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Survival of pathogens in low moisture foods

Professional Doctorate (PhD)

AgriFood Training Partnership

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Abstract

This work investigated the survival and heat resistance of pathogens (*Salmonella* spp and *Listeria monocytogenes*) and a potential surrogate strain (*E. faecium* NRRL B-2354) in a selection of low moisture foods. The pathogens and the potential surrogate bacteria were inoculated into a selection of low moisture products (confectionery formulation, chicken meat powder, pet food and savoury seasoning, paprika powder and rice flour) and survival during storage as well as heat resistance were determined using glass vials and specially designed thermal cells. This study showed that pathogens can survive well in low moisture foods and survival was dependent on many factors such as water activity (a_w), storage temperature and food composition. It was also shown that RpoS regulon plays an important role in *Salmonella* survival in low moisture foods. A strain lacking an active RpoS was significantly less viable in low moisture foods and significantly less heat resistant than the RpoS+ve strain. This study also showed that the use of *E. faecium* NRRL B-2354 as a surrogate is feasible for process validation although it has some limitations. It was shown that *E. faecium* NRRL B-2354 cannot be used as a surrogate in products containing high levels of sugar (confectionery powder) as *Salmonella* was significantly more heat resistant in this type of product than *E. faecium* NRRL B-2354. It was also shown that in paprika powder and in rice flour the two most resistant *Salmonella* strains (*S*. Enteritidis - PT 30 ATCC BAA-1045 and *S*. Typhimurium ST30; both RpoS +ve) in some conditions were more resistant than *E. faecium* NRRL B-2354. This study also showed that survival curves representing microbial survival during storage or during heat processes may not always be linear. In this study, concave upwards, concave downwards and linear curves were recorded and the Weibull model was used to fit raw data and precisely calculate the time required for 5 log reduction in viable numbers.

Declaration of original authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

Grzegorz Rachon

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Chapter 1 - Literature review

1.1 Introduction

Low moisture foods prevent pathogens from growing, but pathogens can survive well during long storage in various dry conditions. Low numbers of pathogens in foods can cause serious illness if ingested and therefore their presence in low moisture foods must be controlled, and eliminated in ready-to-eat foods.

Over the past decades, several outbreaks of salmonellosis have been associated with the consumption of ready-to-eat low-moisture products, including chocolate (Kapperud et al. 1990; Werber et al. 2005), powdered infant formula (Rowe et al. 1987; Brouard et al. 2007), raw almonds (S. Isaacs et al. 2005), toasted oats breakfast cereal (CDC, 1998a), dry seasonings, paprika-seasoned potato crisps (Lehmacher et al. 1995), infant cereals (Rushdy et al. 1998) and, more recently, peanut butter (CDC, 2012b) sprouted chia seed powder (Harvey et al. 2017) or pistachios (CDC, 2016). Costs associated with pathogen outbreaks in food are considerable. The *Salmonella* outbreak in Cadbury's chocolate bars in 2006 cost over £37 million in that year and the company also set aside £5 million for a marketing communications campaign to rebuild consumer confidence (Lindgreen et al., 2009). Loss of withdrawn product, incineration cost of contaminated food, damaging of brand name, hospitalisation of infected people, permanent health damage or, in more extreme cases, death, can be avoided and must be reduced.

According to Public Health England (PHE), the pathogens causing greatest number of gastrointestinal infections excluding Norovirus and rotavirus are *Campylobacter* followed by *Salmonella*, *E. coli* O157 (verocytotoxin producing isolates) and *Clostridium perfringens* (below 600 cases per year, data not shown). The number of recorded infections in England and Wales is shown in Table 1.1 and Figure 1.1.

This indicates that the number of infections caused by *Campylobacter* is significantly greater than for *E. coli* O157 or non-typhoidal *Salmonella*. The prevalence of *Campylobacter* infection in the last 10 years is approximately 63 times greater than *E. coli* O157 and 6.5 greater than *Salmonella*. Furthermore the number of *Campylobacter* infections does not significantly change through the past 10 years, reaching the highest number in 2012 and the lowest in 2006 when total number of *Campylobacter* infections was 28% lower than the number of infections in 2012. The number of *Salmonella* infections dropped; the highest number was recorded in 2006 and the lowest number (43% less) in 2014. The number of gastrointestinal infections are not directly linked to

particular food poisoning or outbreaks but can be good indicators of level of contaminated food consumed. The drop in the total number of *Salmonella* infections may be correlated with the introduction of vaccination program for chicken hens. A raft of control measures were introduced into the poultry industry after the number of salmonella infections rose by 170% in the UK, This was driven primarily by an epidemic of *Salmonella* Enteritidis which peaked in 1993. The control measures included movement restrictions, compulsory slaughter and disinfection procedures, as well as a voluntary industry-led vaccination scheme that began in breeding flocks in 1994 and in laying flocks in 1998.

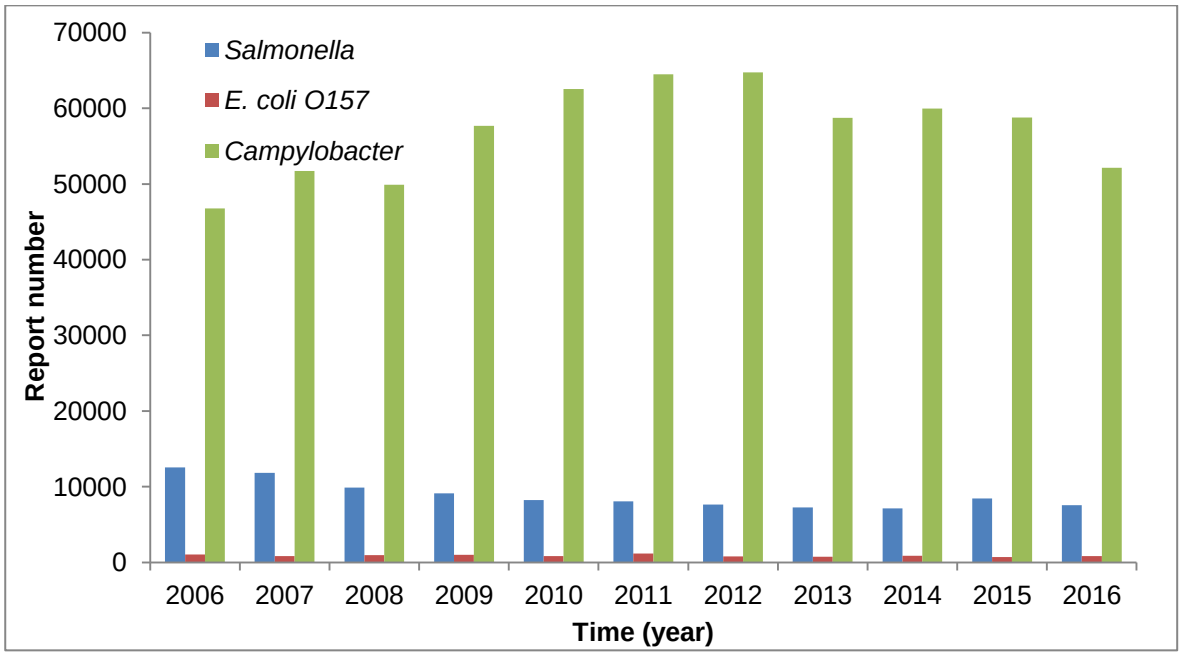


Fig. 1.1 Number of gastrointestinal infections in England and Wales between 2006 and 2016 (PHE)

Year	*Salmonella*	*E. coli* O157	*Campylobacter*
2006	12541	1030	46762
2007	11828	822	51696
2008	9889	948	49904
2009	9119	1000	57691
2010	8237	852	62544
2011	8078	1175	64502
2012	7638	793	64758
2013	7255	770	58742
2014	7119	891	59950
2015	8451	722	58800
2016	7536	814	52129

Table 1.1 Number of gastrointestinal infections in England and Wales between 2006 and 2016 (PHE)

Looking at this problem globally, according to the WHO and data extracted using the online tool (WHO Map Production: Foodborne Disease Burden Epidemology Reference Group, 2010) (WHO Map Production: Foodborne Disease Burden Epidemology Reference Group, 2010) - Fig. 1.2 - 1.5, only in 2010 in Europe there were over 2.3 million cases of food borne illnesses caused by *Campylobacter spp* (245 deaths; 0.01%), 797,668 cases of foodborne illnesses caused by non-typhoidal *S. enterica* (886 deaths; 0.1%), 39,304 cases of illnesses caused by enteropathogenic *E.coli* (0 deaths) and 1495 cases of illnesses caused by *L. monocytogenes* (334 deaths; 22.3%). Globally in 2010 there were over 83 million cases of foodborne illnesses caused by *Campylobacter spp* (20,960 deaths; 0.025%), 60 million cases of foodborne illnesses caused by none-typhoidal *S. enterica* (58644 deaths; 0.1%), over 21 million cases of illnesses caused by enteropathogenic *E. coli* (35301 deaths; 0.17%) and 11,709 cases of illnesses caused by *L. monocytogenes* (2623 deaths; 22.4%).

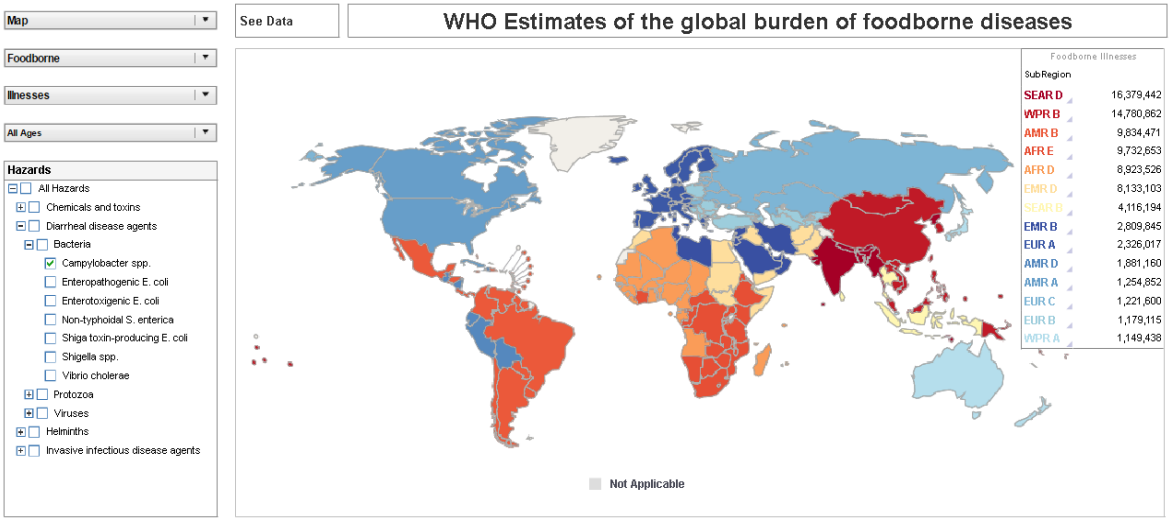


Fig. 1.2 Estimated number of illnesses caused by *Campylobacter spp*

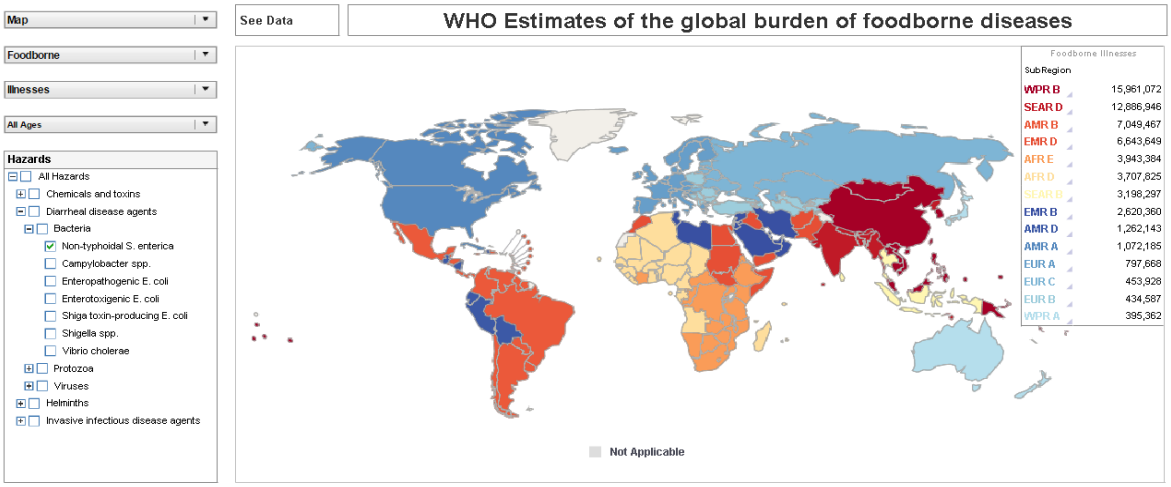


Fig. 1.3 Estimated number of illnesses caused by non-typhoidal *S. enterica*

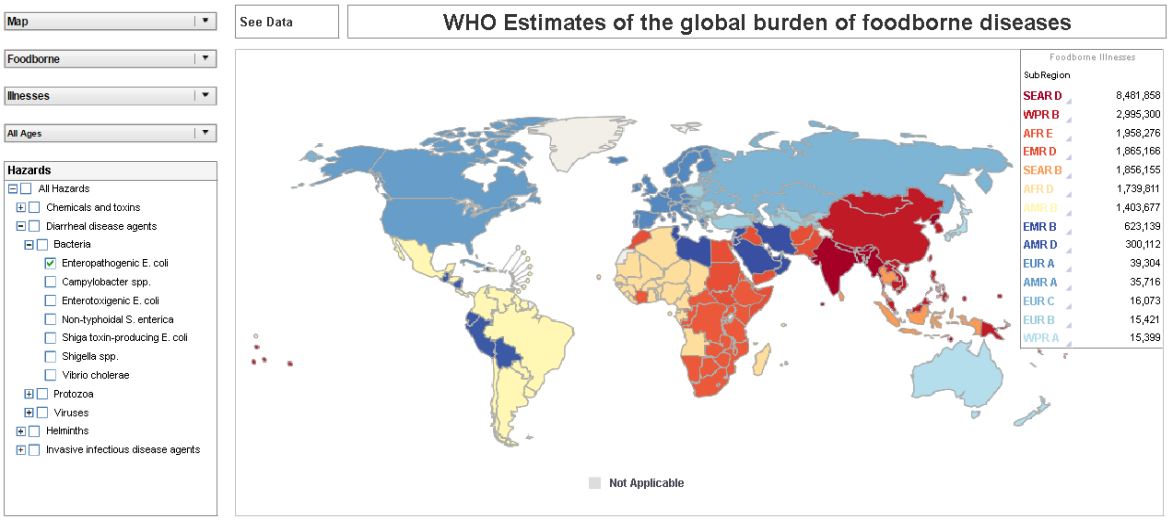


Fig. 1.4 Estimated number of illnesses caused by enteropathogenic *E. coli*

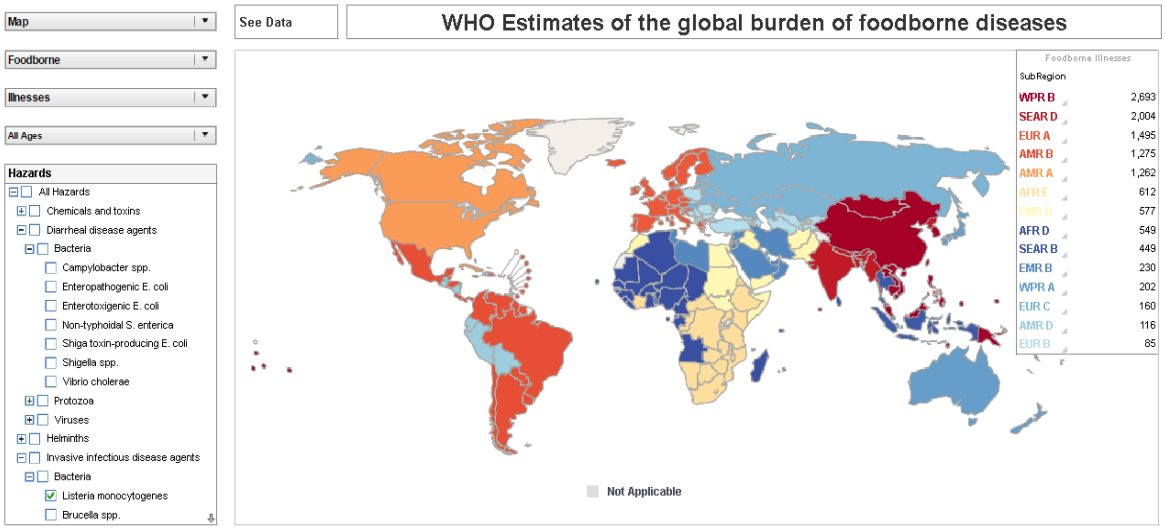


Fig. 1.5 Estimated number of illnesses caused by *L. monocytogenes*

According to Rapid Alert System for Food and Feed (RASFF) (European Commission, 2016) over the past 10 years there were 3897 (Table 1.2) alerts and notifications related to *Salmonella* associated with its presence in all types of food from which, 1575 (40%) were low moisture food, 501 (13%) were classified as food and 1074 (28%) classified as feed. Low moisture food contains the following categories: cereals and bakery products, cocoa and cocoa preparations, coffee and tea, confectionery, dietetic foods, food supplements, fortified foods, dried herbs and spices, powdered food additives and flavourings, dried prepared dishes and snacks, nuts.

Number of Alerts and Notifications recorded on RASFF portal							
Year	Total notifications number	Low Moisture Food and Feed		Food		Feed (including pet food)	
		Notifications number	%	Notifications number	%	Notifications number	%
2007	278	98	35	31	11	67	24
2008	339	133	39	43	13	90	27
2009	319	130	41	45	14	85	27
2010	346	167	48	40	12	127	37
2011	402	161	40	38	9	123	31
2012	419	177	42	52	12	125	30
2013	485	165	34	36	7	129	27
2014	477	213	45	70	15	143	30
2015	517	206	40	100	19	106	21
2016	457	131	29	52	11	79	17
Total in past 10 years	4039	1581	39	507	13	1074	27

Table 1.2 RASFF notifications recorded between 2007 and 2016 for *Salmonella* contamination of Food and Feed (RASFF, EC 2016)

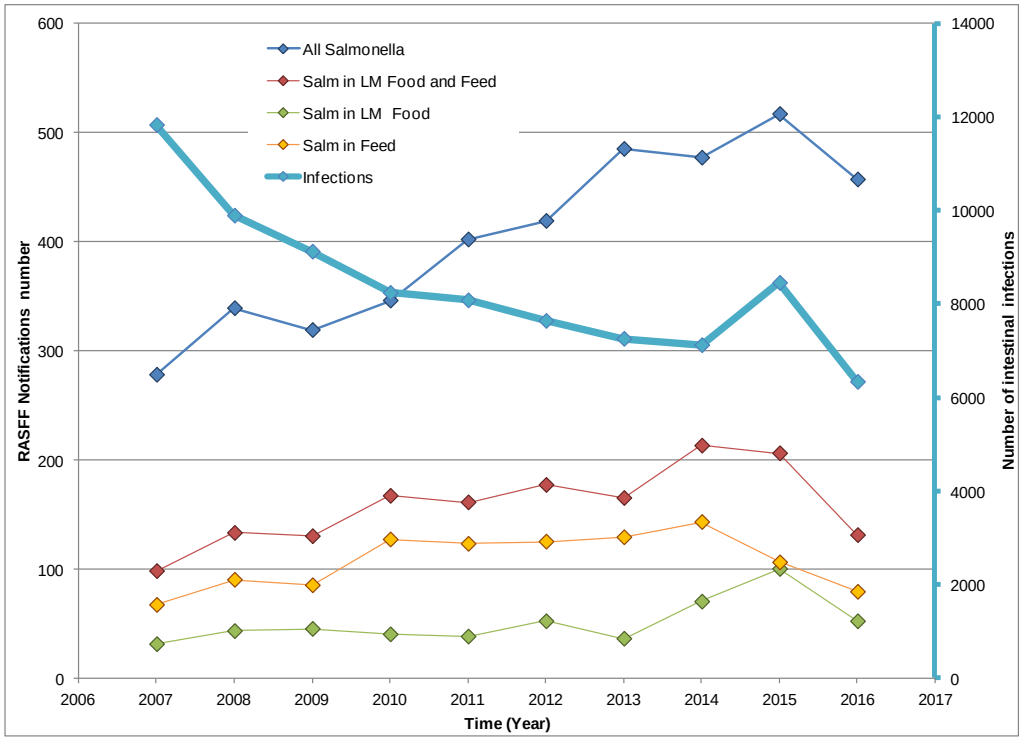


Fig. 1.6 Number of gastrointestinal infection caused by *Salmonella* and RASFF notifications recorded between 2007 and 2016 for *Salmonella* contamination of Food and Feed

Table 1.2 and Fig. 1.6 show that number of notifications are increasing year by year. However, the percentage of volume of rejected food is unknown. Interestingly over the past 10 years, the total number of intestinal infections caused by salmonella reported by HPA is decreasing which means that increased number of notifications may have a negative impact on infection numbers.

Although in general the number of notifications seems relatively small, the volume of contaminated samples/shipments is large. Van Doren at al. (2013) commented that 75,000 kg of Capsicum at a cost of $160,000 and 350,000 kg of Sesame seeds at a cost of $710,000 that entered the United States only between August 2010 and December 2010 were found to be contaminated and in effect withdrawn, reprocessed or incinerated.

Year	*Salmonella* serotype	Product	Location	Number of people affected	Reference
1973	Derby	Powdered Milk	Trinidad	3000	(Weissman et al. 1977)
1974	Eastbourne	Chocolate	Canada	95	(D'Aoustl et al. 1974)
1982	Napoli	Chocolate	UK	245	(Gill et al. 1983)
1985	Ealing	Powdered infant formula	UK	76	(Rowe et al. 1987)
1987	Typhimurium	Chocolate	Norway, Finland	361	(Kapperud et al. 1990)
1993	Rubislaw, Saintpaul, Javiana	Potato chips	Germany	1000	(Lehmacher et al. 1995)
1995	Senftenberg	Infant food	UK	5	(Rushdy et al. 1998)
1996	Enteritidis PT4	Marshmallow	UK	45	(Lewis et al. 1996)
1996	Mbandaka	Peanut butter	Australia	15	(Scheil et al. 1998)
1998	Agona	Toasted Oats Cereal	USA	209	(CDC, 1998a)
2000	Enteritidis PT30	Almonds	USA, Canada	168	(S. Isaacs et al. 2005)
2001	Oranienburg	Chocolate	Germany	439	(Werber et al. 2005)
2001	Stanley, Newport	Peanuts	Australia, Canada, UK	109	(Kirk et al. 2004)
2003	Agona	Tea	Germany	42	(Rabsch et al. 2005)
2005	Agona	Powdered infant formula	France	141	(Brouard et al. 2007)
2006	Tennessee	Peanut butter	USA	628	(CDC, 2007a)
2008	Typhimurium	Peanut butter	USA, Canada	714	(CDC, 2009a)
2009	Montevideo	Red and black pepper	USA	272	(CDC, 2010)
2011	Entiritidis	Turkish pine nuts	USA	43	(CDC, 2011)
2012	Infantis	Dry dog food	USA	49	(CDC, 2012a)
2012	Brendeney	Peanut butter	USA	42	(CDC, 2012b)
2013	Montevideo/Mbandaka	Tahini paste	USA	16	(CDC, 2013)
2014	Newport, Hartford, Oranienburg	Organic Sprouted Chia Powder	USA	31	(CDC, 2014a)
2014	Braenderup	Nut Butter	USA	6	(CDC, 2014b)
2015	Paratyphi	Nut Butter Spreads	USA	13	(CDC,2015)
2016	Montevideo, Senftenberg	Wonderful Pistachios	USA	11	(CDC, 2016)

Table 1.3 List of selected outbreaks of *Salmonella* infection after consumption of low-moisture foods

Although *Salmonella* outbreaks from low-moisture products are relatively rare (Table 1.3), they normally impact a large number of people. It has been estimated that 1,000 people were affected by paprika powdered potato chips in the 1993 outbreak in Germany (Lehmacher et al., 1995). In another outbreak, over 400 cases have been associated with a black pepper in 1981 (Gustavsen and Breen, 1984) and 2009 (Gieraltowski et al., 2013). Furthermore, between April and June 1998 more than 200 cases were attributed to

toasted oat cereals in 11 states (Centers for Disease Control, 1998b). Between August 2006 and May 2007 more than 400 cases were attributed to peanut butter and affected 44 US states (Centers for Disease Control, 2007b). More than 700 cases spread in 46 states between 2008 and 2009 were attributed to peanut butter and peanut butter-containing products (Centers for Disease Control, 2009b). Due to the large number of unreported cases of salmonellosis for all types of products, the actual number of cases is likely to be much higher. The official data for food poisoning cases significantly under-estimates number of infections, as only the most serious cases are reported. Most people do not seek treatment from their GP or A&E departments, and not all GPs carry out tests for specific pathogens, so these unreported cases are not captured in routine surveillance data (FSA, 2014).

1.2 Mechanism of cell death and survival

The precise process leading to cell death is complex and difficult to explain. Complexity of food matrices containing various components both supporting and inhibiting growth, makes predictions very difficult. Even though degradation of bacterial cell wall during heat treatment is well described, the precise prediction of bacterial death in a food environment can be very challenging. Bacteria are not only exposed to heat but also to acids, bases, bacteriocins, different water activity (a_w) values, pH, osmotic pressure differences, oxidation reduction potential, oxidative agents and antimicrobial agents. Furthermore, each of those factors can cause damage to the cell at different degrees and may compromise cell functions at different levels. Conditions required for bacterial growth (optimal and extreme) are well known (Table 1.4) and any conditions outside these will inhibit growth, and may damage the cell.

PATHOGEN	Min a_w (using salt)	min. pH	max. pH	max. % water phase salt	min. Temp.	max. Temp.	Oxygen requirement
Bacillus cereus	0.92	4.3	9.3	10	4	55	facultative anaerobe
Campylobacter jejuni	0.987	4.9	9.5	1.7	30	45	micro-aerophile
Clostridium botulinum, Type A, and proteolitic types B and F	0.935	4.6	9	10	10	48	anaerobe
Clostridium botulinum, Type E, and nonproteolytic types B and F	0.97	5	9	5	3.3	45	anaerobe
Clostridium perfringens	0.93	5	9	7	10	52	anaerobe
Pathogenic strains of E.coli	0.95	4	10	6.5	6.5	49.4	facultative anaerobe
Listeria monocytogenes	0.92	4.4	9.4	10	-0.4	45	facultative anaerobe
Salmonella spp.	0.94	3.7	9.5	8	5.2	46.2	facultative anaerobe
Shigella spp.	0.96	4.8	9.3	5.2	6.1	47.1	facultative anaerobe
Staphylococcus aureus - growth	0.83	4	10	20	4	50	facultative anaerobe
Staphylococcus aureus - toxin formation	0.95	4	9.8	10	10	48	facultative anaerobe
Vibrio Cholerae	0.97	5	10	6	10	43	facultative anaerobe
Vibrio parahaemolyticus	0.94	4.8	11	10	5	45.3	facultative anaerobe
Vibrio vulnificus	0.96	5	10	5	8	43	facultative anaerobe
Yersinia enterococitica	0.945	4.2	10	7	-1.3	42	facultative anaerobe

Table 1.4 Limiting conditions for pathogenic growth (Food and Drug Administration, 2011)

The nature of the microbial interactions in foods or in the environment, with the matrix, or between organisms themselves and their response can determine the ability to survive. Bacterial resistance to potentially lethal treatments can be affected by the state of the cell determined by environmental conditions encountered previously. Adaptation stages can result in a decrease of the harmful effects of unfavourable conditions. Growth conditions

or the exposure of organisms such as *Salmonella* to higher temperatures can increase their heat resistance (Álvarez-Ordóñez et al., 2008; Yang et al., 2014). Furthermore, pre-exposure of organisms to low pH can also increase their resistance to extreme acidic conditions (Foster, 1999). Some reactions to stress can be observed rapidly after exposure while some responses are slower as they require gene transcription and the production of proteins such as heat shock proteins known as chaperones or chaperonins. Heat shock proteins are programmed by genes (Brooks et. al., 2011) and are regulated by sigma factors. Expression of stress-related genes is initiated by specific sigma factors, such as σ^S (encoded by *rpoS* gene) and σ^H (encoded by *rpoH* gene) (Ray and Bhunia, 2008). It is known that σ^S supports the survival of *Salmonella* spp. by controlling expression of up to 50 proteins (Humprey, 2004) while σ^H provides protection against thermal stress by regulating the transcription of the heat shock proteins (Spector and Kenyon, 2012). It is known that RpoS is a universal stress response regulator in many Gram negative bacteria such as *Salmonella* which is upregulated in response to various environmental stresses such as osmotic stress or low pH (Dodd and Aldsworth, 2002). Because RpoS response confers resistance to various stresses, exposure to one stress such as low pH can cause increased resistance to other stresses such as heat (Adams and Moss, 2008; Dodd and Aldsworth, 2002).

1.2.1 Heat

The mode of action of thermal inactivation of bacteria is complex and several factors influence its success (George and Peck, 2000). The composition and pH of the matrix, the type of organism, the growth conditions (medium, growth phase, temperature), the heating method (open systems are less accurate than closed ones) and the recovery conditions are just some of the most important factors. Every component within the cell (outer layers, membrane, enzymes and proteins, DNA, RNA) is expected to be affected to some extent by high temperatures (Table 1.5).

Site	Damage
Cell wall (Gram-positive)	Probably not significant (Peptidoglycan confers some protection)
Outer membrane (Gram-negatives)	Affected to some extent by high temperatures
Cytoplasmic (inner) membrane	Severe damage (heat stability varies with melting point of cell lipids)
Ribosomes and ribosomal RNA	Degradation; preceeds loss of viability
DNA	Single strands breaks (SSB), partly a consequence of nuclease activity; repair of SSB in radiation-resistant but not -sensitive bacteria
Proteins	Denaturation, especially at high temperatures (possible coagulation)
Enzymes	Inactivation, especially at high temperatures

Table 1.5 Sites of damage in non sporulating bacteria exposed to moist heat (Russell, 2003)

Some changes are more distinct than others and some types of damage are not reversible. All changes depend on the intensity of the applied temperature and therefore it is desirable to investigate the effect of a wide range of temperatures (from mild to severe rather than concentrating on a narrow range of temperatures in order to explain the nature of the lethal effects. Damage to the outer membrane or cell wall is different in Gram-positive and Gram-negative bacteria. In Gram-negative bacteria the damage of the outer membrane can occur under mild heat shock (Hitchener and Egan, 1977). Furthermore, significant loss of lipopolysaccharides can occur (Katsui et al., 1982; Tsuchido et al., 1989, 1985). This changes the permeability of the cell and leads to a loss of periplasmic proteins that increases the sensitivity to hydrophobic antimicrobials (Boziaris and Adams, 2001; Mackay, 1983). It is also well documented that the antibacterial effect of moderate heat is significantly enhanced by pH (Gray and Postgate, 1976a; Hitchener and Egan, 1977; Hugo, 1971; Tsuchido et al., 1985). The cell of Gram-positive microorganisms (e.g. *Staphylococcus aureus*) is much more rigid as they contain an increased amount of cross-linked peptidoglycans (Gray and Postgate, 1976a) and therefore the cell wall is less likely to be affected than that of other bacteria. Cell shrinkage and precipitation of intracellular materials, as well as leakage of heated *S. aureus* cells has also been observed (Allwood and Russell, 1969a).

The cytoplasmic membrane (inner) is a delicate, semipermeable lipoproteinous structure situated beneath the cell wall. The cytoplasmic inner membrane controls the transfer of solutes in and out of the cell and, when damaged, it has a profound effect on bacteria. High temperature and membrane-degrading chemical agents, e.g. cationic biocides, can easily damage the inner membrane (Balows and Duerden, 1998). Damage of the membrane can be detected by measuring the levels of leaked K+ ions, nucleotides, denatured proteins, amino acids from the heated cells and the extent determined (Allwood and Russell, 1970; Allwood and Russell, 1976; Beuchat, 1978; Russell, 1984; Russell and Harries, 1968, 1967). According to various scientists (Allwood and Russell, 1970; Skinner and Hugo, 1976; Tomlins and Ordal, 1971) stability of ribosome and rRNA can be affected by heat. Mild heating can cause degradation of rRNA (Allwood and Russell, 1968) and in some cases degradation can depend on additional factors. In *S. aureus*, the degradation of 30S ribosomal subunits caused by heat depends on the concentration of Mg2+ ions (Andrew and Russell, 1984; Hurst and Hughes, 1978). Although the degradation of rRNA is linked to the loss of viability, it is not considered to be the primary cause of cell death in heated cells (Gray and Postgate, 1976b).

Degradation of DNA is linked to the melting temperature (T_m values) of DNA which depends on its G + C content. However, a fundamental factor is the presence of an adequate DNA repair system activity or protection mechanisms that prevent damage (Balows and Duerden, 1998). Mackey and Seymour (1987) have reported that the recovery of repair-deficient *E. coli* mutants is increased by the presence of catalase in the recovery medium. Czechowich et al. (1996) showed that pyruvate is required for the recovery of thermally stressed *E.coli* O157:H7. It has also been reported that heat injured cells have extended lag phase when transferred to nutrient media and the duration depends on the severity of the heat shock applied (Allwood and Russell, 1969b).

Proteins and enzymes are very often complex and structurally heat fragile components. Denatured proteins lose their three dimensional structure and thus their function. Protein denaturation occurs when cells are thermally stressed, with coagulation occurring at very high temperatures (Allwood and Russell, 1970; Harries and Russell, 1967).

1.2.2 Effect of pH

When cells are placed in a low pH environment, undissociated lipophilic acid molecules, unlike protons and other charged molecules, can pass freely through the membrane. Once they enter the cell, the higher intracellular pH shifts the equilibrium towards the production of undissociated molecules in the cytoplasm. The cell tries to maintain its internal pH by neutralizing or exporting the protons released by the dissociation of the acid, but this inhibits growth as energy is diverted from growth-related functions. If the external pH is sufficiently low the burden on the cell becomes high and the cytoplasmic pH drops to a level where damage to cellular structures such as proteins etc. occurs and growth is no longer possible resulting in cell death. Adaptation of bacteria to non-favourable environmental conditions is essential for survival in acidic foods or inside the host stomach where during the gastric passage the foodborne pathogens survive a combination of inorganic and organic acids and pH values as low as 1.5-2.5. In *Salmonella*, there are two main mechanisms responsible for resistance to low pH: acid tolerance responses (ATRs) and acid resistance mechanisms (AR) which are affected by (a) the phase of growth when the ATR is elicited and/or (b) whether certain amino acids are present during exposure to the acidic pH of 2.5 (arginine- or lysine-dependent AR systems) and/or (c) whether acidification of the environment results from inorganic or organic acids (Alvarez-Ordonez et al., 2010a, 2010b; Audia et al., 2001; Park et al., 1996). In *Salmonella* Typhimurium at least four systems of acid tolerance response seem to

exist. Two of them are pH-dependent; the log-phase and the stationary phase acid tolerance response which are induced only at low pH and require protein syntesis (Foster, 1991). The third system is pH-independent but requires the stationary-phase sigma factor encoded by *rpoS* (Hall et al., 1995). The fourth system includes the two component sensor regulator PhoP/Q and the ferric uptake regulator Fur (Wilmes-Riesenberg et al., 1996; Bearson et al., 1998). Besides to have crucial role in acid response, these systems are also important to the virulence. Like other foodborne pathogens, Salmonella also benefits from acid adaptation by having cross-protection against several stress conditions: heat, cold and salt (Foster et al., 1991; Leyer and Johnson, 1993; Greenacre and Brocklehurst, 2006; Xu et al., 2008).

