CRANFIELD UNIVERSITY

Stavros G. Manios

Investigating the impact of retail and household practices on the quality and safety of ready-to-eat and ready-to-cook foods

Cranfield Health

PhD Thesis
Academic Years 2009 - 2012

Supervisors:
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Dr. Panagiotis N. Skandamis (Agricultural University of Athens)

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ABSTRACT

Bacterial responses to environmental stresses may be easily observed and predicted under controlled laboratory conditions. However, realistic conditions encountered during manufacturing, in retail or in households may cause unpredicted responses of spoilage or pathogenic bacteria. Therefore it is essential to identify and understand the microbial dynamics under such conditions. The overall aim of the present study was to simulate the most common environmental conditions and consumer-style practices during storage or preparation of Ready-to-Eat (RTE) and Ready-to-Cook (RTC) products in the domestic environment, and predict the microbial dynamics which may deteriorate the quality or compromise the safety of these foods.

Aiming to develop a unified mathematical model for the prediction of the growth of the specific spoilage microorganisms (SSOs), the spoilage pattern of three RTE acidic spreads of low pH was described in relation to microbial, physicochemical and molecular changes during storage. Results showed that the spoilage profile of the products was primarily affected by the initial pH and the storage temperature, despite the differences in their formulation. These findings enabled the assessment of two unified models (polynomial and Ratkowsky) for the prediction of the growth of lactic acid bacteria (LAB; SSOs) in such acidic spreads, using only the initial pH, the concentration of undissociated acetic acid and the storage temperature. The models were validated under realistic conditions in household refrigerators. Despite the abrupt fluctuations of the temperature during validation procedure, they both were able to adequately predict the growth of LAB in the spreads. However, the initial contamination level was proved to be necessary and crucial for the accurate prediction of microbial dynamics.

The time-temperature profiles of the validation procedure revealed that the suggested storage conditions were not followed promptly and, therefore, concerns were raised on the effect of such consumer mishandlings on the safety of foods. Therefore, the responses of *Salmonella* spp. and *Escherichia coli* O157:H7 to the stresses encountered during frozen storage, thawing and

cooking of ground beef, simulating typical scenarios followed by the consumers, were evaluated. The results revealed that the guidelines issued by the food safety authorities lack of some specific points that may affect the safety of the final product, such as the duration of frozen storage and the method of cooking. In particular, it was found that the heat resistance of *E. coli* O157:H7 was likely increased after long term frozen storage, while cooking in pan-grill did not ensure the safety of the final product, even when cooked at the suggested temperature.

As shown in the first study, the initial contamination level played a significant role on the predictions of the models and further on the shelf-life of the products. Therefore, the dynamics of realistically low initial populations of *Listeria monocytogenes* and *Salmonella* Typhimurium *versus* higher levels of the pathogens (such those used during *in vitro* trials) in RTE fresh-cut salads were compared. In addition, any potential uncertainty sources for the growth potential of the pathogens in broth-based simulations were investigated**.** Results showed that the growth variability of low inocula is highly affected by the marginal storage temperatures, the indigenous microflora and the availability of nutrients. Because of this, growth from low populations showed the likelihood to exceed the growth derived from unrealistically high inocula, suggesting that “fail-dangerous” implications may derive from such challenge tests. Data derived from this part were compared with broth-based simulations and the results showed that high uncertainty should be expected when extrapolating such predictions from low initial populations in fresh-cut salads, due to the various factors affecting the microbial growth on a real food, which are (inevitably) ignored by broth-based models.

Overall, the present Thesis highlights the significant impact of consumer mishandlings on the food safety and quality of foods and contributes to the identification of unpredicted potential risk origins in the domestic environment.

Keywords: Shelf-life, spoilage dynamics, unified model, frozen storage, thawing, cooking, beef patties, single cells modelling, growth variability, fresh produce.

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TABLE OF CONTENTS

ABSTRACT i
ACKNOWLEDGEMENTS v
LIST OF FIGURES x
LIST OF TABLES xiv
LIST OF EQUATIONS xvi
LIST OF ABBREVIATIONS xvii
1 LITERATURE REVIEW AND OUTLINE OF THE THESIS 1
1.1 Principles of microbial behaviour 1
1.1.1 Food quality and Food safety 1
1.1.2 Microbial growth cycle 3
1.1.3 Hurdle technology and stress responses 4
1.2 Effect of food intrinsic, extrinsic and implicit parameters on microbial behaviour 8
1.2.1 Extrinsic parameters 9
1.2.2 Intrinsic parameters 14
1.2.3 Implicit parameters 19
1.3 Common food-handling practices during distribution, in retail or in households 20
1.3.1 Food handling during distribution and in retail 21
1.3.2 Food handling by consumers 23
1.4 Modelling of microbial behaviour 32
1.4.1 Predictive modelling 32
1.4.2 Classification of predictive models 33
1.4.3 Development of mathematical models 34
1.4.4 Practical implications of predictive models 37
1.5 Outline and objective of the Thesis 39
2 ASSESSMENT OF A UNIFIED PREDICTIVE MODEL FOR THE SHELF-LIFE OF ACIDIC SPREADS 45
2.1 Introduction 45
2.2 Materials and Methods 48
2.2.1 Products characteristics 48

2.2.2 Microbiological analysis 49
2.2.3 Physicochemical analysis 49
2.2.4 Sensory analysis 50
2.2.5 Organic acid analysis 50
2.2.6 Molecular analysis of lactic acid bacteria 51
2.2.7 Model development 51
2.2.8 Model validation 53
2.3 Results and Discussion 55
2.3.1 Spoilage pattern 55
2.3.2 Development and validation of models 65
2.4 Conclusions 71
3 EFFECT OF REALISTIC SCENARIOS OF FREEZING, THAWING AND COOKING ON THE SURVIVAL OF *SALMONELLA* SPP. AND *ESCHERICHIA COLI* O157:H7 IN BEEF PATTIES 73
3.1 Introduction 73
3.2 Materials and Methods 75
3.2.1 Bacterial cultures and preparation of inocula 75
3.2.2 Inoculation of ground beef 75
3.2.3 Thawing and cooking of beef patties 76
3.2.4 Microbiological analysis 78
3.2.5 Statistical analysis 79
3.3 Results and Discussion 81
3.3.1 Effect of freezing and thawing 81
3.3.2 Effect of cooking method and amount of cooking 86
3.4 Conclusions 96
4 DYNAMICS OF LOW (1-4 CELLS) *vs.* HIGH POPULATIONS OF *LISTERIA MONOCYTOGENES* AND *SALMONELLA* TYPHIMURIUM IN FRESH-CUT SALADS 99
4.1 Introduction 99
4.2 Materials and Methods 101
4.2.1 Bacteria and culture conditions 101
4.2.2 Isolation of low number of cells 101

4.2.3 Preparation, inoculation and microbial analysis of vegetables ... 103
4.2.4 Preparation, inoculation and microbial analysis of vegetable sterile extracts ... 104
4.2.5 Monte Carlo simulation ... 106
4.2.6 Statistical analysis ... 108
4.3 Results and Discussion ... 108
4.3.1 Growth kinetics on vegetables ... 108
4.3.2 Growth kinetics on sterile extracts of vegetables ... 114
4.3.3 Monte Carlo simulation ... 120
4.4 Conclusions ... 123
5 GENERAL DISCUSSION AND CONCLUSIONS ... 124
6 ONGOING RESEARCH ... 128
REFERENCES ... 134
APPENDIX I ... 172
APPENDIX II ... 174

LIST OF FIGURES

Figure 1-1 The typical bacterial growth curve, consisted of 6 discrete phases (A) lag phase, (B) acceleration phase, (C) exponential phase, (D) retardation phase, (E) stationary phase and (F) death phase. 3

Figure 1-2 Graphical representation of the potential physiological stages of microorganisms during exposure to unfavorable environments (from Yousef and Courtney, 2003). 5

Figure 1-3 Graphical representation of the microbial reaction to adverse environmental conditions (stress response) in order to maintain to a healthy physiological stage (from Cheng Vollmer and Van Dyk, 2004)... 6

Figure 1-4 Effect of storage temperature on the growth rate (μ) of microorganisms (from Adams and Moss, 2008) 11

Figure 1-5 Representation of the mode of action of weak acids against bacteria. The low external pH favors the dissociation of the organic acid and the undissociated form is able to penetrate the cell membrane. When exposed to the high pH of the cytoplasm, the undissociated organic acid dissociates, while in parallel releases a proton. Since H+ cannot naturally exit the cell, the H+ATPase uses energy from the hydrolysis of ATP in order to pump the protons out of the cell (from Lambert and Stratford, 1999). 15

Figure 1-6 Number of isolation reports of vero-toxigenic *Escherichia coli* (O157 or other serotypes) from meat products. Most cases are attributed to common mishandling procedures by the consumers (JNII, 2012)..... 21

