular cellular component which helps them cope with the stress e.g. protein in their cells; therefore the ones which contain this specific component are able to survive, and the ones which lack it, die off. This might explain the 'nonlinear' death kinetics of the analysed *Salmonella* strains. It may be argued that some of the cells entered a viable but not culturable (VBNC) state and could not be detected with the methods used. The recovery method however was validated (data not shown) and additionally an agar incorporation method was used to pick up low cell numbers. The LIVE/DEAD stain was used to establish the relationship between cultured cells and live cells on the surfaces; however the stain did not work well with dried, attached cells.

Some of these hypotheses suggest the existence of two subpopulations (with different resistances) within one *Salmonella* strain and support the findings suggested by the kinetic model used in this paper.

Essentially, with respect to the manufacturers of low-moisture foods, this study has shown that some *Salmonella* strains survived for at least 30 days under desiccation conditions on stainless steel and potentially could survive much longer. In addition, the results give an insight into the survival time and death kinetics for *Salmonella* in dry processing environments, suggesting that once this pathogen is introduced to the factory environment, there is a serious risk of its survival. Good control measures are necessary, therefore, in dry food factory processing environments, to avoid the entry of *Salmonella* and to prevent any cross-contamination from such factory environments to the food product.

A greater understanding of the ability of *Salmonella* strains to survive in the environment is now necessary to effectively control this pathogen. Future studies should focus on the selection of the best strains to study desiccation mechanisms, investigation of different factory environmental conditions and practices (external factors) and their influence on desiccation resistance and examination of *Salmonella*'s molecular responses to desiccation.

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APPENDIX M

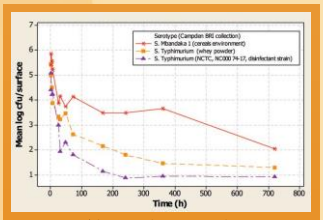
IFT FOOD & EXPO. 27 JUNE 2012. LAS VEGAS, USA

Survival and decontamination resistance of surface adhered *Salmonella* strains

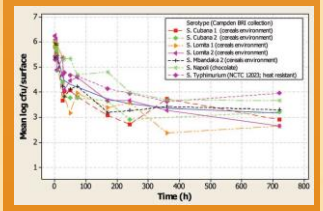
E. Margas[1,2], S. Pineda[1], J. Holah[1] and C. Dodd[2]
1 Campden BRI, Chipping Campden.GL55 6LD, UK
2 University of Nottingham, School of Biosciences, Nottingham LE12 5RD, UK

Campden BRI
food and drink innovation

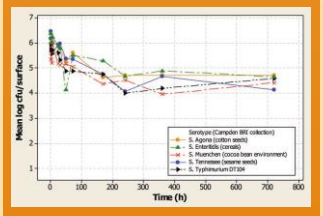
Since the 1970's, a number of large *Salmonella* outbreaks have been associated with low a_w, ready-to-eat food products. A significant factor in these outbreaks was found to be cross-contamination, therefore controlling *Salmonella*'s persistence in the processing environment is a current food safety priority.



a) Low survival rate strains



b) Moderate survival rate strains



c) High survival rate strains

Figure 1. Survival rates of different strains of *Salmonella* suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33% RH and 23ºC for up to 720 hours (30 days).

Aim

This study sought to assess the survival of 15 *Salmonella* strains (Figure 1), isolated from low a_w foods or environments that were associated with the *Salmonella* outbreaks. They were adhered to stainless steel surfaces and the resistance to disinfectant and heat was determined.

Methods

Strains were obtained from the Campden BRI culture collection; they were grown in Nutrient Broth (NB; Oxoid) for 24 h in a shaking incubator at 37˚C and 100 rpm. The broth was then centrifuged at 3000 g for 10 minutes. Pellets were re-suspended in Maximum Recovery Diluent (MRD; LabM) to obtain inoculums of ~ 10^8 cfu/ml. 50µl was inoculated onto stainless steel surfaces and discs were placed in a controlled environment (RH 33% and 23˚C).