1.2.3 Antimicrobial components

Antimicrobial agents are produced by a wide range of microorganisms, plants and mammals as part of their defence against natural enemies or competitors for nutritional resources in their environment. An important role of the antimicrobial compounds is to inhibit the growth of pathogenic or competitive bacteria. A prominent target of numerous antibacterial agents is the bacterial cell envelope, which is unique to bacteria and fulfils many crucial physiological functions. Unlike antibiotics, sanitizers are multi-target antimicrobial agents that usually act on various types of microorganisms in a concentration-dependent manner. Their mechanisms of action are rather general and involve diffusion through the bacterial cell membrane, DNA cross linkage or elimination of spore germination (Doyle et al., 1997). The nature of specific adaptive responses of microorganisms to the presence of certain antimicrobial compounds is often linked to the particular modes of action and characteristic target sites of the stress response-inducing antimicrobials and their major target sites are the bacterial cell envelope, DNA replication and protein biosynthesis.

1.2.3.1 Cell envelope

The bacterial cell envelope accounts for several fundamental functions: a shape-giving structure and diffusion barrier. In addition, the bacterial cell envelope also functions as a vital communication interface between the cell and its surrounding environment. Structurally, the bacterial cell envelope is built with a glycopeptide scaffold of the cell wall plus one or two lipid membranes. Antimicrobial agents interfere with almost every step of cell wall biosynthesis by either inhibiting an enzymatic reaction or isolating an essential substrate of the synthesis reaction, thereby inhibiting the production of new cell wall material which certainly leads to cell death (Wong, 2012).

1.2.3.2 Inhibitors of DNA synthesis

DNA replication represents an additional vital bacterial synthesis pathway that is a common target for antimicrobial agents. Ciprofloxacin and nalidixic acid, block DNA synthesis by targeting topoisomerase II (gyrase) and topoisomerase IV activities, enzymes that coil and uncoil DNA. Gyrase plays at least four roles in chromosome function, including:

- activation of the chromosome for all processes involving strand separation,
- the response to some types of environmental changes by facilitating the movement of replication and transcription complexes through DNA by adding negative supercoils ahead of the complexes
- the removal of knots from DNA
- assistance for the bending and folding of DNA.
  (Drlica and Zhao, 1997; Wong, 2012)

1.2.3.3 Inhibitors of RNA and protein synthesis

The bacterial transcription/translation mechanism represents the third vital biosynthesis pathway that is under attack by numerous antimicrobial agents. These inhibitors may be divided into three different types that target various stages throughout transcription and translation:

- inhibitors of transcription that usually interfere with RNA polymerase (RNAP)
- antibiotics that bind to 30S and 50S ribosomal subunits and subsequently inhibit translation elongation or affect translation accuracy
- antimicrobials that interfere with tRNA synthases and elongation factors, thereby affecting the cellular concentration of charged tRNA molecules or the delivery and release of tRNAs to and from the ribosome
  (Cavalleri et al., 2013; Schlunzen et al., 2001; Gao et al., 2009; Long and Porse, 2003)

1.2.3.4 Sanitizers

The most common biocidal agents used are aldehydes and alcohols. Aldehydes, including glutaraldehyde and formaldehyde, represent important disinfectants that act on bacteria by inducing DNA cross-links as well as protein-DNA and protein-protein cross-links. Those effects result in efficient inhibition of DNA synthesis and agglutination of bacteria.

Alcohols, disrupt bacterial membranes and inhibit DNA, RNA, protein and peptidoglycan synthesis (Russell, 2003). Chlorine compounds affect DNA synthesis from the formation of chlorinated derivatives of nucleotide bases (McDonnell and Russell, 1999).

Antimicrobials known as phytoalexins are produced by many plants in response to microbial invasion such as the antifungal compound phaseollin produced by green beans. The most commonly used antimicrobials are from plants and they can also be used to flavour food as they are present in herbs and spices. Analysis of its volatile flavour and odour fractions, known as essential oils, has frequently identified compounds such as allicin in garlic, eugenol in allspice, cloves and cinnamon, thymol from thyme and oregano and cinnamic aldehyde from cinnamon and cassia which have significant antimicrobial activity (Table 1.6). Despite the fact that herbs and spices may contribute to microbiological stability of foods, in some cases they can be sources of microbial contamination leading to food spoilage or public health problems.

Category	Spices	Plant part	Major flavour component	Bacteria inhibition (%)
Herbs	Basil, sweet (*Ocimum basilicum*)	Leaves	Linalool/methyl chavicol	<50
	Oregano (*Origanum vulgare*)	Leaves/flowers	Carvacrol/thymol	75-100
	Rosemary (*Rosmarinus offinicalis*)	Leaves	Camphor/1,8-cineole/borneol/camphor	75-100
	Sage (*Salvia officinalis*)	Leaves	Thujone, 1,8-cinole/borneol/camphor	50-75
	Thyme (*Thymus vulgares*)	Leaves	Thymol/carvacol	75-100
Spices	Allspice, pimento (*Pimenta diocia*)	Berry/leaves	Eugenol/b-caryophyllene	75-100
	Cinnamon (*Cinnamonum zeylanicum*)	Bark	Cinnamic aldehyde/eugenol	75-100
	Clove (*Syzgium aromaticum*)	Flower bud	Eugenol	75-100
	Mustard (*Brassica*)	Seed	Allyl isothiocyanate	50-75
	Nutmeg (*Myristica fragrans*)	Seed	Myristicin/a-piene/Sabinene	50-75
	Vanilla (Vanilla planifola, V. pompona, V. tahitensis)	Fruit/seed	Vanillin (4-hydroxymethoxybenzaldehyde)/p-OH-benzyl methyl ether)	-
Oils	Olive oil	Fruit	Oleuropein	-
	Tea-tree oil (*Melaleuca alternifolia*)	Leaves	Terpenoids	-

Table 1.6 Spices, herbs and oils with antimicrobial activity and their flavour components (Tajkarimi et al., 2010)

1.2.4 Essential oils

Essential oils (EOs) also called volatile or ethereal oils are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and

roots) (Calo et al., 2015; Sadaka et al., 2014). They can be obtained by fermentation, enfleurage or extraction, but the method of steam distillation is most commonly used for commercial production of EOs.

The mechanism of the antibacterial activity of EOs has been investigated extensively and most of the studies agree that the mode of action is the disruption of cell membrane by constituent molecules in essential oils (Burt, 2004). An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing these structures and rendering them more permeable. However, the mechanism is still unclear and requires further studies.

The “disruption” mechanism is associated with membrane expansion, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration and alteration of the ion transport processes. The lipophilicity of oil constituents, the lipid composition of bacterial membranes and their net surface charge are the major factors influencing the membrane permeability of oil constituents. Oil constituents might also cross the cell membrane by penetrating the interior of the cell and interacting with intracellular targets.

Considering the large number of different groups of chemical compounds present in essential oils, it is most likely that their antibacterial activity is not attributable to one specific mechanism but it affects several targets in the cell (Fig. 1.7). For example, essential oils degrade the cell wall, interact or disrupt the cytoplasmic membrane, damage membrane proteins, cause leakage of cellular components and deplete the proton motive force. Finally, the mode of action of antimicrobial agents essentially depends on the type of microorganisms and is mainly related to their cell wall structure and the outer membrane arrangement.

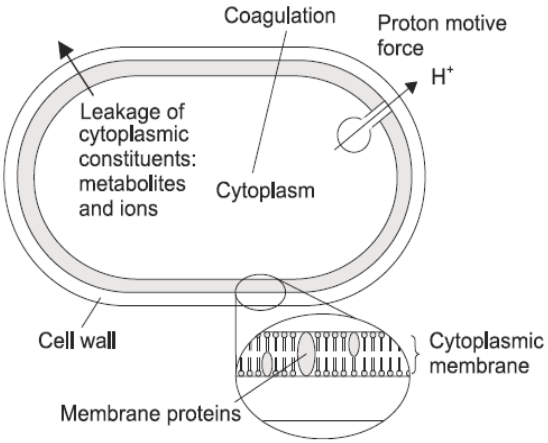


Fig. 1.7 Degradation mechanisms of bacterial cells through actions of EO’s (Burt, 2004)

1.2.5 Osmotic stress

Bacteria can maintain their cellular homeostasis and volume when the external osmotic pressure changes. A decrease in external osmotic pressure causes water influx and swelling or even lysis, whereas an increase in external osmotic pressure causes water efflux and dehydration. Water fluxes simultaneously, and almost instantaneously, perturb many cellular functions and affect various cell properties. These include cell volume (or the relative volumes of the cytoplasm and periplasm); turgor pressure; cell wall strain; and cytoplasmic membrane tension; as well as individual uncharged solutes, salt ion, and biopolymer concentrations. Cells exposed consistently to a very high osmotic pressure must maintain correspondingly high cytoplasmic solute concentrations. Evidence suggests that the regulation of cytoplasmic composition and hydration is a key objective of cellular homeostasis (Wood, 2011). Cells respond to variations in external osmotic pressure by accumulating or releasing solutes, thereby attenuating water fluxes. Those solutes include inorganic ions (often K+), and organic molecules denoted “osmolytes”. The latter are able to minimally perturb cellular functions, even after accumulating to high (up to molar) concentrations. In turn, organisms have adapted to tolerate osmoregulatory solute accumulation. Under extremely high levels of osmolarity, some halophiles accumulate KCl to molar concentrations and their proteins have adapted to function only in high salt environments (Sarwar, et. al., 2015; Wood, 2015). Osmoregulatory solutes build up via active transport or synthesis if the osmotic pressure rises and are released via mechanosensitive channels if the osmotic pressure falls. Multiple enzymes, transporters, and channels with redundant functions and specificities mediate in solute accumulation and release from each organism. The abundance of most osmoregulatory systems is controlled transcriptionally (Altendorf et al., 2009; Krämer, 2010) involving a number of osmoregulatory genes. The genes and enzymes responsible for modulation of osmoregulatory solute levels have been identified in diverse bacteria.

Solute build up can stimulate bacterial growth at high osmotic pressure, and solute release allows cells to survive osmotic down shocks. Although bacterial osmoregulation is the subject of many studies focusing on the enzymes, transporters, and channels mediating solute accumulation and release (Krämer, 2010; Kung et al., 2010; Wood, 2011) it is still unknown how increasing osmotic pressure can inhibit bacterial growth in the absence of solute accumulation. The rate of growth is correlated to cytoplasmic hydration and increasing concentration of solutes differentially affects cellular rehydration and growth. Accumulation of K+ and sodium glutamate can disturb protein - nucleic acid interactions and can partially rehydrate cells (Altendorf et al., 2009; Cayley and Record, 2003; Wood, 1999).

1.2.6 Desiccation stress

All life forms are totally dependent on the presence of water in its liquid state. The aqueous cytoplasmic environment within a bacterial cell surrounded by the permeable membrane allows water molecules to pass freely from the cytoplasm to the environment and from the environment to the cytoplasm. This dynamic two-way flow of water molecules is normally in a steady state and a living organism will be stressed if there is a net flow out of the cytoplasm, leading to plasmolysis, or a net flow into the cell, leading to rupture of the membrane; the latter is normally prevented by the presence of a strong cell wall in bacteria and fungi.

Although the cytoplasm must be in the liquid phase for active growth, cells will survive well in a dehydrated state; freeze drying of bacteria is a very common process used by researchers and food manufacturers for preservation of viable cells.

It is also very well documented that cells will survive longer when exposed to heat processes in a dehydrated state (in an equilibrated state in low moisture food) compared to the fully hydrated state (in an equilibrium state in high moisture food) (Archer et al., 1998; Barrile and Cone, 1970; Garibaldi et al., 1969; Goepfert and Biggie, 1968; McDonough and Hargrove, 1968; Peñaloza and Komitopoulou, 2012, Van Cauwenberge et al., 1981).

The ability of microorganisms, to survive desiccation is dependent on their ability to cope with certain salts and solutes in their environment, reactive oxygen species (ROS), radiation stresses, and temperature extremes (Billi and Potts, 2002, 2000; Deaker et al., 2006; Potts, 1994; Ramos et al., 2001; Welsh, 2000). Desiccation stress can be divided into three main stages: drying, storage and rehydration. During drying, the concentration of salts and solutes increase and when a certain a_w is reached, injury occurs. Furthermore, researchers have observed that drying rates have a significant effect on bacterial survival (Chaot and Alexander, 1984; Mary et al., 1986, 1985; Sleesman and Leben, 1976) and that on rapid drying survival decreases. The increase in survival during slow drying suggests that physiological responses to dry conditions may take place during the drying process. During drying, concentrations of salts and other compounds may reach toxic levels and cause osmotic and salt stress leading to decreased viability (Steinborn and Roughley, 1975; Vriezen et al., 2006). In contrast, the accumulation of certain osmoprotectant compounds, may increase desiccation survival (Fougere and Rudulier, 1990; Gouffi et al., 2000, 1999, 1998; Gouffi and Blanco, 2000; Madkour et al., 1990). Extended drying, to below $a_w < 0.53$ induces cell damage, RNA polymerase ceases

to function, metabolism stalls and only a monolayer of water surrounds the molecules, making further extraction of water more difficult (Brown, 1990).

Following drying, bacteria will reach environmental equilibrium and further extraction of water ceases and the storage stage (holding of the product) begins which is characterized by a slow decline in viable counts of bacteria after slow drying. Viability at this stage depends on the composition of the matrix but also on the speed of drying. Antheunissen et al. (1979) showed that following slow drying bacteria can survive desiccation for up to 4 years. A decline in viable cells during long-term storage under desiccation can be explained by the accumulation of oxygen and radiation-induced damage (Breaks, 1979; Mary et al., 1993; Mattimore and Battista, 1996).

In the rewetting stage, accumulated damage can be repaired and bacterial metabolism restarts and as in earlier stages the rate of this process, (rewetting) has vital consequences for survival. Fast rewetting results in disruption of the cell at the subpolar regions and causes cell death (Bushby and Marshall, 1977; Salema et al., 1982). Kosanke et al. (1992) reported that slow rewetting increase survival rates of *S. meliloti*, *Rhizobium leguminosarum*, and *Pseudomonas putida*.

1.2.7 Osmo-protective agents

Osmo-protective agent (also known as a osmoprotectant) are highly soluble nontoxic at high concentrations compounds which can protect cells from the osmotice stress. Protective additives can be generally classed into two categories: (i) amorphous glass forming, and (ii) eutectic crystallizing salts (Morgan et al., 2006)

Although there are a few protectants that appear to work well with various species (non-fat milk solids, serum, trehalose, glycerol, betaine, adonitol, sucrose, glucose, lactose and polymers such as dextran and polyethylene glycol) (Hubalek, 2003), very often the type of protectant depends on the micro-organism. In freeze drying the level of cell viability varies and depends on numerous factors, including the strain of micro-organism but also the efficacy of the protective agent used during freeze drying. Protective additives can be in general classed into two categories: (a) amorphous glass and (b) eutectic crystallizing salts. The matrix includes substances such as carbohydrates, proteins and polymers. A glass is a supersaturated thermodynamically unstable liquid with a very high viscosity. These glass forming additives have been shown to exert the highest protection during freeze drying (Israeli et al., 1993; Leslie et al., 1995; Linders et al., 1997; Lodato et al., 1999). The formation of a glassy state induces sufficient viscosity within and around a cell

to arrest molecular mobility to a minimum. The inert amorphous glass is also able to hold on to waste products produced by the cells within the glass arrangement before freezing, and therefore electro-chemical changes to the plasma membrane during storage will be inhibited (Orndorff and MacKenzie, 1973).

Sugars like trehalose and sucrose can exhibit enhanced desiccation tolerance in various organisms, replacing the water around polar residues and therefore stabilize membranes and proteins (Rudolph and Crowe, 1985). Trehalose and sucrose can prevent protein denaturation by the formation of hydrogen bonds and preserve structure and function of isolated proteins during drying (Leslie et al., 1995). It has also been shown that sugar mixtures inhibit crystallization and therefore help seeds to survive adverse conditions (Buitink et al., 2000). Many studies have shown that trehalose was a good cryoprotectant but also that presence of trehalose enables higher survival of microorganisms than sucrose (Leslie et al., 1995; Israeli et al., 1993; Crowe et al., 1998; Gomez Zavaglia et al., 2003; Streeter, 2003). Trehalose has higher glass-transition temperature (T_g) than sucrose and it was suggested that glass-transition temperature (T_g) plays significant role in cryoprotectivity. Furthermore, components with a higher glass-transition temperature are more stable in the freeze dried matrix.

Buitink et al. (2000) have shown that polypeptides can significantly alter the glass properties of sugars, furthermore, proteins are more stable above their T_g than sugars. This suggests that proteins may play a more important role in glass formation compared to sugars. For example serum and skimmed milk powder are efficient desiccation protectants (Hubalek, 2003) indicating that mixtures of proteins and sugars are effective desiccation protective agents.

As shown by Abadias et al. (2001) blends of skimmed milk and carbohydrate sugars improve recovery of *Candida sake* from 45 – 85% and Desmond et al. (2002) have shown that *Lactobacillus paracasei* survival can be substantially increased with the addition of 10% acacia gum in 10% reconstituted skim milk. Teixeira et al. (1995) have shown that ascorbic acid and monosodium glutamate increase survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* but only at 4°C, while inhibitory effects were observed at a higher temperature (20°C). Similarly, Golowczyc et al. (2011) proved that storage survival of two *Lactobacillus* kefir strains after spray drying was significantly higher under lower relative humidity (RH = 0 and 11%) when comparing to relative humidity of 23%. Furthermore, they have shown that monosodium glutamate and fructo-oligosaccharides (FOS) were also protective.

1.2.8 RpoS (σ^S) regulon in bacteria – role in survival under starvation and stress

The gene *rpoS* (RNA polymerase, sigma S) plays a key role in the survival of bacteria under starvation or stress conditions. The *rpoS* gene encodes a sigma factor (known as σ^S, RpoS, KatF or σ^{38}) which regulates transcription of essential genes in bacteria. Sigma factors are proteins activated in response to different environmental conditions. RpoS is a primary regulator of stationary phase genes and is a central regulator of genes involved in general stress response. Expression of *rpoS* can also be induced during the late exponential phase when cells are exposed to various stress conditions, e.g. accumulation of metabolic end-products, such as acids. The stress-response genes regulated by RpoS are involved in various functions: stress resistance, metabolism, cell morphology, virulence and lysis. RpoS not only allows the cell to survive environmental challenges, but it also prepares it for the possible subsequent occurrence of other stresses. In fast-growing cells, the level of σ^S are very small, but when cells are exposed to various stress conditions, rapid induction of σ^S is observed (Hengge-Aronis, 2002) and many of them are involved in resistance to stress. Increase of *rpoS* transcription is a consequence of reduced growth rate, in addition, acidic pH, low temperature, high osmolarity, and some late-log-phase signals stimulate the translation of already present *rpoS* mRNA. RpoS translation is controlled by several proteins (Hfq and HU) and small regulatory RNAs that probably affect the secondary structure of *rpoS* mRNA.

1.2.8.1 Stress resistance

RpoS is a key regulator of the acid tolerance response and as mentioned above can be induced by various stress conditions. Resistance of bacteria to acid, heat, oxidative stress, starvation or osmotic shock is not only affected by physico-chemical changes and interactions of cells with their environment, but is also regulated by *rpoS* gene and is dependent on the level of stress. Mechanisms of acid resistance are complex and coordinated by a number of regulatory proteins. In log phase cells, *Salmonella* virulence proteins PhoP, PhoQ, and Fur regulate the cells response to acid (Brenneman et al., 2013). PhoP and PhoQ coordinate protection against nonorganic acid and Fur controls acid shock proteins essential for protecting the cell against organic acids. Production of exonucleases is also regulated by RpoS. Exonuclease participates in DNA repair by removing 5'monophosphates near abasic sites in damaged DNA (Demple et al., 1983). Similarly, catalases HPI and HPII, encoded by *katG* and *katE* that convert harmful hydrogen peroxide molecules to water and oxygen (Schellhorn and Stones, 1992) are

also regulated by RpoS. The *otsBA* gene which is also regulated by RpoS, coordinates production of trehalose which functions as an osmoprotectant and is required for gaining desiccation resistance (Kaasen et al., 1992). Glutathione reductase, an enzyme encoded by the *gsr g*ene catalyzes the reduction of glutathione disulfide to the sulfhydryl form glutathione which is a critical molecule in resisting oxidative stress similar to superoxide dismutase encoded by *sodC* (Becker-Hapak and Eisenstark, 1995). Table 1.7 below shows a number of various regulators and their roles (Spector and Kenyon, 2012)

Regulator (s)	Stress										
	C-starvatior	Acid	Oxidative	Heat	Envelope	AP	Bile	Multi-drug	Osmotic	Dessication	Iron
σ^H			✓	✓							
σ^S	✓	✓	✓	✓					✓	✓	
σ^E	✓	✓	✓	✓	✓	✓					
AdiY		✓									
BaeRS					✓		✓	✓			
CpxRA				✓	✓	✓					
cAMP-CRP	✓										
CsgD										✓	
DksA		✓	✓								
Fur		✓	✓								✓
LexA			✓								
MarA							✓	✓			
OmpR-EnvZ		✓							✓		
PhoPQ		✓				✓	✓				
PmrAB						✓					✓
OxyR			✓				✓				
RamRA							✓	✓			
RcsBCD					✓	✓					
RecA		✓	✓				✓				
SoxRS			✓				✓	✓			
SlyA			✓			✓					

Table 1.7 Regulatory proteins/systems playing roles in stress resistance in *Salmonella enterica*

1.2.8.2 Resistance to heat

RpoS controls the synthesis of heat resistance (chaperone) proteins induced under stress conditions. These proteins provide protection of DNA and many enzymes, making cells more resistant to higher temperatures. Expression of stress genes is initiated by σ^S and σ^H sigma factors which are encoded by *rpoS* genes. σ^S supports the survival of *Salmonella* spp. in stationary phase and under environmental stress or changes such as pH and temperature and controls expression of up to 50 proteins (Humphrey, 2004). σ^H provides protection against cytoplasmic thermal stress by regulating the transcription of the heat shock proteins which function as chaperones for protease (Spector and Kenyon, 2012).

1.3 Mathematical modelling

Thermal and non-thermal inactivation curves are not always linear, and there are four types of survival curves commonly observed: linear curves (Fig. 1.8, curve A), curves with a shoulder (Fig. 1.8, curve B), curves with a tailing (or biphasic curves) Fig. 1.8, curves C and D) and sigmoidal curves (Fig. 1.8, curves E and F).

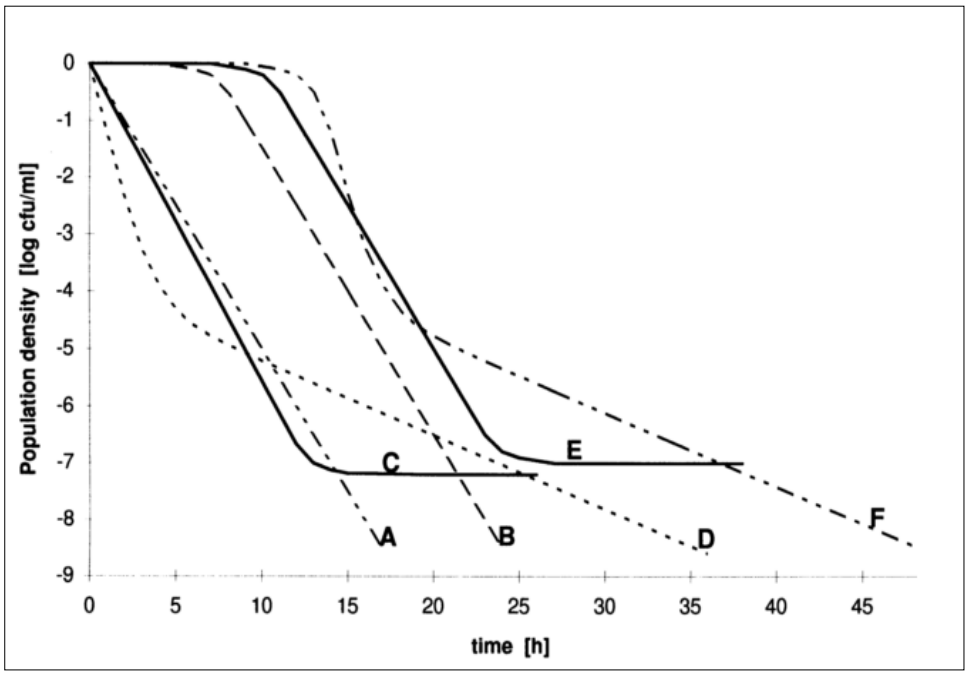


Fig. 1.8 Types of non linear regression curves (Xiong et al., 1999)

There are a number of different mathematical models (Table 1.8) used to describe raw data and therefore behaviour of microorganisms in particular experimental conditions can be predicted.

First order kinetic model is used for linear survival curves as has been proposed by Chick in 1908 (Chick, 1908). This model assumes a linear relationship between the decline in the logarithm of the number of survivors over treatment time. Decimal reduction time (Parameter D) (decimal reduction time) can be calculated with the use of this model which represents the time required to inactivate 90% of the organisms (min), in t the treatment time (min).

In 1977 Cerf (Cerf, 1977) proposed a two-fraction model for describing biphasic curves, which are normally considered to characterize a mix of strains having different heat resistances. The model was developed by Kamau et al. (1990) and shown good fit to linear and non-linear survival curves for *Listeria monocytogenes* heated in milk. This model was modified by Whiting and Buchanan (Sun, 2012) and could describe regression

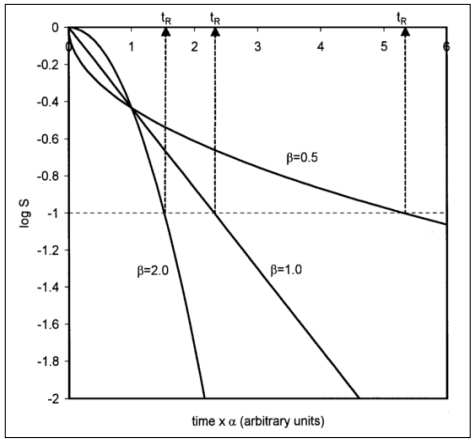
with a significant shoulder and tail. This model was used to describe non-thermal inactivation of *L. monocytogenes* and *Staphylococcus aureus* (Buchanan et al., 1994; Buchanan and Golden, 1995; Whiting et al., 1996). A modified Gompertz equation has been used for sigmoidal curves (Fig. 1.8, curve E). Bhaduri et al. (1991) demonstrated that this equation can model the non-linear survival curves of *L. monocytogenes* heated in liver sausage slurry. A modified Gompertz equation provided a more accurate estimation of a microorganism's thermal resistance than the first order kinetic model. Linton et al. (1996, 1995) used this equation to fit non-linear survival curves for *L. monocytogenes* Scott A and proved that it could be used for both linear curves and curves containing a shoulder and tail. The Cole model was used for the thermal destruction kinetics of *L. monocytogenes*, *Salmonella* Typhimurium or *Yersinia enterocolitica* (Cole et al., 1993; Ellison et al., 1994; Little et al., 1994; Stephens et al., 1994). Buchanan et al. (1993) proposed a step equation to fit survival curves with a shoulder which has been applied in the non-thermal inactivation of *L. monocytogenes* (Buchanan et al., 1997, 1994; Buchanan and Golden, 1995). Membre et al. (1997) proposed an equation describing survival curves with a shoulder for non-thermal inactivation of *S.* Typhimurium in reduced calorie mayonnaise. More recently the Weibull model has been frequently used to describe various regression curves. The Weibull model can describe three types of regression curves: linear, concave upward and concave downward. From all those models only the Whiting–Buchanan model can be used to fit all of the six different shapes of survival curves shown in Fig. 1.8

Model	Mathematical formula	Curves fitted by model	Reference
First order kinetics	$N(t) = N_0 e^{-kt}$ or $log\frac{N(t)}{N_0} = -\frac{t}{D}$	A	(Chick, 1908; Block, 2001)
Cerf	$\frac{N(t)}{N_0} - fe^{-k_1 t} + (1-f)e^{-k_2 t}$	A,C,D	(Cerf, 1977)
Kamau	For linear survival curves $\frac{N(t)}{N_0} = \frac{2}{1+e^{bt}}$	A	(Kamau et al., 1990)
	For survival curves with lag phase $log\frac{N(t)}{N_0} = \log\left(\frac{2f}{1+e^{b_1 t}} + \frac{2(1-f)}{1+e^{b_2 t}}\right)$	A, C, D	
Whiting-Buchanan	$log\frac{N(t)}{N_0} = \log\left(\frac{f(1+e^{-b_1 t_{lag}})}{1+e^{b_1(t-t_{lag})}} + \frac{(1-f)(1+e^{-b_2 t_{lag}}}{1+e^{b_2(t-t_{lag})}}\right)$	A, B, C, D, E, F	(Sun, 2012)
Gompertz	Modified Gompertz equation $logN(t) = A - Ce^{-e^{-B(t-M)}}$ or $log\frac{N(t)}{N_0} = Ce^{-e^{BM}} - Ce^{-e^{-B(t-M)}}$	B, C, E	(Bhaduri et al., 1991) (Bhaduri et al., 1991; Linton et al., 1996, 1995)

Cole	$logN(t) = \alpha + \frac{\omega - \alpha}{1 + e^{4\sigma(\tau - logt)/(\omega - \sigma)}}$	B, C, E	(Cole et al., 1993)
Buchanan	$\log(N(t) = \begin{cases} logN_0 & (t \le t_{lag}) \\ logN_0 - \frac{t - t_{lag}}{D} & (t > t_{lag}) \end{cases}$	A, B	(Buchanan et al., 1993)
Membre	$logN(t) = (1 + logN_0) - e^{kt}$	B	(Membre et al., 1997)
Weibull	$\log\left(\frac{N}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta}$	A, B, D	(Van Boekel, 2002)

Table 1.8 Various mathematical models used to inactivation curves

Weibull distribution corresponds to a concave upward survival curve if $\beta < 1$, concave downward if $\beta > 1$ and reduces to an exponential (linear) distribution if $\beta = 1$. α represents a scale parameter (a characteristic time) Fig 1.9. Both parameters, α and β can be calculated and fitting of the model to raw data can be performed. Time (t_R or t_D) required to achieve certain level of inactivation can then be precisely calculated (Van Boekel, 2002).



$$t_D = \alpha(-\ln(10^{-d})^{\frac{1}{\beta}})$$

Fig. 1.9 Regression curves represented by the Weibull Model

Where:

Log S - Survival level ($LogN/N_0$)

t_R - reliable life; time of reduction of 90% of population

t_D - time required to achieve required log reduction (min)

d – number of required decimal log reductions (i.e. 5D = 5)

α and β – parameters as described above

Possible explanation for nonlinear kinetics can be summarised into two classes: those due to limitations in experimental procedures and those due to normal features of inactivation process. The first class include limitations and variability in heating procedures; use of mixed cultures or populations, clumping, protective effect of dead cells, method of enumeration and statistical design. The second class includes natural variability in heat sensitivity or heat adaptation (McKellar and Lu., 2004). The linear survival curves are representing inactivation of a homogeneous suspension of microorganisms at constant rate. If however clumps of cells are present or if inactivation of microbial suspension is measured during ramp time non linear curves are observed. All cells in the clump must be inactivated prior to the colony forming ability of the clump being inactivated (Adams and Moss, 2008). If the lag phase represents a time in which the cells resynthesize a vital component, death ensues only when the rate of destruction exceeds the rate of resynthesis (Mossel et al., 1995). Survival curves with a tail represent a suspension of microorganisms in which some cells are intrinsically more resistant than others or are protected by various factors (Cerf, 1977). There are two types (shapes) of tailing: level tailing (C) or slope tailing (D). These curves are also called biphasic and generally represent a mixture of two fractions or sub-populations of different heat resistance (Buchanan et al., 1994; Cerf, 1977; Kamau et al., 1990). The first straight section of the curve describes inactivation of the less resistant microorganisms and the second section describes the death of the more resistant ones. The Cerf model (Cerf, 1977) called also two-fraction model, is based on the assumption that two sub-populations or fractions exists and that inactivation rate for each subpopulation or fraction is constant and follows first order kinetics. In some cases, survival curves contain both a shoulder and a tailing and therefore there are two corresponding sigmoidal curves, i.e. curves E and F (Fig. 1.8).

1.4 Process validation and use of surrogates

Although scientists have substantial knowledge of survival of bacteria, spores yeast or moulds in various environments, precise predictions of kinetics of growth or death are still very difficult. Therefore, it is necessary to conduct process validation or challenge testing. While in a laboratory environment those trials can be conducted using any pathogenic microorganism (*E. coli* O157, *Salmonella*, *L. monocytogenes*, *Cl. botulinum* etc.) or natural isolate (associated with a case or an outbreak), in pilot plants or factory environments only non pathogenic microorganisms can be used. In conducting validation trials, heat penetration, heating time, and cooling are the main contributors to the unpredictability of results. The main direction in this case is to establish if the trial is performed in 'worst case' conditions, which are likely to be experienced during normal production.

1.4.1 Challenge Testing

Challenge testing involves spiking food products with microorganisms in order to understand the issues that may arise during processing, distribution and storage.
As a result, it is possible to determine microbial growth, survival and death in foods; evaluate the effectiveness of packaging, food preservatives and additives; and determine the extent of lethality, or kill, delivered by the process or treatment. Essentially, challenge testing asks the question: "what if this product became contaminated with a particular organism? What would the consequences be?" The results allow the producer and the regulator to determine a safe shelf-life for a product. Challenge testing isn’t the only approach, as there are various predictive models available, which aim to forecast microbiological growth, death or survival in food. These models, however, can carry significant risk of error as interactions of micro-organisms in food matrices are complex and cannot be described comprehensively by simple mathematical formulae. Challenge testing is still the only empirical way of evaluating the impact of contamination in a food product. It is also still believed to be the best way to predict the shelf life of the product, validate product processing and understand the behaviour of bacteria in food (Augustin et al., 2011; Beaufort, 2011; Beaufort et al., 2014; Health Canada, 2012; Rachon, 2016; Uyttendaele et al., 2004).

1.4.1.1 Organisms

A product can be spiked with any contaminant: however it is important to know which are relevant to the product or process being tested. An understanding of the historic illness outbreaks connected with the product or process, as well as an awareness of current foodborne outbreaks is crucial.

1.4.1.2 Inoculation

Both the level of inoculation and the method of inoculation can be challenging. The composition of the food must not be changed during inoculation, whilst it is important to achieve the desired dispersal of organisms throughout the sample. Dairy products are a good example of this. The level of fat content in cheese makes injecting it with liquid not viable. At Leatherhead Food Research, in these cases, the glass beads method is used. Viable bacteria are attached to the surface of beads and those are pressed into the product. The trade-off is that although the bacteria are not always evenly distributed, the product composition remains unchanged (no additional liquid is injected), (challenge testing of butter at Leatherhead Food Research - data not published).

1.4.1.3 Time of the study

The product should be tested for the whole of its shelf-life and for a period beyond, as it is important to know the impact of the product if consumed after its best-before date. The challenge is to obtain adequate data from different stages of a product's shelf life.

1.4.1.4 Environment

Test samples should preferably be stored and packaged as they would be in the commercial marketplace so that the testing is truly representative. It may also be necessary to test at non-optimal conditions, such as testing refrigerated products at different (abused) temperatures, to assess the impact of these on the safety and shelf-life of the product.

1.4.1.5 Sample numbers

The levels of viable challenge microorganisms are counted immediately after inoculation and at each sampling point. Typically, it is required to have at least duplicate and, preferably, triplicate samples for analysis at each time point.