Figure 1-7 Post-manufacturing stages that may affect the quality and safety of foods, due to improper handling. (A) Transportation from processing plant to retail, (B) Retail storage, (C) Transportation from retail to home, (D) Storage in domestic refrigerators, (E) Thawing practices of frozen foods, (F) Food handling (e.g. slicing, washing), (G) Cooking, (H) Reheating. Red circles highlight the stages that have been examined in the present Thesis.. 40

Figure 1-8 Schematic outline of the present study...................................... 42

Figure 2-1 Changes of lactic acid (■) or acetic acid (■) concentration, in association with lactic acid bacteria growth (△), during storage of pepper-based salad (A) at 18°C (i) and 20°C (ii) or fava beans-based salad (B) at 10°C (i) and 15°C (ii). 61

Figure 2-2 Diversity of *Lactobacillus* species during storage in pepper-based salad (A) or fava-beans salad (B) during storage at 12°C. (■) *L. brevis*, (■) *L. plantarum*, (□) *L. buchneri* 63

Figure 2-3 Linear regression of the maximum specific growth rates of LAB and storage temperatures in pepper-based (●) and fava beans-based spread (■). 68

Figure 2-4 Growth of lactic acid bacteria in pepper-based spread under four different time-temperature profiles (- - -) in four household refrigerators (A-D), as predicted by the unified model (▬) or the product-specific model (▬), in conjunction with the observed populations (●)................ 70

Figure 2-5 Growth of lactic acid bacteria in fava beans-based spread under four different time-temperature profiles (- - -) in four household refrigerators (A-D), as predicted by the unified model (▬) or the product-specific model (▬), in conjunction with the observed populations (●)... 71

Figure 3-1 Time temperature profile during freezing of ground beef blocks of 400 g (●) or 90 g beef patties (○). 76

Figure 3-2 Flow diagram of the experimental procedure that was followed. 80

Figure 3-3 Populations of *Salmonella* (A) and *E. coli* O157:H7 (B) recovered on non-selective (TSA) or selective (XLD, CT-SMAC) media immediately after inoculation (day 0) and after 5 or 75 days of storage at -22°C and before thawing. Each bar represents the mean (± standard deviation) of 8 independent samples. 83

Figure 3-4 Changes of temperature during thawing of ground beef blocks (400 g) on counter (A; 20°C for 12 hours), in refrigerator (B; 4°C for 16 hours) or in microwave (C; defrosting mode for 22-24 minutes). Each point represents the mean (± standard deviation) of 8 independent samples... 85

Figure 3-5 Logarithmic reductions of *Salmonella* spp. in beef patties after storage at -22°C for 5 (A) or 75 days (B), thawing with four different methods (counter, refrigerator, microwave, no thawing) and cooking in a kitchen oven-broiler or in a pan-grill until the internal temperature of the patties reached 60 or 71°C. Similar lower case letters for a particular thawing method indicate non-significant differences among the two different cooking methods and amount of cooking. Similar upper case letters indicate no significant effect of the different thawing methods on the same cooking method and amount of cooking. 88

Figure 3-6 Logarithmic reductions of *Escherichia coli* O157:H7 in beef patties after storage at -22°C for 5 (A) or 75 days (B), thawing with four different methods (counter, refrigerator, microwave, no thawing) and cooking in a kitchen oven-broiler or in a pan-grill until the internal temperature of the patties reached 60 or 71°C. Similar lower case letters for a particular thawing method indicate non-significant differences among the two different cooking methods and levels of doneness. Similar upper case letters indicate no significant effect of the different thawing methods on the same cooking method and amount of cooking. 89

Figure 3-7 Time-temperature profiles during cooking of beef patties in oven-broiler (open symbols) and pan-grill (closed symbols) until 71°C and after thawing on countertop (A), in microwave (B), in refrigerator (C) or without prior thawing (D). Each point represents the mean (± standard deviation) of 16 independent samples. 91

Figure 3-8 pH changes during cooking of beef patties in oven-broiler or in pan-grill up to two levels of doneness (60°C and 71°C). Columns with asterisk indicate no significant differences (p>0.05) of the pH of the treatment in comparison with that before cooking of the samples. 92

Figure 3-9 Weight loses (%) of beef patties during cooking in oven-broiler or in pan-grill up to two levels of doneness (60°C and 71°C). Within a thawing method, different letters indicate statistically significant differences (p<0.05). 94

Figure 4-1 Percentage (%) relative frequency (bars) and cumulative probability (line) of cells contained in a microplate well (in 200 μl total volume per well) and used to inoculate samples of lettuce or cabbage salad or the corresponding extracts. Counts were estimated by plating the whole content of an additional microplate column. 102

Figure 4-2 Populations (log CFU/g) of the indigenous microflora of lettuce (top) and cabbage (bottom) before and after treatment with 200 ppm sodium hypochlorite for 5 min and washing with sterile water for 15 min. 109

Figure 4-3 Growth of 1000 (i) or 1-4 cells (ii) of *Listeria monocytogenes* per sample of lettuce (A) or cabbage (B) salad at 8°C. (◆) 1000 cells of *L. monocytogenes*, (■) total viable counts, (▲) pseudomonads, (✕) Enterobacteriaceae, (●) 1-4 cells of *L. monocytogenes*. Counts of the indigenous microflora (A-i, B-i) also refer to (A-ii, B-ii). The initial levels of *L. monocytogenes* in samples inoculated with 1-4 cells (-1 to -0.4 log CFU/g; graph A-ii, B-ii), are rounded to 0 log CFU/g, with detection limit of 0.7 log CFU/g. 110

Figure 4-4 Growth of 1000 (i) or 1-4 cells (ii) of *Salmonella* Typhimurium on lettuce (A) and cabbage (B) salad at 8°C. (◆) 1000 cells of *S.* Typhimurium, (■) total viable counts, (▲) Pseudomonadaceae, (✕) Enterobacteriaceae, (●) 1-4 cells of S. Typhimurium. Counts of the indigenous microflora (A-I, B-i) also refer to (A-ii, B-ii). The initial levels of *S.* Typhimurium in samples inoculated with a 1-4 cells (-1 to -0.4 log CFU/g; graph A-ii, B-ii), are rounded to 0 log CFU/g.......................... 110

Figure 4-5 Growth of 1000 (i) or 1-4 cells (ii) of *Listeria monocytogenes* on two different batches (batch A, ●,○; batch B, ▲,△) of LEA (A), LEB (B), CEA (C) and CEB (D) stored at 8°C (closed symbols) or 10°C (open symbols). For readability reasons, results of two representative batches are presented. The initial levels of L. monocytogenes in samples inoculated with 1-4 cells/sample (-1.2 to -0.6 log CFU/g and -0.7 to -0.1 log CFU/g for solidified and liquid extracts, respectively) are rounded to 0 log CFU/g or ml.. 115

Figure 4-6 Growth of 1000 (i) or 1-4 cells (ii) of *Salmonella* Typhimurium on two different batches (batch A, ●,○; batch B, ▲,△) of LEA (A), LEB (B), CEA (C) and CEB (D) stored at 8°C (closed symbols) or 10°C (open symbols). For readability reasons, results of two representative batches are presented. The initial levels of *S.* Typhimurium in samples inoculated with 1-4 cells/sample (-1.2 to -0.6 log CFU/g and -0.7 to -0.1 log CFU/g for solidified and liquid extracts, respectively) are rounded to 0 log CFU/g or ml.. 116

Figure 4-7 Predicted (—) and observed (···) cumulative distribution of *L. monocytogenes* on lettuce (A) and cabbage (B) after 12 days of storage. 122

Figure 6-1 Populations of *Salmonella* spp. habituated in different food residues and recovered from different food-related surfaces with the swab method... 130

Figure 6-2 Populations of Salmonella transferred to tomato sauce, beef patties or lasagna, after habituation in different FR and types of surfaces for 24 h at 25°C . (■) Glass, (■) Metal, (■) Plastic.............................. 131

Figure 6-3 Lag time of *Salmonella* spp. cells recovered from (■) glass, (■) metal or (■) plastic surfaces after habituation to different food residues, in comparison with a freshly activated culture (control, ■) 132

LIST OF TABLES

Table 1-1 Suggested temperatures for the storage of different categories of foods (Source: Laguerre et al., 2002) 24

Table 1-2 Percentage (%) of consumer practices which are commonly followed for the thawing of frozen foods, as revealed from surveys in different countries 28

Table 1-3 Suggested minimum internal temperatures which ensure the elimination of pathogenic bacteria in different food commodities (Source: USDA/FSIS, 2011b) 30

Table 1-4 List of the most commonly used primary, secondary and tertiary models 34

Table 1-5 Summary of the major practical implications of predictive microbiology in the food industry environment (from Fakruddin et al., 2011) 38