Survivors were assessed at various time points from 1 hour to 30 days, by vortexing the surfaces with 10 ml of MRD and plating out using Tryptone Soya Agar (TSA; Oxoid). The four most resistant strains were attached to discs for 30 days or for 80 minutes, and then subjected to direct flame by a propane-butane torch or to a surface disinfection treatment using sodium hypochlorite (50, 100, 250, 500 and 1000 ppm). A modified protocol of EN 13697:2001, the current EU surface disinfectant test, was followed.

Results

Following an initial reduction in viability during the first few days, no further reduction was seen over the rest of the 30 day period (Figure 1 a, b and c). This suggested that time was not a factor influencing prolonged survival and confirmed reports of *Salmonella*

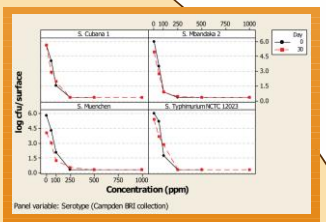


Figure 2. Sodium hypochlorite concentration dependent survival of *Salmonella* strains attached to surfaces for 30 days or 80 minutes (0 days).

persisting in food processing environments. There was no survival of *S.* Typhimurium NCTC (PHLS), NC000 74-17 after 7 days under experimental conditions, suggesting that survival was not serotype related (Figure 1). There was no survival of any strain after thermal decontamination (> 6 log cfu/ surface reduction). A standard concentration of sodium hypochlorite (250 ppm) was needed to achieve a >4 log reduction for all strains for both attachment periods (Figure 2).

Conclusion

The results indicate that whilst there is a risk of *Salmonella* surviving in food processing environments, standard concentrations of disinfectant should achieve appropriate control levels. These results will be incorporated into a Campden BRI guidance document on the survival of *Salmonella* in dry process environments with relation to strain, temperature, humidity, water and soiling, which would help the industry to control the endurance of *Salmonella* in the environment and thus in the final product.

Campden BRI continues to investigate this problem and can help industry partners who wish to better control the risk of cross-contamination from the factory environment to food products.

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APPENDIX N

CURRICULUM VITAE

Edyta Margas MSc Eng



Gartenstrasse 5, 9000 St. Gallen, Switzerland
Mobile: +447824369103. Email: edyta.margas@gmail.com
Nationality: Polish

PROFILE

I am an ambitious, creative, competent, experienced and determined individual, and my work and development at a highly respected company are proof to my commitment and ability. I have a strong educational background together with international work experience that has required team work, problem solving and analytical abilities, organisational skills and the ability to be a successful leader.

AREAS OF EXPERTISE

- Prerequisites plans
- Hygienic design
- Factory hygiene
- People and projects management
- Troubleshooting and auditing
- Novel processing technologies
- Research and innovation
- Training and consultancy
- Dry foods processing

PROFESSIONAL DEVELOPMENT

PhD candidate	Food Microbiology, The University of Nottingham, UK; School of Biosciences, Thesis: *The survival and control of Salmonella in low a_w foods' environments*	2010-2015 (expected)
FCE/ESOL level 2	First Certificate in English; Evesham Collage, UK	2007-2008
MSc	Food Technology; Wroclaw University of Environmental and Life Science; Poland. Thesis: *Implementation of HACCP prerequisites into a restaurant's system*	2005-2006
BEng.	Food Quality Management; Wroclaw University of Agriculture; Poland	2001-2005

Training courses:

• Creating business opportunities (IpOp model)	2014
• HACCP advanced (with RSPH certificate)	2012
• Communication skills for managers	2012
• Principles of canning	2011
• Internal auditing; HACCP workshop	2009
• Advanced Microbiology; Improving your presentation techniques	2008
• An introduction to statistical analysis ; Minitab graphs ; Sample size	2008
• Hygienic engineering; Cleaning and disinfection	2007

PROFESSIONAL EXPERIENCE AND SIGNIFICANT ACHIEVEMENTS

FOOD SAFETY AND HYGIENIC DESIGN; BÜHLER AG, SWITZERLAND ***10/2014 - PRESENT***
Project manager in a corporate research department. Responsible for training and support of various business areas accountable for processing technologies for low moisture foods