1.4.1.6 Interpretation

Interpreting the results of the challenge tests and drawing meaningful conclusions is the final challenge. Trend analysis and suitable graphical plotting of the data will show whether the challenge organisms died, remained stable or increased in numbers over time. At Leatherhead Food Research an increasing number of challenge tests for clients are being carried out. One of the drivers for this has been product reformulation.

Food matrices are complex, with products containing a range of additives and preservatives and interactions between them and the various microorganisms are complicated. Any product reformulation affects this food matrix and manufacturers want to understand how this impacts the behaviour of micro-organisms. With the continued focus on food safety, challenge testing remains a vital tool for the food industry. Complexity of Challenge testing is shown on diagram in Fig. 1.10.

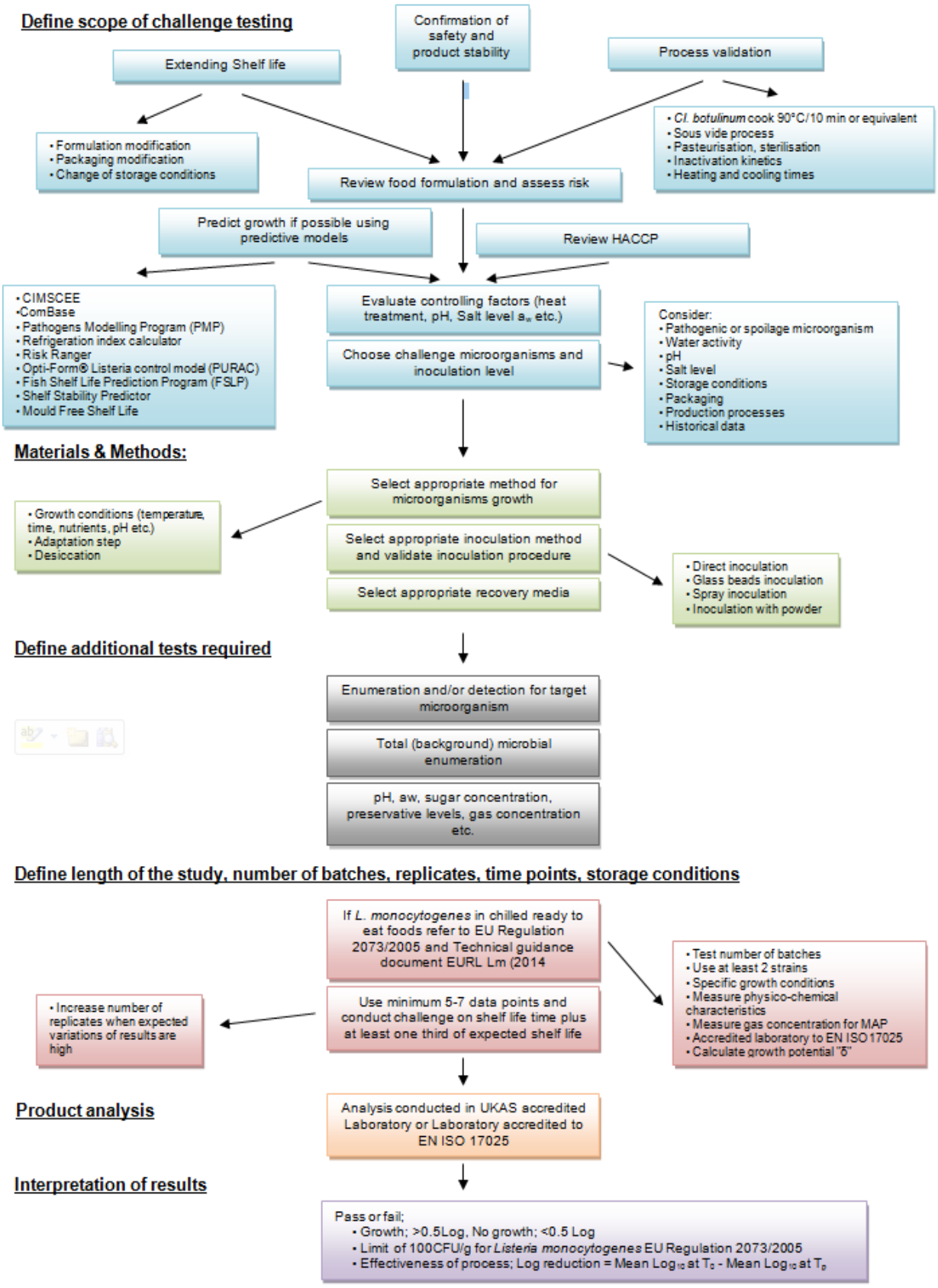


Fig. 1.10 Complexity of challenge testing

1.4.2 Process Validation

Process validation is a study that determines if the particular process is safe (providing sufficient reduction of number of microorganisms) and compatible with the current regulations or guidance. The levels of microbial reduction in the food manufacturing process, can be expressed as a “log reduction” value. When conducting process validation product samples, surface or tested material are inoculated either with a pathogen of concern, bacteria causing spoilage, or a surrogate culture and exposed to a process or an activity. Samples, surface or tested material are analyzed before the process and immediately after and the overall log reduction of the inoculated microorganisms is determined. It is very important that microorganisms are adapted to the new environment and the worst case scenario is tested. The log reduction required for a process depends on the particular regulatory or customer expectations. For example, in the U.S., there is a requirement for a 2-log reduction of *E. coli* O157:H7 in sausage, a 4-5 log reduction of *Salmonella* in roasted almonds, and a 6.5 log reduction of *Salmonella* and *Listeria monocytogenes* in cooked meats, poultry, and seafood or 12 log kill for *C. botulinum* in sterilisation processes. If legislation doesn't cover a particular process or product, a 5-log reduction of the target pathogen is the acceptable default level applied to pasteurization processes in the U.S. based on public health risk levels.

Common examples of process validations are: establishing safety of cooking rare or medium rare burgers, validation of pasteurizer or steriliser units, validation of safety of production of dried or cured meats, evaluation of safety of Sous-vide processes, evaluation of safety of cooling processes, validation of various cleaning and disinfection procedures.

Prior to validation, preliminary trials are typically conducted and processing variations are identified and process parameters for the worst case scenario are selected. In the first stage, validation of a surrogate organism is necessary and normally this must be conducted in a laboratory of containment Level 2, 3 or higher. Typically inactivation kinetics/growth rates and level of reduction are determined for surrogate and microorganism of interest which are compared and statistical analysis is performed. If survival of a surrogate is statistically similar to the microorganism of concern or greater, the surrogate can be used in further studies in the pilot plant. The inoculated with a surrogate samples, are then exposed to the process and the level of reduction of microorganisms is determined. It is crucial that the preparation of inocula is standardised as small changes of conditions during growth, storage, equilibration of cultures, may have significant influences on bacterial resistance.

It is also crucial that a validation be performed using the worst-case scenario parameters for the process. For instance, in a heat process, validation should be conducted at the absolute lowest temperature, at the shortest exposure, and the product throughput should be at its greatest degree. This ensures that any parameters used during normal processing are more effective than those that were validated. The parameters established during the process validation at the worst-case scenario, can then be used in an HACCP plan as validated critical limits. Process validations performed during normal processing parameters often lead to a breach of critical limits as a result of normal process variation.

1.4.3 Use of Surrogates

Prior to the use of a particular surrogate in a pilot plant, validation in a laboratory environment must be conducted in identical material and in process conditions similar to or the same as those used during a real process and results should be compared with pathogens or spoilage microorganisms. If results show that the surrogate microorganism is more resistant or as resistant as the target pathogenic or spoilage microorganism, then this surrogate can be used in a pilot plant and the process validated. Although surrogates can be used in both stability trials and validation trials, they are mostly utilised in plant validations as conditions of processing cannot always be easily replicated in a laboratory environment, compared to stability trials or challenge testing for which storing conditions can be easily replicated. The most common surrogates are spores of *Cl. sporogenes* PA 3679 used as a *Cl. botulinun* surrogates for validation of thermal processes (Brown et al., 2012) and *E. faecium* NRRL B-2354 (Almond Board of California, 2014) used as surrogate for *Salmonella* or other pathogens for mild thermal processes, e.g. in line pasteurisation. A list of strains used in Validation trials and challenge testing is shown in Table 1.9.

Surrogate strains	Target pathogen	Experiment trials	Reference
Listeria innocua, (NCTC 11288)	*Listeria monocytogenes*	Survival in a dry fermented sausage	(Hospital et al. 2012)
Listeria innocua, (ATCC 33090, ATCC 33091, ATCC 51742)		Ozone treatment of salmon fillets and various other trials	(Crowe et al. 2012)
*Listeria ivanovii (*ATCC 19119)		Testing activity of pediocin from *E. faecium*	(Todorov et al. 2010)
Lactobacillus plantarum, (ATCC 8014), *Lactobacillus leichmannii,* (ATCC 4797), *Lactobacillus acidophilus,* (ATCC 19992)		Process validation for ultrahigh pressure and pulsed electric field. *Lb. plantarum* gave the most comparable thermal resistance.	(Waite-Cusic et al. 2011)
Enterococcus faecium B-2354, *Pediococcus acidilactici, Pediococcus parvulus*	*Listeria monocytogenes*	Thermal inactivation in ground beef	(Ma et al. 2007)
Enterococcus faecium B-2354, *Pediococcus acidilactici, Pediococcus parvulus*	*Salmonella* spp	Thermal inactivation in ground beef	(Ma et al. 2007)
Enterococcus faecium, (NRRL B-2354)	*Salmonella* Enteritidis PT30	Roasting of almonds; moist air convention heating of almonds	(Yang et al. 2010)
E. coli K12 (ATCC 23716)	*Salmonella* Enteritidis PT30	Pasteurisation of liquid egg	(Jin et al. 2008)
E. coli ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1430, ATCC BAA-1431	*Salmonella* (5 serotypes)	Processing on meat	(Niebuhr et al. 2008)
E. Coli ATCC 11775, ATCC 25253, ATCC 35695, ATCC 25922	*Escherichia coli* O157, Salmonella spp.	Thermal inactivation and attachment studies	(Eblen et al. 2005)
E. coli K12 (ATCC 23716), *Lb. casei* (ATCC 393), *Lb. fermenti* (ATCC 9338), *Lb. plantarum* (ATCC 49445), *L. Lactis* (ATCC 11454)	*E. coli* O157:H7, *Salmonella* Typhimurium	Inactivation by pulsed field in orange juice – tested range of surrogates.	(Gurtler et al. 2010)
E. coli non-pathogenic ATCC 35218	*E. coli* O157:H7	Inactivation in strawberry juice by heat treatment with and without preservatives	(Gurtler et al. 2011)
E. coli K12 ATCC 23716	*E. coli* O157:H7	Supercritical carbon dioxide effects on *E. coli* in apple cider	(Yuk et al. 2010)
Commercially available LAB starter cultures, non-*E. coli* coliforms and ATCC strains	*E. coli* O157:H7	Beef carcass intervention trials	(Ingham et al. 2010)
Cl.sporogenes	*Cl. botulinum*	Wide selection of foodstuff	(Brown et al. 2012)

Table 1.9 Surrogate used in process validation

1.5 Conclusions

The literature review showed that pathogens especially *Salmonella* are very common contaminants of low moisture food. Although *Salmonella* outbreaks associated with low-moisture products are relatively rare (Table 1.3), they often impact large numbers of people as a result of the long-term stability and widespread distribution of such commodities. Studies of the survival of bacteria in low moisture foods have been the subject of research by many scientists and therefore mechanisms of inactivation, responses of microorganisms to various stresses and the ability to survive in various environments are well known. However, due to the fact that inactivation of microorganisms depends on many factors and very complex food matrices, precise prediction of survival or inactivation kinetics of particular microorganisms in food is still challenging. Therefore, challenge testing or process validation is still the only one way to precisely determine the rate of inactivation. A substantial number of studies have shown that use of surrogate bacteria is a very useful method to validate various processes and the use of various mathematical modelling procedures, is a very effective tool and can describe various nonlinear regression curves.

1.6 Aims and research hypothesis

Aims of this research were to investigate the survival of pathogens in low moisture foods. As such, the ability of pathogens to survive throughout storage at various conditions and also their heat resistance was investigated. There is substantial evidence showing that pathogens can survive prolonged storage in low moisture foods and that heat resistance of pathogens is significantly greater than within foods containing a high content of water; however, the survival kinetics and mechanisms of survival are not fully understood. The paprika powder was chosen as a first product and survival of Salmonella was evaluated. The selection was made as in 1993 over 1000 of people were infected with *Salmonella* following consumption of chips sprinkled with *Salmonella* contaminated paprika powder. Furthermore, no follow up research looking into survival patterns of *Salmonella* in the paprika powder was conducted. Moreover sporadically *Salmonella* can be found in herbs, spices or other low moisture foods. It was expected that paprika powder will have some antimicrobial properties and *Salmonella* would not survive as well as in other low moisture foods. The survival of *Salmonella* in the rice flour and survival of *Salmonella* and *L. monocytogenes* in other low moisture products was conducted.

Furthermore, the survival of pathogens in different low water activity food was explored, these are confectionery powder (containing high level carbohydrates; starch, sucrose, maltodextrin, wheat flour), seasoning powder (containing high levels of salts), pet food powder (containing corn, rice, wheat flours, and protein-rich materials like corn gluten, soybean meal, fish meal, but also chicken by-product) and chicken powder (containing high levels of protein). The aim was therefore to explore the survival kinetics of pathogens in this wide selection of food matrices would; to determine which food components are most and less likely to support the survival of bacteria during storage and heat processing. In addition, to investigating the survival of pathogens in the low moisture foods the performance of *E. faecium* NRRL B-2354 (potential *Salmonella* surrogate) was also investigated. From previous reports it was expected that *E. faecium* NRRL B-2354 would survive much better than *Salmonella* or *L. monocytogenes* in all products.

As such, the hypotheses of this research were:

- Survival of *Salmonella* in low moisture foods will be significantly greater in low a_w
- Survival patterns of different *Salmonella* strains will be different
- RpoS +ve *Salmonella* will survive in low moisture foods significantly better than RpoS -ve *Salmonella*

- The composition of low moisture foods (carbohydrates, protein, fat or capsaicin) will have a significant impact on the survival of *Salmonella*
- *E. faecium* NRRL B-2354 (potential *Salmonella* surrogate) will survive in low moisture foods similarly to *Salmonella* or significantly better and can be used in process validations

1.7 References

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Chapter 2 - Survival of *Salmonella* and *Enterococcus faecium* NRRL B-2354 in paprika powder and in rice flour

2.1 Introduction

Public Health England reported 5,937 *Salmonella* gastrointestinal-infection cases in England and Wales in 2014 and 2,986 infections so far in 2015 (until end of June 2015). RASFF (the Rapid Alert System for Food and Feed) recorded a total of 477 notifications related to *Salmonella* in all types of food in 2014, of which 101 were related to low moisture foods – 21.2%. In 2015, 334 notifications were recorded (until end of August 2015) of which 95 were related to low moisture food – 28.4% (mainly sesame seeds, dried herbs and spices, nuts). In 2014 the highest number of reports of *Salmonella* contaminated dry foods were recorded. In a total of 103 notifications, the two most commonly reported were spice powders (33) and sesame seeds (24). Although the number of notifications seems relatively small, the volume of contaminated samples/shipments is large. Van Doren et al. (2013) commented that 75 tonnes of Capsicum at a cost of $160,000 and 350,000 kg of Sesame seeds at a cost of $710,000 offered for entry to the United States only between August 2010 and December 2010 were found to be contaminated.

Paprika powder is a dried spice that is commonly used in many products world-wide. There are two main functions of paprika powder added to foods; firstly it is used as a spice to give a mild peppery hot flavour and secondly may be used as a colourant and both uses are recognised by FDA. Paprika is listed by FDA as a spice (21CFR182.10), as a spice oleoresin (21CFR182.20), and as a colour additive (21CFR73.340 for ground paprika and 21CFR73.345 for paprika oleoresin). Its dual function in food as a spice and a colour additive is also recognised (21CFR101.22.(a)(2)) (FDA, 2016). In Europe paprika powder is used as a spice and as a colorant under symbol E160c. The a_w of paprika powder is a_w <0.6 that makes it a very shelf stable raw product as no microbial growth will occur in paprika powder in those conditions. However paprika powder like most raw products and ingredients can be an excellent carrier of unwanted bacteria including pathogens. The microbiological status of dried herb and spice samples is assessed using criteria in Recommendations 2004/24/EC (EC, 2004) and the European Spice Association (ESA, 2004) specifications (see Appendix 1).

No *Salmonella* presence is allowed in 25 g of paprika powder and other pathogens are allowed at certain level. Acceptable level of *Bacillus cereus* is between 10^3 -10^4 CFU/g, *Clostridium perfringens* between 10^2 -10^3 CFU/g and *E. coli* between 10 -10^2 CFU/g. Aerobic colony counts (ACCs) are not routinely required but may be performed for spoilage investigation and labelled as "Unsatisfactory" if the predominant organism is > 10^6 yeasts, >10^7 Gram-negative bacilli or *Bacillus* spp., or >10^8 lactic acid bacteria unless added as a processing aid (Health Protection Agency (HPA, 2009; European Communities, 2004).

In critical cases where paprika powder is cross contaminated with pathogenic bacteria, controlling these bacteria and understanding their survival patterns is necessary to formulate possible improvements to the processes and practices.

Rice flour, also known as a rice powder, is a form of flour made from finely milled rice. Rice flour is a particularly good substitute for wheat flour, which causes irritation in the digestive systems of those who are gluten-intolerant. Rice flour is also used as a thickening agent in recipes that are refrigerated or frozen since it inhibits liquid separation. The increasing popularity and use of rice flour, but also its uniformity as a powder, makes it a great matrix for the study of survival of pathogenic bacteria and heat resistance in low moisture foods.

Aims of this study were to:

- investigate survival of various *Salmonella* strains in the paprika powder and in the rice flour.
- investigate extent of differences in survival patterns between *Salmonella* strains
- to show if RpoS +ve *Salmonella* strains can survive much better in the low moisture foods than RpoS -ve *Salmonella* strains
- investigate impact of storage temperature and a_w on survival of *E. faecium* and *Salmonella*
- to validate *E. faecium* as a *Salmonella* surrogate in selected foods

2.2 Materials and methods

2.2.1 Paprika powder

One batch of paprika powder purchased from a local supermarket in 400 g packets was used in storage experiments. The measured pH was 5.00, a_w = 0.474, and moisture content 11% w/w. The natural microbiological background of this paprika powder was enumerated in the range 10^2 - 10^3 cfu/g. Background micro flora was confirmed by optical microscopy. The presence of spores and aerobic growth indicated presence of *Bacillus* spp..

2.2.2 Rice flour

Two batches of rice flour were kindly provided by Dr Walter Peñaloza, Nestle Research Centre, Lausanne at a_w = 0.365 and moisture 9.35 %. Background microflora was eliminated by irradiation conducted at Nestle Research Centre. Samples were stored at 15°C in sealed aluminium foil-lined plastic bags and used within six months.

2.2.3 Strains used

Eight strains of *Salmonella* were used: *S.* Senftenberg 775 W (NCTC 9959) well-known as a highly heat resistant strain (Anellis et al., 1953), *S.* Enteritidis PT30 (BAA-1045) – a strain isolated from a raw almonds outbreak 2000-2001 (Isaacs et al., 2005), *S.* Montevideo – UK chocolate outbreak strain in 2006, *S.* Napoli – UK chocolate outbreak strain, 1982 (Gill et al., 1983), *S.* Typhimurium ATCC 14028, *S.* Tennessee S778 – butter isolate (provided by Dr Walter Peñaloza, Nestle Research Centre, Lausanne), *S.* Typhimurium ST30 (RpoS+ve strain), *S.* Typhimurium ST10 (the natural mutant RpoS-ve) (both strains were provided by Dr Andreas Karatzas, University of Reading).

All strains were stored at -70°C in cryopreservation fluid: Beef Extract Peptone Sodium chloride Glycerol (20%) and De-ionised Water, and recovered on Tryptone Soya Broth (TSB, Oxoid, UK) and Tryptone Soya Agar (TSA, Oxoid, UK).

A single strain of *Enterococcus faecium* was used in this study: *Enterococcus faecium* ATCC 8459 - NRRL B-2354 (strain used for process validation).

2.2.4 Preparation of inoculum

A number of studies (Uesugi and Harris, 2006; Uesugi et al., 2006; Komitopoulou and Peñaloza, 2009) have shown that cells grown on lawn plates are able to survive storage

and heat treatments much better that those grown in broth; therefore the lawn methodology described by Uesugi (Uesugi and Harris, 2006) was adopted in these experiments. Strains required for the experiment were recovered by transferring an inoculated stored frozen bead onto TSA (Oxoid, UK) and incubated aerobically at 37 ± 1°C for 24 h. A single colony was picked using a loop to inoculate 20 mL of TSB (Oxoid, UK) and incubated aerobically at 37 ± 1°C for 24 h. An aliquot (100 µL) of resulting growth was used for inoculating 20 mL portions of TSB and incubated aerobically at 37°C for 24 h. Finally, 1 mL of this culture was poured onto large (140 mm) TSA plates, spread and incubated aerobically at 37 ± 1°C for 24 h. Plates were then flooded with 20 mL of Phosphate buffer (0.1 M; pH = 7.00); cells were harvested using an L-shaped spreader and the resulting suspension collected. Cell suspensions, containing *ca*. 1-3 x 10^{10} cfu/mL prepared in this way were used immediately.

2.2.5 Inoculation of paprika powder

Paprika powder (*Capsicum annum*) was inoculated using a direct spraying inoculation method. 120 g of inoculated paprika powder was spread onto a large (140 mm) Petri dish and was placed in the desiccator. Inoculated samples were then held there until the desired a_w was reached. Desiccators contained saturated solutions of lithium chloride (LiCl) (Greenspan, 1977) that generated 11% RH (relative humidity). A saturated solution of LiCl was prepared by adding 83 g of LiCl to 100 mL of water at 20°C.

A portion of paprika powder spread onto a large petri dish was inoculated with an adequate volume of inoculum to provide *ca.* 10^8 cfu/g. Inoculation validation was performed and sorption isotherms (Brett et al., 2009) created. It was established that to produce inoculated samples at final a_w = 0.55, 1.5 mL of inoculum must be added to 120 ± 0.1 g portion of paprika powder. Therefore, six 120 ± 0.1 g portions of paprika powder were spray-inoculated with 1.5 mL of inoculum, packed and sealed in EVOH bags (Weald Packaging Supplies Ltd., UK) then three 120 ± 0.1 g portions (three independent replicates) were stored at 15 ± 1°C and three at 25 ± 1°C. A further six 120 ± 0.1 g portions were inoculated and the water added during inoculation, was removed in a desiccator to obtain a required level of a_w = 0.45. Preliminary trials confirmed that 120 ± 0.1 g portion of paprika powder inoculated with 1.5 mL of inoculum, spread onto two large (140 mm) Petri dishes and held in a desiccator over a saturated solution of Lithium Chloride, would require 4 h at 30 ± 1°C to reach a_w = 0.45.

2.2.6 Inoculation of rice flour

Rice flour was also inoculated using a direct spraying inoculation method. Inoculated rice flour was spread onto a large (140 mm) Petri dish and was placed in the desiccator. Inoculated samples were then held there until the desired a_w was reached. Desiccators contained saturated solutions of Lithium Chloride (Greenspan, 1977; Archer et al., 1998). A saturated solution of LiCl was made as described in section 2.2.5.

A portion of rice flour spread onto a large Petri-dish was inoculated with an adequate volume of inoculum. Inoculation validation was performed and sorption isotherms (Brett et al., 2009) were created. It was established that to produce inoculated samples at final a_w= 0.55, 1.35 mL of inoculum must be added to 50 ± 0.1 g portion of rice. For rice flour, the survival and heat inactivation experiments were conducted separately.

Six 50 ± 0.1 g portions of rice flour were spray-inoculated with 1.35 mL of inoculum, packed and sealed in EVOH bags then three 50 ± 0.1 g portions (three independent replicates) were stored at 15 ± 1°C and three at 25 ± 1°C. A further six 50 ± 0.1 g portions were inoculated and the water added during inoculation was removed in a desiccator to obtain a required level of a_w = 0.20. Preliminary trials confirmed that a 50 ± 0.1 g portion of rice flour inoculated with 1.35 mL of inoculum and spread onto two large (140 mm) Petri dishes and held in a desiccator over a saturated solution of LiCl, would require 48 h at 30 ± 1°C to reach a_w = 0.20.

2.2.7 Survival trial

Survival of *Salmonella* in paprika powder and in rice flour was performed over the period of 12 weeks with sampling every 2 weeks. At each time point three independent replicates of two storage conditions and at two a_w values, were tested. A sample (1 ± 0.05 g) of paprika powder or rice flour was mixed with 9 ± 0.1 mL of BPW (Buffered Peptone Water; Oxoid, UK) and surviving cells enumerated. Appropriate decimal dilutions in volumes of 100 µL were spread- plated on TSA incubated aerobically at 37°C for 48 h and colonies were counted. To achieve maximum recovery of all cells both healthy and injured, TSA was used instead of XLD (Xylose Lysine Deoxycholate agar; Oxoid, UK). Chang et al. (Chang et al., 2010) showed that using non-selective TSA to recover heat treated cells results in a higher (over 1 log higher) recovery rate compared to using XLD as a recovery medium. Greater recovery of all cells was also confirmed during the recovery validation at Leatherhead Food Research (data not shown). Sporadically, several colonies were confirmed as *Salmonella* using API 20E. The a_w measurements were taken every two

weeks over the storage period using an Aqua Lab Water Activity Meter – Series 3TE (Labcell Ltd. UK), and moisture content using an Ohaus MB25 Moisture Meter (Ohaus, UK).

2.2.8 Surrogate validation

Enterococcus faecium NRRL B-2354 was selected as a primary strain for validation. *E. faecium* NRRL B-2354 is used by Almond Board of California for almond process validations (Almond Board of California, 2014), and limited published data suggest that *E. faecium NRRL B-2354* can be used as a *Salmonella* surrogate (Jeong et al., 2011). Both survival and heat resistance experiments were performed using this strain in paprika powder and rice flour by the methodology described (sections 2.2.4, 2.2.5, 2.2.6 and 2.2.7).

2.2.9 Evaluation of effect of capsaicin on survival of *S.* strains.

Initially, a hypothesis that capsaicin plays a significant role in the survival of *Salmonella* in paprika powder was evaluated. Effects of capsaicin (Sigma - Aldrich) on survival of *S.* Enteritidis PT30 (BAA-1045) was evaluated using 7 different standard methods (Andrews, J. 2008; Klančnik et al., 2010; Matuschek et al., 2014):

2.2.9.1 Disk diffusion assay; 0.5 mg of capsaicin per disc (5 replicates), inhibition compared to control discs.

Capsaicin in form of powder (≥95%) was purchased from Sigma Aldrich and primary solution (ethanol suspension) was prepared by dissolving 50mg of powder in 2mL of ethanol (≥99.9%). Five sterile paper disks (6 mm in diameter; Becton, Dickinson & Co.) were inoculated with 20µL of capsaicin solution. Impregnated discs were then placed on the sterile metal mesh and were left to dry for 1 h in the safety cabinet. Following, five control discs (impregnated only with ethanol) and five capsaicin impregnated discs (impregnated with capsaicin in ethanol solution) were placed on the surface of inoculated TSA agar plates. Agar plates were inuculated with 1mL of overnight growth, diluted to approximately 10^5 - 10^6 cfu/mL and spread using sterile swab. Plates were incubated aerobically for 24 h and zone of inhibition around the capsaicin impregnated discs were measured. Preliminary trials shown that control discs did not inhibit surface growth of tested *Salmonella*.

2.2.9.2 Agar well diffusion method; 0.5 mg of capsaicin per well (5 replicates), inhibition compared to control well.

Capsaicin solution prepared as described above (2.2.9.1) was used in this study. 20 µL of capsaicin solution was inoculated into five agar wells cut from the inoculated (as described above) TSA agar plates. Following incubation, zone of inhibition around agar wells inoculated with capsaicin solution was measured. Preliminary trials shown that 20 µL of ethanol inoculated into agar wells did not affect growth of *Salmonella* around the wells.

2.2.9.3 Agar dilution method:

In this experiment, the effect of capsaicin on the recovery of the *Salmonella* inoculated on the surface of the TSA agar plates was establish. Two approaches were evaluated: a) when capsaicin was spread on the surface of the agar and b) when capsaicin was mixed with the molten agar just before pouring and solidification. 100 µL (2.5mg per plate) of primary solution was spread on the surface of the TSA plates or added to the molten agar just before pouring and solidification. Following addition of capsaicin both types of plates were dried in the safety cabinet for 1 h a. The overnight growth of *Salmonella* in TSB was serially diluted and 100 µL of three decimal dilutions were spread plated onto surface of the TSA agar plates. In parallel, the same dilutions were spread plated on the control (not containing capsaicin) TSA agar plates. Following incubation at 37°C for 24 h plates containing 10 to 300 colonies were counted, number of recovered colonies compared and the effect of capsaicin on the recovery of the *Salmonella* established. This experiment was conducted in three replicates.

2.2.9.4 Broth dilution method;

In this experiment the effect of capsaicin on the growth of *Salmonella* in the liquid matrices (BPW) was evaluated. The 20 µL of the primary solution was added to 20 mL of BPW (25 µg/mL). In parallel, two additional sets were prepared, one with addition of 20 µL of ethanol and one with addition of 20 µL of sterile distilled water. All samples were inoculated with the overnight growth of *Salmonella* at initial level of 10^4cfu/mL. All samples were then incubated at ambient temperature (ca. 20°C) and the number of *Salmonella* cells was enumerated at day 0, 5, 7 and 24 by spread plate technique. At each time point three replicates of each set were tested and the effect of capsaicin on the growth/survival of *Salmonella* establish.

2.2.9.5 Broth dilution method;

In this experiment the effect of paprika powder on the *Salmonella* in the liquid medium (BPW) was evaluated. Three sets of broths were prepared in this experiment. First, BPW broth not containing addition of paprika powder, second, BPW broth containing 1% of paprika powder and third, BPW containing 10% of paprika powder. Following addition of paprika powder all three sets were autoclaved at 121°C for 15 min and inoculated with Salmonella at initial level of 10^4cfu/mL. All samples were stored at ambient temperature (ca. 20°C) and the number of *Salmonella* cells was enumerated immediately after inoculation and after 24 hours. At each time point three replicates of each set were tested and the effect of capsaicin on the growth/survival of *Salmonella* establish.

2.2.9.6 Paprika powder method;

In this experiment 0.5 mg of capsaicin per g of paprika powder was added and samples were inoculated with *Salmonella* at the level of ca. 10^7cfu/g . Samples were stored at 25 ± 1°C and number of Inoculated *Salmonella* was enumerated immediately after inoculation, after 2, 6 and 10 days. At each time point three replicates of each set were tested and the effect of capsaicin on the survival of *Salmonella* in the paprika powder establish.

2.2.9.7 Optical density method;

This method was eliminated at the early stages due to significant changes in OD of the broth caused by addition of diluted capsaicin.

2.3 Results

The results showed that microbial inactivation occurring during storage, is not always linear. A number of concave upwards, concave downwards and linear inactivation curves inactivation curves were observed, Fig 2.1 (A-H) for rice flour and Fig 2.2 (A-G) for paprika powder. Furthermore, in a couple of cases, inactivation did not occur at all, reduction of *E. faecium* NRRL B-2354 in paprika powder at 15°C at both water activities was not seen Fig 2.2 G.

All curves were fitted to the Weibull model:

$$\log\left(\frac{N}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta}$$

Where: N_0 - initial number of viable counts before heating
N - number of viable counts at heating time t
t - time (min)
α - scale parameter (a characteristic time)
β - shape parameter

Weibull distribution corresponds to a concave upward survival curve if $\beta < 1$, concave downward if $\beta > 1$ and reduces to an exponential (linear) distribution if $\beta = 1$.
Parameters α and β were estimated using Excel equation solver and GRG (Generalized Reduced Gradient) Nonlinear Solving Method. Fitting of the model to raw data was confirmed by conducting an F-test using Excel (Microsoft Office) (Drosinos et al., 2006) and R^2 (Brown, 2001). Parameters α and β were estimated for mean values.
In addition, the standard log-linear model was fitted to the data, R^2 calculated and linear fit compared to Weibull model fit.