Table 2-1 Physicochemical properties (pH and total acetic acid concentration) and storage temperature of the different types of spreads that permitted the growth of lactic acid bacteria. The kinetic parameters (μ_{max}, lag time), which derived during fitting the LAB populations (log CFU/g) of each combination by the Baranyi model, were further used for the development of the unified models. Combinations that did not permit microbial growth are not included. 54

Table 2-2 Growth parameters (lag time, growth rate) of specific spoilage microorganisms (lactic acid bacteria) in two batches of pepper-based spread, fava beans-based spread and eggplant-based spread and final product shelf-life during storage under constant temperature conditions 57

Table 2-3 Changes of pH (standard deviation) and titratable acidity, and LAB populations at the end of shelf-life of pepper-based salad, fava beans-salad and eggplant salad after storage under isothermal conditions..... 59

Table 2-4 Species of lactic acid bacteria that dominated the microbial association of pepper-based salad, fava beans-based salad and eggplant-based salad at the point of product rejection, after storage under different isothermal conditions 63

Table 2-5 Significant ($p<0.05$) parameter estimates (± 95 % confidence intervals) and goodness-of-fit criteria of polynomial and Ratkowsky

equations fitted as two alternative “unified” models to collective data (log CFU/g) for the growth of LAB in various acetic acid-based spreads. 67

Table 2-6 Bias (B_f) and accuracy (A_f) factors of the models predicting the growth of LAB in pepper spread and fava beans spread, after exposure to fluctuating time-temperature profiles 69

Table 3-1 Means (Log CFU/g ± standard deviation) of *Salmonella* spp. and *E. coli* O157:H7 populations after storage at -22°C for 5 or 75 days and subsequent thawing in a household refrigerator, a microwave or on kitchen counter. 82

Table 3-2 Total duration (minutes) of thermal process during cooking of 90 g beef patties in the oven-broiler or in pan-grill, up to two levels of doneness, and after thawing with four different methods. 86

Table 3-3 Mean values of thermal process lethality (F value) of *Escherichia coli* O157:H7 in beef patties which were cooked in oven-broiler or in pan-grill at 60°C or 71°C, after thawing with four different practices. 90

Table 3-4 Mean values of thermal process lethality (F value) of *Salmonella* spp. in beef patties which were cooked in oven-broiler or in pan-grill at 60°C or 71°C, after thawing with four different practices. 90

Table 4-1 Approximation of experimental conditions in salads by the broth-based study of Francois et al. (2006a) and corresponding parameters of Weibull distributions of individual lag times, used for the simulation of *Listeria monocytogenes* growth on lettuce or cabbage salad, starting from 1-4 cells/sample. 107

Table 6-1 Initial and final pH of different food residues, which were inoculated with Salmonella, during storage at 25°C for 24 hours 129

LIST OF EQUATIONS

(1-1) 13

(1-2) 13

(2-1) 45

(2-2) 52

(2-3) 52

(2-4) 52

(2-5) 52

(2-6) 53

(3-1) 78

(4-1) 106

(4-2) 106

LIST OF ABBREVIATIONS

Caps	Cold shock acclimation proteins
CEA	Cabbage extract agar
CEB	Cabbage extract broth
Csps	Cold shock
CT	Column target
ES	Eggplant spread
FS	Fava beans spread
GB	Ground beef
GB+L	Ground beef + Lettuce
GHPs	Good Hygiene Practices
GMPs	Good Manufacturing Practices
HACCP	Hazard Analysis and Critical Control Point
Hsps	Heat shock proteins
L	Lettuce
LAB	Lactic acid bacteria
LEA	Lettuce extract agar
LEB	Lettuce extract broth
LI	Log increase
M	Mayonnaise
O.D.	Optical density
PS	Pepper spread
RTC	Ready to Cook
RTE	Ready to Eat
SSOs	Specific spoilage microorganisms
TAC	Total acetic acid
T_d	Temperature decrease
T_i	Temperature increase
TTA	Total titratable acidity
UAC	Undissociated acetic acid
VTEC	Verotoxigenic *Escherichia coli*
YOPI	Young old pregnant immunocompromised

1 LITERATURE REVIEW AND OUTLINE OF THE THESIS

1.1 Principles of microbial behaviour

1.1.1 Food quality and Food safety

The microbiological quality and safety of foods are two different terms which may indirectly describe the microbiological profile of a product. Both terms are associated with the physiological activity of spoilage or pathogenic microorganisms. Although the spoilage and virulence may be caused by different types of microorganisms, interaction between these two conditions may be observed; for instance, the indigenous microflora of a food could inhibit or stimulate the behaviour of the contaminant (Mellefont et al., 2008). Therefore, the term “Total Food Quality” is used in order to adequately describe the qualitative and safety condition of a product (Guisti et al., 2008).

The “food quality” of a product is a rather complex and difficult term to define as it may include various variables regarding sensory characteristics, microbiological patterns, nutritional characteristics, ethical requirements and marketing issues (Peri, 2006). According to Grunert (2005), “food quality” may be either an objective or a subjective term. From the aspect of food microbiologists and engineers, food quality refers to the microbiological profile and/or the physical characteristics of a product. On the other hand, according to the subjective opinion of the consumers a general definition for food quality is “the combination of attributes or characteristics of a product that have significance in determining the degree of acceptability of the product to a user” (Cardello, 1998). The deterioration of the quality of foods is a result of physical, chemical or microbiological changes during storage, with the latter being the main objective of the present review. In particular, microbial spoilage of foods is caused by the metabolic activity of various microorganisms, such as lactic acid bacteria, pseudomonads, enterobacteria, moulds or fungi, depending on the type of food (Baird-Parker, 2000). These

microorganisms usually originate from the ingredients which compose a specific food, as a part of their physiological flora. In addition, these microorganisms may also derive from cross-contamination of foods during preparation, as they may be permanent inhabitants of soil or water. Under favorable conditions, the microbial proliferation results in increasing amounts of metabolites, which may further cause significant changes in the organoleptic characteristics of the fresh product (Doyle, 2007, Jacxsens et al., 2003). Despite that, deteriorated food due to microbial activity may still be safe to eat, although such foods are usually perceived by consumers as unacceptable or undesirable for consumption.

On the other hand, microbiological food safety refers to all the principles which describe handling, preparation and storage of foods which may prevent the potential contamination or survival of pathogenic microorganisms. All these principles of food hygiene before consumption are included in the "Codex Alimentarius", which was published by the Food Agriculture Organization and World Health Organization in 2003 (FAO-WHO, 2003), in order to ensure the safety of foodstuff. The most common species of bacteria associated with the origin of foodborne illnesses are *Listeria monocytogenes* and various serotypes of *Salmonella* spp. or *Escherichia coli*. These pathogens may naturally occur in or on various biotic or abiotic substrates and thus, they can easily contaminate foods or food ingredients. Indeed, numerous outbreaks of foodborne diseases associated with different types of foods such as fresh produce (CDC, 2012a; 2011a; 2011bHarris et al., 2003), meat products (CDC, 2012b; 2011b; 2010a) or Ready-To-Eat foods (CDC 2010b; 2010c), have been reported in recent years. Except for their ubiquitous nature, these microorganisms may also survive and/or grow even under adverse environmental conditions, rendering their control in foods more complex. Another matter of concern is that contrary to the spoilage microorganisms, pathogens require very low initial numbers of cells to cause illnesses, highlighting the necessity of good hygienic practices during handling of foods (Gandhi and Chikindas, 2007; Jemmi and Stephan, 2006; Kathariou, 2002; Pui et al., 2011; Bell and Kyriakides, 2002; Nataro and

Kaper, 1998). The mode of virulence of each pathogen may differ, however, between vulnerable groups of humans with the young, old, pregnant and immune-compromised (YOPI) being in the highest danger against all cases of foodborne illnesses. It is easily understandable that the major difference between food quality and food safety is that retrenchments in the first one may just lead to economic losses, while any compromise in food safety principles may cost human lives.