EXPERT ON DRY MATERIALS HANDLING, EHEDG, EUROPE ***08/2012 - PRESENT***
Active member of the Dry Materials Handling subgroup of the European Hygienic Engineering Design Group (www.ehedg.org) contributing to hygienic practices guidelines for the food industry

EXPERT ON DRY FOOD SAFETY; FAO AND WHO, GLOBAL ***03/2014 -11/2014***
Part of the joint FAO/WHO working group focused on ranking of microbiological hazards associated with low moisture foods

SCIENTIFIC ADVISOR ***01/2013 - 10/2014***
Providing a consultancy in the field of food safety and food processing. Scientific advisor for Bühler AG (technology provider for low moisture food manufacturing). Competent in molecular and classical microbiology.

HYGIENE AND NOVEL TECHNOLOGIES SPECIALIST; CAMPDEN BRI, UK ***08/2011 – 12/2012***

Part of the Innovative Process Technologies section at food research and services company.

- Responsible for managing research projects, contract work and clients enquiries, carrying out consultancy and training in areas related to new processing methods and factory hygiene.
- Specialist in high pressure processing, ultrasound, pulsed light, dry ingredients pasteurization systems, ozone, cold plasma, UV etc. Including validation of new technologies
- Specialist in hygienic design, building design including zoning, environmental sampling, personnel hygiene, cleaning and disinfection etc.
- In charge of contamination control in dry food processing environments and for a PhD project focused on the survival and control of *Salmonella* in low a_w food product's environments
- Involved in development of new HACCP prerequisites programme based on risk assessment
- Responsible for undertaking food safety and troubleshooting audits in food factories
- Event director of training courses (personnel hygiene, hygienic design) and chairmen of a food safety symposium at IAFP'12 European conference
- Representative of Campden BRI at international meetings, conferences, technical panels etc.
- Manager of a team of people
- In charge of business development, proposals writing and achieving financial targets
- Author of: scientific reports, book chapter, guidelines for the food industry, peer reviewed papers and trade press articles (see Appendix 1)
- Participant in European COST action on bacterial colonisation and persistence

HIGHER RESEARCH OFFICER; CAMPDEN BRI, UK ***02/2011 – 08/2011***

The New Products & Technologies Section. Responsible for writing a 'New Technologies Bulletin', carrying out research, contract work, consultancy and training in a wide range of areas related to new food processing methods (e.g. High Pressure, ultrasound, pulsed light, ozone).

RESEARCH OFFICER; CAMPDEN BRI, UK ***08/2008 – 02/2011***

The Food Hygiene Department. Responsible for the design, undertaking and reporting of high quality research and technical work related to HACCP prerequisites.

- Departmental quality co-ordinator: procedures, reviews, improvements, audits
- Teaching of environmental microbiology and factory hygiene on training courses
- Development of junior staff

MICROBIOLOGY LABORATORY TECHNICIAN T4; CAMPDEN BRI, UK ***01/2008 – 08/2008***
MICROBIOLOGY LABORATORY TECHNICIAN T3; CAMPDEN BRI, UK ***07/2006 – 01/2008***

Part of the Food Hygiene Department team. Responsible for undertaking technical and research work in a areas related to factory hygiene and day to day microbiological laboratory duties.

- Calibration officer and chemical and sample receipt steward
- Proficient in a range of European Standard disinfectant tests
- Carried out validation of the aseptic process

RESEARCH PROJECTS

- New Technologies for food production: Campden BRI member funded; 2011-2012; project manager
- SUCLEAN: Sustainable Cleaning and Disinfection in Fresh-Cut Food Industries, EU Framework VII, priority: Food Quality and Safety; 2012; task leader
- The control and survival of *Salmonella* in low water activity foods and their environments: Campden BRI member funded research project; 2010-2013; work package leader/project manager
- NovelQ: Novel Processing Methods for the Production and Distribution of High-Quality and Safe Food, EU Framework VI funded, priority Food Quality and Safety; 2006-2011
- Effective surface decontamination during hot filling; Campden BRI member funded; 2008-2010