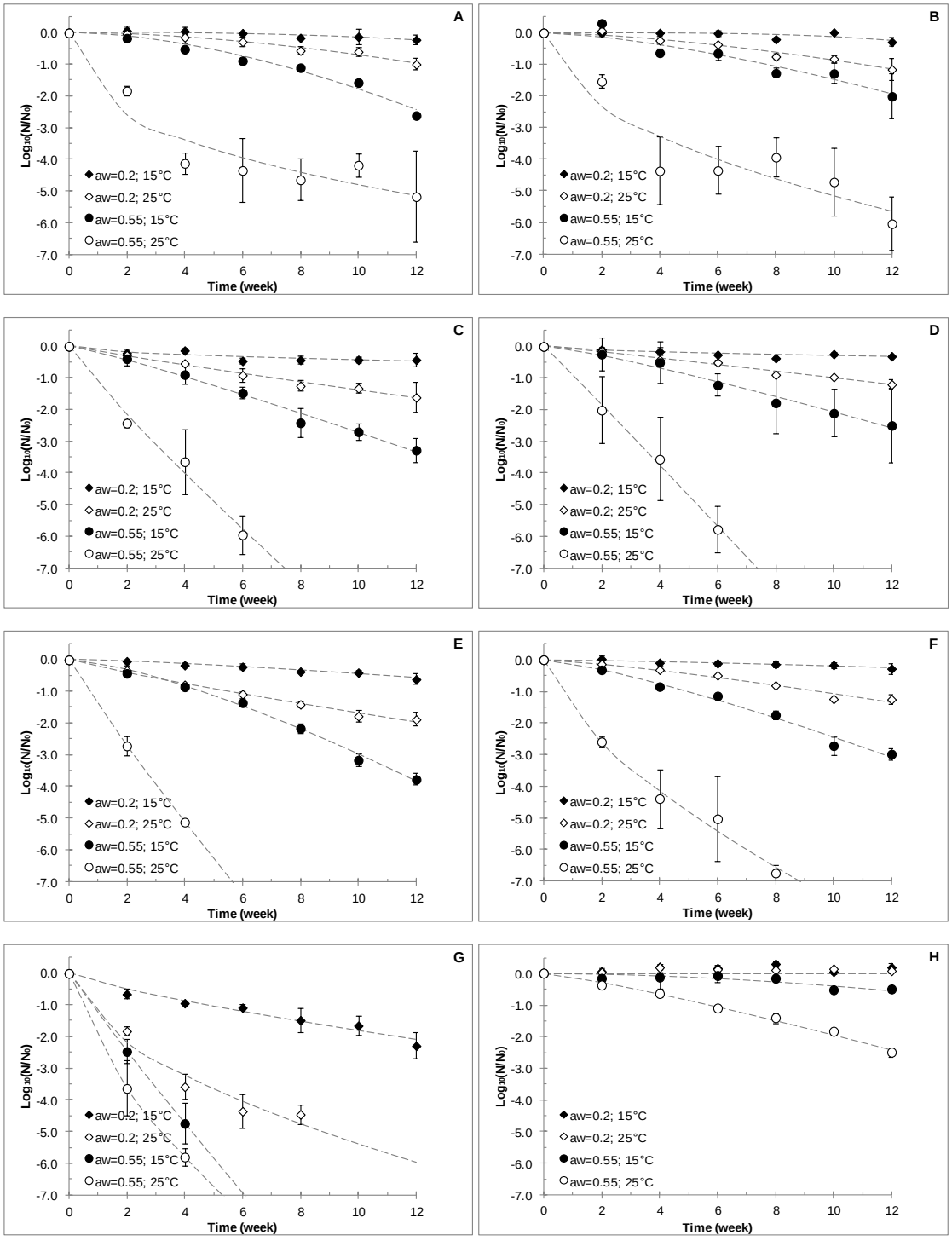


Fig. 2.1 (A-H) Survival of Salmonellae and *E. faecium NRRL B-2354* in rice flour; A - *S.* Enteritidis PT 30, B - *S.* Tennessee S778, C - *S.* Montevideo, D - *S.* Typhimurium - ATCC 14028, E - *S.* Senftenberg 775W, F - *S.* Typhimurium ST30, RpoS +ve, G - *S.* Typhimurium ST10, RpoS -ve, H - *E. faecium* NRRL B-2354

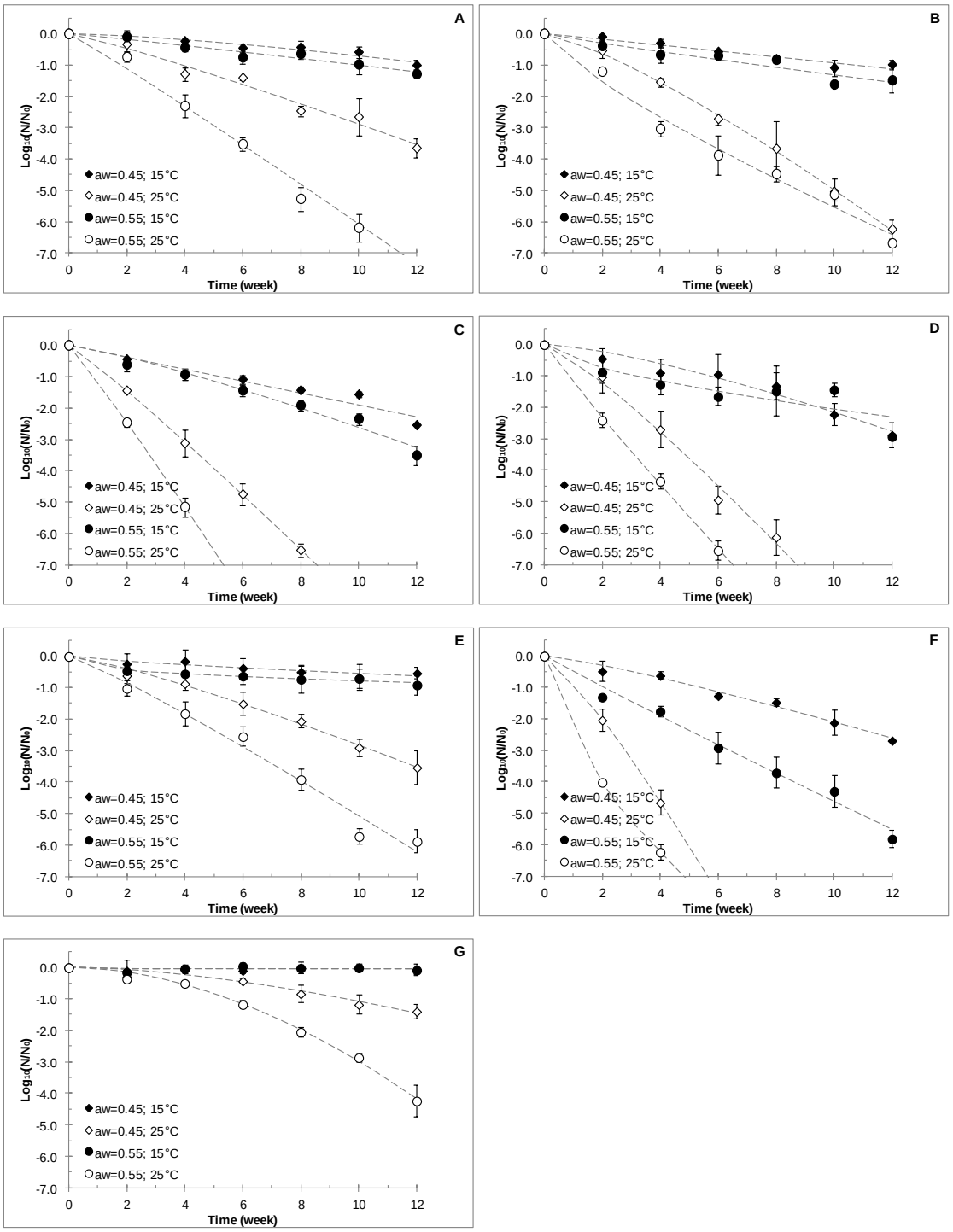


Fig. 2.2 (A-G) Survival of Salmonellae and *E. faecium NRRL B-2354* in paprika powder; A - *S.* Enteritidis PT 30, B - *S.* Montevideo, C - *S.* Napoli, D - *S.* Senftenberg 775W, E - *S.* Typhimurium ST30, RpoS +ve, F - *S.* Typhimurium ST10, RpoS -ve, G - *E. faecium* NRRL B-2354

Calculated parameters for Weibull model, R^2 for both non-linear and linear fit are presented in Table 2.1 and Table 2.2

Strain	aw	Temp.	α	β	R^2 Weibull	R^2 Linear
S. Enteritidis PT 30 ATCC BAA-1045	0.2	15°C	15.571	2.520	0.778	0.758
		25°C	7.599	1.724	0.975	0.930
	0.55	15°C	4.350	1.703	0.973	0.924
		25°C	0.018	0.381	0.917	0.742
S. Tennessee S778	0.2	15°C	13.946	3.687	0.517	0.423
		25°C	6.332	1.542	0.966	0.949
	0.55	15°C	4.343	1.481	0.910	0.904
		25°C	0.069	0.497	0.893	0.805
S. Montevideo	0.2	15°C	10.090	0.496	0.799	0.714
		25°C	2.793	0.909	0.988	0.985
	0.55	15°C	1.930	1.119	0.989	0.988
		25°C	0.332	0.894	0.987	0.986
S. Typhimurium ATCC 14028	0.2	15°C	20.081	0.526	0.825	0.739
		25°C	4.515	1.044	0.982	0.982
	0.55	15°C	2.669	1.188	0.984	0.982
		25°C	0.481	1.019	0.996	0.996
S. Senftenberg	0.2	15°C	9.567	1.317	0.977	0.964
		25°C	2.094	0.866	0.992	0.986
	0.55	15°C	2.472	1.381	0.995	0.975
		25°C	0.269	0.914	1.000	0.999
S. Typhimurium ST30, RpoS +ve	0.2	15°C	17.258	1.380	0.918	0.910
		25°C	4.889	1.267	0.972	0.964
	0.55	15°C	2.572	1.276	0.985	0.974
		25°C	0.133	0.663	0.991	0.960
S. Typhimurium ST10, RpoS -ve	0.2	15°C	1.632	0.789	0.967	0.964
		25°C	0.111	0.560	0.970	0.900
	0.55	15°C	0.316	0.941	1.000	0.999
		25°C	0.086	0.675	1.000	0.979
E. faecium ATCC 8459	0.2	15°C	2.9E+03	5.4E+07	-1.923	0.219
		25°C	7.5E+08	2.5E+08	-2.459	0.210
	0.55	15°C	10.626	1.790	0.772	0.713
		25°C	2.855	1.193	0.992	0.985

Table 2.1 Weibull model parameters (α and β) and R^2 for Weibull model fit and R^2 for linear regression fit calculated for regression curves of Salmonellae and *E. faecium* NRRL B-2354 in rice flour

Strain	aw	Temp.	α	β	R^2 Weibull	R^2 Linear
S. Enteritidis PT 30 ATCC BAA-1045	0.45	15°C	7.061	1.403	0.937	0.917
		25°C	1.856	1.126	0.976	0.974
	0.55	15°C	4.482	1.057	0.947	0.947
		25°C	0.962	1.149	0.988	0.991
S. Montevideo	0.45	15°C	4.734	1.027	0.947	0.949
		25°C	1.468	1.272	0.999	0.990
	0.55	15°C	2.953	0.909	0.905	0.905
		25°C	0.415	0.800	0.983	0.972
S. Napoli	0.45	15°C	2.260	0.990	0.943	0.944
		25°C	0.649	1.079	1.000	0.999
	0.55	15°C	2.213	1.194	0.976	0.968
		25°C	0.391	1.064	1.000	0.999
S. Senftenberg	0.45	15°C	3.063	1.358	0.962	0.941
		25°C	0.827	1.181	0.989	0.986
	0.55	15°C	0.837	0.628	0.802	0.785
		25°C	0.332	0.934	0.999	0.998
S. Typhimurium ST30, RpoS +ve	0.45	15°C	7.362	0.741	0.867	0.854
		25°C	2.115	1.205	0.994	0.986
	0.55	15°C	2.064	0.373	0.975	0.826
		25°C	1.082	1.106	0.979	0.977
S. Typhimurium ST10, RpoS -ve	0.45	15°C	2.621	1.184	0.986	0.979
		25°C	0.549	1.194	1.000	0.995
	0.55	15°C	0.849	0.961	0.986	0.986
		25°C	0.060	0.635	1.000	0.973
E. faecium ATCC 8459	0.45	15°C	2.7E+19	0.104	-1.037	0.010
		25°C	5.694	1.628	0.940	0.894
	0.55	15°C	1.0E+20	0.053	0.041	0.008
		25°C	3.486	1.830	0.995	0.931

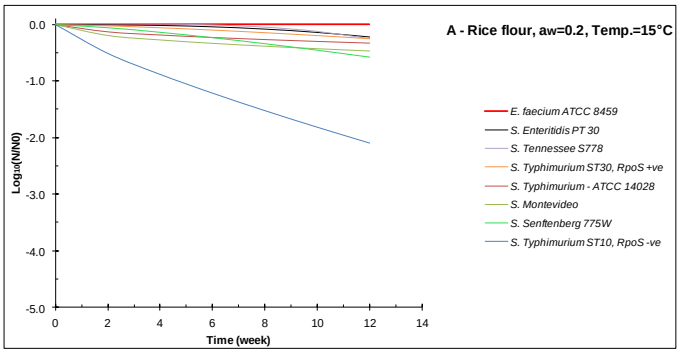
Table 2.2 Weibull model parameters (α and β) and R^2 for Weibull model fit and R^2 for linear regression fit calculated for regression curves of Salmonellae and *E. faecium* NRRL B-2354 in paprika powder

Results showed that the Weibull model is the correct mathematical model to fit raw survival data. R^2 values calculated for nonlinear curves for Weibull model were mainly close to 1 which indicated a satisfactory fit. Furthermore, R^2 values for the Weibull model, except in a few cases, are greater than the R^2 values for first order kinetics model which indicates the suitability of Weibull model. Inactivation curves (Fig. 2.1 A-H and Fig. 2.2 A-H) and calculated levels of inactivation of each tested strain at week 6 and week 12 (Fig. 2.3 A-D, and Fig. 2.4 A-D) have shown that *Salmonella* and *E. faecium* NRRL B-2354 survived best at lower a_w and at lower storage temperature. In paprika powder, the best survival for all tested strains was observed at $a_w = 0.45$ and 15°C followed by $a_w = 0.55$ at 15°C, by $a_w = 0.45$ at 25°C and finally by $a_w = 0.55$ and 25°C. In rice flour, this pattern was only applicable for *E. faecium* NRRL B-2354 which survived best at $a_w = 0.2$ at 15°C followed by $a_w = 0.55$ at 15°C followed by $a_w = 0.2$ and 15°C and $a_w = 0.55$ and 25°C but

all salmonellae tested in rice flour survived best at a_w = 0.2 and 15°C followed by a_w = 0.2 and 25°C followed by a_w = 0.55 and 15°C followed by a_w = 0.55 and 25°C which indicates that storage temperature is not greatly affecting the viability of salmonellae at lower a_w (a_w = 0.2) but has a greater impact on survival at higher a_w (a_w = 0.45 or 0.55).

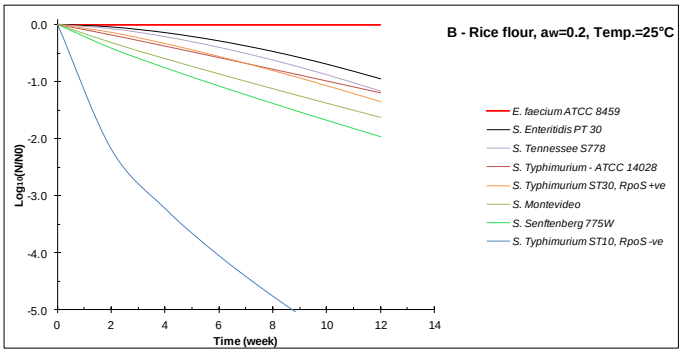
Survival characteristics of each strain, including *E. faecium* NRRL B-2354, were determined using curves defined by the Weibull model and calculated parameters *α* and *β.* Statistical significance of differences was calculated and expressed as a p-value, comparing all *Salmonella* strains as well as comparing the RpoS +ve strains to the RpoS-ve strain, and comparing *E. faecium* NRRL B-2354 with the most resistant *Salmonella* strain at each condition. The set of Figs. 2.3 (A - D) and Figs. 2.4 (A-D) show those survival curves, respectively for rice flour and paprika powder. Results showed that *S.* Typhimurium RpoS -ve was the most sensitive strain tested and survived significantly less compared to *S.* Typhimurium RpoS +ve ($p<0.05$) or when compared to any other strain under all tested conditions. This confirms the significant role of the RpoS gene in increased survival of salmonellae. In rice flour, at lower a_w (a_w = 0.2) and at both storage temperatures (15 and 25°C) the most resistant strains were *S.* Enteritidis PT30, *S.* Tennessee S778, *S.* Typhimurium ST 30 and *S.* Typhimurium ATCC 14028. *E. faecium* NRRL B-2354 was one of the poorest surviving strain while at higher a_w (a_w = 0.55), *E faecium* NRRL B-2354 was the strain surviving best, following by *S.* Tennessee S778, *S.* Enteritidis PT30, *S.* Typhimurium ATCC 14028, and *S.* Typhimurium ST30. The difference between *E. faecium* NRRL B-2354 and the most resistant *Salmonella* strain was statistically significant ($p < 0.05$) at lower a_w (a_w = 0.2) but not statistically significant at higher a_w (a_w = 0.55).

In the paprika powder, *E. faecium* NRRL B-2354 was the most resistant strain in all tested conditions and *S.* Typhimurium ST30 (RpoS +ve) was significantly more resistant than *S.* Typhimurium ST10 (RpoS -ve). Following *E. faecium* NRRL B-2354, the most resistant strain was *S.* Typhimurium ST30 (RpoS +ve), followed by *S.* Enteritidis PT30 and *S.* Montevideo. In all cases, the resistance of *E. faecium* NRRL B-2354 was significantly greater than resistance of the most resistant *Salmonella* strain ($p < 0.05$).



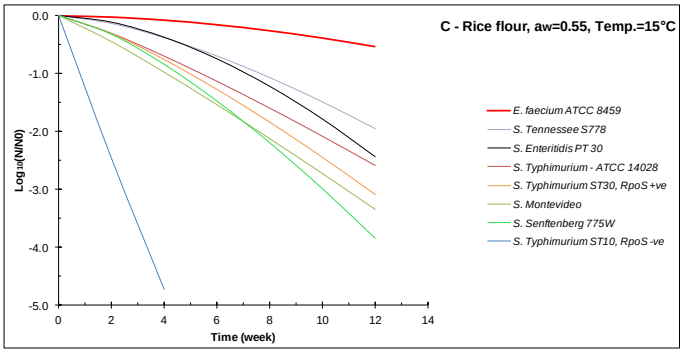
Level of inactivation during storage (δLog)		
Strain	6 weeks	12 weeks
E. faecium ATCC 8459	0.0	0.0
S. Enteritidis PT 30	0.0	0.2
S. Tennessee S778	0.0	0.2
S. Typhimurium ST30, RpoS +ve	0.1	0.3
S. Typhimurium ATCC 14028	0.2	0.3
S. Montevideo	0.3	0.5
S. Senftenberg 775W	0.2	0.6
S. Typhimurium ST10, RpoS -ve	1.2	2.1

Compared strains			p-value
RpoS +ve	vs.	RpoS-ve	0.005
E. faecium	vs.	*S.* Enteritidis	0.016
All Salmonellas			0.000



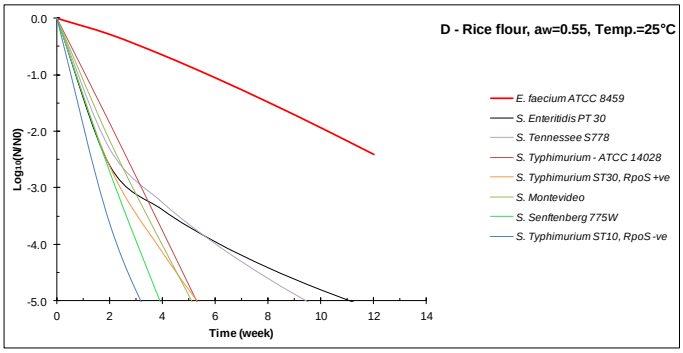
Level of inactivation during storage (δLog)		
Strain	6 weeks	12 weeks
E. faecium ATCC 8459	0.0	0.0
S. Enteritidis PT 30	0.3	1.0
S. Tennessee S778	0.4	1.2
S. Typhimurium ATCC 14028	0.6	1.2
S. Typhimurium ST30, RpoS +ve	0.6	1.4
S. Montevideo	0.9	1.6
S. Senftenberg	1.1	2.0
S. Typhimurium ST10, RpoS -ve	4.1	4.6

Compared strains			p-value
RpoS +ve	vs.	RpoS-ve	0.002
E. faecium	vs.	*S.* Enteritidis	0.010
All Salmonellas			0.000



Level of inactivation during storage (δLog)		
Strain	6 weeks	12 weeks
E. faecium ATCC 8459	0.2	0.5
S. Tennessee S778	0.7	2.0
S. Enteritidis PT 30	0.8	2.4
S. Typhimurium ATCC 14028	1.1	2.6
S. Typhimurium ST30, RpoS +ve	1.3	3.1
S. Montevideo	1.5	3.4
S. Senftenberg 775W	1.5	3.8
S. Typhimurium ST10, RpoS -ve	4.8	5.8

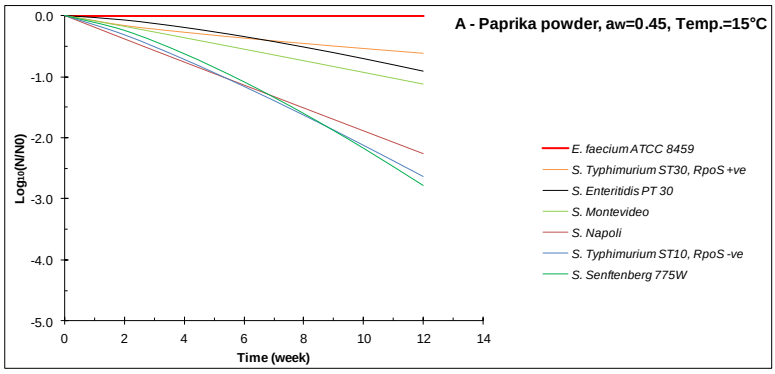
Compared strains			p-value
RpoS +ve	vs.	RpoS-ve	0.008
E. faecium	vs.	*S.* Tennessee	0.092
All Salmonellas			0.000



Level of inactivation during storage (δLog)		
Strain	6 weeks	12 weeks
E. faecium ATCC 8459	1.1	2.4
S. Enteritidis PT 30	4.0	5.2
S. Tennessee S778	4.0	5.6
S. Typhimurium ATCC 14028	5.7	7.7
S. Typhimurium ST30, RpoS +ve	5.4	8.0
S. Montevideo	6.0	7.3
S. Senftenberg 775W	6.1	7.1
S. Typhimurium ST10, RpoS -ve	4.8	5.8

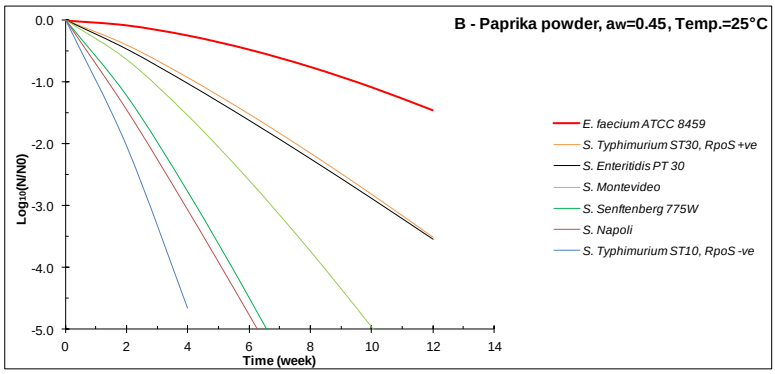
Compared strains			p-value
RpoS +ve	vs.	RpoS-ve	0.005
E. faecium	vs.	*S.* Enteritidis	0.791
All Salmonellas			0.261

Fig. 2.3 (A-D) Survival of *Salmonellae* and *E. faecium* NRRL B-2354 in rice flour at; A - a_w=0.2/Temp.=15°C, B - a_w=0.2/Temp.=25°C, C - a_w=0.55/Temp.=15°C and D - a_w=0.55/Temp.=25°C



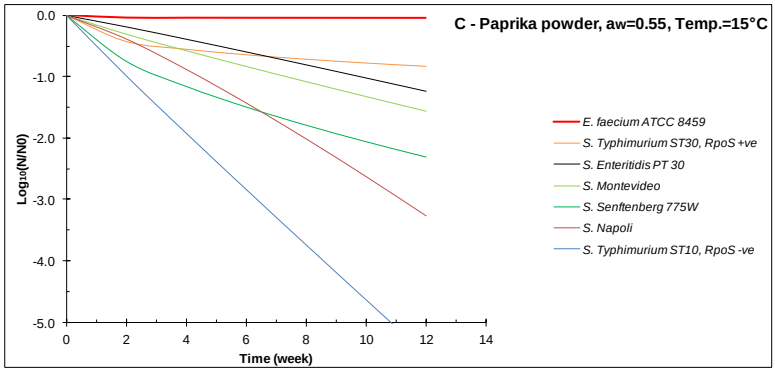
Level of inactivation during storage (δ Log)		
Strain	6 weeks	12 weeks
E. faecium ATCC 8459	0.1	0.1
S. Typhimurium ST30, RpoS +ve	0.4	0.6
S. Enteritidis PT 30	0.3	0.9
S. Montevideo	0.6	1.1
S. Napoli	1.1	2.3
S. Typhimurium ST10, RpoS -ve	1.2	2.6
S. Senftenberg 775W	1.1	2.8

Compared strains			p-value
RpoS +ve	vs.	RpoS-ve	0.021
E. faecium	vs.	*S.* ST30	0.008
All Salmonellas			0.046



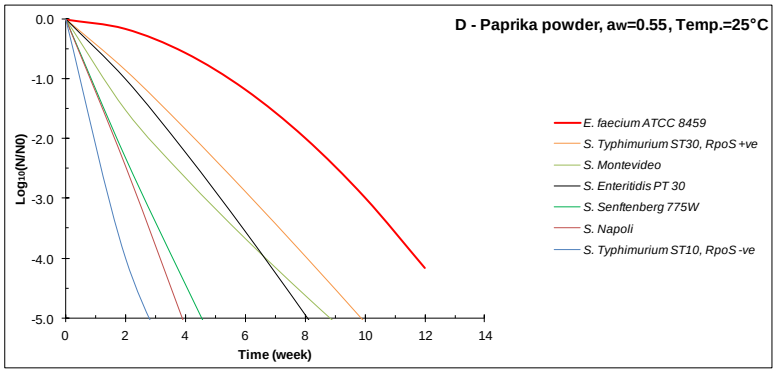
Level of inactivation during storage (δ Log)		
Strain	6 weeks	12 weeks
E. faecium ATCC 8459	0.5	1.5
S. Typhimurium ST30, RpoS +ve	1.5	3.5
S. Enteritidis PT 30	1.6	3.6
S. Montevideo	2.6	6.3
S. Senftenberg 775W	4.5	6.1
S. Napoli	4.8	6.3
S. Typhimurium ST10, RpoS -ve	6.4	6.4

Compared strains			p-value
RpoS +ve	vs.	RpoS-ve	0.015
E. faecium	vs.	*S.* ST30	0.010
All Salmonellas			0.015



Level of inactivation during storage (δ Log)		
Strain	6 weeks	12 weeks
E. faecium ATCC 8459	0.04	0.04
S. Typhimurium ST30, RpoS +ve	0.65	0.84
S. Enteritidis PT 30	0.59	1.23
S. Montevideo	0.83	1.55
S. Senftenberg 775W	1.08	2.77
S. Napoli	1.43	3.27
S. Typhimurium ST10, RpoS -ve	2.84	5.53

Compared strains			p-value
RpoS +ve	vs.	RpoS-ve	0.014
E. faecium	vs.	*S.* ST30	0.002
All Salmonellas			0.002



Level of inactivation during storage (δ Log)		
Strain	6 weeks	12 weeks
E. faecium ATCC 8459	1.17	4.17
S. Typhimurium ST30, RpoS +ve	2.89	6.21
S. Montevideo	3.68	6.41
S. Enteritidis PT 30	3.56	6.25
S. Senftenberg 775W	4.51	7.34
S. Napoli	6.31	7.31
S. Typhimurium ST10, RpoS -ve	7.33	7.33

Compared strains			p-value
RpoS +ve	vs.	RpoS-ve	0.002
E. faecium	vs.	*S.* ST30	0.003
All Salmonellas			0.035

Fig. 2.4 (A-D) Survival of *Salmonella* and *E. faecium* NRRL B-2354 in Paprika powder at; A - a_w=0.45/Temp.=15°C, B - a_w=0.45/Temp.=25°C, C - a_w=0.55/Temp.=15°C and D - a_w=0.55/Temp.=25°C

2.3.1 Survival of *Salmonella* and *E. faecium* NRRL B-2354 in rice flour vs. paprika powder

Although experiments were performed at the same storage temperatures, 15°C and 25°C, the a_w of samples was different (paprika powder; 0.45 and 0.55 and rice flour; 0.2 and 0.55). Therefore, only survival at the higher a_w (a_w = 0.55) could be compared for strains tested in both commodities. Fig.2.5 shows the reduction level (mean log ± standard deviation) of *Salmonella* and *E. faecium* NRRL B-2354 recorded on week 6 and week 12 of storage at a_w = 0.55 in both powders at 15°C. Furthermore, both trials were not conducted in parallel. Trials with rice flour were conducted approximately three months later that trials in paprika powder. Moreover, while key the same *Salmonella* strains were used in both experiments, *S.* Tennessee S778 and *S.* Typhimurium ATCC 14028 was used only in the rice flour and *S.* Napoli was used only in the paprika powder. Decision of not using *S.* Montevideo in rice flour was made after low survival rate of this strain was observed in the paprika powder. Performing trials in the paprika powder using *S.* Tennessee S778 and *S.* Typhimurium ATCC 14028 were not conducted due to error and running out of the tested material (paprika powder) from the same batch.

Salmonella and the surrogate survived better at the lower temperature and lower a_w, in both powders. At the lower temperature (15°C) and same a_w (0.55), survival was better in paprika than rice flour, but at the higher temperature (25°C), full comparison could not be completed as most of the bacteria did not survive for the whole storage period and were eliminated before week 6 or week 12. The most resistant strains (*S.* Enteritidis PT 30, *S.* Montevideo and *S.* Typhimurium ST30) survived better in paprika powder rather than in rice flour.

This indicates that powder composition may have a significant role in the survival of bacteria in low-moisture foods. It is suggested that antimicrobial compounds (antimicrobial essential oils) that could be present in paprika powder are not active at the lower temperature and the higher concentration of fat, sugars and proteins in the paprika powder may protect cells from desiccation and death.

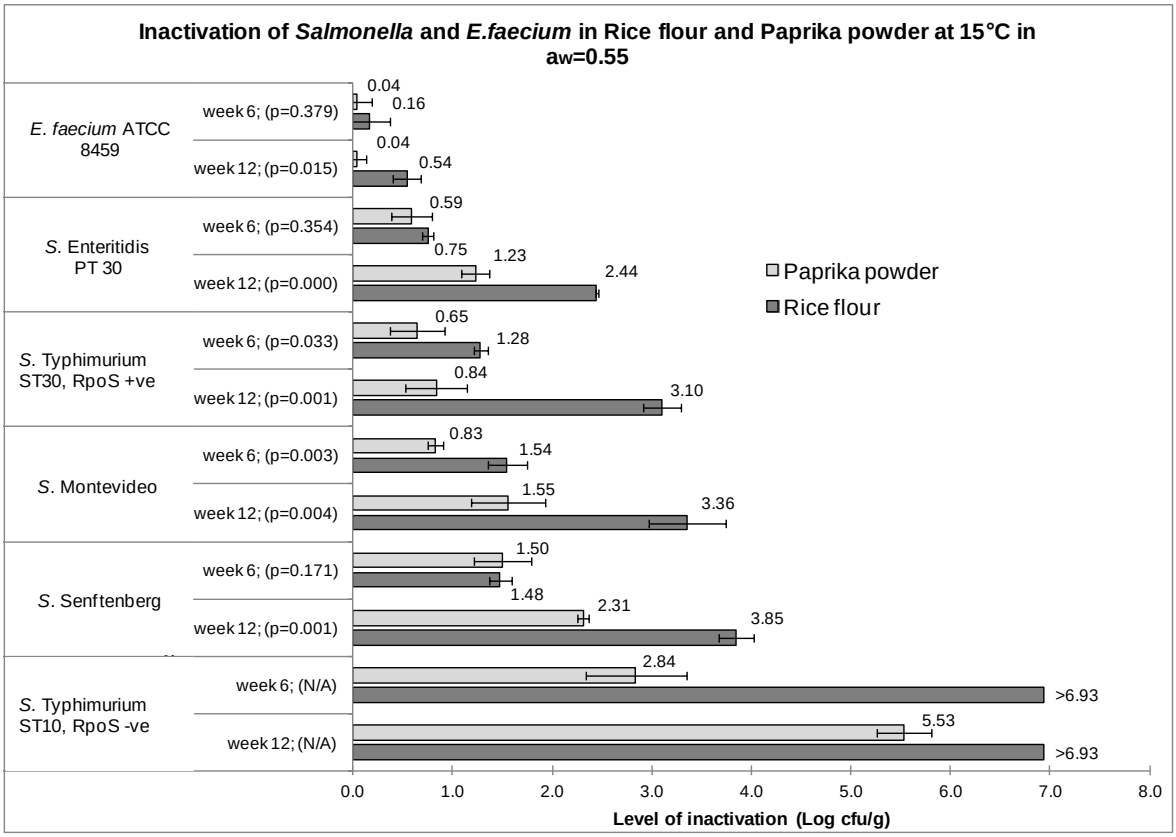


Fig. 2.5 Comparison of survival of *Salmonellae* and *E. faecium* NRRL B-2354 in paprika powder and rice flour at a_w=0.55 and 15°C

2.3.2 Surrogate validation – survival of *E. faecium* NRRL B-2354 in paprika powder and rice flour

Survival of *E. faecium* NRRL B-2354 in paprika powder and in rice flour was conducted in parallel with *Salmonella* trials and results indicate that although *E. faecium* NRRL B-2354 can be used as *Salmonella* surrogate for survival trials in paprika powder with some limitations if used for rice flour. For paprika powder, in all tested conditions, survival of *E. faecium* NRRL B-2354 was greater than the survival of *Salmonella* but in rice flour, survival of *E. faecium* NRRL B-2354 was greater only at the higher a_w (a_w = 0.55) while at lower a_w (a_w = 0.2) *E. faecium* NRRL B-2354 could not be used as a *Salmonella* surrogate.

2.3.3 Effect of capsaicin on survival of *Salmonella* strains

An initial hypothesis that capsaicin contained in paprika powder would have a significant role in the survival of *Salmonella* in these storage conditions, was not confirmed (see 2.2.9). Several experiments were performed to evaluate this; effect of various levels of capsaicin on survival/recovery of *Salmonella* was investigated (0.5 mg of capsaicin per

disc and per well, 25 µg of capsaicin per 1 mL of broth, 0.5 mg of capsaicin per 1 g of paprika powder) but none of them showed a deleterious effect on survival of *Salmonella* (results not shown). No inhibition zones of Salmonella were observed for diffusion assay discs method and agar well diffusion method. Recovery of the *Salmonella* inoculated at various levels on the surface of the agar or in the broths containing the capsaicin was the same the recovery from control agars and broth (not containing any additives). *Salmonella* inoculated into broths containing two levels of paprika powder grew at the same rate always the same. Therefore, it was concluded that survival of *Salmonella* in paprika powder is mostly dependent on a_w, pH, and other components. However, only one strain (*S.* Enteritidis PT30; BAA-1045) was tested in these sets of experiments. Therefore, further work is required. No correlation between capsaicin and survival of the tested strain was observed.

2.4 Discussion

Results have shown that *Salmonella* can survive well both in paprika powder and rice flour over a period of 12 weeks storage. Survival was greater when a_w of those products was lower and the bactericidal effects of low a_w on *Salmonella* were greater at higher storage temperatures. Over 5 logs reduction of one of the most resistant strains tested (*S.* Typhimurium RpoS+ve) was recorded when paprika powder with $a_w = 0.55$ was stored at 25°C, but only 0.6 log reduction was recorded when stored at $a_w = 0.45$ and 15°C (Fig. 2.4A). Similar patterns were observed in rice flour; over 6 log reduction accrued at 25°C and $a_w = 0.55$ (Fig. 2.3D) but only 0.3 log reduction when stored at 15˚C (Fig. 2.3A.). *Salmonella* survived significantly better in paprika powder than in rice flour despite initial assumptions that paprika powder may have antibacterial properties. Regardless of rice flour containing a significantly higher percentage of carbohydrates (80 %), when compared to paprika powder (55 %), fat content may have a significant impact on survival of bacteria during storage. Paprika powder on average contains 13 % fat compared to rice flour containing only 1 %. Furthermore, the level of protein is significantly higher in paprika powder (15 %) compared to rice flour (6 %).

A small number of studies evaluating survival of *Salmonella* in low moisture foods and ingredients are published and available but, to our knowledge no study using paprika powder or rice flour has been published. Most of the studies were conducted under different experimental conditions and therefore results are difficult to compare. The conditions of inoculum preparation (temperature, medium used) the method of inoculum preparation (centrifugation of broth growth or collection of cells from lawn plate), as well as the method of inoculation or storage conditions are usually different from study to study. Uesugi et al. (Uesugi et al., 2006), Komitopoulou and Peñaloza (Komitopoulou and Peñaloza, 2009; Peñaloza and Komitopoulou, 2012) or Blessington *et al.* (Blessington et al., 2012) have shown greater survival of *Salmonella* when cells were prepared using lawn plates while others, (Archer et al., 1998; Shachar and Yaron, 2006; Torlak et al., 2013) prepared an inoculum using centrifuged broth growth.

The ability of bacteria to survive in low moisture food at low storage temperatures is well documented. Komitopoulou and Peñaloza (Komitopoulou and Peñaloza, 2009) showed increased survival of various *Salmonella* strains in cocoa butter oil at lower temperatures, and Uesugi et al., (Uesugi et al., 2006) showed increased survival of *Salmonella* on almonds at low storage temperatures. Similarly Beuchat el al. (2013) and Podolak et al. (2010) postulate that a_w is a significant influencing factor on survival of *Salmonella* and lower a_w levels offer a protective effect.