1.1.2 Microbial growth cycle

The foundations of food microbiology are set on the typical growth curve exhibited by all bacteria. Regardless of their spoilage or pathogenic nature, microorganisms reproducing in a medium suitable for growth, follow a sigmoidal growth curve consisting of 6 phases; (i) lag phase, (ii) acceleration phase, (iii) exponential or logarithmic phase, (iv) retardation phase, (v) stationary phase and (iv) death phase (Figure 1-1; Monod, 1949). Each of

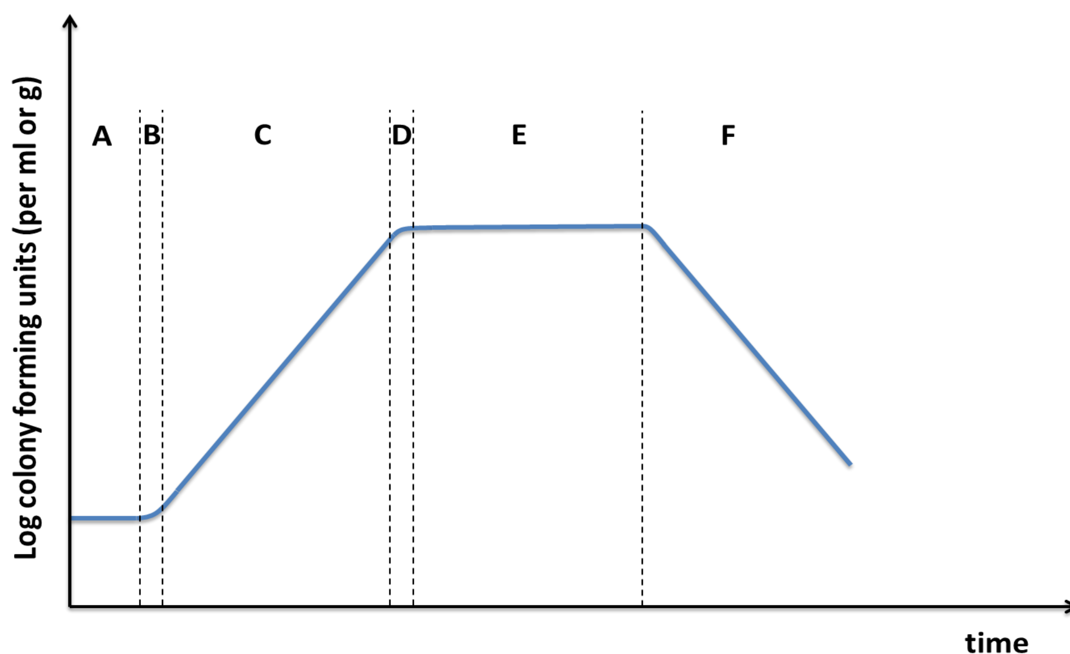


Figure 1-1 The typical bacterial growth curve, consisted of 6 discrete phases (A) lag phase, (B) acceleration phase, (C) exponential phase, (D) retardation phase, (E) stationary phase and (F) death phase.

these phases represents the different physiological activities occurring in a cell during its life. The phases that are of the highest importance from the aspect of food safety and quality are the lag and the exponential phase. During lag time, bacteria gradually adapt to the new environmental conditions, before growth initiation. In contrast, during the exponential or logarithmic phase, the metabolic activity of bacteria increases, resulting in cell division at the maximal rate. These two phases are the only two along the bacterial growth curve that permit any interference, in order to decelerate the undesirable microbial growth; prolonging the lag phase of a microorganism or decreasing its rate of growth results in more stable or safe products. To achieve this, it is mandatory to understand the use and the mode action of most common applied hurdles.

1.1.3 Hurdle technology and stress responses

The control of spoilage microorganisms or pathogens in foods is based on the well-known theory of “Hurdle Technology”, which was primarily described by Leistner (1978; 2000). This theory implies that different growth inhibiting factors are introduced in a food system in order to ensure the safety and stability of the product. According to McMahon et al. (2007), as an environmental stressful factor may be defined as any “external factor that has an adverse effect on the physiological welfare of bacterial cells, leading to reduction in growth rate, or in more extreme circumstances, to inhibition and/or death, at individual cell or population levels”. Among others, hurdles such as low water activity, high acidity, chilled or freezing temperatures, chemical or natural antimicrobials, microbial competition, packaging under vacuum or modified atmospheres may be applied or physically occur during food manufacturing, storage or preparation before consumption (Capozzi et al., 2009; Tiganitas et al., 2009; Wesche et al., 2009; Beuchat, 2002; Yousef, 2000). The aim of an applied stress is either to prolong the duration required by the cells to adapt to the new environment (lag time), to decrease the rate

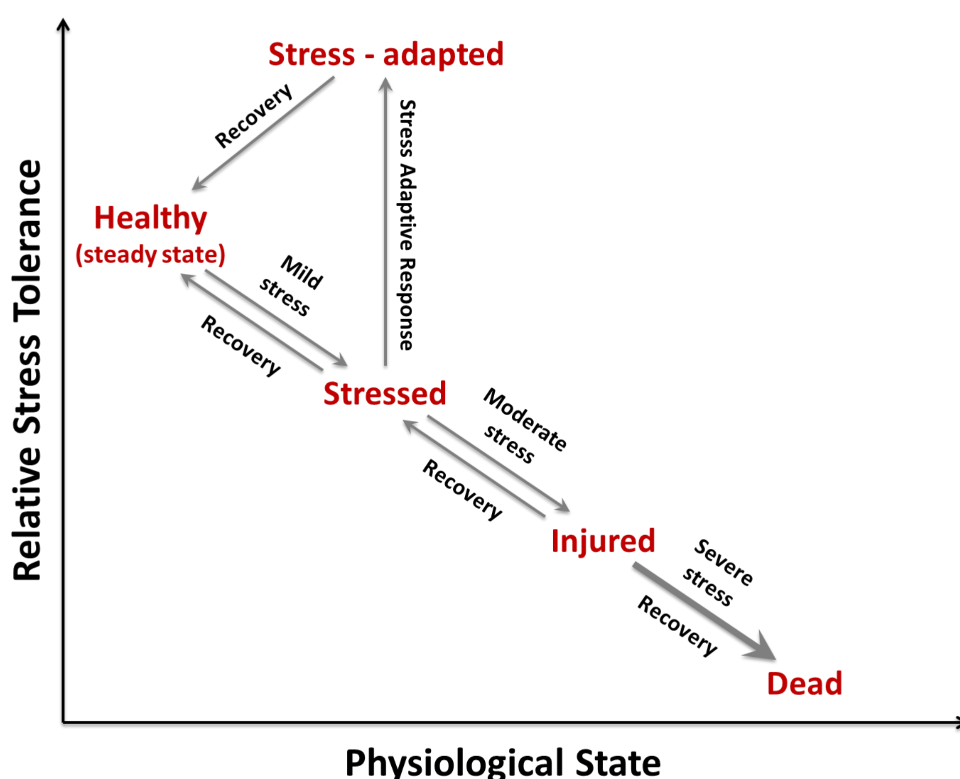


Figure 1-2 Graphical representation of the potential physiological stages of microorganisms during exposure to unfavorable environments (from Yousef and Courtney, 2003).

of cell division or cause a reduction in numbers. Considering spoilage microorganisms, retarding their growth may extend the shelf-life of the product, while the growth deceleration or inhibition of pathogens improves the overall product safety. The intensity of an applied “hurdle” may also have different impacts on the physiological state of microorganisms (Figure 1-2; Yousef and Courtney, 2003; Russell et al., 1995; Ray 1986). Stresses termed “severe” are those which can cause irreversible injuries and death to the majority of the population of a microorganism. Following a stress of “moderate” level, the cells are firstly injured and, depending on the degree of the injury, and the conditions of the environment or changes that occur to that environment, they may either die or slowly recover to the earlier healthy

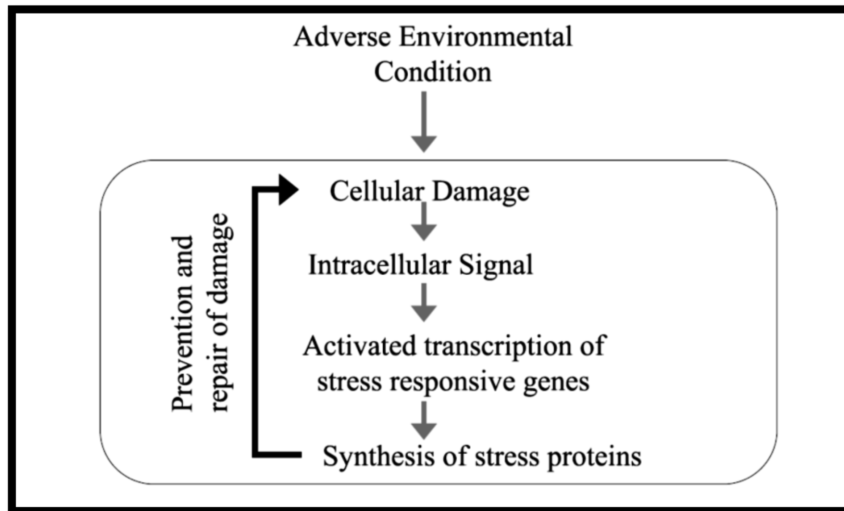


Figure 1-3 Graphical representation of the microbial reaction to adverse environmental conditions (stress response) in order to maintain to a healthy physiological stage (from Cheng Vollmer and Van Dyk, 2004).

state. “Mild” stresses are sublethal conditions that do not cause any loss of the viability of the bacteria but inhibit microbial growth, either by introducing lag to the microorganism or by decreasing its growth rate (Yousef and Courtney, 2003; Storz and Hengge-Aronis, 2000).