HONOURS/AWARDS

- Finalist of Bühler AG 'Innovation challenge', Switzerland, 2014
- 2nd place in the Developing Scientist Award Competition at the IAFP'11 conference, Milwaukee
- Finalist of Marks & Spencer Millennium Prize for early career scientist, employed at Campden BRI with the potential to positively impact on the food, drink and allied industries, UK, 2012.
- Recipient of IAFP'13 Student Travel Scholarship, North Carolina, Charlotte, USA

Languages:	Polish (native), English (fluent), Spanish (basic), German (basic)
Interests and Pastimes:	food safety, sports, reading, travelling, dancing
Membership:	IAFP, IFT, EHEDG

Presentations and publications

Publications

- Burgess C.M, Gianotti, A, Grusdev, N, Holah, J, Knochel, S, Lehner, A, Margas, E, Schmitz Esser S, Sela (Saldinger), S and Tresse, O. 2015. The response of foodborne pathogens to osmotic and desiccation stresses in the food, submitted to International Journal of Food Microbiology, May 2015
- Expert panel, E. Margas. 2014. Ranking of low moisture foods in support of microbiologial risk management. Report of an FAO/WHO consultation process, http://ucfoodsafety.ucdavis.edu/files/209893.pdf
- Bahr, K.H., M. Louwe Kooijmans, K. Mager, **E. Margas**, K. Masters, G.M.H. Meesters, S. Multer, E. Polman, J. Roels, W. Rumpf, M. Stephan, E. Verplanke, M. Waskow. Hygienic Engineering of Pack-off Systems in Process Lines for Dry Particulate Materials, EHEDG Guidelines, EHEDG press (in preparation)
- Bahr, K.H., M. Louwe Kooijmans, K. Mager, **E. Margas**, K. Masters, G.M.H. Meesters, S. Multer, E. Polman, J. Roels, W. Rumpf, M. Stephan, E. Verplanke, M. Waskow. 2014. General Hygienic Design Criteria for the Safe Processing Of Dry Particulate Materials, EHEDG Guidelines doc. no 22, second edition, March 2014, EHEDG press
- Margas, E., Meneses, N., Holah, J. and Dodd, C. (2014) Survival and death kinetics of *Salmonella* strains attached to the surfaces. Journal of Food Microbiology 187:33–40
- Margas, E., McFarland, S. and Leadley, C. 2013. Power Ultrasound and its Use for Modification of Food Viscosity. R&D Report no 340, Campden BRI press
- Margas, E., Maguire, E., Holah, J., Berland, C. and Welander, F. 2013. Assessment of the environmental microbiological cross-contamination following hand drying with paper hand towels or an air blade dryer. Journal of Applied Microbiology, 115(2):572-582
- Margas, E. and Holah, J. 2014. Personal hygiene, *In: Hygiene in Food Processing, Principles and Practice. Second Edition.* Lelieveld, H.L.M, Holah, J. and Napper, D.Woodhead Publishing Ltd., UK
- Margas, E., et al. 2011-2012. New technologies bulletins no. 40, 41, 42 and 43. Campden BRI.
- Holah, J., Margas, E., Hagberg, R., Warren, B., Fraser-Heaps, J. and Mortimore, S. (2012) Identifying and controlling microbiological cross-contamination. Food Safety Magazine, February-March 2012: 54-61.
- Holah, J., Margas, E., Hagberg, R., Warren, B., Fraser-Heaps, J. and Mortimore, S. (2011) The rise of prerequisites. New Food, 6.
- Margas, E. and Holah, J. 2011. Guideline no. 67. Guidelines on good manufacturing practice for high pressure processed foods. Chipping Campden: Campden BRI press.
- Margas, E., Alstrom-Moore, A., Dodd, C. and Holah, J. (2011) *Salmonella* survival in low a_w food environments. 11th International Congress on Engineering and Food. 22-26 May 2011. Athens, Greece. Conference proceedings – 6 and 2 pages papers.