No influence of capsaicin on the survival of *Salmonella* in paprika powder has been observed in our work while, studies on this subject are limited. Cichewicz and Thorpe 1996 investigated antimicrobial properties of various *Capsicum* species against a wide selection of microorganisms including *S.* Typhimurium. Although an inhibitory effect on various microorganism was clearly shown, results for *Salmonella* were not presented. Kemin Industries, Inc. patent application (1999) presented an invention of a method of extraction and the effect of a crude protein extract from the seeds of *Tagetes* and *Capsicum* showed an inhibitory effect for *Salmonella* and *E. coli.* However, only an optical density method was performed with no confirmation on cells' recovery. Molina-Torres *et al.* (Molina-Torres et al., 1999) evaluated antimicrobial properties of affinin and capsaicin, against various Gram negative microorganisms but no *Salmonella* strains were included. Dorantes et al. (2000) tested the inhibitory effect of three chilli extracts against *Listeria monocytogenes*, *Staphylococcus aureus*, *S.* Typhimurium and *Bacillus cereus,* and concluded that *S.* Typhimurium was the most resistant of all 4 species tested. Antimicrobial activity of *Capsicum* extract against *S.* Typhimurium and *Pseudomonas aeruginosa* inoculated in raw beef was evaluated by Careaga et al. (2003) and showed a 3 log reduction with minimum lethal concentration (MLC) of bell pepper extract 1.5 mL/100 g of raw meat.

2.5 Conclusions

This study shown that *Salmonella* and *E. faecium* NRRL B-2354 survived well in low moisture foods and survival was greater at lower storage temperature and at lower a_w. Differences in survival patterns of *Salmonella* strains and those RpoS+ve and RpoS-ve were significant. Finally, in all cases *E. faecium* NRRL B-2354 survived significantly better than *Salmonella* what indicate that *E. faecium* NRRL B-2354 is suitable strain for validation study and can be used as a *Salmonella* surrogate.

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Chapter 3 - Heat resistance of *Salmonella* and *Enterococcus faecium* NRRL B-2354 in paprika powder and in rice flour

3.1 Introduction

Second only to the ability of microorganisms to survive in low moisture food during storage, heat resistance is an additional important characteristic of bacteria which can determine their persistence in foods, especially in those where a mild heat treatment is applied. Knowledge of heat resistance of pathogens in low moisture food, mechanism of changes and kinetics of changes are very important as these are major factors essential in choosing the correct parameters of heat treatment. Furthermore, it is still unclear if heat resistance of bacteria dispersed in the low moisture foods is changing throughout the storage period or remains stable. Therefore it is crucial to answer this question as this will help food manufacturers to schedule processing at the right time (when the heat resistance is lower) and adjust level of processing if needed. Although the fact that bacteria survive heat treatment better in low moisture food is well documented (Archer et al., 1998; Barrile and Cone, 1970; Garibaldi et al., 1969; Goepfert and Biggie, 1968; McDonough and Hargrove, 1968; Peñaloza and Komitopoulou, 2012, Van Cauwenberge et al., 1981), precise calculations are not always possible, especially in complex food matrices when time required to eliminate pathogenic bacteria is dependent on many factors. In those situations, studies of heat resistance of particular bacteria in a particular food matrix in a range of temperatures and different water activities, are necessary to attain full confidence in selection of heat process parameters to ensure a sufficient reduction of bacteria was achieved with minimum energy use and minimum damage to the food material.

Aims of this study were to:

- investigate heat resistance of various *Salmonella* strains in the paprika powder and in the rice flour.
- to show if heat resistance of RpoS +ve *Salmonella* strains is significantly different than RpoS -ve *Salmonella* strains
- to validate *E. faecium* NRRL B-2354 as a *Salmonella* surrogate in selected foods

3.2 Materials and methods

3.2.1 Paprika powder

The paprika powder which was used in previous experiments (Chapter 2) was used in this study. One batch of paprika powder purchased from a local supermarket in 400 g packets was used in storage experiments. The measured pH was 5.00, a_w = 0.474, and the moisture content was 11% w/w. The natural microbiological background of this paprika powder was enumerated in the range 10^2 - 10^3 cfu/g (confirmed mainly as *Bacillus* spp. spores). Several other paprika powders were tested with similar results except one type of paprika powder contained *Bacillus* spp. at levels > 10^6cfu/g.

3.2.2 Rice flour

The rice flour which was used in previous experiments (Chapter 2) was used in this study. Two batches of rice flour were kindly provided by Dr Walter Peñaloza, Nestle Research Centre, Lausanne at a_w = 0.365 and Moisture 9.35 %. Background microflora was eliminated by irradiation conducted at Nestle Research Centre. Samples were stored at 15°C in sealed aluminium foil-lined plastic bags and used within six months.

3.2.3 Strains used

Eight strains of *Salmonella* were used: *S.* Senftenberg 775 W (NCTC 9959) well-known as a highly heat resistant strain (Anellis et al., 1953), *S.* Enteritidis PT30 (BAA-1045) – a strain isolated from a raw almonds outbreak 2000-2001 (Isaacs et al., 2005), *S.* Montevideo – UK chocolate outbreak strain in 2006, *S.* Napoli – UK chocolate outbreak strain, 1982 (Gill et al., 1983), *S.* Typhimurium ATCC 14028, *S.* Tennessee S778 – butter isolate (provided by Dr Walter Peñaloza, Nestle Research Centre, Lausanne), *S.* Typhimurium ST30 (RpoS+ve strain), *S.* Typhimurium ST10 (RpoS-ve) (both strains were provided by Dr Andreas Karatzas, University of Reading).

All strains were stored at -70°C in cryopreservation fluid: Beef Extract Peptone Sodium Chloride Glycerol (20%) and De-ionised Water, and recovered in Tryptone Soya Broth (TSB, Oxoid, UK) and Tryptone Soya Agar (TSA, Oxoid, UK).

A single strain of *Enterococcus faecium* was used in this study: *Enterococcus faecium* ATCC 8459 - NRRL B-2354 (strain used for process validation).

3.2.4 Preparation of inoculum

A number of studies (Uesugi and Harris, 2006; Uesugi et al., 2006; Komitopoulou and Peñaloza, 2009) have shown that cells grown on lawn plates were able to survive storage and heat treatments much better that those grown in broth; therefore the lawn methodology described by Uesugi (Uesugi and Harris, 2006) was adopted and used. Strains required for the experiment were recovered by transferring an inoculated stored frozen bead onto TSA (Oxoid, UK) and incubated aerobically at 37 ± 1°C for 24 h. A single colony was picked using a loop to inoculate 20 mL of TSB (Oxoid, UK) and incubated aerobically at 37 ± 1°C for 24 h. An aliquot (100 µL) of resulting growth was used for inoculating 20 mL portions of TSB and incubated aerobically at 37°C for 24 h. Finally, 1 mL of this culture was poured onto large (140 mm) TSA plates, spread and incubated aerobically at 37 ± 1°C for 24 h. Plates were then flooded with 20 mL of Phosphate buffer (0.1 M; pH = 7.00); cells were harvested using an L-shaped spreader and the resulting suspension collected. Cell suspensions, containing *ca*. 1-3 x 10^{10} cfu/mL prepared in this way were used immediately.

3.2.5 Inoculation of paprika powder

Paprika powder (*Capsicum annum*) was inoculated using a direct spraying inoculation method as described in the Chapter 2. One set of Inoculated paprika powder was used for both; storage survival trials (Chapter 2) and heat inactivation trial (Chapter 3). Inoculated paprika powder was spread onto a large (140 mm) Petri dish and was placed in the desiccator. Inoculated samples were then held there until the desired a_w was reached. Desiccators contained saturated solutions of lithium chloride (LiCl) (Greenspan, 1977) what generated 11 % RH (relative humidity). A saturated solution of LiCl was prepared by adding 83 g of LiCl to 100 mL of water at 20°C.

A portion of paprika powder spread onto a large petri dish was inoculated with an adequate volume of inoculum to provide *ca.* 10^8cfu/g. Inoculation validation was performed and sorption isotherms (Brett et al., 2009) have been created. It was established that to produce inoculated samples at final a_w = 0.55, 1.5 mL of inoculum must be added to 120 ± 0.1 g portion of paprika powder. Therefore, six 120 ± 0.1 g portions of paprika powder were spray-inoculated with 1.5 mL of inoculum, packed and sealed in EVOH bags (Weald Packaging Supplies Ltd., UK) then three 120 ± 0.1 g portions (three independent replicates) were stored at 15 ± 1°C and three at 25 ± 1°C. A further six 120 ± 0.1 g portions were inoculated and the water added during inoculation, was removed in a

desiccator to obtain a required level of a_w = 0.45. Preliminary trials confirmed that 120 ± 0.1 g portion of paprika powder inoculated with 1.5 mL of inoculum, spread onto two large (140 mm) Petri dishes and held in a desiccator over a saturated solution of Lithium Chloride, would require 4 h at 30 ± 1°C to reach a_w = 0.45.

3.2.6 Inoculation of rice flour

Rice flour was also inoculated using a direct spraying inoculation method. Inoculated rice flour was spread onto large (140 mm) Petri dish and was placed in the desiccator. Inoculated samples were then held there until and desired a_w was reached. Desiccators contained saturated solutions of Lithium Chloride (Greenspan, 1977; Archer et al., 1998). A saturated solution of LiCl was made as described above.

A portion of rice flour spread onto a large Petri-dish was inoculated with an adequate volume of inoculum. Inoculation validation was performed and sorption isotherms (Brett et al., 2009) have been created. It was established that to produce inoculated samples at final a_w = 0.55, 1.35 mL of inoculum must be added to 50 ± 0.1 g portion of rice. For rice flour, the survival and heat inactivation experiments were conducted separately.

Six 50 ± 0.1 g portions of rice flour were spray-inoculated with 1.35 mL of inoculum, packed and sealed in EVOH bags then three 50 ± 0.1 g portions (three independent replicates) were stored at 15 ± 1°C and three at 25 ± 1°C. A further six 50 ± 0.1 g portions were inoculated and the water added during inoculation, was removed in a desiccator to obtain a required level of a_w = 0.20. Preliminary trials confirmed that a 50 ± 0.1 g portion of rice flour inoculated with 1.35 mL of inoculum and spread onto two large (140 mm) Petri dishes and held in a desiccator over a saturated solution of LiCl, would require 48 h at 30 ± 1°C to reach a_w = 0.20.

3.2.7 Heat resistance

Heat resistance of *Salmonella* strains in paprika powder and in rice flour was performed on the day of inoculation and after 6 weeks and 12 weeks of storage. Heat resistance was determined at three temperatures, in three independent replicates using the glass vial method. Glass vials (33-Expansion clear glass - USP Type 1 borosilicate, Class A; Verex) were filled with 1 ± 0.05 g of paprika powder or rice flour and inactivation trials performed as described by Peñaloza and Komitopoulou, (Peñaloza and Komitopoulou, 2012).

Survivors were recovered using the spread plate method as described above (2.2.7). Briefly, a sample (1 ± 0.05 g) of paprika powder or rice flour was mixed with 9 ± 0.1 mL of BPW (Buffered Peptone Water; Oxoid, UK) and surviving cells enumerated. Appropriate decimal dilutions in volumes of 100 µL were spread- plated on TSA incubated aerobically at 37°C for 48 h and colonies were counted. To achieve maximum recovery of all cells both healthy and injured, TSA was used instead of XLD (Xylose Lysine Deoxycholate agar; Oxoid, UK).

Each trial was conducted in three replicates, and for each replicate eight vials containing inoculated product were prepared and tested. With each trial, core temperatures of three control vials filled with tested product were measured using t-type thermocouples and logged using a DaqPro 5300 logger (Fourtec, USA). The temperature was measured during the come up time, during holding and during the cooling time. For each product, three treatment temperatures were selected and the z-values were calculated. For each temperature, time intervals were preselected based on preliminary trials in order to achieve 4-5 log cfu/g reductions of inoculated bacteria. Exceptionally when heat inactivation trials were performed on heat resistant strains at low temperatures; for example *S*. Typhimurium ST30 RpoS +ve at 75°C - inactivation treatment time was 150 min and calculated D_{75} = 83.1 min. In cases where initial inocula were low (below 5 logs due to poor storage survival, especially at a_w = 0.55 and at storage temperature T = 25°C) levels of inactivation between 3-4 logs cfu/g were accepted. During each trial, all vials were immersed at the same time in preheated oil using Grant (W28) thermostatic bath/circulator (Grant Instruments Ltd., UK); the circulator correction factor was set to give an accuracy of ± 0.05 °C. Instead of monitoring the inactivation kinetics during come up time (heating up time) and holding time the inactivation of *Salmonella* was always measured at the target temperature only. Therefore once core temperatures of the vials reached target temperature, a single vial from each replicate was removed, cooled down to approximately 25°C in ice water and the level of surviving bacteria were enumerated. This represented the initial number of bacteria (N_0). At each subsequent time interval, the next set (R1, R2 and R3) of vials was removed from the oil bath and cooled in ice water. Subsequently, the contents of vials (1 ± 0.05 g) were removed, mixed with 9 ± 0.1 mL BPW (Buffered Peptone Water; Oxoid, UK) and surviving cells (N_t) were enumerated. Appropriate decimal dilutions in volumes of 100 µL were spread-plated on TSA, incubated aerobically at 37°C for 48 h and colonies were counted. To achieve maximum recovery of all healthy and injured cells, TSA was used instead of XLD (Xylose Lysine Deoxycholate agar; Oxoid, UK). Chang et al. (Chang et al., 2010) showed that using non-selective TSA to recover heat treated cells resulted in a higher (over 1 log higher) recovery rate

compared to XLD as a recovery medium. Greater recovery of all cells was also confirmed during the recovery validation step at Leatherhead Food Research. Sporadically, several colonies were confirmed as *Salmonella* using API 20E. Survival curves were prepared for each trial ($f(t) = \log_{10}(N_t/N_0$; t - time, N_t - number of surviving bacteria at time t, N_0 - initial number of bacteria) and mean D-values (decimal reduction time) ± SD (Standard deviation) were calculated based on the three replicates. Then, for each replicate ($f(T) = \log_{10}D_T$) curves were prepared and z-values (increase of temperature required to decrease D-value by 10-fold) were calculated and expressed as a mean z-value ± SD.

3.2.8 Statistical analyses

All experiments were performed in triplicate. Mean values of D ± SD (Standard deviation) and z ± SD were calculated. The statistical significance ($p \leq 0.05$) of the differences in D-values and z-values amongst strains and products was tested using the Student's t test. In addition, statistical significance of the difference in D and z-values of strains tested at the beginning, middle and end of storage was calculated. All calculations were performed using Microsoft Excel 2000 software (Microsoft Corp., WA).

3.2.9 Surrogate validation

Enterococcus faecium NRRL B-2354 was selected as a primary strain for validation. *E. faecium* NRRL B-2354 is used by the Almond Board of California for almond process validations (Almond Board of California, 2014), and limited published data suggest that *E. faecium* NRRL B-2354 can be used as a *Salmonella* surrogate (Jeong et al., 2011). Heat resistance experiments were performed using this strain in paprika powder and rice flour by the methodology described above.

3.3 Results

Heat inactivation curves for both rice flour and paprika powder were linear and the first order kinetics equation was used. R^2 values for linear inactivation were between 0.95 and 0.99. Examples of the inactivation curves are presented on Figure 3.1. (A and B).

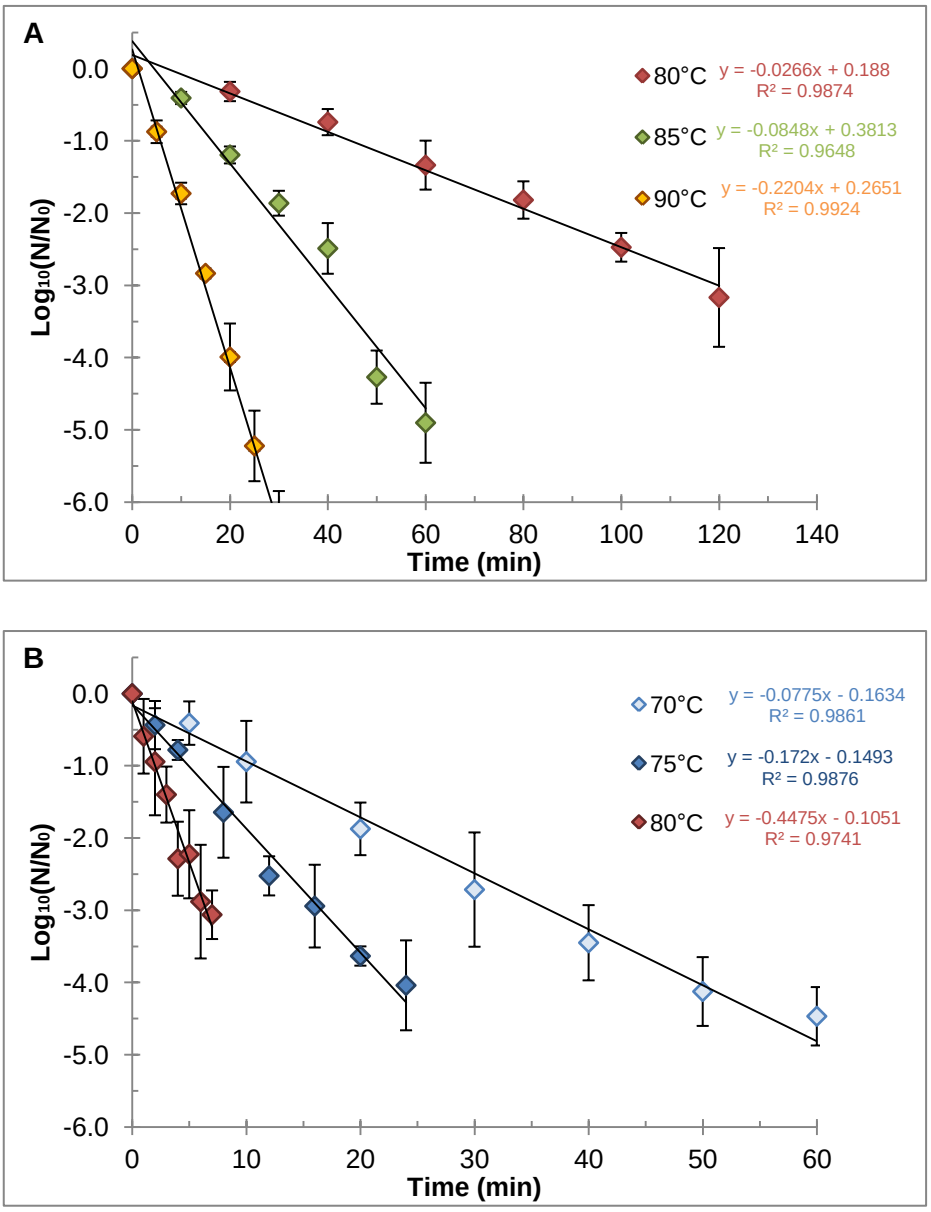

Fig. 3.1 (A, B) Linear inactivation curves; A) *E. faecium* in rice flour stored at 15°C (week 0) and B) *S.* Typhimurium ST30 RpoS +ve in paprika powder stored 15°C (week 12)

Linearity of inactivation curves may be explained by the fact that the thermal inactivation was only measured during holding time and the thermal inactivation during ramp time and cooling time was not measured. The starting point for all inactivation curves was the time when core temperature of a tested product had reached inactivation temperature. Inactivation temperatures were achieved after 5 and 5.5 min of heating in the oil bath. D-

values were calculated for each replicate and z-values calculated from three temperature points. All parameters were calculated in the beginning of storage, the middle (6 weeks) and the end of storage (12 weeks) but due to poor survival of salmonella in both powders at higher a_w (a_w = 0.55) and at higher storage temperature (T = 25°C), inactivation rates could not be measured (insufficient numbers of cells were enumerated at t = 0 following ramp time). Table 3.1 and Table 3.2 show calculated D and z-values for paprika powder and rice flour respectively. Results of this study show that heat resistance of *Salmonella* and *E. faecium* NRRL B-2354 in rice flour is significantly greater than in paprika powder. Higher resistance was recorded at lower a_w (a_w = 0.2 and a_w = 0.45 for rice flour and paprika powder respectively). Furthermore, while in rice flour at lower a_w (a_w = 0.2) differences between heat resistance at different storage temperatures (15 and 25°C) were not significant, whereas in paprika powder storage temperature had a significant impact on heat resistance.

In paprika powder, the most heat resistant strains were *E. faecium* NRRL B-2354, *S.* Typhimurium ST30 RpoS +ve and *S.* Enteritidis PT30. The most heat sensitive strain was also in this case, *S.* Typhimurium RpoS -ve which again confirms the importance of the *rpoS* gene in heat resistance. D-values for RpoS +ve *S.* Typhimurium are almost four times higher (D_{70} = 14.2 min) than D-values for RpoS -ve *S.* Typhimurium (D_{70} = 3.8 min). The RpoS -ve strain was also the most sensitive strain tested in this study.

In rice flour the most heat resistant strain tested was *S.* Typhimurium RpoS +ve and *E. faecium* NRRL B-2354. This showed that in this product and in these testing conditions the *E. faecium* NRRL B-2354 is not a suitable surrogate and cannot be used for thermal process validation. The most heat sensitive strain was *S.* Typhimurium RpoS -ve which confirms the importance of the RpoS gene in heat resistance. D-values for RpoS +ve *S.* Typhimurium are over 8 times higher (D_{75} = 83.1 min) than D-values for RpoS -ve *S.* Typhimurium (D_{75} = 10.1 min). The RpoS -ve strain is also the most sensitive strain tested in this study.

Overall, the heat resistance of *Salmonella* was much greater (over 4 times) in rice flour than in paprika powder. For example, D_{75} for *S.* Enteritidis in paprika powder (a_w = 0.55) at storage time T = 0 was D_{75} = 5.9 min but in rice flour D_{75}=26.5 min, D_{75} for *S.* Typhimurium in paprika powder (a_w = 0.55) at storage time T = 0 was D_{75} = 5.5 min but in rice flour D_{75} = 28.8 min or D_{80} for *E. faecium* NRRL B-2354 in paprika powder (a_w = 0.55) at storage time T = 0 was D_{80} = 2.07 min but in rice flour D_{80} = 9.3 min.

Strain	aw	Temp. (°C)	Time (week)	D values				z - value
Salmonella Enteritidis - PT 30 ATCC BAA-1045 (S108)				70°C	75°C	80°C		
	0.45	15	0	12.6 ± 0.3	5.9 ± 0.2	2.8 ± 0.2		15.4 ± 0.8
			6	10.2 ± 1.0	5.5 ± 0.3	2.9 ± 0.3		18.5 ± 2.6
			12	12.3 ± 0.5	5.9 ± 0.6	2.6 ± 0.4		14.8 ± 1.3
				p=0.009	p=0.369	p=0.528		p=0.093
		25	0	12.6 ± 0.3	5.9 ± 0.2	2.8 ± 0.2		15.4 ± 0.8
			6	11.4 ± 1.3	5.4 ± 0.4	1.8 ± 0.3		12.3 ± 2.1
				p=0.209	p=0.158	p=0.024		p=0.044
	0.55	15	0	9.5 ± 0.9	3.8 ± 0.2	2.3 ± 0.2		16.2 ± 0.7
			6	9.4 ± 1.7	4.9 ± 0.9	2.2 ± 0.2		15.8 ± 0.8
			12	7.9 ± 1.1	3.7 ± 0.4	2.1 ± 0.5		17.4 ± 3.7
				p=0.322	p=0.086	p=0.923		p=0.330
Salmonella Montevideo - (S110)				70°C	75°C	80°C		
	0.45	15	0	13.7 ± 0.4	6.7 ± 0.6	2.8 ± 0.3		14.5 ± 1.2
			6	12.1 ± 1.8	6.1 ± 0.7	3.0 ± 0.2		16.8 ± 1.2
			12	12.3 ± 0.2	6.3 ± 0.5	3.1 ± 0.2		16.8 ± 0.8
				p=0.215	p=0.535	p=0.290		p=0.061
	0.55	15	0	9.0 ± 0.5	4.3 ± 0.4	2.0 ± 0.1		15.2 ± 1.2
			6	8.8 ± 1.0	2.8 ± 0.2	2.4 ± 1.0		15.6 ± 3.3
			12	9.3 ± 2.8	2.8 ± 0.4	1.5 ± 0.6		12.6 ± 3.1
				p=0.939	p=0.002	p=0.264		p=0.181
Salmonella Napoli - (S111)				70°C	72°C	75°C	77°C	
	0.45	15	0	8.7 ± 0.8		4.0 ± 0.3	3.2 ± 0.2	15.8 ± 0.7
			6	7.0 ± 1.1		3.7 ± 0.6	3.2 ± 0.2	19.9 ± 6.0
			12	9.6 ± 2.7		6.9 ± 1.2	2.6 ± 1.2	15.9 ± 7.2
				p=0.165		p=0.005	p=0.604	p=0.435
	0.55	15	0	6.6 ± 0.0		2.6 ± 0.1	1.8 ± 0.1	12.5 ± 0.6
			6	5.6 ± 1.2	2.3 ± 0.2	1.2 ± 0.1		7.3 ± 0.9
			12	5.5 ± 2.2	4.3 ± 1.0	1.6 ± 0.1		9.9 ± 3.5
				p=0.730	p=0.051	p=0.000		p=0.051
Salmonella Senftenberg 775W - NCTC 9959 (S117)				65°C	70°C	72°C		
	0.45	15	0	14.1 ± 4.6	5.9 ± 0.7	4.4 ± 0.3		14.9 ± 4.2
			6	9.9 ± 0.6	3.7 ± 0.3	3.2 ± 0.3		13.7 ± 1.7
			12	8.8 ± 0.0	4.1 ± 0.3	2.7 ± 0.2		13.9 ± 1.0
				p=0.103	p=0.002	p=0.001		p=0.849
	0.55	15	0	9.0 ± 0.8	2.6 ± 0.1	2.0 ± 0.2		10.3 ± 0.9
			6	7.3 ± 0.8				
			12	7.7 ± 0.8				
				p=0.098				
Salmonella Typhimurium ST30; RpoS +ve (S1126)				70°C	75°C	80°C		
	0.45	15	0	14.1 ± 1.5	7.0 ± 0.6	2.9 ± 0.2		14.7 ± 1.3
			6	13.9 ± 1.4	6.0 ± 0.8	2.6 ± 0.7		13.9 ± 1.9
			12	12.9 ± 0.8	5.8 ± 0.5	2.2 ± 0.2		13.2 ± 0.6
				p=0.518	p=0.113	p=0.243		p=0.464
		25	0	14.1 ± 1.5	7.0 ± 0.6	2.9 ± 0.3		14.7 ± 1.3
			6	17.7 ± 2.6	4.6 ± 1.6	2.8 ± 0.5		13.9 ± 1.9
			12					
				p=0.107	p=0.068	p=0.658		p=0.183
	0.55	15	0	12.2 ± 0.7	5.6 ± 0.3	2.6 ± 0.2		15.0 ± 0.2
			6	9.4 ± 0.4	3.2 ± 0.1	2.2 ± 0.5		14.9 ± 1.8
			12	7.1 ± 0.9	3.2 ± 0.3	1.1 ± 0.1		12.2 ± 1.1
				p=0.000	p=0.000	p=0.002		p=0.050
Salmonella Typhimurium ST10; RpoS -ve; (S1130)				60°C	65°C	70°C		
	0.45	15	0	17.3 ± 1.9	9.3 ± 0.6	3.8 ± 0.8		15.5 ± 3.1
	0.55	15	0	13.2 ± 2.6	6.3 ± 0.2	3.3 ± 0.4		17.1 ± 3.7
Enterococcus faecium ATCC 8459 (CO1234)				70°C	75°C	80°C		
	0.45	15	0	18.6 ± 1.5	9.3 ± 0.6	2.7 ± 0.2		11.9 ± 0.9
	0.55	15	0	12.6 ± 0.6	4.8 ± 0.2	2.1 ± 0.1		12.7 ± 0.2

Table 3.1 D and z-values of Salmonellae and *E. faecium* NRRL B-2354 in paprika powder.

Strain	aw	Temp. (°C)	Time (week)	D values				z - value
Salmonella Enteritidis - PT 30 ATCC BAA-1045 (S108)				75°C	80°C	85°C		
	0.2	15	0	41.1 ± 5.0	24.1 ± 3.6	11.3 ± 1.0		18.3 ± 3.3
			6	60.0 ± 11.7	26.5 ± 3.9	10.7 ± 0.8		13.5 ± 1.0
			12	50.8 ± 5.7	23.7 ± 1.9	11.3 ± 0.9		15.4 ± 1.6
				p= 0.074	p= 0.569	p= 0.687		p= 0.096
		25	0	41.1 ± 5.0	24.1 ± 3.6	11.3 ± 1.0		18.3 ± 3.3
			6	58.1 ± 2.2	26.8 ± 1.9	12.5 ± 2.6		15.1 ± 2.3
			12	57.4 ± 1.3	25.9 ± 1.5	13.1 ± 1.4		15.6 ± 0.9
				p= 0.001	p= 0.470	p=0.506		p= 0.290
	0.55	15	0	26.5 ± 1.9	11.6 ± 1.3	3.7 ± 0.6		11.8 ± 1.1
			6	18.0 ± 2.6	8.8 ± 2.5	3.7 ± 0.7		14.6 ± 0.4
			12	23.1 ± 2.9	10.6 ± 4.0	6.5 ± 1.7		18.7 ± 4.8
				p= 0.016	p= 0.503	p=0.035		p= 0.063
Salmonella Typhimurium - ATCC 14028 (S123)				75°C	80°C	85°C		
	0.2	15	0	25.0 ± 1.8	12.6 ± 0.9	6.6 ± 1.0		17.3 ± 1.5
			6	24.7 ± 2.8	12.1 ± 0.6	6.3 ± 0.5		16.9 ± 1.3
			12	20.2 ± 1.6	11.3 ± 0.7	5.4 ± 0.6		19.3 ± 2.0
				p= 0.064	p= 0.196	p=0.183		p= 0.233
		25	0	25.0 ± 1.80	12.6 ± 0.9	6.6 ± 1.0		17.3 ± 1.5
			6	21.5 ± 0.53	12.2 ± 0.3	6.7 ± 0.6		19.7 ± 1.1
			12	19.1 ± 1.29	9.4 ± 1.0	5.4 ± 0.9		18.3 ± 2.7
				p= 0.005	p= 0.006	p=0.193		p= 0.383
				65°C	70°C	75°C		
	0.55	15	0	13.5 ± 0.79	7.9 ± 1.2	3.1 ± 0.4		15.7 ± 1.0
			6	3.6 Log red. during ramp time	4.3 Log red. during ramp time	4.6 Log red. during ramp time		
			12	<10^2 at the end of ramp time	<10^2 at the end of ramp time	<10^2 at the end of ramp time		
Salmonella Tennesse S778 (S1131)				75°C	80°C	85°C		
	0.2	15	0	43.5 ± 6.8	15.1 ± 1.0	9.6 ± 2.7		15.0 ± 1.0
			6	30.9 ± 2.6	17.1 ± 0.8	7.1 ± 0.7		15.6 ± 0.1
			12	29.5 ± 3.5	14.9 ± 1.5	7.4 ± 0.6		16.1 ± 2.1
				p= 0.098	p= 0.245	p=0.376		p=0.632
		25	0	43.5 ± 6.8	15.1 ± 1.0	9.6 ± 2.7		15.0 ± 1.0
			6	25.4 ± 1.7	13.6 ± 0.3	6.5 ± 0.5		16.5 ± 0.3
			12	26.0 ± 1.9	11.8 ± 2.0	6.2 ± 0.3		15.7 ± 0.5
				p= 0.037	p= 0.169	p=0.211		p=0.055
	0.55	15	0	11.2 ± 2.5	3.8 ± 0.7	2.9 ± 1.2		16.2 ± 0.6
			6	16.4 ± 8.5	5.7 ± 0.7	2.8 ± 0.2		11.5 ± 2.7
			12	15.1 ± 0.8	7.3 ± 4.5	2.8 ± 1.4		15.5 ± 2.3
				p= 0.864	p= 0.160	p=0.271		p=0.360
Salmonella Senftenberg 775W - NCTC 9959 (S117)				75°C	80°C	85°C		
	0.2	15	0	39.3 ± 3.6	19.1 ± 1.4			17.0 ± 1.1
			6	45.4 ± 3.6	17.4 ± 1.6	9.2 ± 0.8		14.4 ± 0.1
			12		16.6 ± 1.3	8.5 ± 0.7		15.4 ± 0.8
				p= 0.107	p= 0.175	p=0.320		p=0.018
		25	0	39.3 ± 3.6	19.1 ± 1.4			17.0 ± 1.1
			6	33.0 ± 3.2	13.5 ± 0.1	8.6 ± 1.1		17.1 ± 0.8
			12		15.0 ± 2.1	6.4 ± 0.5		14.4 ± 1.1
				p= 0.088	p= 0.008	p=0.037		p=0.027
				65°C	70°C	75°C		
	0.55	15	0	20.6 ± 1.0	14.1 ± 0.1	9.3 ± 2.5		30.5 ± 10.4
			6	21.5 ± 1.3	10.6 ± 0.6	8.0 ± 1.6		23.6 ± 4.8
			12	18.8 ± 5.2	12.7 ± 2.5	4.8 ± 1.0		17.1 ± 0.9
				p= 0.584	p= 0.073	p=0.054		p=0.121
Salmonella Typhimurium ST30; RpoS +ve (S1126)				80°C	85°C	90°C	95°C	
	0.2	15	0	45.7 ± 4.4	21.0 ± 1.0			16.7 ± 0.3
			6	43.0 ± 3.6	19.1 ± 2.2	8.7 ± 0.6		14.4 ± 0.1
			12		16.9 ± 1.0	8.5 ± 0.1	4.8 ± 0.5	18.4 ± 0.5
				p= 0.450	p= 0.043	p=0.700		p=0.000
		25	0	45.7 ± 4.4	21.0 ± 1.0			16.7 ± 0.3
			6	42.7 ± 2.1	17.2 ± 0.9	8.5 ± 0.5		14.3 ± 0.3
			12		16.6 ± 1.6	7.8 ± 1.0	4.8 ± 0.5	18.8 ± 3.0
				p= 0.336	p= 0.009	p=0.314		p=0.056
	0.55	15	0	75°C	80°C	85°C		
			6	28.8 ± 0.6	16.8 ± 1.3	8.2 ± 0.9		18.3 ± 1.9
			12	34.0 ± 1.7	14.2 ± 1.5	4.8 ± 0.3		11.8 ± 0.4
				36.4 ± 0.5	18.4 ± 1.0	5.8 ± 0.4		12.5 ± 0.4
				p= 0.000	p= 0.020	p=0.001		p=0.001
Salmonella Typhimurium ST10; RpoS -ve; (S1130)				65°C	70°C	75°C	80°C	
	0.2		0		22.2 ± 1.7	10.1 ± 1.1	5.6 ± 0.4	16.7 ± 0.8
	0.55		0	8.89 ± 2.12	3.65 Log red. during ramp time	6.44 Log red. during ramp time		
Enterococcus faecium ATCC 8459 (CO1234)				75°C	80°C	85°C	90°C	
	0.2	15	0		38.1 ± 4.8	11.8 ± 0.7	5.1 ± 0.2	11.5 ± 0.6
			6		29.8 ± 1.6	11.5 ± 0.9	4.5 ± 0.2	12.2 ± 0.4
			12		29.8 ± 1.2	10.9 ± 0.5	4.6 ± 0.2	12.3 ± 0.5
					p= 0.023	p= 0.382	p=0.031	p= 0.162
		25	0		38.1 ± 4.8	11.8 ± 0.7	5.1 ± 0.2	11.5 ± 0.6
			6		28.4 ± 1.3	10.7 ± 0.7	4.8 ± 0.2	13.0 ± 0.4
			12		26.5 ± 1.2	11.3 ± 0.5	4.4 ± 0.4	12.8 ± 0.4
					p= 0.006	p= 0.197	p=0.086	p= 0.012
	0.55	15	0		9.3 ± 0.1	3.4 ± 0.5	1.5 ± 0.1	12.8 ± 0.3
			6		12.5 ± 0.1	2.8 ± 0.4	1.5 ± 0.0	10.7 ± 0.1
			12	24.5 ± 1.3	9.6 ± 0.6	3.9 ± 0.1		12.6 ± 0.2
					p= 0.000	p= 0.033	p=0.146	p= 0.000

Table 3.2 D and z-values of Salmonellae and *E. faecium* NRRL B-2354 in rice flour.