The response of microorganisms to any adverse environmental conditions is termed “stress response” and it refers to any microbial action to repair or minimize any internal damage, and restore the microorganism to its previous physiological state (Figure 1-3; Cheng Vollmer and Van Dyk, 2004). Following exposure to extreme conditions, microorganisms invoke particular physiological and/or genetic mechanisms, in order to regulate the activation and expression of necessary genes whose products (proteins) assist in the survival of the cell, while in parallel the synthesis of unnecessary proteins is halted (Fig 3; Boor, 2006; Cheng Vollmer and Van Dyk, 2004; Abee and Wouters, 1999; Chowdhury et al., 1996). Potential results of microbial stress responses include the production of stress protective proteins, the induction of tolerance to the same or other stresses, the passage of cells to a lethargic

phase (i.e., spore formation, viable but nonculturable state) and the increased resistance to the defensive mechanisms of the host organism (Yousef and Courtney, 2003). However, the exact reaction of bacteria to stress is rather complicated and depends on the type of microorganism and the type of the applied stress.

Under the consumers' pressure for less processed products of higher quality and with limited or no chemical additives, the preservation techniques followed by the food industries are becoming milder (Fellows, 2000). However, it has been found that microorganisms that have been extensively exposed to sublethal (mild or moderate) stresses may adapt to such conditions and further induce tolerance to lethal levels of the same or other stresses (Fig 2; Xu et al., 2008; Hill et al., 2002; Phan-Thanh et al., 2000; Abee and Wouters, 1999; Lou and Yousef, 1997). The adaptive responses of pathogenic bacteria to stresses and the potential induction of protection is of high practical interest; microorganisms which have been hardened by insufficient preservation techniques or during exposure to sublethal food-related stresses may further tolerate common lethal stresses. For instance, Yuk and Schneider (2006) reported that habituation of different *Salmonella* serotypes to the low pH of apple, orange or tomato juice enhanced their subsequent survival in the extremely low pH of a simulated gastric fluid. Similarly, Shen et al. (2011a) observed induced heat tolerance of acid adapted cells of *Escherichia coli* O157:H7 after cooking at 65°C in a pan-broiler. Belessi et al. (2011) studied the effect of abrupt shifts of pH and water activity (a_w) above and close to the growth boundaries of *L. monocytogenes* on the adaptive responses and further survival of the pathogen, simulating the fluctuating conditions that the microorganisms may be exposed during food manufacturing. In that study, it was shown that the lag time of the pathogen was reduced when transferred from long–term incubation under no-growth conditions of aw to growth permitting conditions of aw or pH, indicating potential adaptation of the cells to the osmotic stress and further induction of tolerance to pH or a_w. Relevant studies of increased tolerance of acid adapted *L. monocytotgenes* and *Salmonella* spp. cells in acidic foods

have also been reported by other researchers (Gahan et al., 1996; Leyer and Johnson, 1992).

Except for pathogens, spoilage microorganisms may also encounter adverse conditions in a food system and further induce adaptive responses to these stresses. Contrary to the fact that the adaptive stress responses of pathogens may have significant impact on the safety of the products, the potential accelerated growth of stress hardened spoilage bacteria may result in significant economical losses by reducing the predetermined shelf-life of the product (Beales, 2004; Piper et al., 2001; Wouters et al., 2000) or changing the desired behaviour of a starter culture (De Angelis and Cobbetti, 2004; Sanders et al., 1999).

In order to avoid phenomena of stress hardening, the microbial hurdles may be applied in combinations (multiple hurdle technology) simultaneously or in sequence because, in this manner, the continuous exposure to sublethal conditions may exhaust and further kill the cells (Sofos, 1993). There are two different theories about the interaction among these hurdles; the Gamma hypothesis suggests that hurdles act independently and additively (Bidlas and Lambert, 2008), while there is also the theory that the applied hurdles interact (Augustin and Carlier, 2000). Nevertheless, even the order and the intensity of these stresses may determine the adaptive responses of a microorganism. For example, Skandamis et al. (2008) reported that the application of NaCl, low pH and heat stress in sequence resulted in less survivors of *L. monocytogenes* compared with those seen after simultaneous exposure to the same stresses.

1.2 Effect of food intrinsic, extrinsic and implicit parameters on microbial behaviour

The ability of a microorganism to grow in a food system is affected by various environmental and food-related parameters. These parameters are divided

into three categories. Extrinsic parameters refer to factors in the environment external to the food, which affect both the microorganisms and the food itself during processing and storage (e.g. temperature, relative humidity of the environment, gas composition of food package). Intrinsic parameters refer to factors that are inherent to the food product and determine its physical and chemical characteristics (e.g. pH, a_w, redox potential (Eh), availability of nutrients, food structure, antimicrobial additives). Finally, implicit parameters refer to the microbial interactions in the food system (e.g. microbial antagonism, synergism or commensalism). In this paragraph, the effect of some of the most common intrinsic, extrinsic and implicit parameters which are associated with the manufacturing and further handling of foods, on the survival of bacteria, as well as the response of microorganisms to these inhibiting factors, will be discussed.

1.2.1 Extrinsic parameters

The environmental or extrinsic parameters include non-substrate dependent factors which, however, may affect the viability and/or the growth potential of a microorganism. The temperature of the growth environment of the microorganisms plays a significant role in their growth or survival potential, when referring to low or high temperatures, respectively. Other extrinsic factors (e.g. composition of modified atmosphere packaging, and relative humidity) have also a significant impact on the behaviour of microorganisms.

1.2.1.1 Frozen and chilled storage

Bacterial microorganisms are able to grow under a wide range of temperatures from below 4^oC to over 60^oC. Depending on their temperature requirements for growth, microorganisms are categorized in three groups: *psychrotrophs* are characterized as organisms which are able to grow at or below 7^oC, while their optimum growth temperature ranges between 20^oC and 30^oC; microorganisms that have an optimum growth temperature

between 30°C and 40°C but they may also grow well between 20°C and 45°C are called *mesophiles*; *thermophiles,* are defined as microorganisms that grow at and above 45°C, with optimum growth temperature between 55°C and 65◦C (Jay et al., 2005). The higher and lower limits of these temperatures (4-60°C) constitute the so-called *danger zone* in which the proliferation of microorganisms is favored and thus the quality and/or the safety of the products may be compromised. In contrast, below the danger zone microbial growth is stopped, while at high temperatures (>60oC) death also occurs. However, the ability of *L. monocytogenes* to grow at low temperatures (even below 4°C; Walker et al., 1990) may be of concern, especially for RTE foods which require refrigeration for long term storage.

Chilled and frozen temperatures are widely used as an additive hurdle for the growth of pathogenic or spoilage bacteria. By decreasing the storage temperature, the bacterial mechanisms of growth are significantly decelerated, until growth is totally halted at temperatures below the T_{min} (Figure 1-4). Growth ceases primarily due to the slowing of enzymatic reactions within the cell (Adams and Moss, 2008). In addition, the fluidity of the cytoplasmic membranes is decreased at low temperatures, and therefore the internalization of nutrients to the enzymes within the cell is suppressed. In order for microorganisms to maintain their membrane fluidity and remain capable of growth even at low temperatures, they lower the melting point of the membrane lipids by decreasing their chain length. The membranes fluidity may also be favored by the slight increase of the concentration of unsaturated lipids which occurs after exposure to low temperatures (Hébraud and Potier, 1999; Russell et al., 1995).

Except for the mechanisms activated by the bacteria to maintain the fluidity of the phospholipid membrane, another reaction of microorganisms after exposure to low temperatures is the synthesis of the so-called cold-shock proteins (Jones, 2012; Abee and Wouters, 1999; Sanders et al., 1999). The induction of these proteins may increase the efficiency of DNA translation, transcription and replication, which is inhibited due to the stabilization of the