- Margas, E., Holah, J. Milanov, A. and Ahme, L. 2010. Hygienic design of novel processing equipment. New Food 5: 50-54
- Margas, E., Holah, J. 2010. Novel processing methods for the production and distribution of high-quality and safe foods (NovelQ). Research Summary Sheet 2010-19
- Smith, D. L., Margas, E., Holah, J. 2010. Novel processing methods for the production and distribution of high-quality and safe foods (NovelQ). Research Summary Sheet 2009-40

Presentations at international conferences

- Margas, E. (2014) The survival and control of *Salmonella* on surfaces. Water Activity Seminar. Campden BRI, 25th November 2014, UK
- Margas, E., Meneses, N., Holah, J. and Dodd, C. (2013) The long term survival of *Salmonella* cells adhered to stainless steel under various environmental conditions and their resistance to disinfectants. IAFP'13. 29-31 July 2013. Charlotte, North Carolina, USA
- Margas, E. (2013) Survival of microorganisms in low aw foods and their environments. Safe Production of Dried Foods Conference. Campden BRI, 11-12 July 2013. Chipping Campden, UK
- Margas, E. and Holah, J. 2012. A history of biofilms. IBBS Meeting – Antimicrobial strategies for biofilm control. 10-11 September 2012. Winchester, UK
- Margas, E. (2012) Recent Developments in Process Technologies to Improve Shelf-life. Impact of Novel Technologies on Shelf-Life of Food Conference. 13 September 2012. Leatherhead Food Research, London, UK
- Margas, E., Pineda, S., Dodd, C., and Holah, J. (2012) Survival and decontamination resistance of surface adhered Salmonella strains (poster). Institute of Food Technologies Food & Expo' 12. 25-28 June 2012. Las Vegas, USA
- Margas, E., McFarland, S., and Leadley, C.2012. Ultrasonic viscosity modification for better quality and clean label products (poster). Institute of Food Technologies Food & Expo' 12. 25-28 June 2012. Las Vegas, USA
- Margas, E. 2012. Microbial controls in food processing environment (oral presentation). Thermal Processing Conference. Campden BRI. 12-13 June 2012. Chipping Campden, UK.
- Margas, E. and Leadley, C. (2011) Ultrasonic viscosity modification of food products. Ifood, IFT Nonthermal Processing Division Workshop. 10-12 October 2011. Osnabruck, Germany.
- Margas, E. 2011. Pulsed light for surface decontamination. Cibustec, European Food Institutes (EFI) Forum: Technological Innovation and Control Systems in the Food Industry, 19 October 2011. Parma, Italy
- Margas, E. 2011. New Technologies as the key to product innovation. Food and Health Innovation Service Conference, 29 September 2011, Perth, Scotland, UK
- Margas, E. Survival and death kinetics of *Salmonella* strains attached to the surfaces. IAFP Annual Meeting, 31 July – 3 August 2011, Milwaukee, WI, USA

- Margas, E., Alstrom-Moore, A., Dodd, C. and Holah, J. (2011) *Salmonella* survival in low a_w food environments (oral presentation). 11th International Congress on Engineering and Food. 22-26 May 2011. Athens, Greece.
- Margas, E. and Holah, J. (2010) Hygienic building design including zoning (oral presentation). 1st EHEDG Conference for Food Factories in Russia. 4-5 October 2010. St. Petersburg, Russia.
- Margas, E. and Holah, J. (2010) Survival of microorganisms in HP systems and potential leaker spoilage (poster). 5th International conference on the Food Factory of the Future. 30 June - 2 July 2010. Gothenburg, Sweden
- Margas, E. and Gaze, J. (2010). *Salmonella* management in low a_w food products (poster). Campden BRI open day. 3 June 2010. Chipping Campden, UK.
- Margas, E. and Holah, J. (2009) Hygienic design and microorganisms in biofilms on high pressure processing equipment (oral presentation). Food Microbiology International Conference. 23 - 24 September 2009. Chipping Campden, UK
- Margas, E. and Holah, J. (2009) Potential for microbial spoilage in high pressure processed food (oral presentation). IAFP 2009, 96th annual meeting. 12-15 July 2009. Grapevine, Texas, USA.