Differences in D values during storage are shown in a set of figures: Fig 3.1 (A- G), Fig 3.2 (A-G), Fig 3.3 (A- G), Fig 3.4 (A-G) and Fig 3.5 (A-G). In most cases, heat resistance did not change during storage ($p > 0.05$) but in some cases the difference was significant, but no pattern or explanation was found. It is believed that sporadic significant differences in the heat resistance was caused by variation in heat penetration within the glass vials, as pressure within the glass vials was not controlled.

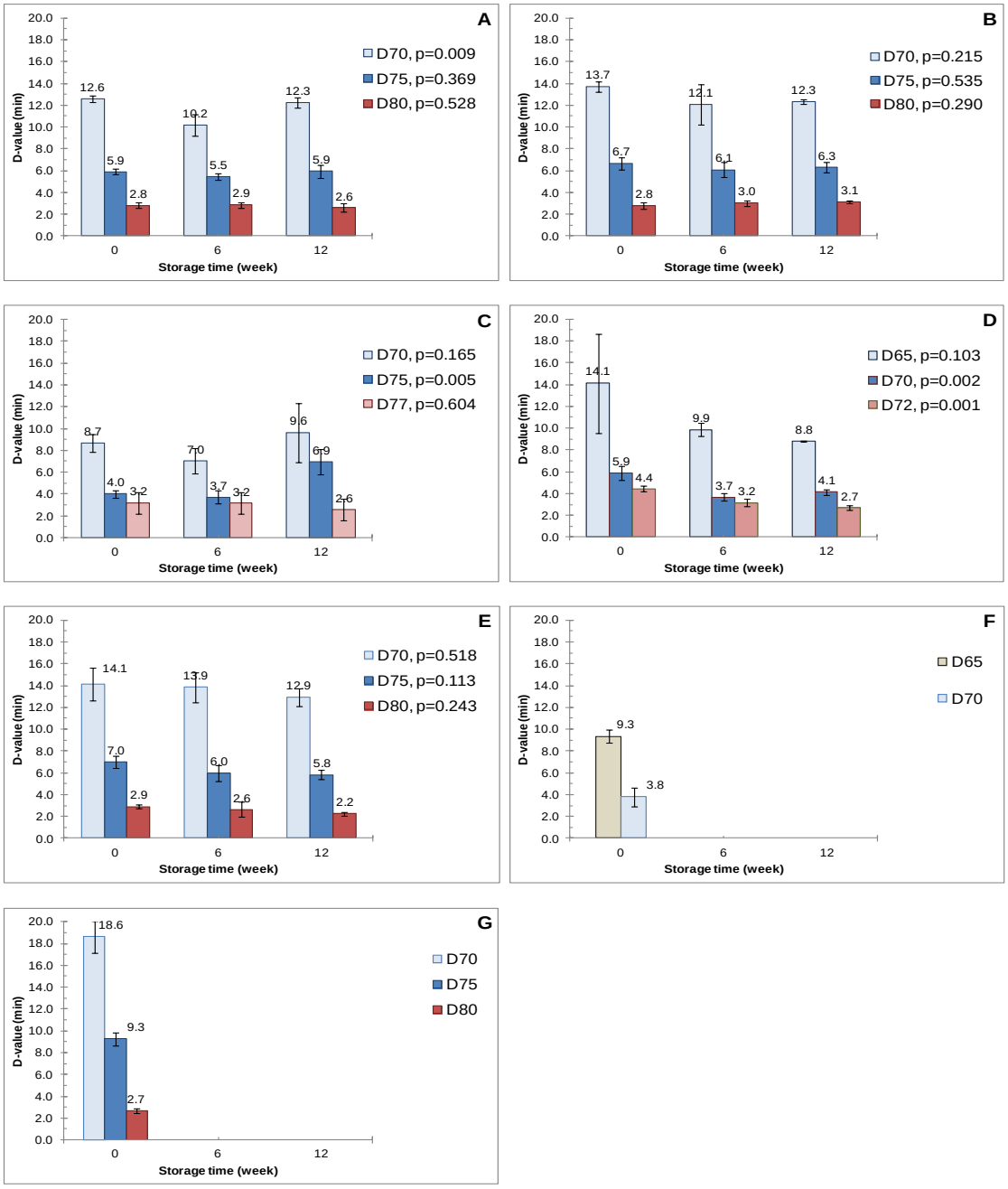

Fig. 3.2 (A-G) Heat resistance of Salmonellae and *E. faecium* NRRL B-2354 in paprika powder at a_w=0.45 and storage temperature T=15°C; A - *S.* Enteritidis PT 30, B - *S.* Montevideo, C - *S.* Napoli, D - *S.* Senftenberg 775W, E - *S.* Typhimurium ST30, RpoS +ve, F - *S.* Typhimurium ST10, RpoS -ve, G - *E. faecium* NRRL B-2354

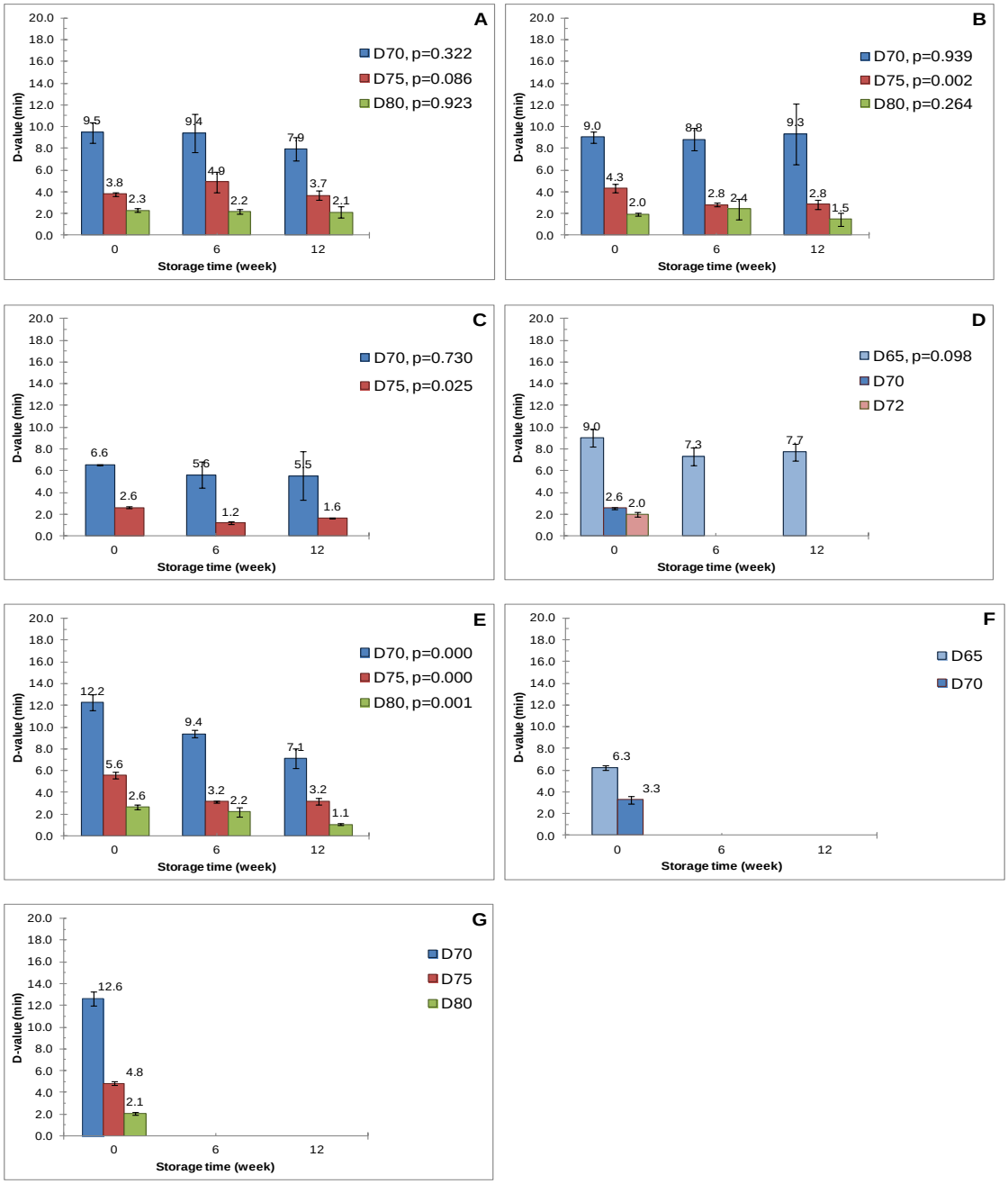

Fig. 3.3 (A-G) Heat resistance of Salmonellae and *E. faecium* NRRL B-2354 in paprika powder at a_w=0.55 and storage temperature T=15°C; A - *S.* Enteritidis PT 30, B - *S.* Montevideo, C - *S.* Napoli, D - *S.* Senftenberg 775W, E - *S.* Typhimurium ST30, RpoS +ve, F - *S.* Typhimurium ST10, RpoS -ve, G - *E. faecium* NRRL B-2354

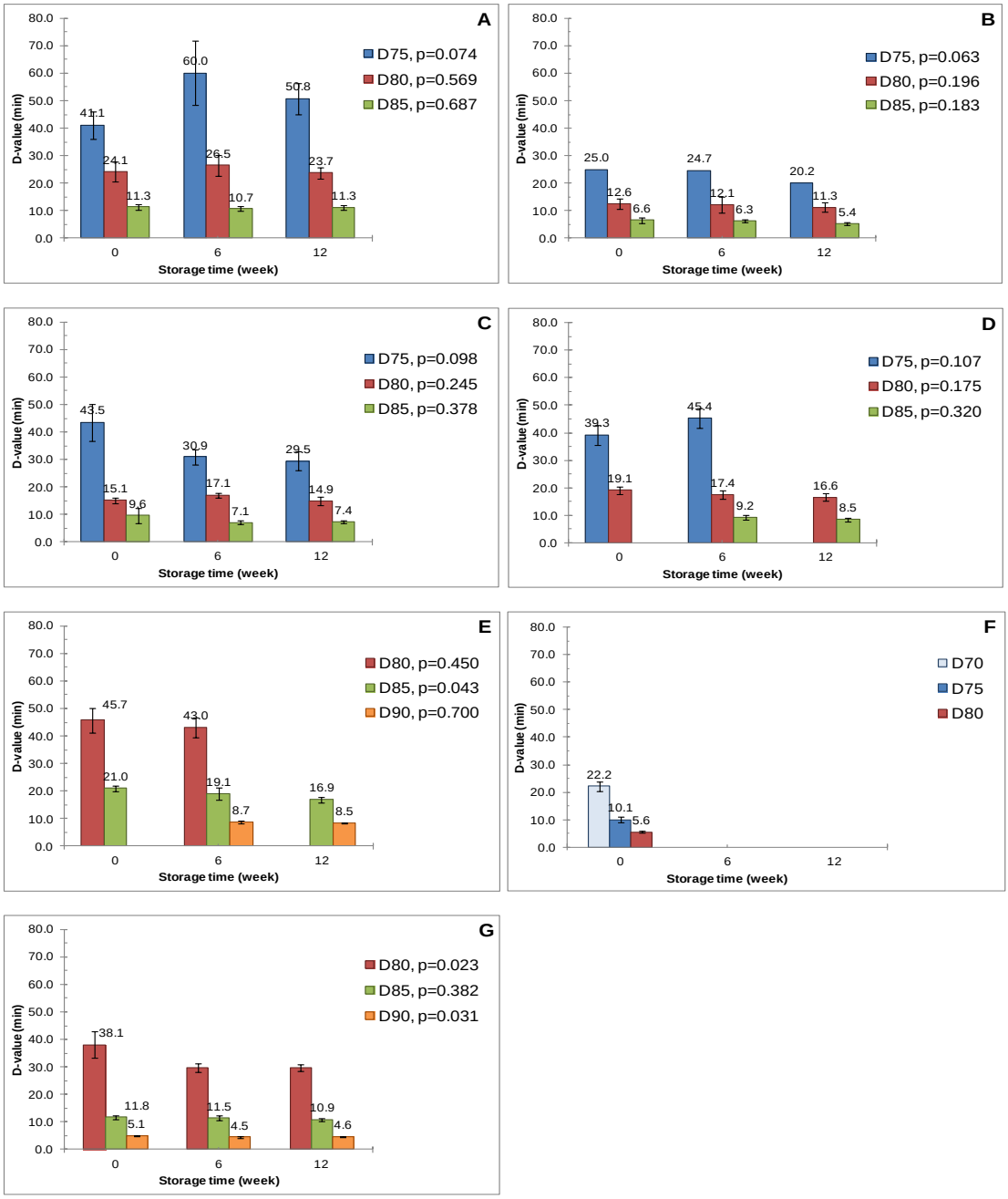

Fig. 3.4 (A-G) Heat resistance of Salmonellae and *E. faecium* NRRL B-2354 in rice flour at a_w=0.2 and storage temperature T=15°C; A - *S.* Enteritidis PT 30, B - *S.* Montevideo, C - *S.* Napoli, D - *S.* Senftenberg 775W, E - *S.* Typhimurium ST30, RpoS +ve, F - *S.* Typhimurium ST10, RpoS -ve, G - *E. faecium* NRRL B-2354

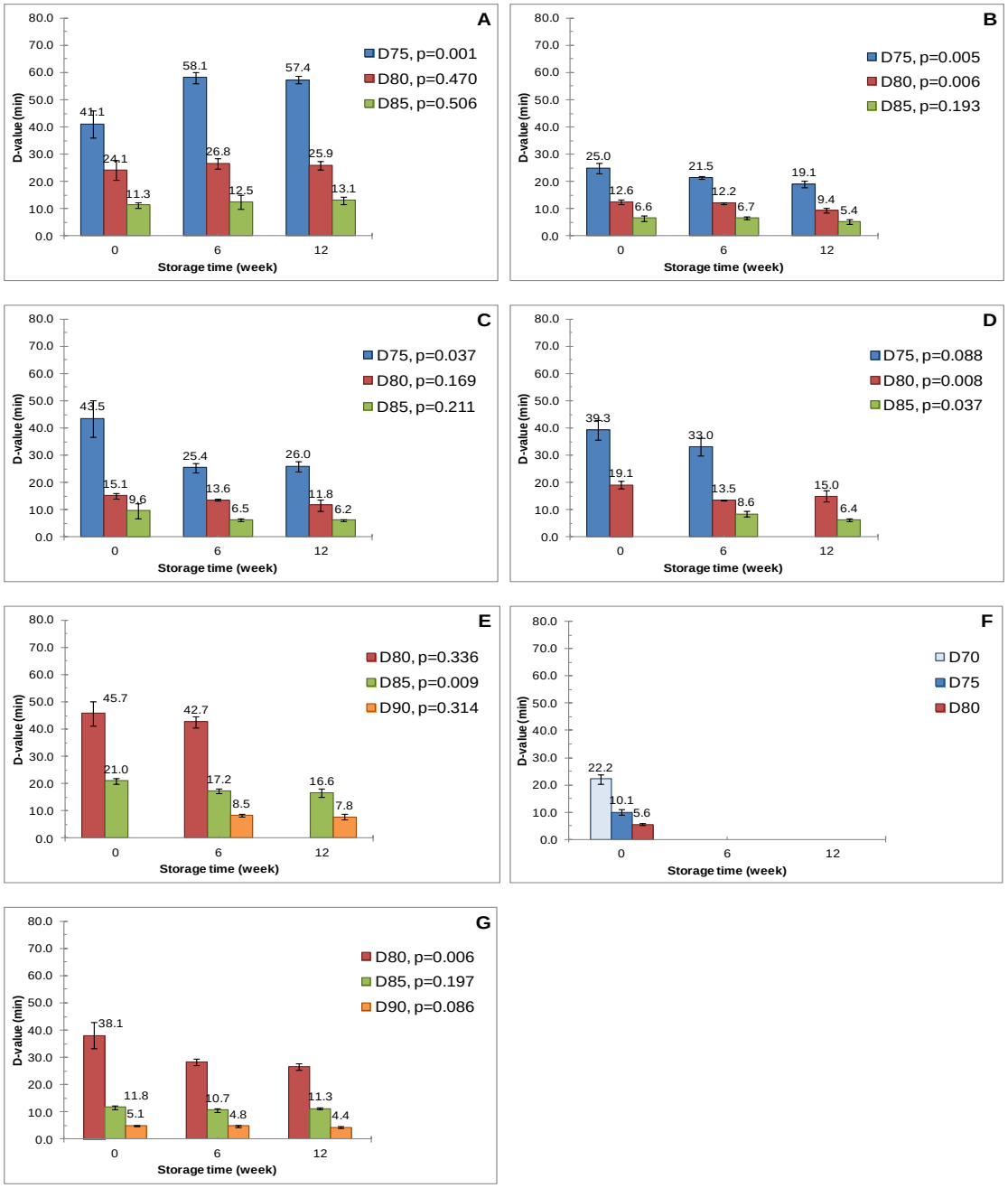

Fig. 3.5 (A-G) Heat resistance of Salmonellae and *E. faecium* NRRL B-2354 in rice flour at a_w=0.2 and storage temperature T=25°C; A - *S.* Enteritidis PT 30, B - *S.* Montevideo, C - *S.* Napoli, D - *S.* Senftenberg 775W, E - *S.* Typhimurium ST30, RpoS +ve, F - *S.* Typhimurium ST10, RpoS -ve, G - *E. faecium* NRRL B-2354

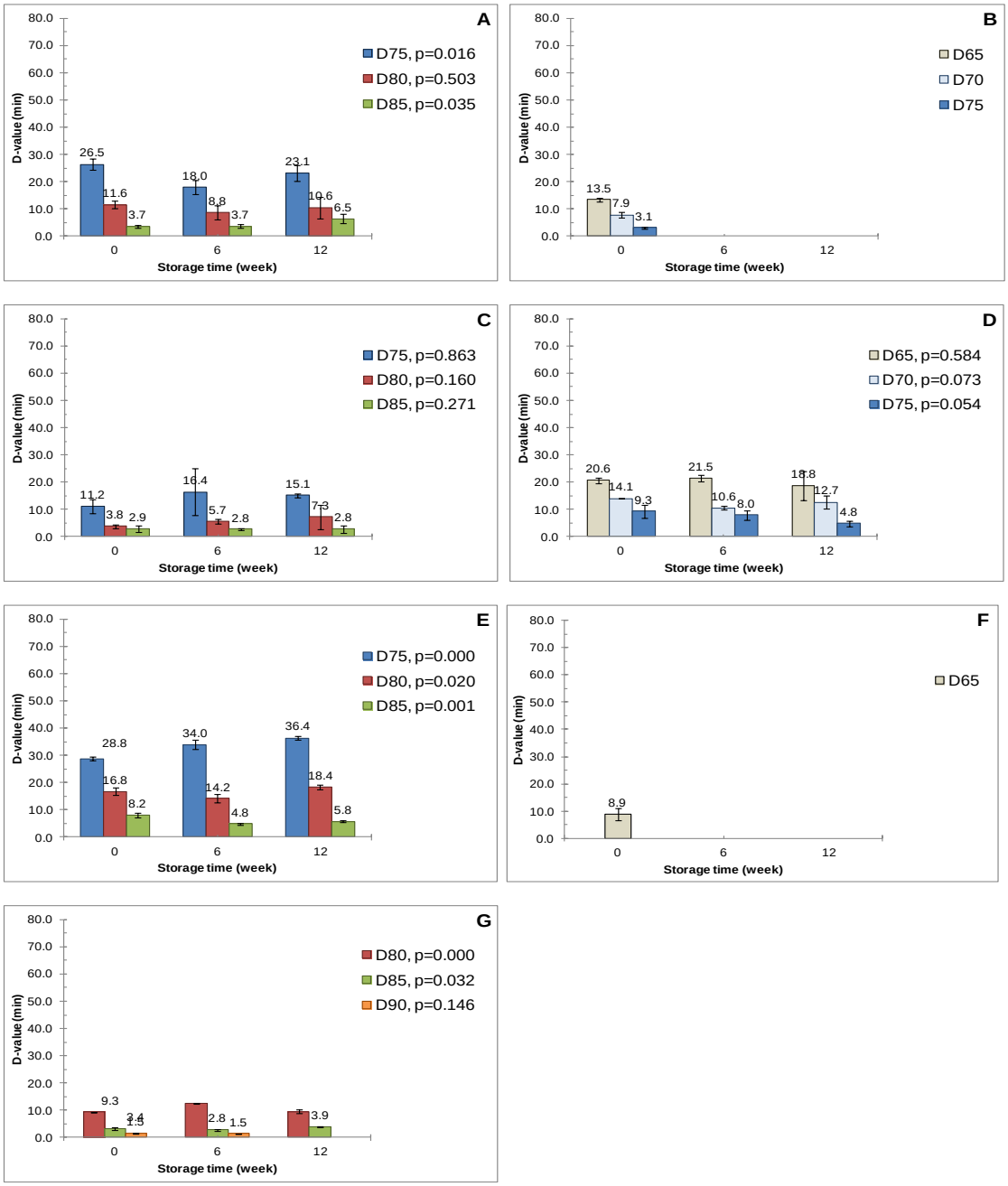

Fig. 3.6 (A-G) Heat resistance of Salmonellae and *E. faecium* NRRL B-2354 in rice flour at a_w=0.5 and storage temperature T=15°C; A - *S.* Enteritidis PT 30, B - *S.* Montevideo, C - *S.* Napoli, D - *S.* Senftenberg 775W, E - *S.* Typhimurium ST30, RpoS +ve, F - *S.* Typhimurium ST10, RpoS -ve, G - *E. faecium* NRRL B-2354

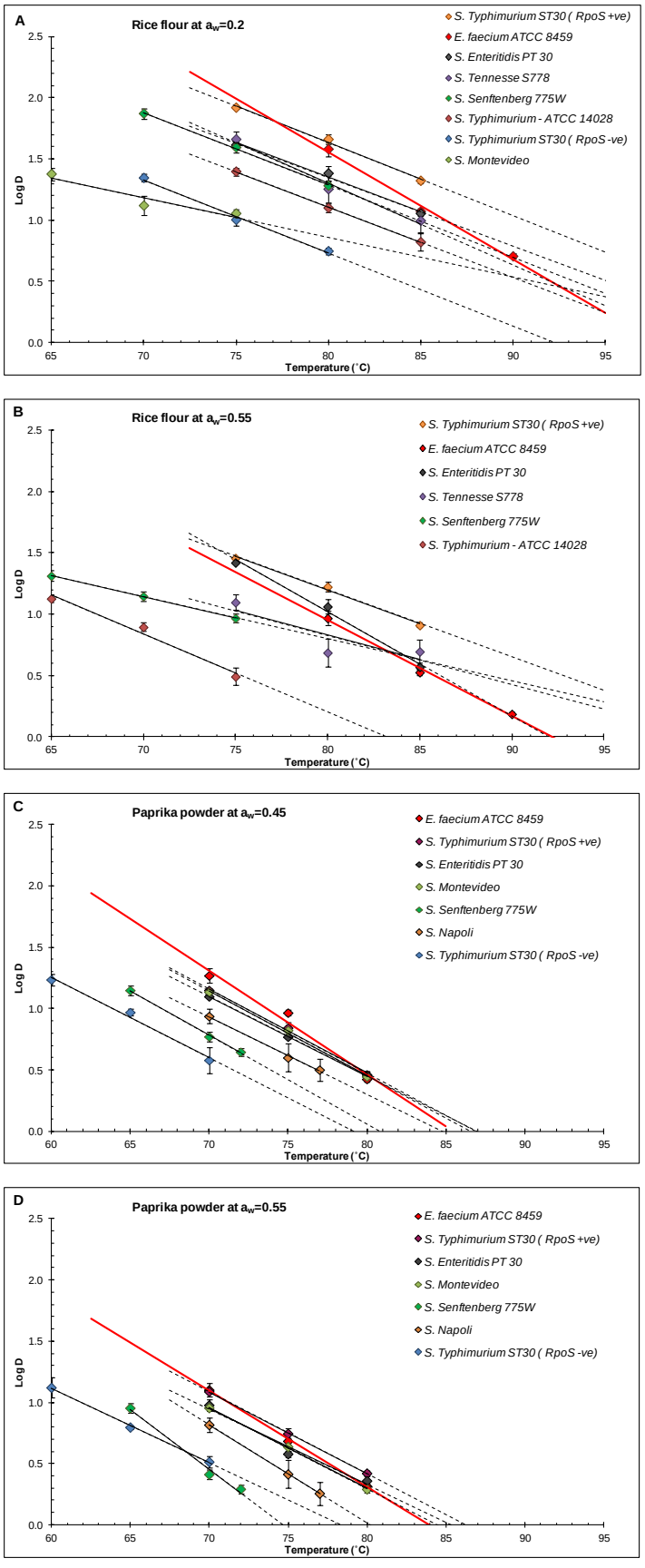

Fig. 3.7 (A-D) Comparison of *Salmonella* and *E. faecium* NRRL 2354 z-values in paprika powder and rice flour

Although the thermal resistance (D-value) of *E. faecium* NRRL B-2354 seems to be similar to the thermal resistance of the most resistant *Salmonella* strains, z-values calculated from inactivation curves ($f(T) = \log_{10}(D)$) shown in Fig 3.6, and Table 3.1 and 3.2, revealed that z-values are significantly different. The z-values for *E. faecium* NRRL B-2354 were between 10.7 and 13.0°C and for *Salmonella* between 10.3 and 30.5°C. Microorganisms with higher z-values (*Salmonella*) survive much better at higher temperatures than microorganisms with lower z-values (*E. faecium* NRRL B-2354) what is shown in Fig 3.6 A. In this circumstance *E. faecium* NRRL B-2354 was inactivated much faster than *Salmonella* at temperatures greater than *ca.* 77°C but at temperatures lower that *ca.* 77°C *E. faecium* NRRL B-2354 was more resistant than *Salmonella* spp. This indicates that in this product and in these conditions *E. faecium* NRRL B-2354 can only be used for process validation at lower temperatures (< 77°C). In contrast heat resistance of *E. faecium* NRRL B-2354 in rice flour at higher a_w (a_w = 0.55) is lower than heat resistance of two of the most resistant *Salmonella* strains and therefore *E. faecium* NRRL B-2354 cannot be used in this condition at any temperature. In paprika powder, however, *E. faecium* NRRL B-2354 can be used for process validation but this is dependent on the inactivation temperature. At a_w = 0.45 *E. faecium* NRRL B-2354 can be used at the temperatures < 80°C and at a_w = 0.55 at the temperatures < 70°C.

3.4 Discussion

In this work we have investigated the heat resistance of *Salmonella* in various low water activity products. Heat inactivation trials were performed using glass vials. Each of the glass vial was filled with one gram of the inoculated material and vials were capped with the plastic screw cup containing rubber seal. Although all the efforts were made to fill and close all vials the same way it was believed that same small variation in tightness of the vial cups may have influence on the results. Moreover vials were heated up in the oil bath and inactivation of microorganisms was measured from the moment at which the core temperature of the samples reached the target temperature (temperature of the oil bath). This mean that inactivation which has occurred during rump time (time required to heat core of the sample to the target temperature) was not measured. Therefore, starting point for each inactivation was slightly different. However, since all the inactivation curves were linear it was assumed that initial microbial load would not have significant impact on the inactivation results (D-values).

Results showed the significant differences in heat resistance between the different *Salmonella* serovars. For example, in rice flour, D-values of *S*. Typhimurium ST30 were almost twice greater than D-values of *S.* Enteritidis PT30 and almost four times greater than D-values of *S.* Typhimurium (ATCC 14028). The most heat-resistant *Salmonella* strains were: *S.* Enteritidis PT30, *S.* Typhimurium RpoS +ve, S. Montevideo and *S.* Tennessee and the most sensitive: *S.* Typhimurium RpoS -ve which was over eight times more sensitive than *S*. Typhimurium ST30 RpoS +ve. Variation of heat resistance of serovars is well documented. Podolak et al (2010), Goepfert and Biggie (1968) or Lee et al. (1989) have reported significant differences in the heat resistance of *Salmonella* in various low moisture foods. On the other hand, others (Shachar and Yaron, 2006) have shown no significant differences in heat resistance between *Salmonella* serovars. However, it is possible that some strains show similar heat resistance which might depend on the selection of the specific serovars used. Overall variability in heat resistance between *Salmonella* serovars is a well-documented characteristic (Sherry et al. 2004).

Furthermore, our work shows that heat resistance of *Salmonella* is dependent on the product composition and a_w. Archer et al., (1998), Barrile and Cone (1970), Garibaldi et al. (1969), Goepfert and Biggie, (1968), McDonough and Hargrove (1968), Peñaloza and Komitopoulou, (2012) or Van Cauwenberge et al. (1981) have shown that the a_w of products has a significant impact on the survival of bacteria during heat treatment. However, Li et al. (2014) showed that a_w was not the only factor affecting the heat

resistance. They showed that the highest heat resistance of *Salmonella* was in samples containing increased levels of carbohydrates and decreased levels of fat. These findings coincide with our results as heat resistance of *Salmonella* in rice flour which in general contains a higher percentage of carbohydrates (80%) was over four times greater than the heat resistance of *Salmonella* in paprika powder which in general has a lower concentration of carbohydrates (55%). The protective function of sugars has been previously well documented; Sumner et al. (1991) showed that heat resistance of *S.* Typhimurium and *L. monocytogenes* increased as sucrose concentration increased and a_w decreased. Mattick et al. (2001) also showed the great impact of sucrose and glucose-fructose solutions on heat resistance of *Salmonella*.

Our work also demonstrates that *S.* Typhimurium RpoS +ve strain was significantly more heat resistant than an RpoS -ve strain. The role of RpoS in the resistance to heat was extensively studied by various scientists. The increased heat resistance of the RpoS +ve strains was linked to heat-resistant proteins induced by the RpoS regulon under stress conditions (Dodd et al. 2002; Humphrey, 2004; Spector and Kenyon, 2012).

The present work also investigated that possibility that *E. faecium* could be used a surrogate for *Salmonella* in paprika powder. The results show that *E. faecium* was a suitable surrogate strain if used for process validation of paprika powder at low temperatures (< 80°C for $a_w = 0.45$ and <7 0°C for $a_w = 0.55$) but not suitable if used at higher temperatures. *E. faecium* NRRL B-2354 was also not suitable for rice flour validation as *Salmonella* strains were more resistant than *E. faecium* NRRL B-2354 at various conditions. Although *E. faecium* is considered as a suitable surrogate for *Salmonella* in process validation (Almond Board of California, 2014) many studies focus on low process temperatures (< 80°C). Ceylan and Bautista (2015) successfully validated *E. faecium* NRRL B-2354 against *Salmonella* cocktail in the low moisture pet food at temperature range 65.6 - 87.6°C. Bianchini et al. (2014) concluded that *E. faecium* NRRL B-2354 is a suitable surrogate for extrusion process, their data showed that in some cases the log reduction of *E. feacium* NRRL B-2354 at temperatures between 75°C and 80°C was greater than the log reduction of *Salmonella* at temperatures around 68°C. Furthermore, Jeong et al (2011) validated *E. faecium* NRRL B-2354 against *S.* Enteritidis PT 30 in almonds at high temperatures (121-204°C) but used moist-air convection heating and in a couple of occasions, *Salmonella* survived better than *E. faecium* NRRL B-2354.

Similarly as discussed in the Chapter 2 to our knowledge there is no published data for survival of *Salmonella* and *E. faecium* NRRL B-2354 in the paprika powder or rice flour. Furthermore, most of the studies have been conducted under different experimental conditions and therefore results are difficult to compare. The Experimental conditions for inoculum preparation, as well as the method of inoculation or storage conditions are usually different from study to study (Uesugi et al., 2006, Komitopoulou and Peñaloza, 2009; Peñaloza and Komitopoulou, 2012; Blessington et al., 2012; Archer et al., 1998; Shachar and Yaron, 2006; Torlak et al., 2013).

3.5 Conclusions

This study shown that the heat resistance of *Salmonella* and *E. faecium* NRRL B-2354 was significantly greater in rice flour than in paprika powder. It was shown that heat resistance of different *Salmonella* serovars was statistically significant in both powders and that at lower a_w heat resistance was significantly greater. The heat resistance didn't significantly change throughout the storage period. Finally, it was shown that using *E. faecium* NRRL B-2354 as a *Salmonella* surrogate has same limitations as z-values for *E. faecium* NRRL B-2354 were much lower than z-values for *Salmonella*.

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Chapter 4 - Inactivation of *Salmonella*, *Listeria monocytogenes* and *Enterococcus faecium*-NRRL B-2354 in a selection of low moisture foods

4.1 Introduction

Although low moisture foods cannot support microbial growth and are historically considered as 'low risk' in terms of pathogen contamination and no growth potential compared to high a_w animal- or vegetable-derived products, they significantly contribute to the total number of food-borne infections and outbreaks (Chen, et al., 2009; Podolak et al., 2010; Beuchat, et al., 2013). For example, it has been estimated that 1,000 people were infected by contaminated paprika present on potato chips in the 1993 outbreak in Germany (Lehmacher et al., 1995); over 400 cases (126 in 1981 and 283 in 2009) were associated with black pepper outbreaks (Gustavsen and Breen, 1984; Gieraltowski et al., 2013); and more than 200 cases were attributed to toasted oats cereal in the USA between April and June 1998 (Centers for Disease Control, 2001). Contaminated peanut butter was responsible for more than 400 cases in the USA between August 2006 and May 2007 (Centers for Disease Control, 2007), and again between 2008 and 2009 in 46 states resulting in a further 700 cases. It is generally recognised that many cases of food poisoning e.g. of salmonellosis, are unreported or not investigated, for all types of products; this in turn suggests that association of food-borne infections from dry ingredients, is much higher. According to RASFF (the Rapid Alert System for Food and Feed) a total of 477 notifications related to *Salmonella* in all types of food were recorded in 2014, of which 101 were related to low moisture foods – 21.2 %. In 2015, 517 notifications were recorded of which 116 were related to low moisture food – 22.4 %. Notifications related to *Listeria monocytogenes* are very much lower; 91 in 2014 with only two related to low moisture foods (butter and halva with pistachio nuts) and 99 in 2015 with three recorded notifications related to low moisture food (dry ham, sesame pasta and dried pork sausage).

The high percentage of *Salmonella* notifications in low moisture foods indicates that current methods of harvesting e.g. of seeds, drying and primary processing for control or elimination of *Salmonella*, are not efficacious or are not correctly implemented. Attention should therefore be focused on improving harvesting methods, and

evaluating the ability of pathogens to survive in low moisture foods both during storage and throughout processes. Appropriate and validated, processes and processing conditions should be developed and applied industrially, to control or eliminate food-borne pathogens in dry foods and ingredients for ready-to-eat products that are not heat treated before packaging and distribution. A number of studies relating to survival of pathogens in low moisture foods have been published, (e.g. Danyluk, et al., 2005; Uesegi, et al., 2006; Komitopoulou and Peñaloza, 2009; Blessington, et al., 2012), but the product range, product composition, storage conditions and heating methods differ; therefore obtaining data for specific products, organisms and conditions is necessary.