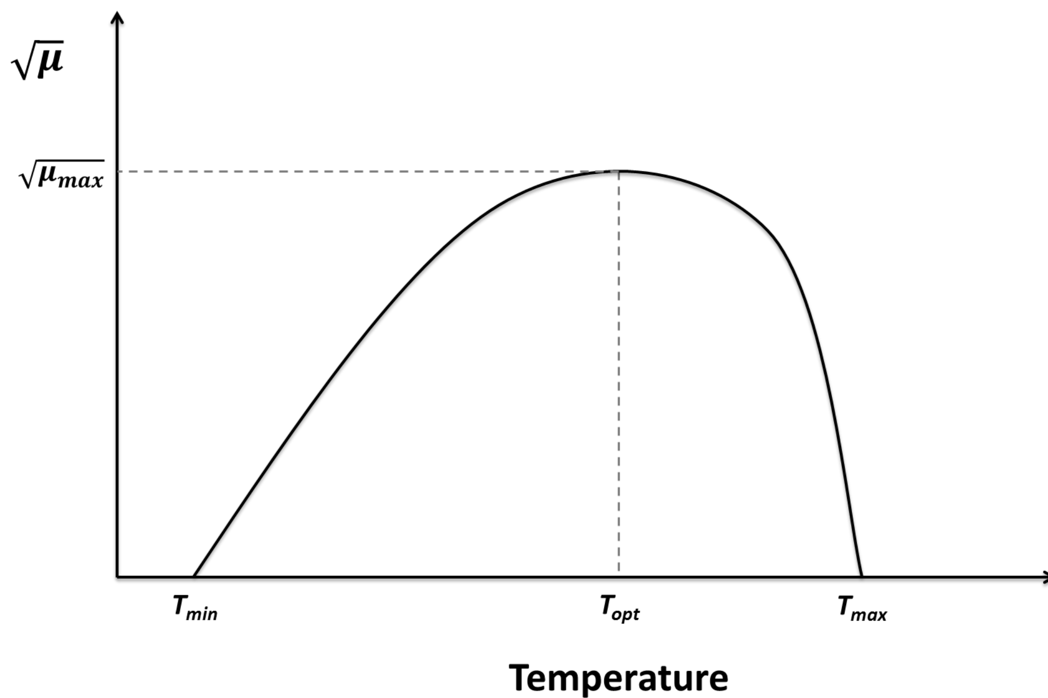


Figure 1-4 Effect of storage temperature on the growth rate (μ) of microorganisms (from Adams and Moss, 2008)

hydrogen bonds in nucleic acid secondary structures after any abrupt decrease of temperature (Yousef and Courtney, 2003). In general, the proteins that are synthesized by most bacteria in response to cold shock can be classified as *cold- shock proteins* (Csps) and *cold-shock acclimation proteins* (Caps). The main difference between these two protein categories is that Csps are instantly coded and transiently overexpressed after a cold-shock, while Caps are synthesized during continuous growth at cold temperatures and remain overexpressed several hours after the reduction of temperature (Phadtare et al., 1999). Despite that Csps coded by different microorganisms may have high similarities (Abee and Wouters, 1999), their complete pattern of the encoded cold-shock proteins may differ (Jones, 2012; Datta and Bhadra, 2003; Sanders et al., 1999).

Upon further lowering the storage temperature at levels below 0°C (frozen storage), the microorganisms have also to tolerate the structural damages

that may occur due to the formation of ice crystals on the surrounding of the cells. Although frozen conditions are not used as a method of microbial destruction, mechanical injuries may be caused and thus, the viability of the microorganisms may be compromised (Mazur, 1984). Indeed, Dominquez and Schaffner (2009) reported significant structural injury of *Salmonella* spp. after inoculation on poultry and frozen storage (-20°C) for 16 weeks. Likewise, the higher populations of *E. coli* O157:H7 that were recovered on TSA supplemented with pyruvate compared with those on SMAC, after frozen storage in apple juice, indicate potential damage effect of frozen storage on the pathogen (Yamamoto and Harris, 2001).

1.2.1.2 Thermal treatments

Food thermal treatments during manufacturing constitute a mean to control the presence of pathogenic or spoilage bacteria in the products, by contributing to the reduction of the initial contamination levels. By these means, the microbiological safety of the products is ensured and their shelf life may be extended (Uyttendaele et al., 2008; Huss et al., 2000). Depending on the type of food and the sensitivity of its quality characteristics to heat, different methods of heat treatment may be applied, including pasteurization, commercial sterilization caused by Ultra High Temperature treatment (UHT), blanching of fresh vegetables and application of hot steam on meat carcasses. In addition to these, microorganisms may also be exposed to high temperatures in the domestic environment, during cooking or reheating of meals.

The efficacy of each thermal treatment depends on the final temperature, the duration of the application and the rate that temperature is increased, as well as the inherent thermo-tolerance of the microorganism (Shen et al., 2010; Aberle et al., 2001; Quintavalla and Campanini, 1991). The evaluation of the ability of a thermal application to reduce a microbial population is usually based on the calculation of two parameters; D and z. The D value describes the time a process requires to cause a decrease of one decimal log in

bacterial numbers, at a given temperature (Eq. (1-1). Whereas, the z value is the temperature difference required for the D value to change by a factor of ten (1 log change) (Eq. (1-2). These values are very useful means for the comparison of the thermotolerance of different microorganisms under the same conditions (Rajkowski, 2012; Stopforth et al., 2008; van Asselt and Zwietering, 2006; Murphy et al., 2002), or of the same microorganism under different conditions (Juneja and Marmer, 1999; Juneja et al., 1997; Carlier et al., 1996). In addition to *D* or *z* values*,* the total lethality process parameter (*F* value) is also used to evaluate the efficacy of a thermal treatment. This parameter represents the minimum required time to eliminate a known population of microorganisms in a given food and under specified conditions (van Doornmalen and Kopinga, 2009).

$$D = \frac{t}{LogN_0 - LogN_t} \quad \textbf{(1-1)}$$

where t is time N_0 and N_t the populations of the microorganism at time 0 and t, respectively

$$z = \frac{T_2 - T_1}{logD_1 - logD_2} \quad \textbf{(1-2)}$$

where T is the temperature and D the corresponding D-value.

The abrupt increase of temperature during a heat-shock can cause damage to the membrane of the cell, as well as to the macromolecular cell components, i.e. ribosomes, nucleic acids, enzymes and proteins which are located within the cell or on the cell membrane (Abee and Wouters, 1999; Yousef and Courtney, 2003). The microbial response to this stress is generally similar to the response to a cold-shock; on the one hand, the membrane damage is avoided by increasing the saturation and the length of the fatty acids, which is thought to recover the fluidity of the membrane that has been increased by the elevated temperatures (Cronan, 2002; Yura et al., 2000). On a molecular base, the microorganisms invoke the up-regulation of

heat protective proteins, called Heat Shock proteins (HSPs). The HSPs are either protein chaperones which assist in the repair of heat damaged proteins or are ATP-dependent proteases that may destroy the damaged components (Sergelidis and Abrahim, 2009; Arsène et al., 2000).

1.2.2 Intrinsic parameters

1.2.2.1 Low pH

Microorganisms may encounter stressful conditions in foods during manufacturing, during fermentation and in the gastrointestinal tract after the consumption of the contaminated foods. The natural pH of most foods ranges from neutral to highly acidic, as alkaline pH often renders the taste of the products rather unpleasant (soapy). In addition, food industries may regularly use acidic compounds (mostly organic acids) to improve the sensory characteristics of the final product. Given that the low pH conditions have been reported to have either bacteriostatic or bactericidal effect, depending on the pH value, the understanding of the response of microorganisms to acidic conditions may be of crucial importance for the food industries.

The mode of action of low pH against bacteria is generally focused on the lowering of the internal pH of the cell and/or the damage of the cell membrane. However, it may differ, depending on the type of the acidified agent (organic or inorganic acid). On the one hand, inorganic acids are not able to penetrate the cell membrane. Therefore, the acids increase the membranes permeability to protons (H^+) by denaturing the enzymes present in its outer membrane (Beales, 2004). By these means, the internal pH of the cell decreases and the cell metabolic activity and function are gradually reduced until the complete cessation of active metabolism and subsequently death of the cell. Organic acids, on the other hand, may be used either as taste improvers (e.g. citric or acetic acid) or as preservatives in the form of salts (e.g. salts of sorbic or benzoic acid). Depending on the external pH, these acids usually exist both in undissociated and dissociated form, with the

concentration of undissociated acid being higher at lower levels of pH. In the dissociated form, organic acids may not penetrate the cell membrane, while in the undissociated one they may freely pass to the cell interior. Due to the high pH in the internal environment of the cells, the undissociated acid form dissociates. However, the charged ions may not leave the cells through the membrane, resulting in the accumulation of high amounts of anions in the inner of the cell and thus, decrease of the internal pH could be expected (Figure 1-5). Such conditions are inhibitory for the microbial metabolism and gradually exhaust the cell (Theron and Lues, 2007; Lambert and Stratford, 1999). Organic acids have been also reported to affect the viability of the cells by causing damage on the phospholipid membrane (Stratford and Anslow, 1998).