Although the number of cases of listeriosis is low, and those causally related to dry foods, very low, the infection can be life-threatening (20-30 % mortality). For this reason, the survival and heat resistance of *L. monocytogenes* in a selection of four dried foods was investigated. These data are necessary for evaluating potential hazards and taking data-based decisions in HACCP studies. The use of clinical strains is a prudent option as there seems to be some evidence that strains isolated from foods and food-processing environments tend to exhibit reduced virulence (Liu et al., 2007). While most publications show no limitations in using *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* (Almond Board of California, 2007, 2014; Jeong et al., 2011; Enache et al., 2015) other studies have shown some limitations of using this surrogate (Rachon and Gibbs, 2015).

Survival curves obtained during heat inactivation studies are not always linear. Non-linear curves are very common in both laboratory experiments and in pilot plant scale trials (Humpheson et al. 1998; Drosinos et al., 2006; Leguérinel et al. 2007). While for linear curves a first order kinetic model has been used and D- and z-values calculated, for non-linear curves, several different models have been proposed (Smith, 1991; Xiong et al. 1999; Juneja et al. 2001; Pasquali et al., 2016).

The current study was undertaken to obtain data and information on the viability of two important pathogens, *Salmonella* and *L. monocytogenes*, in four dried food materials of different compositions during storage for 21 days. In addition, the utility of a non-pathogenic organism – *E. faecium* NRRL B-2354 – as a surrogate for these pathogens for potential use in food processing environments was evaluated. Moreover , the heat resistance of the pathogens and surrogate in the four low moisture foods, was determined to evaluate the kinetics of inactivation to achieve a 5 log or greater inactivation levels.

The finding of this study was published in the International Journal of Food Microbiology in 2016. Copy of this publication is attached to this thesis in Appendix 1. Over 95 % of technical work associated with this project was conducted by myself.

Sporadic help from lab technician was required with a preparation of microbiological media and plating out. Both co-authors took part in the initial discussion when the scope of the project was drawn and acceptable criteria set. Subsequently, the project proposal was prepared by myself, checked by Dr Paul Gibbs and then accepted by Dr Walter Penaloza. It was estimated that total input of each co-author did not exceed 5% of the total time spent.

4.2 Materials and Methods

4.2.1 Low moisture foods

Four low moisture food formulations in powder form, were supplied by Nestec Ltd in sealed, flexible aluminised plastic pouches and stored at 16°C. The samples were decontaminated at 25-50 kGy for this study by an external private company and were delivered to Leatherhead Food Research.

The composition of the products and a_w before inoculation are shown in Table 4.1. In addition to the proximate composition, the confectionery formulation contained starch (35 %), sucrose (20 %), maltodextrin (20 %), wheat flour (20 %), and natural flavouring ingredients (5 %). The savoury seasoning contained salt (30 %), glutamate (30 %), sucrose (20%), rice flour, chicken meat, egg, spices. The chicken meat powder is an industrial raw material mix of chicken meat meal (85 %) and salt (15 %). The pet food formulation contained corn, rice, wheat flours (40 %), and protein-rich materials like corn gluten, soybean meal, fish meal (35 %), chicken by-product meal (20 %), mineral/vitamin premixes and natural flavouring (5 %).

Composition of the powders	Confectionery powder	Seasoning powder	Chicken meat powder	Pet food powder
Moisture (%)	8.36	8.95	3.63	10.94
a_w	0.434	0.648	0.235	0.576
Protein (N_2% x 6.25)	3	24.2	69.5	30
Fat (%)	1	1.2	25	6
Carbohydrate (%)	87.5	26	3	53.8

Table 4.1 Composition and a_w of low moisture foods

4.2.2 Bacterial strains

A cocktail of six *Salmonella* strains was used in this study: *S.* Enteritidis PT 30; ATCC BAA-1045 (a strain associated with the first recorded food-borne outbreak linked to consumption of raw almonds, USA/Canada, 2001), *S.* Senftenberg 775W; ATCC 43845 (heat resistant in moist foods), *S.* Typhimurium; ATCC 14028 (chicken isolate), *S.* Anatum; ATCC BAA-1592 (a strain isolated from a tomatoes linked outbreak in the USA, 2004), *S.* Montevideo; ATCC BAA-710 (tomato isolate), *S.* Tennessee; K4643 (a human isolate from the 2006 peanut butter outbreak in the United States). These

strains were selected for their survival above average among more than 30 strains in selected low moisture foods (data not shown). Selection was focused on the most frequently used strains with heat resistance above average, and strains from outbreaks linked to low moisture foods.

All strains were obtained from American Type Culture Collection (ATCC) except for *S.* Tennessee K4643 which was supplied by Nestec Ltd. All strains were recovered on Tryptone Soya Agar (TSA, Oxoid, UK) incubated aerobically for 18 h at 37 ± 0.5°C and a number of colonies (< 20) were dispersed in Cryo-preservation beads (TS/80-BL; TSC Ltd, UK) containing Cryopreservative fluid: beef extract, peptone, sodium chloride, glycerol (20 %), de-ionised Water. Three vials of each strain were prepared and stored at -70°C and used for preparing three independent replicates.

Preliminary screening of seventeen *L. monocytogenes* strains for ability to survive in low moisture foods identified five suitable strains with survival above average (data not shown). A cocktail of the five *L. monocytogenes* strains was used in this study: *L. monocytogenes* ATCC 15313 - 53 XXIII, DSMZ 20600 (serovar 1a, mammal isolate), *L. monocytogenes* ATCC 49594 – Petite Scott A (serovar 4b human isolate, the strain widely used as a reference strain for efficacy testing of food processing and preservation techniques, establishment of detection methods in foods, growth and heat resistance studies, and virulence studies, (Briers et al., 2011), *L. monocytogenes* ATCC 35152 – NCTC 7973 (serovar 1a, isolated from mammal), *L. monocytogenes* ATCC 13932 - LMG 21264 (isolated from child with meningitis, Germany; serotype 4b), DSMZ 27575 (serovar 4b, human isolate) and *L. monocytogenes* - FRRB 2542 (piezotolerant salami isolate). Strains were obtained from ATCC and Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and *L. monocytogenes* FRRB 2542 was supplied by Nestec Ltd. All strains were grown and stored at -70°C as described above.

A single strain of *Enterococcus* was used in this study: *E. faecium* NRRL B-2354 (ATCC 8459) - (strain most frequently used in heat inactivation studies as a surrogate for *Salmonella*). This strain was obtained from ATCC and grown and stored at -70°C as described above.

4.2.3 Inocula preparation

This study was conducted using a cocktail of *Salmonella* strains, a cocktail of *L. monocytogenes* strains and an *E. faecium* NRRL B-2354 inoculum. The *Salmonella* cocktail combined all 6 strains (grown as individual cultures); *L. monocytogenes*

cocktail combined all 5 strains (grown separately), and *E. faecium* NRRL B-2354 was used as a single strain inoculum. Previous studies have shown that cells prepared on lawns on agar plates are more resistant to heat than those prepared in broth (Uesugi et al. 2006; Komitopoulou and Peñaloza, 2009); the lawn plate technique described by Danyluk et al. (2005), Blessington et al. (2012) and the Almond Board of California (2014), was therefore adopted and used for preparation of both cocktails and the *E. faecium* NRRL B-2354 inoculum. Cocktails and *E. faecium* NRRL B-2354 inoculum were prepared as three independent trials. Strains were activated by inoculating 4 mL of Brain Heart Infusion broth (BHI, Oxoid Ltd, Basingstoke UK) with 1 frozen bead followed by incubation at 37°C for 18-24 h. A second culture was prepared by inoculating 4 mL of BHI with 0.1 mL of the first culture and incubating at 37°C for 18-24 h. The turbidity of each BHI broth culture after incubation provided a visual indication of adequate culture activation. An aliquot (0.5 mL) of the 10^{-2} dilution of each of the second cultures was spread onto separate plates of Tryptone Soya Agar (TSA, Oxoid) and incubated aerobically at 37°C for 24 h. After incubation, cell lawns were harvested as a slurry by gently scraping the agar surface with a sterile L-shaped plastic spreader and 2 mL of sterile Tryptone Salt diluent (TS). TS was prepared by mixing 1 g of Tryptone powder (Oxoid) and 8.5 g of NaCl in 1 L of deionized water and autoclaving for 15 min at 121°C. Equal volumes from each of the cell slurries were mixed into a cocktail, vortexed for 10 s and used within 30 min. The slurries were mixed prior to the direct inoculation of the 4 low moisture foods. A dry inoculum technique was not applied to avoid further reduction of initial viable counts. The initial source of low moisture food contamination incidents is mostly wet, and the equilibration or acclimatization time has been shown to be short in dry products (Smith and Marks 2015).

4.2.4 Sample inoculation

Prior to inoculation, each of the low moisture food (LMF) samples were mixed by hand massage in large stomacher bags and 3 x 100 g replicate samples were evenly spread within large Petri dishes (140 mm diameter). Samples were placed in a safety cabinet and inoculated with 1 mL (1 % v/w) of inoculum using needled syringes. Inoculation was conducted in two stages; first, 0.5 mL was distributed over the sample; the sample was carefully mixed and then the remaining 0.5 mL was added and mixed in the same way. Immediately after mixing, inoculated samples were sealed in stomacher bags and mixed by a vigorous external massage for 5 min in a safety cabinet until a lump free, homogenous mix was achieved. Inoculated samples were packed in aluminised plastic

pouches and stored at 16°C. The inoculation method and mixing to achieve a homogenous distribution, was validated by enumeration of viable counts in at least triplicate sub-samples of inoculated powders in preliminary trials (SD < 0.2 log CFU/g). Further confirmation of a homogenous distribution was obtained from enumeration of viable counts in food powders during storage (maximum SD < 0.25 log CFU/g).

4.2.5 Viability and changes in heat resistance during storage

The viability of *Salmonella*, and *L. monocytogenes* cocktails and *E. faecium* NRRL B-2354 was evaluated during storage up to 21 days at 16°C following one day of moisture equilibration. At each time point, 1 ± 0.01 g of each replicate was weighed and serially diluted in Tryptone Salt diluent. Aliquots (0.1 mL) of appropriate dilutions were spread on TSA plates. Plates were incubated aerobically at 37 ± 0.5°C and colonies counted after 48 h. Periodically, colonies were confirmed by streaking on appropriate selective agars (Xylose Lysine Deoxycholate agar - for *Salmonella* and Oxoid Chromogenic Listeria Agar for *Listeria*) or using API or Microgen *Listeria* confirmation kits to confirm that no contamination had occurred during inoculations and laboratory manipulations of the food powders.

In addition to evaluating viability during storage the heat tolerance at 80°C of the surviving population was evaluated. The temperature of 80°C was chosen after preliminary testing showed that inactivation levels of 2-4 log reduction would enable to quantitatively compare heat resistance changes between the selected storage times. At each time point, 1.2 ± 0.01 g of each replicate was weighed and placed into solid aluminium chambers (thermal cells) used for heat inactivation experiments. These thermal cells allow come-up times to shorten when heating powders. Some of the thermal cells had incorporated built-in platinum thermocouples (Pt 100) designed and supplied by the Nestlé Research Center (Lausanne, Switzerland). Samples (1.2 g) were packed into the thermal cells (0.8 mm deep, 48 mm diameter). In each trial, temperature profiling was conducted and the core temperature of samples was recorded using a data logger (PicoLog TC-08; St Neots, UK).

Temperature and time combinations used in this evaluation, were specific for the products and bacteria under investigation and it was expected that 2-4 log reductions would be achieved by the specific treatments. Changes of log reduction achieved at each time point would indicate if the heat resistance of bacteria was changing or remaining stable during storage of the inoculated samples.

After the heat treatment, viable counts were carried out on TSA plates incubated aerobically at 37 ± 0.5°C for 48 h for *Salmonella,* or 72 h for *L. monocytogenes* and *E. faecium* NRRL B-2354.

4.2.6 Inactivation during low moisture food heat treatments at various temperatures

The heat resistance of *Salmonella, L. monocytogenes* and *E. faecium* NRRL B-2354 was determined within the first week after inoculation following a minimum of 4 days of moisture equilibration at 16°C. Samples were weighed and placed into thermal cells and heat treated at four temperatures between 70 and 140°C depending on the product and organism under investigation. In the pet food, inactivation trials were performed at 70, 80, 90 and 100°C for all microorganisms. In the chicken meat powder and seasoning, inactivation trials were performed at 80, 90, 100 and 120°C for all microorganisms. In the confectionery, inactivation trials were performed at 80, 100, 120 and 140°C for *Salmonella*, at 70, 80, 90 and 100°C for *Listeria monocytogenes*, and at 80, 90, 100 and 120°C for *E. faecium* NRRL B-2354. Heat inactivation experiments were performed using an oil bath (BAT3930; Grant Instruments, Cambridge, UK) filled with thermal conducting oil (Technical oil, VWR, Lutterworth, UK). In each trial, a minimum of 5 log reductions was aimed for and achieved using at least five time points. Experiments were conducted using three independent replicates. In addition, each replicate was tested twice. Thermal cells containing inoculated material were submerged in the oil bath and held for the pre-selected times. Even though a circulating oil bath was used, preliminary trials indicated that times required to reach target temperatures were significantly decreased when additional oil circulation was introduced and therefore immersed thermal cells were moved back and forth at ~1 s intervals during the come-up time (~1.5 - 2 min). Immediately after removing thermal cells from the oil, samples were cooled in cold water to temperatures below 30°C within 30 s. Following heat treatment, powders were removed from thermal cells and serial decimal dilutions prepared using Tryptone Salt diluent. Viable counts were enumerated within 10 min of preparing serial dilutions and volumes of 0.5 and 0.1 mL of appropriate dilutions were spread on the surface of TSA plates and incubated aerobically at 37 ± 0.5°C for 48 h for *Salmonella* and 72 h for *L. monocytogenes* and *E. faecium* NRRL B-2354. After each heat trial, thermal cells and sealing rings were disinfected with Virkon (Day-Impex, Colchester, UK), washed twice using Greenline Plus - GP Mild Detergent and rinsed twice in sterile deionised water. The washed thermal cells were then dried in a drying cabinet at 55 ± 2°C for a minimum of 2 h.

Preliminary trials confirmed that these treatments were effective in removing the inoculated organisms and no sterilisation (autoclaving) was necessary.

4.2.7 Water activity (a_w) and moisture content

The a_w and moisture content of inoculated samples was measured at the beginning of the trial (after one day of equilibration) and at the end of the storage at day 21. Water activities of the powders were measured using an AquaLab Series 3, a_w meter (Decagon Devices, Inc. Pullman, USA) and two samples of each replicate were tested. Moisture content was measured using an Ohaus MB25 (Ohaus Corporation, Parsippany, USA) moisture tester at 133°C for 2 h.

4.2.8 Data analysis

For each of the three independent storage trials, viable counts data of *Salmonella,* and *L. monocytogenes* cocktails and *E. faecium* NRRL B-2354 during storage in the four products, were expressed as mean log values with standard deviations for each trial (SD). Changes in log values (Δ log) for each time point were compared to log values at day 0.

The heat resistance of *Salmonella, L. monocytogenes* cocktails and *E. faecium* NRRL B-2354 in the low moisture food samples at 80°C during the storage, was expressed as levels of inactivation (in mean log value) that was achieved at each sampling day (day 0, 3, 7 and 21) of the storage. The inactivation level was then calculated by subtracting the mean log value of viable counts after the heat treatment from the mean log value of non-heat treated samples. In addition, D_{80} value (from one time point data) at each storage time was calculated. Viable counts from replicate heat inactivation trials were also expressed as mean log+/-SD and calculated for each time point.

The heat inactivation curves of *Salmonella, L. monocytogenes* and *E. faecium* NRRL B-2354 in the low moisture food samples heated in thermal cells exposed to temperatures in the range of 70 - 140°C were used to calculate the heating time to reduce the initial population by 5 log. This time was calculated using the Weibull model fitting (Boekel, 2002): log (N/N_0) and expressed as a function of heating time (t) in the inactivation curves, where N = number of viable counts at time t, N_0 = initial number of viable counts before heating. The following Weibull distribution equation was used to fit survival curves:

$$\log\left(\frac{N}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta}$$

Where: **t** - time (min)

α - scale parameter (a characteristic time)

β - shape parameter

The Weibull distribution corresponds to a concave upward survival curve if $\beta < 1$, concave downward if $\beta > 1$ and reduces to an exponential (linear) distribution if $\beta = 1$. Parameters α and β were estimated using Excel equation solver and GRG (Generalized Reduced Gradient) Nonlinear Solving Method. Fitting of the model to raw data was confirmed by conducting an F-test using Excel (Microsoft Office; Drosinos et al. 2006) and R^2 (Brown, 2001). Parameters α and β were estimated for each replicate and mean values and standard deviations calculated.

The time required to reduce the initial population of pathogens by 5 log (5D - as generally applied in the food industry) was calculated using the equation below (Van Boekel, 2002):

$$t_D = \alpha(-\ln(10^{-d})^{\frac{1}{\beta}})$$

Where:

t_D - time required to achieve required log reduction (min)

d – number of required decimal log reductions (i.e. 5D = 5)

α and β – parameters as described above

Time t_D was calculated for each replicate separately and the average value (mean) and standard deviation (SD) was calculated.

In addition, the standard log-linear model was fitted to the data of product at temperature higher than 70°C. The D values and time required to reduce the initial population of pathogens by 5 log were calculated. Times calculated from Weibull model and D values were then compared.

4.3 Results

4.3.1 Microbial viability during storage of low moisture foods

4.3.1.1 a_w and moisture changes

During the inoculation step, 1 % v/w of inoculum slurry was introduced to samples. This significantly changed a_w of samples. The a_w of samples increased from 0.434 to 0.565 for confectionery, 0.648 to 0.655 for seasoning, and 0.235 to 0.383 for chicken meat powder and from 0.576 to 0.653 for pet food. Although this increase is statistically significant when calculated using the a_w values, only 1ml of inoculum was added to 100 g of sample and the measured moisture increased by an average of 0.82 % of moisture in all samples. The moisture content of confectionery powder increased from 8.36 to 9.14 %, seasoning powder from 8.95 to 9.84 %, chicken meat powder from 3.63 to 4.69 % and pet food powder from 10.94 to 11.49 %. The only small increase of a_w in seasoning can be explained by the fact that the water introduced in the inoculum quickly reacted with the salt (salt concentration in seasoning was in a range of 20-30 %) and, therefore, the a_w did not change significantly.

During storage (21 days at 16°C), inoculated samples showed small but statistically significant differences ($p < 0.05$) of a_w and moisture content. Maximum recorded changes of a_w were; Δ a_w = -0.040 and maximum changes of moisture were; Δ Moisture content = -0.61 %. Those changes were expected and no action was taken to stop them as it was believed that some changes may occur during storage and preventing them (storage in desiccators with adjusted humidity) would not be representative for ordinary storage.

4.3.1.2 Viability of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 during storage

Salmonella, *L. monocytogenes* and *E. faecium* NRRL B-2354 survived within the same log level during the 21 day storage at 16°C in the inoculated low moisture foods (Fig.4.1A – 4A). The *Salmonella* viable counts were significantly lower ($p < 0.01$) in all foods at the end of storage. However, the largest reductions observed were only 0.5 and 0.4 log in confectionery and pet food respectively. Normally, differences in viable cell counts of < 0.5 log are generally regarded as non-significant in microbiological

analysis (ISO 4833:2013). *Salmonella* viable counts at day 3 or 7 remained statistically ($p > 0.05$) within the same levels of inoculation.

The viable counts of *L. monocytogenes* progressively decreased significantly ($p < 0.05$) and over storage while the largest reduction of 0.8 log was observed in the confectionery formulation by day 21. Reductions by day 3 and 7 in confectionery and pet food were similar and below 0.4 log. Surprisingly in culinary seasoning, viable counts remained stable over storage ($p = 0.317$, $p = 0.580$ and $p = 0.094$ for 3 d, 7 d and 21 d respectively compared to 0 d).

E. faecium NRRL B-2354 viable counts remained stable over storage ($p > 0.05$) and its decrease was lower than 0.2 log amongst all products tested, indicating that this strain was a suitable fail-safe indicator for *Salmonella* or *L. monocytogenes* viability in low moisture foods upon storage before processing.

Despite the fact that the culinary seasoning a_w ($a_w = 0.655$) after inoculation was the highest, *Salmonella* and *L. monocytogenes* survived notably better than in the other three products. In the culinary seasoning, only 0.2 log reduction was recorded for *Salmonella* and 0.1 log for *L. monocytogenes*.

The survival of *Salmonella* and *L. monocytogenes* in culinary seasoning and chicken meat powder was slightly greater than in confectionery and pet food formulations during storage for 21 days at 16°C.

The results indicate that microbial viability during storage in dried foods depends on the particular organism and can vary both with product composition and time. The largest differences in viability were observed for *Salmonella* < 0.54 log and *L. monocytogenes* < 0.8 log. Although those differences were statistically significant ($p < 0.01$), they were considered as a small change when comparing to initial high level of inoculum. Furthermore, those small differences would leave practically no impact on storage before processing of dry raw materials. Initial contamination levels were expected to remain at the same log level.

The microbial viability within seven days of inoculation and storage at 16°C remained statistically ($p > 0.01$) at the same level (decrease < 0.4 log). Thus, short storage of maximum seven days of inoculated foods was adopted for the heat inactivation tests in this study.

4.3.1.3 D_{80} of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 during storage in low moisture foods

Product	Time (day)	*Salmonella* Δ log ± SD	D_{80}	*Listeria monocytogenes* Δ log ± SD	D_{80}	*E.faecium* NRRL B2354 Δ log ± SD	D_{80}
Confectionery		80°C / 20 min		80°C / 2 min		80°C / 20 min	
aw = 0.565	0	-3.3 ± 0.5	6.15	-2.6 ± 0.2	0.77	-4.4 ± 0.1	4.59
	3	-2.9 ± 0.5	6.97	-2.2 ± 0.3	0.91	-4.3 ± 0.1	4.70
	7	-2.8 ± 0.3	7.07	-2.3 ± 0.1	0.89	-4.3 ± 0.1	4.68
	21	-3.0 ± 0.3	6.69	-1.7 ± 0.1	1.17	-4.4 ± 0.0	4.52
		$p = 0.628$		$p = 0.006$		$p = 0.179$	
Culinary		80°C / 5 min		80°C / 5 min		80°C / 25 min	
aw = 0.655	0	-2.7 ± 0.1	1.85	-2.5 ± 0.1	2.25	-2.9 ± 0.1	8.66
	3	-2.9 ± 0.3	1.71	-2.8 ± 0.1	1.80	-2.9 ± 0.1	8.52
	7	-2.4 ± 0.1	2.05	-3.0 ± 0.0	1.66	-3.2 ± 0.0	7.78
	21	-2.8 ± 0.1	1.77	-3.4 ± 0.1	1.49	-3.3 ± 0.1	7.63
		$p = 0.015$		$p < 0.001$		$p = 0.126$	
Chicken meat powder		80°C / 20 min		80°C / 5 min		80°C / 60 min	
aw = 0.383	0	-2.2 ± 0.1	8.93	-2.6 ± 0.1	1.95	-2.5 ± 0.2	23.75
	3	-2.5 ± 0.1	8.03	-2.2 ± 0.1	2.31	-2.7 ± 0.2	22.62
	7	-2.4 ± 0.0	8.30	-2.6 ± 0.1	1.92	-2.7 ± 0.2	21.88
	21	-2.6 ± 0.1	7.79	-2.8 ± 0.2	1.82	-2.3 ± 0.1	25.71
		$p = 0.039$		$p = 0.029$		$p = 0.120$	
Pet food		80°C / 2 min		80°C / 2 min		80°C / 25min	
aw = 0.653	0	-2.9 ± 0.2	0.67	-3.1 ± 0.3	0.64	-2.9 ± 0.1	1.72
	3	-2.7 ± 0.1	0.74	-3.0 ± 0.5	0.66	-2.4 ± 0.1	2.06
	7	-2.8 ± 0.1	0.71	-3.3 ± 0.1	0.62	-2.6 ± 0.1	1.95
	21	-2.7 ± 0.1	0.75	-3.6 ± 0.2	0.56	-2.8 ± 0.2	1.79
		$p = 0.148$		$p = 0.211$		$p = 0.006$	

Table 4.2 Changes in heat tolerance of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 in all products during storage; Δlog ±SD - logarithmic change of counts (and standard deviation) achieved by heat treatment at 80°C, D_{80}- decimal reduction time at 80°C, *p*-value representing statistical significance of difference

The temperature (80°C) was selected from preliminary tests to inactivate only a fraction of the microbial population and obtain quantitative results to compare heat resistance over time after inoculation and therefore determine the maximum keeping time at 16°C. The microbial inactivation in each product fluctuated randomly within a narrow range of variability (< 0.5 log) between the different storage times (Table 4.2), and no significant correlation of D_{80} value over time was observed, except for *L. monocytogenes* D_{80} increase ($p < 0.01$) from 0.77 to 1.17 min in the confectionery formulation and a significant decrease ($p < 0.01$) from 2.25 to 1.49 min in the culinary seasoning. These differences are of relatively minor relevance, and are similar to the standard deviation of the D_{80} values at other experimental conditions.

The mean D_{80} values of *Salmonella* were greater in the chicken meat powder (8.3 ± 0.4 min) and confectionery formulation (6.8 ± 0.5 min) compared to culinary seasoning (1.8 ± 0.2 min) and pet food (0.71 ± 0.04 min). The D_{80} values of *L. monocytogenes*

were greater in the culinary seasoning (2.06 ± 0.072 min) and chicken meat powder (2.0 ± 0.17 min) than in confectionery (0.94 ± 0.14 min) and pet food (0.62 ± 0.04 min). *Salmonella* had a greater heat resistance (D_{80} values) than *L. monocytogenes* in the low moisture foods tested, except in the culinary seasoning where both D_{80} values were in the same range. The D_{80} values of the *E. faecium* NRRL B-2354 exceeded by approximately 3 to 4 times those of *Salmonella* in chicken meat powder, culinary seasoning and pet food. However, its D_{80} value in the confectionery formulation (4.62 ± 0.07 min) was lower than *Salmonella* (Table 4.1).

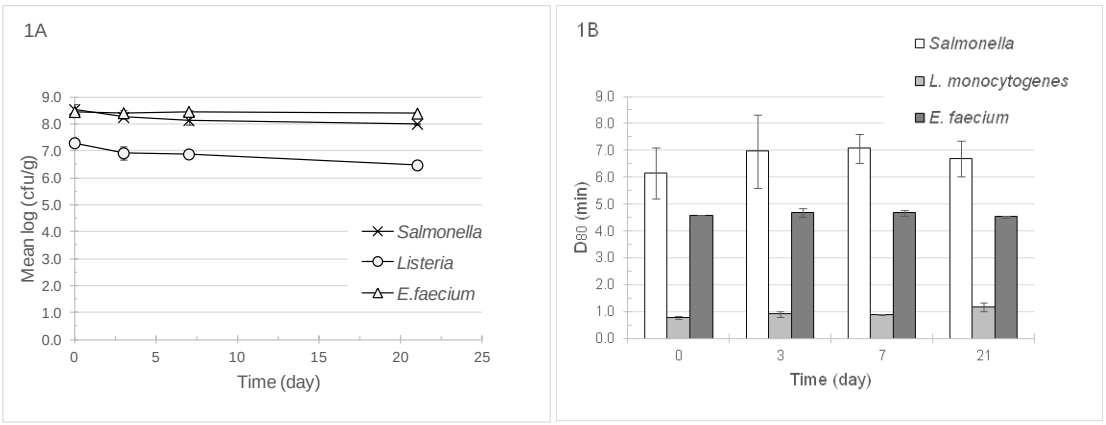

Fig. 4.1 Viability (1A) and heat resistance (1B) - (D_{80}) of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 in confectionery during storage

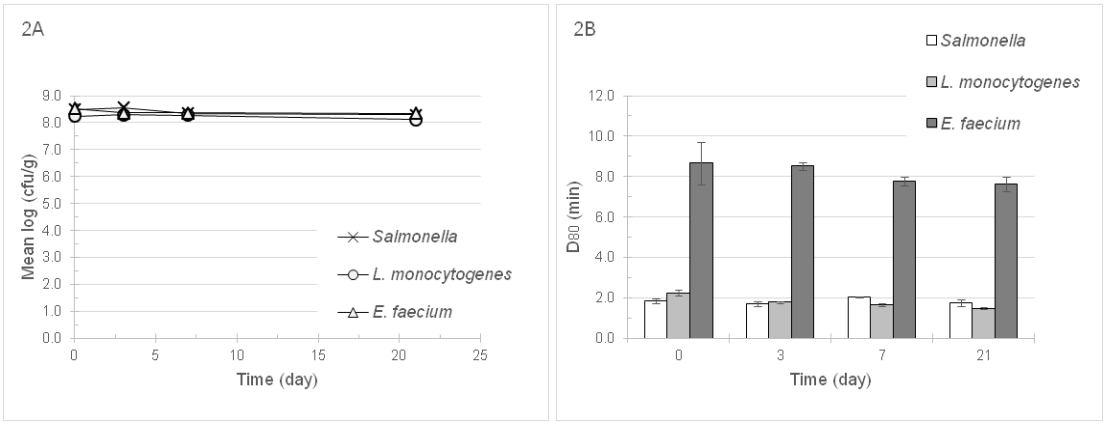

Fig. 4.2 Viability (2A) and heat resistance (2B) - (D_{80}) of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 in culinary seasoning during storage

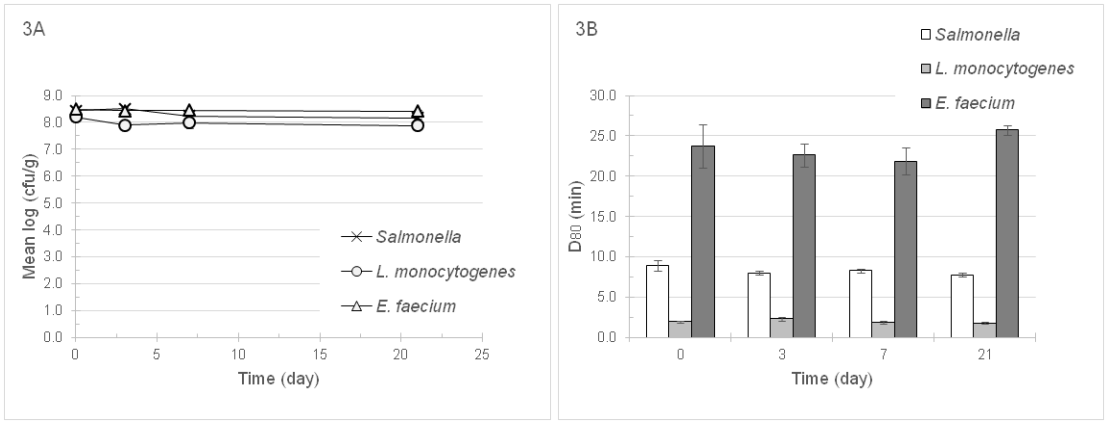

Fig. 4.3 Viability (3A) and heat resistance (3B) - (D_{80}) of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 in chicken meat powder during storage

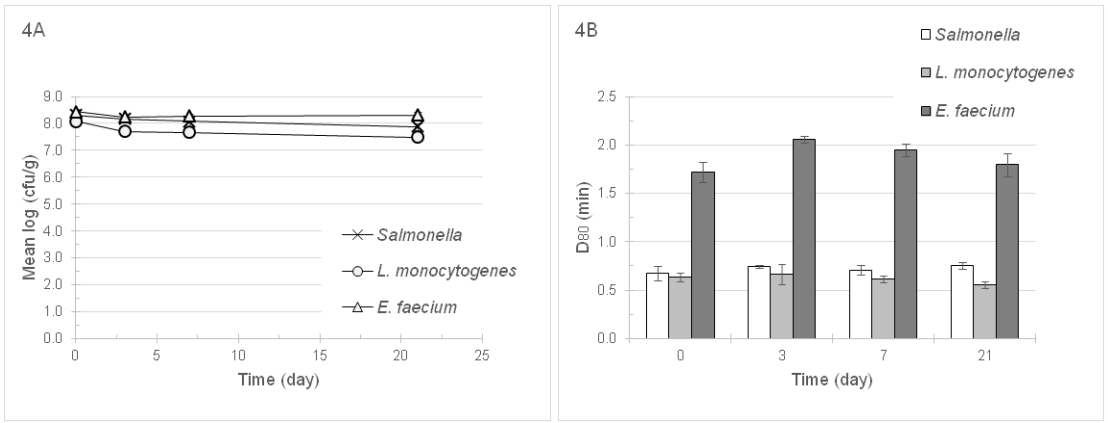

Fig. 4.4 Viability (4A) and Heat resistance (4B) - (D_{80}) of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 in pet food during storage

4.3.2 Inactivation during heat treatments at various temperatures

The results showed that most of the microbial inactivation curves were log linear. However, a number of concave upward inactivation curves were observed and also a number of concave downward curves. Three examples of data fitting with the Weibull model to raw data are presented below (Figure 4.5):

Knowing the parameters β and α, the heating time to achieve a 5 log reduction of each organism was calculated from the following equation:

$$t_D = \alpha(-\ln(10^{-d})^{\frac{1}{\beta}})$$

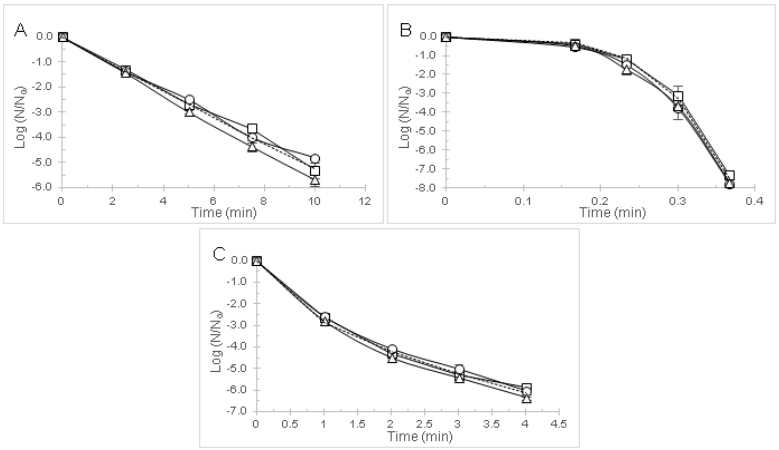

Fig. 4.5 Examples of inactivation curves and fitting of Weibull Model. A; linear curve (*Salmonella* in seasoning at 80˚C), B; downward concave (*Salmonella* in seasoning at 120˚C), C; upward concave (*Salmonella* in confectionery at 100˚C). Replicate 1 (□), Replicate 2 (○), Replicate 3 (Δ) and (---) Weibull Model. Examples of inactivation curves and fitting of Weibull Model. A; linear curve, B; downward concave, C; upward concave

Product	Temp. (°C)	*Salmonella*			*Listeria monocytogenes*			*E.faecium* NRRL B2354		
		α Mean ± SD	*β* Mean ± SD	R^2	*α* Mean ± SD	*β* Mean ± SD	R^2	*α* Mean ± SD	*β* Mean ± SD	R^2
Confectionery	70				0.106 ± 0.043	0.393 ± 0.017	0.997			
aw=0.565	80	4.750 ± 1.233	1.136 ± 0.110	0.996	0.086 ± 0.037	0.533 ± 0.031	0.993	0.280 ± 0.035	0.544 ± 0.011	0.981
	90				0.173 ± 0.040	1.073 ± 0.099	0.998	0.142 ± 0.021	0.703 ± 0.027	0.995
	100	0.109 ± 0.036	0.837 ± 0.070	0.998	0.140 ± 0.010	1.406 ± 0.047	0.999	0.246 ± 0.019	1.673 ± 0.076	0.993
	120	0.309 ± 0.015	4.055 ± 0.281	0.990				0.211 ± 0.026	3.496 ± 0.499	0.958
	140	0.260 ± 0.001	7.333 ± 0.299	0.989						
Culinary	80	0.763 ± 0.078	0.970 ± 0.055	1.000	0.338 ± 0.068	0.687 ± 0.044	0.997	7.272 ± 0.312	1.545 ± 0.030	0.996
aw=0.655	90	0.278 ± 0.015	1.451 ± 0.064	0.999	0.117 ± 0.015	0.944 ± 0.035	0.992	1.018 ± 0.086	1.451 ± 0.054	0.991
	100	0.310 ± 0.010	3.299 ± 0.197	0.996	0.214 ± 0.007	2.198 ± 0.071	0.986	0.507 ± 0.004	3.069 ± 0.025	0.993
	120	0.183 ± 0.006	4.114 ± 0.145	0.995	0.175 ± 0.005	2.813 ± 0.068	0.996	0.215 ± 0.017	3.585 ± 0.327	0.936
Chicken meat powder	80	1.378 ± 0.152	0.628 ± 0.013	0.983	0.442 ± 0.180	0.790 ± 0.085	0.991	4.498 ± 1.160	0.730 ± 0.053	0.980
aw=0.383	90	0.449 ± 0.111	0.696 ± 0.066	0.995	0.195 ± 0.049	1.107 ± 0.113	0.995	0.816 ± 0.355	0.728 ± 0.096	0.993
	100	0.030 ± 0.008	0.541 ± 0.025	0.997	0.074 ± 0.025	1.146 ± 0.162	0.996	0.242 ± 0.071	0.908 ± 0.094	0.999
	120	0.221 ± 0.002	2.853 ± 0.157	0.993	0.232 ± 0.006	3.720 ± 0.152	0.977	0.251 ± 0.016	2.906 ± 0.138	0.990
	130	0.155 ± 0.019	2.595 ± 0.257	0.979						
Pet food	70	0.189 ± 0.057	0.457 ± 0.035	0.984	0.157 ± 0.026	0.425 ± 0.009	0.998	2.680 ± 0.756	0.593 ± 0.039	0.998
aw=0.653	80	0.280 ± 0.026	0.904 ± 0.032	0.947	0.113 ± 0.132	0.620 ± 0.221	0.931	0.325 ± 0.036	0.631 ± 0.023	1.000
	90	0.225 ± 0.022	1.549 ± 0.080	0.992	0.185 ± 0.015	1.430 ± 0.071	0.990	0.109 ± 0.020	0.852 ± 0.060	0.998
	100	0.266 ± 0.024	3.044 ± 0.268	0.998	0.182 ± 0.017	2.234 ± 0.173	0.994	0.270 ± 0.049	2.528 ± 0.378	0.978

Table 4.3 α and β parameters for Weibull model for *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 per product and temperature.

In some cases standard deviations were high (Table 4.2) due to small variations between replicates that resulted in great differences in the parameters *α* and *β.* The greatest variation was observed for inactivation of *L. monocytogenes* in pet food at 80°C (Table 4.2). Heat inactivation curves between replicates were not significantly different ($p > 0.05$). Some changes in the shape of survival curves were clearly visible (not shown) and thus significantly affected parameters *α* and *β* (Table 4.2). In this case additional adjustment of mean values of *α* and *β* were performed and calculated following the fitting of a Weibull model to mean values and not to individual replicates. Although adjusted parameters *α* and *β* significantly improved fitting of the Weibull model, standard deviations remained high. The goodness of fit of the Weibull model to experimental data was confirmed by calculating the R^2 values presented in Table 4.2.

High values (R^2 = 0.931 - 1.000) indicated exceptionally good fit of the inactivation curves of minimum 5 data points, selected in preliminary tests for each temperature, product and organism.

The heating time, including the come up time, to achieve a 5 log reduction in samples heated in thermal cells was calculated using the Weibull model. Applying the log-linear inactivation kinetics, the traditional heat resistance parameters D- and z- values were calculated from the inactivation curves. The early stages of these inactivation curves corresponded to increasing product temperature during the come up time and did not comply with sample isothermal conditions. In cases where R^2 representing fitting of the linear inactivation was < 0.95, linear regression curves were fitted by omitting the initial data point, that generally corresponded to sample temperature lower than 70°C.

Product	Temp. (°C)	*Salmonella*			*Listeria monocytogenes*			*E.faecium* NRRL B-2354		
		Weibull model	First order kinetics		Weibull model	First order kinetics		Weibull model	First order kinetics	
		Mean ± SD	Mean ± SD	p - value	Mean ± SD	Mean ± SD	p - value	Mean ± SD	Mean ± SD	p - value
Confectionery	70				51.61 ± 13.58	78.91 ± 7.60	0.039			
aw=0.565	80	40.57 ± 2.04	40.07 ± 1.83	0.768	7.99 ± 1.79	11.23 ± 0.77	0.045	24.95 ± 1.80	36.17 ± 1.42	0.001
	90				1.68 ± 0.05	1.67 ± 0.05	0.950	4.55 ± 0.13	5.55 ± 0.13	0.001
	100	1.99 ± 0.15	2.12 ± 0.09	0.249	0.79 ± 0.01	0.62 ± 0.01	0.000	1.06 ± 0.04	0.89 ± 0.05	0.009
	120	0.57 ± 0.01	0.25 ± 0.02	0.000				0.43 ± 0.01	0.15 ± 0.01	0.000
	140	0.36 ± 0.005	0.14 ± 0.02	0.000						
Seasoning	80	9.51 ± 0.70	9.55 ± 0.74	0.956	11.81 ± 0.94	13.29 ± 0.82	0.110	35.34 ± 0.44	28.90 ± 0.28	0.000
aw=0.655	90	1.50 ± 0.05	1.53 ± 0.07	0.579	1.55 ± 0.06	1.53 ± 0.07	0.757	5.48 ± 0.13	4.56 ± 0.08	0.001
	100	0.65 ± 0.03	0.27 ± 0.08	0.001	0.65 ± 0.003	0.37 ± 0.01	0.000	1.12 ± 0.02	0.46 ± 0.001	0.000
	120	0.33 ± 0.004	0.11 ± 0.003	0.000	0.42 ± 0.002	0.11 ± 0.003	0.000	0.43 ± 0.01	0.15 ± 0.004	0.000
Chicken meat powder	80	67.27 ± 5.00	77.39 ± 3.64	0.047	9.46 ± 0.69	10.34 ± 0.91	0.158	126.2 ± 6.16	133.5 ± 3.99	0.158
aw=0.383	90	15.10 ± 1.38	17.76 ± 1.54	0.090	1.76 ± 0.05	1.68 ± 0.10	0.280	22.53 ± 2.05	23.40 ± 1.53	0.591
	100	2.73 ± 0.20	4.44 ± 0.16	0.000	0.61 ± 0.04	0.59 ± 0.01	0.397	3.52 ± 0.18	3.65 ± 0.15	0.397
	120	0.52 ± 0.02	0.24 ± 0.04	0.000	0.45 ± 0.001	0.14 ± 0.02	0.000	0.58 ± 0.01	0.23 ± 0.02	0.000
	130	0.44 ± 0.06	0.16 ± 0.01	0.001						
Pet food	70	39.53 ± 4.37	56.55 ± 8.39	0.036	49.49 ± 8.46	15.53 ± 1.22	0.002	162.2 ± 7.06	190.4 ± 1.83	0.003
aw=0.653	80	4.19 ± 0.34	4.35 ± 0.28	0.562	4.62 ± 0.38	6.41 ± 1.25	0.079	15.65 ± 0.56	4.35 ± 0.28	0.000
	90	1.09 ± 0.02	0.89 ± 0.02	0.000	1.02 ± 0.02	0.80 ± 0.03	0.000	1.91 ± 0.07	2.04 ± 0.18	0.327
	100	0.59 ± 0.01	0.23 ± 0.01	0.000	0.54 ± 0.004	0.34 ± 0.01	0.000	0.71 ± 0.02	0.37 ± 0.01	0.000

Table 4.4 Comparison of heating times (min) for a 5 log reduction calculated using Weibull model and first order kinetics (D-values).

Salmonella showed a significantly higher ($p < 0.5$) heating time to reach a 5 log reduction than *L. monocytogenes* in high sugar formulation (confectionery) and high protein (chicken meat powder), whereas the heating time for *Salmonella* inactivation was in the same range as *L. monocytogenes* in the high salt-containing formulation (seasoning) or a rich nutrient formulation (pet food; Table 4.3).

In a high sugar formulation (confectionery), the heating time for a 5 log reduction of *Salmonella* was significantly ($p < 0.05$) longer (40.6 min at 80°C or 2 min at 100°C) than for *E. faecium* NRRL B-2354 (36.2 min at 80°C and 0.9 min 100°C; Table 4.3). However, this surrogate exhibited significantly higher heating times than the pathogens in all other formulations

At low temperatures, e.g. 70 and 80°C, the differences in heating time for a 5 log reduction among the tested organisms was more noticeable, whereas at high temperatures such differences became smaller.

The heating time differences between the two models (D values (first order kinetics) or Weibull Model) are statistically significant at temperatures above 100°C ($p < 0.01$) except a few sporadic cases like *E. faecium* NRRL B-2354 in chicken meat powder.

4.3.2.1 Product temperature profile during heating

Despite significant differences in chemical compositions of the low moisture foods tested in this study, the actual sample temperature logged during the thermal inactivation trials showed no significant differences in ramp up times and highest temperatures attained, amongst products. Temperature profiling conducted on all products at all temperatures, showed that samples were heated at the same rate and no significant differences were observed. At 1.5 min heating time to 70 - 90°C, sample temperature was within 1°C of the target temperature and at 2 min of heating time, sample temperature was within approximately 0.5°C below the target temperature (Figure 4.6). At higher set temperatures (100, 120, 130 and 140°C) 5 log reduction was achieved during the come up time and before reaching the target temperature. For example; 5.5 log reduction of *Salmonella* was achieved in confectionery samples in thermal cells submerged in thermo-fluid bath at 120°C within 35 s, when product temperature was 111.2°C.

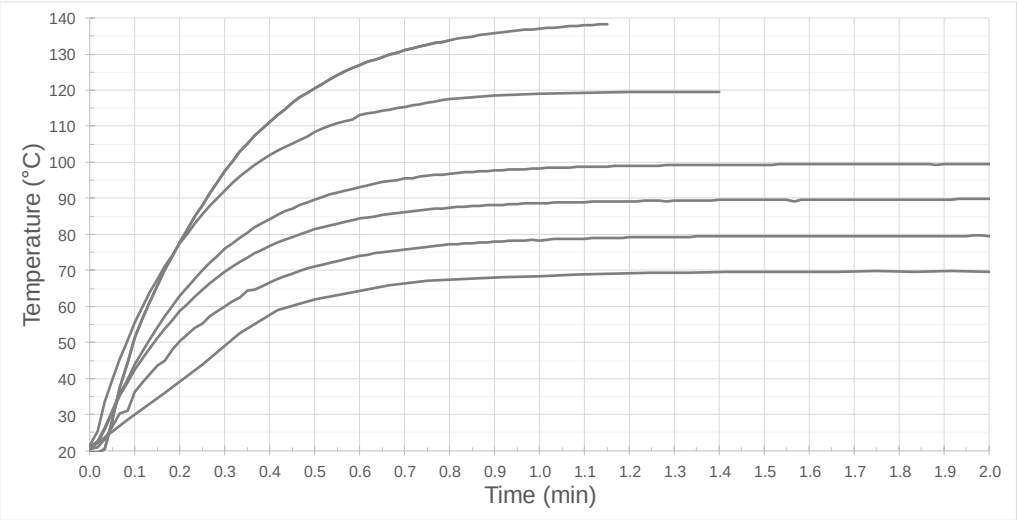

Fig. 4.6 Temperature of low moisture foods during heating in the thermal cells exposed to various set temperatures (70-140°C)

Product	Temp. (°C)	*Salmonella*	*L. monocytogenes*	*E. faecium* NRRL B2354
Confectionery	100	99.5	96.6	98.5
	120	111.2		104.3
Seasoning	100	94.4	94.4	98.8
	120	96.0	103.2	104.3
Chicken meat powder	100	99.7	93.2	99.8
	120	109.3	105.3	111.8
Pet food	100	92.7	91.0	95.4

Table 4.5 Actual temperature of low moisture products to achieve a 5 log reduction of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354

The product temperature that was recorded indicated that a 5 log inactivation occurred at different temperatures (and holding times at lower temperatures) depending on the product and bacteria. Table 4.4 shows the various heat treatment conditions to achieve a 5 log reduction in the four low moisture foods. *Salmonella* could be inactivated when heating pet food, seasoning, chicken powder and confectionery to 92.7, 96.0, 109.3 and 111.2°C respectively (Table 4.4). *L. monocytogenes* was inactivated when heating pet food, confectionery, seasoning and chicken meat powder to 91.0, 96.6, 103.2 and 105.3°C respectively (Table 4.4). *E. faecium* NRRL B-2354 was inactivated at slightly higher temperatures than *Salmonella* and *L. monocytogenes* except in confectionery; 104.3°C was required to inactivate *E. faecium* NRRL B-2354 and 111.2°C to inactivate *Salmonella*.

4.3.3 Additional surrogate validation

The use of *E. faecium* NRRL B-2354 as a surrogate seemed to have some limitations due to the increased heat resistance of *Salmonella* in confectionery powder and therefore additional trials have been conducted. It was hypothesised that modification of *E. faecium* NRRL B-2354 inoculum preparation (growth conditions; nutrient, temperature, time etc.) may increase heat resistance of *E. faecium* NRRL B-2354 in confectionery powder to levels equal to *Salmonella*. Therefore the following conditions were tested:

- *E. faecium* NRRL B-2354 (*E.f.*) grown at 25 (*E.f.* / 25°C), 30 (*E.f.* / 30°C), 35 (*E.f.* / 35°C), 37 (*E.f.* / 37°C), 42 (*E.f.* / 42°C), 44 (*E.f.* / 44°C) and 50°C (*E.f.* / 50°C) using standard methodology described in: 4.2.3 Inocula preparation.

- *E. faecium* NRRL B-2354 grown at 25, 30 and 37°C using NA (Nutrient Agar) instead of TSA (Tryptone Soya Agar) for lawn plate incubated for 24h (*E.f.* / 25°C on NA; one day), (*E.f.* / 30°C on NA; one day), (*E.f.* / 37°C on NA; one day),
- *E. faecium* NRRL B-2354 grown at 30, 37°C using NA instead of TSA for lawn plate incubated for 24h (*E.f.* / 25°C on NA; two day), (*E.f.* - 30°C on NA; one day), (*E.f.* - 37°C on NA; one day),
- *E. faecium* NRRL B-2354 grown at 30 and 37°C on media containing increased level of Sucrose (20%) in both, broth (TSB+20%sucrose) and lawn plate (TSA+20%sucrose); *E.f.* in 20%Sucrose / 30°C and *E.f.* in 20%Sucrose / 37°C.

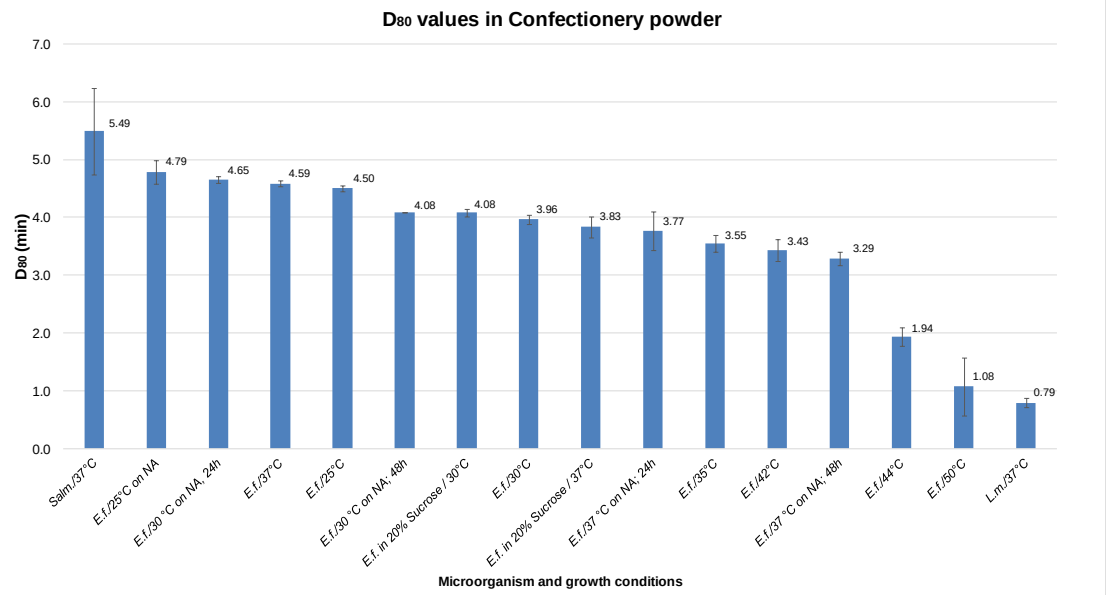

Fig. 4.7 D-values of *E. faecium* NRRL B-2354 (*E.f*), *Salmonella* (*Salm.*) and *L. monocytogenes (L.m.)* in confectionery powder grown at various conditions.

As shown in Fig. 4.7. heat resistance increase was not achieved under any growth conditions. *E. faecium* NRRL B-2354 remained significantly more sensitive to heat than *Salmonella*. Elevated growth temperature, changed nutrient composition of agar, did not increase heat resistance, and even more, increased growth temperature to and above 42°C (42, 44 and 50°C) reduced heat resistance of *E. faecium* NRRL B-2354.

4.4 Discussion

This study has shown that *Salmonella, L. monocytogenes* and *E. faecium* NRRL B-2354 survived very well for 21 d at 16°C in all four low moisture foods. This storage regime was a simulation of temporary storage in warehouses before processing; bacteria survive better in low moisture foods at low storage temperatures as documented by Komitopoulou and Peñaloza (2009), where the counts of various *Salmonella* strains remained stable in cocoa butter oil at low temperatures. Rachon and Gibbs (2015) showed no significant reduction of *Salmonella* in paprika powder (a_w = 0.45) and rice flour (a_w = 0.2) during 12 weeks of storage at 15°C, and Uesugi et al. (2006), reported no decrease of *Salmonella* on almonds at low storage temperatures. Overall, survival of *E. faecium* NRRL B-2354 in low moisture foods confirmed that *E. faecium* NRRL B-2354 was desiccation resistant and showed less reduction in viable counts than *Salmonella* and *L. monocytogenes* in low moisture foods during storage at 16°C for 21 d. The results in general, show that microbial viability during storage is dependent on the particular organism and can vary both with product composition and bacterial species.

Overall, the D_{80} values of *Salmonella*, *L. monocytogenes* and *E. faecium* NRRL B-2354 in low moisture food samples did not change markedly during 21 d of storage. Small changes in heat resistance were observed for *L. monocytogenes* in seasoning and confectionery formulations. The increase in heat resistance (D_{80}) of *L. monocytogenes* by 0.4 min in confectionery was statistically significant as well as the decrease of 0.76 min in the culinary seasoning.

Inactivation curves obtained through the series of heat inactivation experiments confirmed that inactivation was not always linear (Fig. 4.5). Non-linearity was greater at higher temperatures especially when microbial inactivation occurred during come up times, but non-linear inactivation curves also occurred at lower temperatures when come up time was not a significant fraction of the whole inactivation time. The Weibull model in these cases was shown to be an appropriate tool and times required for a 5 log reduction were calculated with precision.

Heat inactivation experiments indicated that there were some limitations when using *E. faecium* NRRL B-2354 as a surrogate, since in the sugar-containing confectionery formulation, heat resistance (D_{80}) (Fig.4.1B) and the time to reach 5 log reduction (Table 4.4) for *E. faecium* NRRL B-2354 was shorter than for *Salmonella* at all tested temperatures.

As demonstrated in many studies, (Archer et al., 1998; Barrile and Cone, 1970; Garibaldi et al., 1969; Goepfert and Biggie, 1968; McDonough and Hargrove, 1968; Peñaloza and Komitopoulou, 2012, Van Cauwenberge et al., 1981) the a_w of products has a significant impact on survival of bacteria during heat treatment. Therefore, it was expected that survival of all tested bacteria during heating would be greatest in the inoculated chicken meat flour (a_w = 0.383) followed by confectionery (a_w = 0.565) and culinary seasoning (a_w = 0.655) or pet food (a_w = 0.653). While at lower heating temperatures (≤ 100°C) this general rule was confirmed in this study, at higher temperatures, the 5 log reduction time for *Salmonella* was slightly greater in confectionery than in chicken flour, indicating that components of the confectionery formulation (sugars) may have a greater protective effect on *Salmonella* at higher temperatures. The protective function of sugars is well documented; Sumner et al. (1991) showed that heat resistance of *S*. Typhimurium and *L. monocytogenes* increased as sucrose concentration increased and a_w decreased. Mattick et al. (2001) also showed the great impact of sucrose and glucose-fructose solutions on heat resistance of *Salmonella* while Li et al. (2014) showed increased heat resistance in samples containing high levels of carbohydrates. They also observed that a_w was not the sole factor affecting the thermal resistance in those samples as the highest resistance of *Salmonella* was observed in samples with higher a_w, increased carbohydrate level and decreased fat concentration. Culinary seasoning, despite its high a_w (~ 0.655) was found to be the most protective product for *L. monocytogenes* (Table 4.4). The 5 log reduction time at 80°C in culinary seasoning was the highest when compared to other products including chicken meat powder. A 5-log reduction at a set heating temperature of 100°C required between *ca.* 1.0 and 3.5 min, for the three target organisms in the four dried food powders, considerably in excess of times and temperatures necessary for pasteurisation in high a_w foods.

Salmonella showed a higher heat resistance than *L. monocytogenes* in the high sugar formulation (confectionery) and high protein (chicken meat powder), whereas the heat resistance of *Salmonella* was just slightly higher or not significantly different from *L. monocytogenes* in the high salt-containing formulation (seasoning) or the rich nutrient formulation (pet food) (Table 4.4).

Comparison of heating times to achieve 5 log reductions calculated from the Weibull model and D-values showed significant differences (Table 4.4). At higher temperatures (≥ 100°C) heating times to achieve 5 log reduction based on calculated D-values (first order kinetics) were significantly lower than those calculated using the Weibull model. This shows the inadequacy of forcing the application of first order kinetics when product temperature increases and when the holding times at target temperatures,

cannot reliably be controlled, in food processes like extrusion and continuous heat treatments without moisture evaporation.

The Weibull prediction was an appropriate mathematical model for fitting actual survival curves including the come up time and calculating more accurately 5 log reduction times than the traditional, forced linear kinetics based on D-values. Heating low moisture foods, similar to the ones used in this study, in moisture-tight environments (thermal cells) to 111.2, 105.3 or 111.8°C can inactivate 5 log of *Salmonella, L. monocytogenes* or *E. faecium* NRRL B-2354 respectively. Therefore using the Weibull model would be a more appropriate tool when inactivation kinetics of non-isothermal heating processes (e.g. extrusion) are assessed.

4.5 References

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Chapter 5 - Discussion

5.1 General Discussion

The literature review showed that pathogens and more specifically *Salmonella* can be common contaminants of low moisture food. Although *Salmonella* outbreaks from low-moisture products are relatively rare, they often impact large numbers of people. Study of survival bacteria in low moisture food was the subject of study of many scientists and therefore mechanisms of inactivation, responses of microorganisms to various stresses and ability to survive in various environments are well known. However, inactivation of microorganisms depends on many factors and the complexity of food matrices, and therefore precise prediction of survival of particular microorganisms in food or precise prediction of the kinetics of inactivation is still challenging. Therefore, challenge testing or process validation is still the only way to precisely determine the rate of inactivation or survival. A substantial number of studies have shown that the use of surrogate bacteria is a very useful method to validate various processes and the use of various mathematical modelling methods is a very effective tool that can describe different nonlinear regression curves.

The experimental work concentrated on measuring (a) the survival ability of various individual strains (*Salmonella* and *E. faecium* NRRL B-2354) and a cocktail of *Salmonella* and *L. monocytogenes* in various low moisture powders, (b) the heat resistance and (c) the changes of heat resistance during storage. Those three measurements are crucial to evaluate the safety of the storage and effectiveness of processing. It is also important to understand if heat resistance changes during storage and if it does, which storage conditions can influence heat resistance during storage significantly. It's also important to establish if storage time affects the heat resistance of bacteria. That information is critical for selecting the most effective and most energy efficient parameters of heat treatment that would be economically and ecologically beneficial.

Survival of *Salmonella* strains in paprika powder and rice flour during storage at 15 and 25°C for 12 weeks (Chapter 2) was completed. Also, the effect of a_w of matrices on survival of *Salmonella*, in samples adjusted and equilibrated to a_w = 0.45 and 0.55 for paprika powder and to 0.2 and 0.55 for rice flour was shown. In addition, the heat resistance of bacterial cells was monitored throughout 12 weeks storage and the impact of storage conditions on the heat resistance was determined (Chapter 3).

Furthermore, survival of *Salmonella* and *L. monocytogenes* cocktails and a single strain of *E. faecium* NRRL B-2354 was determined in four low moisture powders: chicken flour, confectionery powder, pet food powder, and seasoning.

In order to replicate the worst-case scenario, samples were inoculated with bacterial suspensions containing cells with enhanced resistance (Komitopoulou and Peñaloza, 2009; Uesugi and Harris, 2006). Enhanced resistance was achieved by preparation of inocula on lawn plates and harvesting cells at the stationary phase (24h of growth). Results from these studies have shown that heat resistance did not significantly change during storage. It was concluded that the lawn plate technique used for the preparation of bacterial cultures (inocula) and further steps (inoculation and equilibration) of preparation, inoculated powders were adequate for this study and this represented the worst case scenario. Although not confirmed in this study, it is believed that preparation of cultures on the lawn plate was liable for most of the changes associated with generation of resistance occurred. It is well documented that bacteria grown in a less aqueous environment (lawn plates) are exposed to osmotic stress that triggers and activates stress response systems resulting in more stress-resistant cells. A similar effect can be achieved in broth, but only if growth time is significantly extended (2-7 days) allowing the cells to get into the late stationary phase (Uesugi and Harris, 2006).

This study has also shown that bacteria can survive well in low moisture foods during storage. The storage study in Chapter 2 has shown that bacteria can survive significantly better at lower a_w and together with storage temperature they are the most significant factors influencing survival. Furthermore, the composition of food has a significant influence. For example, at the same storage temperature and a_w of powders, salmonellae survived significantly better in paprika powder than in rice flour despite initial assumptions that paprika powder may have antibacterial properties. Regardless of rice flour containing a significantly higher percentage of carbohydrates (80 %), when compared to paprika powder (55 %), fat content may have a significant impact on survival of bacteria during storage. Paprika powder on average contains 13 % fat compared to rice flour containing only 1 %. Furthermore, the level of protein is significantly higher in paprika powder (15 %) compared to rice flour (6 %). Interestingly, during thermal inactivation of these two products, the pattern reversed and *Salmonella* and *E. faecium* NRRL B-2354 were inactivated significantly faster in paprika powder than in rice flour. This indicates that compounds of paprika powder have increased bactericidal effect at higher temperatures in contrast to protective properties at lower

(storage) temperatures. Although no effect of capsaicin on the viability of cells during storage was confirmed, it is believed that capsaicin may contribute to the viability of cells during heat processes (this study was not performed). In this study, a selection of various *Salmonella* strains was tested. In parallel to strains isolated from low moisture foods, two other *Salmonella* strains were used, namely *S.* Typhimurium ST30 (possessing an active RpoS) and *S.* Typhimurium ST10 (possessing an inactive RpoS). *S.* Typhimurium ST 30; RpoS +ve showed increased viability and heat resistance while viability and heat resistance of the RpoS -ve *S.* Typhimurium ST10 was significantly compromised. In fact, the RpoS-ve strain (*S.* Typhimurium ST10) did not survive the preparation (inoculation) process as well as RpoS +ve (*S.* Typhimurium ST 30) or any other *Salmonella* strain tested in this study.

In addition to paprika powder and rice flour, survival of cocktails of *Salmonella* and *L. monocytogenes* and *E. faecium* NRRL B-2354 was evaluated in four other low moisture products: confectionery powder, chicken flour, seasoning and pet food (Chapter 4). These products were used at their own natural form and a_w was not adjusted after inoculation. In all powders, all tested microorganisms survived well during 21 d storage at 16°C and no significant decrease of inoculated bacteria was recorded. During storage, heat resistance was tested at T_0 (day trial started), 3, 7 and 21 d after inoculation and equilibration; no significant decrease in resistance was observed except in two occasions where *L. monocytogenes* D_{80} increased ($p < 0.01$) from 0.77 to 1.17 min in the confectionery formulation and showed a significant decrease ($p < 0.01$) from 2.25 to 1.49 min in the culinary seasoning. As this trial was conducted at low storage temperature (16°C), it is believed that at this temperature, changes to the intracellular structure of bacteria were not significant and therefore, heat resistance remained stable throughout storage. This study has also shown that heat resistance is significantly higher at lower a_w but it is not always the a_w that is the main factor determining heat resistance. All tested bacterial strains shown significantly higher heat resistance in seasoning powder than in pet food, despite very similar values of a_w. At 80°C, the time to achieve a 5 log reduction was over twice longer in seasoning than in pet food despite the fact that seasoning contained high levels of salt.

This study showed that in pet food, culinary and chicken powder, the heat resistance of *E. faecium* NRRL B-2354 was higher than that of *Salmonella* or *L. monocytogenes* and therefore *E. faecium* NRRL B-2354 can be used as a surrogate in these products. However, in confectionery powder, the heat resistance of *E. faecium* NRRL B-2354 was significantly lower than *Salmonella* and therefore alternative surrogates must be

investigated. This was confirmed at all tested temperatures but also it was confirmed during storage experiments when D_{80} was measured during 21 days of storage. This study has also shown that a_w is not always the major controlling factor of heat resistance and food composition and temperature can play an important role too. It was expected that survival of all tested bacteria during heating would be greatest in the inoculated chicken meat flour (a_w = 0.383) followed by confectionery (a_w = 0.565), pet food (a_w = 0.653) and culinary seasoning (a_w = 0.655). While at lower heating temperatures (≤ 100°C) this general rule was confirmed in this study, at higher temperatures the 5 log reduction time for *Salmonella* was slightly greater in confectionery than in chicken flour, indicating that components of the confectionery formulation (possibly sugars) may have a greater protective effect on *Salmonella* at higher temperatures.

This study, similar to many others, demonstrated that exposure of bacteria to low a_w environments increases their survival and heat resistance. Similar results were obtained by Archer et al. (1998), in wheat flour, Van Cauwenberge et al. (1981), in corn flour, by Barrile and Cone (1970) and Goepfert and Biggie (1968) in chocolate, by Penaloza and Komitopoulou (2012) in cocoa and hazelnut shells, by Garibaldi et al. (1969) in egg products or by McDonough and Hargrove (1968) in dried milk. Also, the role of *rpoS* gene and RpoS regulon is well understood and these studies clearly showed that if RpoS regulon is inactivated (RpoS -ve *S.* Typhimurium ST10), both viability and heat resistance are greatly affected (Chapter 2 and 3). This study also showed that validation of surrogates is very important and it must be performed on the whole spectrum of temperatures, storage conditions and on a variety of products compositions. This study showed that *E. faecium* NRRL B-2354 was a suitable surrogate strain when used for process validation of paprika powder at low temperatures (< 80°C for a_w = 0.45 and < 70°C for a_w = 0.55) but not suitable if used at higher temperatures. *E. faecium* NRRL B-2354 was also not suitable for rice flour validation as *Salmonella* strains were more resistant than *E. faecium* NRRL B-2354 at various conditions. Furthermore, *E. faecium* NRRL B-2354 cannot be used as a surrogate in products containing high levels of sugars (confectionery powder; Chapter 4) as the heat resistance of *Salmonella* is significantly greater than heat resistance of *E. faecium* NRRL B-2354.

5.2 Future recommended studies

This study has shown that although mechanisms of survival and heat inactivation of bacteria in low moisture foods are well known, prediction of inactivation of bacteria in low moisture food is still challenging. Food matrices are very complex and there is still not enough data to support and explain the process of inactivation in detail. More research is required to explain the role of the main food components (fat, protein carbohydrates *etc.*) in survival and inactivation that will help us understand how various levels of essential oils, sugars, proteins or fat can protect cells or increase their inactivation in food. Further research in this area will focus on investigating heat resistance of *Salmonella* and *E. faecium* NRRL B-2354 in the low moisture food matrices containing various levels of key food components. The food matrices containing the various levels of fat, protein, carbohydrates will be inoculated with *Salmonella* strains and *E. faecium* NRRL B-2354 and heat resistance will be measured at various a_w so interaction of bacteria with the environment (food) and responses to environmental stresses in food matrices will be investigated. Future study should also evaluate effect of essential oils or extract of paprika powder on the microorganisms tested as initial hypothesis that capsaicin had substantial antimicrobial effect was not confirmed in this study. Although it is well documented that at low a_w, heat resistance is significantly increased, there is still little evidence on how a_w changes at higher temperatures and how this may influence microbial inactivation. A recent study by Syamaladevi et al. (2016) shows that a_w at elevated temperatures may be affected and this depends on the product composition. The a_w of all-purpose flour measured at 20 and 80°C increased from 0.45 to 0.80 respectively while that of peanut butter decreased from 0.45 to 0.04. Furthermore, D_{80} of *S*. Enteritidis PT30 in flour was 6.9 min and 17.0 min in peanut butter that positively correlates with a decrease of a_w.

Further studies should also focus on validation of alternative surrogates which could be used in a product containing high concentrations of sugars like confectionery powder. Despite some attempts being made in this study to elevate the resistance of *E. faecium* NRRL B-2354 in confectionery powder, no satisfactory results have been achieved.

5.3 References

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Appendixes

Appendix 1

Microbiological criteria for paprika powder

Table 1: Classification of dried herbs and spices from production premises as recommended by microbiological criteria within Recommendation 2004/24/EC[13] and ESA[12]

Microorganism	Microbiological quality*
Bacillus cereus	n=5, c=1, m= 10^3 cfu/g, M =10^4 cfu/g
Clostridium perfringens	n=5, c=1, m= 10^2 cfu/g, M =10^3 cfu/g
Salmonella spp.	n = 5, c = 0, Absent in 25 g
Escherichia coli	n = 5, c=1, m= 10 cfu/g, M = 10^2 cfu/g

*Parameters defined as follows:

n = number of units comprising the sample
m = limit below which all results are considered satisfactory
M = limit above which all results are considered unsatisfactory
c = number of sample units that may fall between m and M

For *B. cereus* and *C. Perfringens* the status of a batch is considered to be:

- Satisfactory where all the values are m or less
- Acceptable where the maximum of c values are between m and M
- Unsatisfactory if one or more values is/are equal or above M or more than c values between m and M

For *Salmonella* spp. the status of a batch is considered to be:

- Satisfactory where all the values are not detected in 25g
- Unsatisfactory where one or more values are detected in 25g

Table 2: Classification of dried herbs and spices from retail premises as recommended by microbiological criteria within Recommendation 2004/24/EC[13] and ESA[12]

	Microbiological quality (cfu per gram unless stated)		
Microorganism	**Satisfactory**	**Acceptable**	**Unsatisfactory**
Bacillus cereus	$<10^3$	10^3 -$<10^4$	$\geq 10^4$
Clostridium perfringens	$<10^2$	10^2 -$<10^3$	$\geq 10^3$
Salmonella spp.	*ND in 25g	–	Detected in 25g
Escherichia coli	<10	10-$<10^2$	$\geq 10^2$

*ND = not detected in 25g

Appendix 2

Rachon, G., Peñaloza, W., Gibbs, P.A., 2016. Inactivation of *Salmonella*, *Listeria monocytogenes* and *Enterococcus faecium* NRRL B-2354 in a selection of low moisture foods. Int. J. Food Microbiol. 231, 16–25.