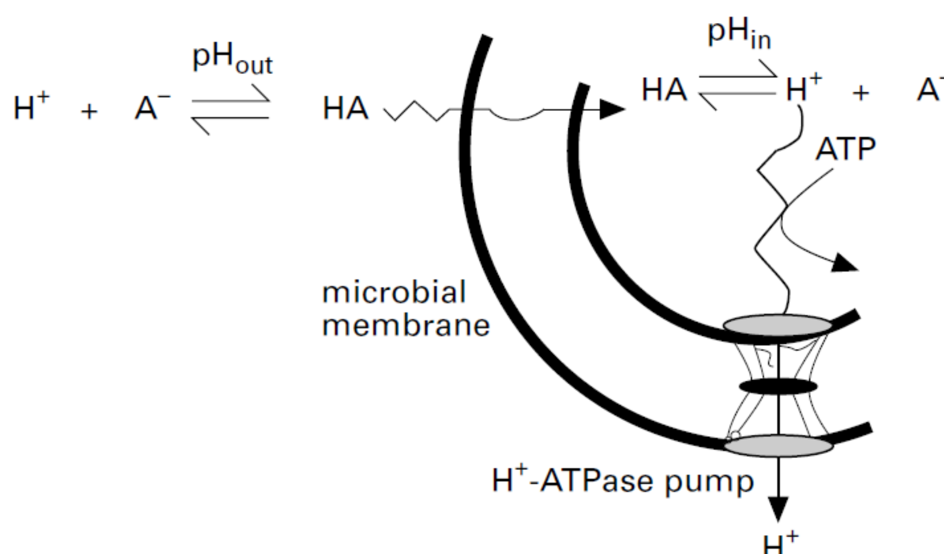


Figure 1-5 Representation of the mode of action of weak acids against bacteria. The low external pH favors the dissociation of the organic acid and the undissociated form is able to penetrate the cell membrane. When exposed to the high pH of the cytoplasm, the undissociated organic acid dissociates, while in parallel releases a proton. Since H+ cannot naturally exit the cell, the H+ATPase uses energy from the hydrolysis of ATP in order to pump the protons out of the cell (from Lambert and Stratford, 1999).

In an attempt to survive the acidic environmental conditions, microorganisms aim to maintain their internal pH (pH homeostasis; Beales, 2004). This may be achieved in many ways such as changes in membrane composition, induction of enzymes to repair macromolecules damages or discarding of protons from the cytoplasm (Yousef and Courtney, 2003; Foster, 2001). Regarding the latter, H+ATPase is an enzyme which utilizes energy from the hydrolysis of ATP in order to transfer the protons to the exterior of the cell and thus, maintain the internal pH (Figure 1-5; Gandhi and Chikindas, 2007). In addition, the response of bacteria to moderate acid stresses included also the synthesis of proteins, named acid shock proteins (ASPs) which promote survival at extremely low pH values (Abee and Wouters, 1999).

1.2.2.2 Water activity (a_w)

Water activity is a parameter for the quantification of freely available water in a food or else, the ratio between the water pressure of the environment above the food and the vapour pressure of distilled water under the same temperature (Jay et al., 2005). This parameter is frequently used to ensure the safety and quality of foods, as without available water the microbial growth is inhibited. By decreasing the a_w of a food the bacteria may perceive an osmotic stress or shock. As osmotic stress is termed any change in the concentration of compatible solutes or salts around a cell. In foods, osmotic stress conditions may be caused due to the addition of salts, sugars or other water-binding solutions such as starch or gelatine (Yousef and Courtney, 2003). Under such conditions, the phenomenon of passive osmosis takes place. In particular, water is drawn from the cytoplasm out of the cell in order to equilibrate the concentration of solutes in both environments. However, this procedure limits the available water inside the cell and inhibits the transport of nutrients from outside the cell. Thus, the microbial activity is suppressed. Under extreme conditions osmotic stress may also lead to

structural damages and finally kill the cells (Wesche et al., 2009; Csonka, 1989).

The response of microorganisms to hyperosmotic conditions is primarily based on either the intracellular production or import from the environment of compatible solutes such as carnitine, glycine betaine, proline, ectoine and trehalose. The accumulation of these compounds increases their concentration in the cytoplasm and thus, equilibrates with the concentration of those outside the cell, without however, affecting normal cellular functions (Jay et al., 2005; Yousef and Courtney, 2003). If the solutes are not imported from the cellular environment, their production is regulated by the activation and expression of specific genes or by modifying enzyme activity. The coding of such genes is generated by specific sensors and signal transduction networks providing information to the cell about the osmolarity of its surroundings (Kültz and Burg, 1998).

1.2.2.3 Nutrient availability and food structure

The availability of nutrients in foods plays a crucial role in the growth and survival of spoilage or pathogenic bacteria. Microorganisms require adequate amounts of essential nutrients to utilize for reproduction or at the very least maintenance of cellular composition. However, energy sources may either be absent or limited and thus, microbial proliferation is itself absent or limited. The continuous exposure of cells to low nutrient conditions produces the condition of starvation stress (Jones, 2012; Spector, 1998). Starvation stress may mostly occur on abiotic but contaminated surfaces such as kitchen counters, cutting boards, kitchen sponges, walls and floors, but also in water, on the surface of animal carcasses or fresh produce and other foods. Under these conditions, the energy sources are rapidly exhausted or are unavailable and cells limit their growth activity. The mechanism by which cells respond to starvation stresses is called stringent response. First cells enter the stationary phase where they are more capable to withstand further harsh conditions. At this phase, the size of the cells is reduced in order to

constrain their need to nutrients, but after exposure to optimum conditions the size and the functionality of the bacteria is restored. In parallel, the expression of specific genes is induced in order to code starvation stress proteins, which may further enhance the resistance of the cells and/or promote the utilizing of alternative sources of nutrients (Ravishankar and Juneja, 2003). Stressed cells responding to carbon exhaustion may also cross protect to others stresses, but this is not evident in nitrogen or phosphorus starved cells (Moat et al., 2002).

Food structure does not possess any antimicrobial activity by itself, but it may indirectly affect the growth potential of microorganisms by controlling the availability of nutrients and the diffusion of toxic metabolites (Ongeng et al., 2007). Depending on their rheological properties, foods may be distinguished into two categories; (i) liquid and (ii) structural (Theys et al., 2008). In liquid foods, nutrients are equally diffused in the whole substrate and they may be easily accepted by the microorganisms. Likewise, the products of the bacterial metabolic activity diffuse away, without affecting the surrounding micro-environment, where growth occurs. On the contrary in structural foods, cells are immobilized and may not easily reach the available nutrients and thus, starvation stress conditions are more common. In addition, the accumulation of toxic metabolic products may create adverse conditions for the growth or even the survival of the microorganisms (Noriega et al., 2010a; Wilson et al., 2002). However, cells which grow in liquid foods are more susceptible to antimicrobial compounds compared to those which grow in structured foods (Skandamis et al., 2000). In addition, in solid products bacteria grow in colonies and therefore, interactions between closely located cells may affect the growth kinetics of the population (e.g. *crowding effect*; Noriega et al., 2010a; Thomas et al., 1997). It has to be stressed though, that in a real food system both liquid and solid forms may be present, which makes the understanding of microbial behaviour in such products more complex.

1.2.3 Implicit parameters

1.2.3.1 Microbial interactions

In most research studies experiments are conducted on sterile nutrient media. In this manner, however, the interactions between the tested microorganism and the indigenous microflora of a food are excluded. In practice, products of animal or plant origin are carriers of a complex pattern of microbial flora, if no previous sterilization process has been followed. The behavior of each specific group of this pattern is highly associated with the activity of the remaining groups (Skandamis and Nychas, 2003; Samelis and Sofos, 2002). The interactions between microorganisms in a food system may be classified in to 5 major groups; (a) amensalism, (b) competition or antagonism, (c) commensalism, (d) parasitism, and (e) mutualism or synergism (Sieuwerts et al., 2008; Fleet, 1999). Amensalism describes the condition that a microbial group inhibits the growth of another closely located microorganism, without though affecting its own behavior. A classic example of amensalism is the behavior of commensal flora against immigrants (Al-Zeyara et al., 2011; Hwang and Sheen, 2011). During competitive or antagonistic activity both concerned microorganisms are competing for the uptake and utilization of nutrients, especially when some particular energy sources are in limited availability (Duffy et al., 1999), as well as for the available space for growth. During commensalism, the one organism benefits from the action of the second without however, affecting its behavior. This phenomenon may be observed when the available nutrients in a substrate may not be easily utilized by a microorganism, but the metabolic activity of the second microorganism may convert these compounds to a more acceptable form (i.e. disruption of macromolecules to smaller molecules) (Cooley et al., 2006). Parasitism is an interaction between two organisms, during which one species benefits against another (e.g. the activity of bacteriophages) (Sturino and Klaenhammer, 2004). Finally, synergism or mutualism describes the condition that two species are acting synergistically or else, each one benefits from the action of the other (Cheirsilp, et al., 2003). Overall, the interest in microbial interactions is not only focused on the

subsequent behavior of pathogens but also on the behavior of spoilage bacteria and starter cultures for food fermentation.

1.3 Common food-handling practices during distribution, in retail or in households

The quality and safety of final products depend on the constant manufacturing processes which are followed, according to the Good Manufacturing and Hygiene Practices (GMPs and GHPs), which constitute the pre-requisites of Hazard Analysis and Critical Control Points (HACCP) plans in the food industry. However, final products may be further exposed to less controlled conditions along the distribution chain and in the domestic environment. In particular, improper handling after the product release and during distribution, in retail or in households may result in significant deterioration of the quality and compromise the safety of the products (Figure 1-7). These handlings mainly refer to abuse temperatures that the products may be exposed to, cross-contamination in the retail or domestic environment and insufficient thermal treatment during cooking or reheating. For instance, Figure 1-6 illustrates reports of isolated VTEC from meat products from 1991 to 2011, with most of the incidents being linked to improper consumer handlings (JINN, 2012). It is concluded that such inappropriate post-production handling may affect not only perishable products (e.g. meat or fresh produce) but also shelf-stable foods, as the contamination of the latter with any pathogen may lead to serious foodborne diseases and, especially if no further antimicrobial process (e.g. sanitation, cooking, or reheating) is required.

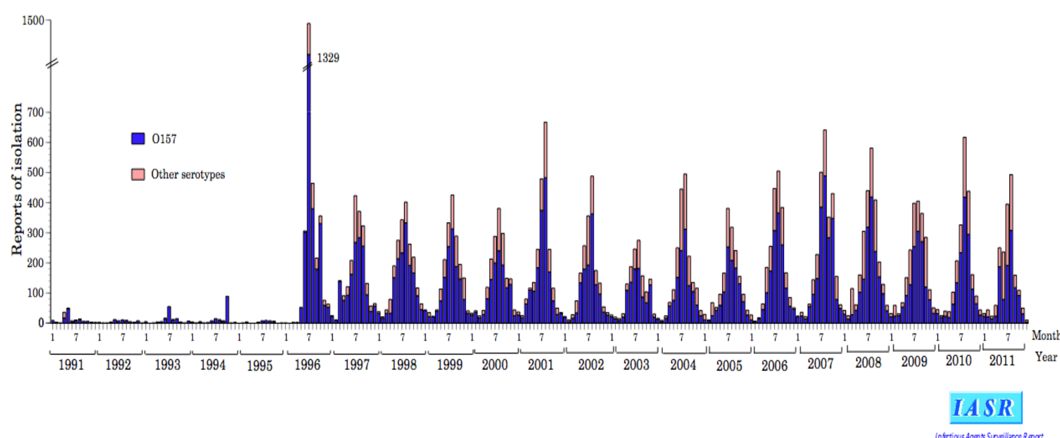


Figure 1-6 Number of isolation reports of vero-toxigenic *Escherichia coli* (O157 or other serotypes) from meat products. Most cases are attributed to common mishandling procedures by the consumers (JNII, 2012).

1.3.1 Food handling during distribution and in retail

Food transportation of foods from the processing plant to the retail outlet is the first stage of the distribution chain that may affect the quality and safety of the final product, especially when referring to foods whose microbial profile is highly dependent on storage temperature (Nychas et al., 2008). In addition, if the hygienic conditions are not maintained, the contamination of stable foods with pathogenic bacteria is possible, resulting also in unsafe products. These risks are even more significant, given that during this stage the products may sometimes be handled by untrained personnel (i.e. truck drivers).

The temperature during distribution of perishable foods such as raw meat, poultry, fishes, vegetables and fruits should not exceed the suggested temperatures (Panozzo and Cortella, 2008). However, especially in the summer period, the environmental temperature may affect the cooling capacity of the refrigerator used (Koutsoumanis and Taoukis, 2005). Indeed, Manios et al. (2009) monitored the temperature during the transportation of acidic spreads from northern to southern Greece in refrigerator trucks. In that study it was revealed that the temperature in the trucks ranged from above

5°C to 8°C, while the recommended temperature for such products was 4°C. Similarly, the temperature in refrigeration trucks during distribution of pasteurized milk reached 10.9^{o}C, while the average temperature of 12 to 15 trucks was 6.7^{o}C (Koutsoumanis et al., 2010). At the end point of distribution of fresh produce, Nunes et al. (2009) reported that the truck temperature ranged from 3.3^{o}C to 8.8^{o}C. Conversely, Derens et al. (2006) and Morelli and Derens (2009) reported that the average temperature (2.9 ± 1.4^{o}C and 3.6 ± 2.7^{o}C, respectively) during transportation did not exceed the suggested temperatures. Although the results of all the above studies reveal that the exposure of products to abuse temperatures during transportation is likely, the rather short transfer duration (from hours for perishable to a few days for more stable foods) could theoretically reduce the risk of significant quality deterioration. However, due the low infection dose of most pathogens, even one cell division cycle could raise safety issues.

Great variability of temperature is also observed in retail cabinets, with the mean temperatures being higher compared with those during distribution. This is primarily attributed to the type of the displays that are being used; open cabinets with no front doors show higher temperatures compared to those with covers. However, even if the latter occurs, regular opening of the doors can also affect the environmental temperature in the cabinet. Several surveys have been conducted on the temperature profile of retail displays (Koutsoumanis et al., 2010; Manios et al., 2009; Dallaire et al., 2006; Likar and Jevšnik, 1996; Willocx et al., 1994). The results of all studies showed that products may be regularly exposed to abuse temperatures (even above 15°C), due to improper handling or constant defrosting cycles of the cabinets. Another significant factor that should be taken into consideration for the temperature settings in retail cabinets is that the temperatures may vary, depending on the location of the load and the loading amount. Top shelves in the displays may have significantly different temperatures compared with the bottom or the middle shelves, while the position on each shelf (front or back) may also affect the temperature to which the products are exposed. (Koutsoumanis et al., 2010; Nunes et al., 2009; Willocx et al., 1994). Contrary

to the distribution stage, products may remain in the retail display for longer periods and thus, any upshifts in temperature may significantly affect both their microbial quality and safety.

1.3.2 Food handling by consumers

1.3.2.1 Transportation from retail

During transportation from the processing plant to the retail and storage in the retail cabinet, the storage temperature may be regularly checked, and corrected in case of any variation. In contrast, such control may be lost when the products leave the retail store. Given that the environmental temperature may reach high levels during the summer period, the most common problem of this handling is the duration that the products are exposed to such conditions, before they are placed in the domestic refrigerator. Relative studies showed that 38.1% of correspondents in China (Gong et al., 2011), 35% in Turkey (Karabudak et al., 2008) and 5.6% in New Zealand (Gilbert et al., 2007a) needed more than 1 hour to transfer the products to their home. In addition, the corresponding percentages of the consumers that used cooler bags or boxes for the transportation were only 10.2%, 4.8% and 18.6%. The duration between the retail and the home refrigerator includes also the time spent in the market, which acts additively to the total exposure time of the products to abuse temperatures. In the survey of Jevšnik et al. (2008) only 9.9% of the respondents purchased raw meat just before checkout, while only 58.1% of them found the duration of transport very important. The significance of the above improper handlings has been highlighted in the survey of James and Evans (1992), which showed that the temperature of products which were transferred for 1 hour in common bags, ranged from 18 to 38°C, but even if cooler bags were used the corresponding temperatures were 4-18°C. Overall, it is concluded that the behavior of the consumers is associated with their personal convenience and their poor education on food safety issues, and therefore, Foods Authorities should

invest more on this topic which may affect the shelf-life and the safety of a product.

1.3.2.2 Home storage

Following transportation to the domestic environment, products which require refrigerated or frozen storage conditions should immediately be placed in the cooler or the freezer, in order to inhibit the potential of any further microbial growth. Concerns have been raised though, about the awareness of the consumers on the necessary temperature that has to be kept in domestic refrigerators (Redmond and Griffith, 2003), as well as if this temperature is checked or calibrated regularly (Trepka et al., 2007). From a food safety aspect, temperatures in domestic refrigerators should not exceed 4.4^{o}C (40°F; USDA/FSIS, 2010a), which should be even lower when referring to particular types of foods such as fish or ground meat (Laguerre et al., 2002; Table 1-1). However, the average temperature of domestic refrigerators may deviate significantly from the suggested (Garrido et al., 2010; James et al., 2008; Carrasco et al., 2007; Gilbert et al., 2007b; Sergelidis et al., 1997), with some surveys reporting average temperatures even above 10°C (Willocx et al.; 1994). Similarly to the refrigerators in retails, differences in temperature

Table 1-1 Suggested temperatures for the storage of different categories of foods (Source: Laguerre et al., 2002)

Type of food	Suggested storage temperatures
Fish and seafood	0 to + 2°C
Ground meat	+ 2°C
Various types of meat and meat preparations, sausage, chicken, fresh milk, cheese	+ 4°C
Eggs	+ 5°C
Stable meat products, dairy products	+ 8°C

are also observed among the shelves in the domestic coolers, with the highest average temperatures being monitored at the doors shelves (Koutsoumanis et al., 2010).

The storage in domestic refrigerators or freezers is the last method to inhibit the deterioration of quality characteristics of a product. If the chill chain was followed properly and the expiration date not exceeded, the quality of the product should have remained above acceptable levels. Any further handling after this step (e.g. thawing, washing, sanitation, cutting, cooking) mainly affects the safety of the products (e.g. contamination transfer, growth of pathogens), as long as they are consumed immediately.

1.3.2.3 Hygienic conditions during handling of raw or Ready to Eat products

In the kitchen environment, poor hygienic practices may lead to the contamination with pathogenic bacteria of foods that have been treated and stored promptly until this stage. Higher concerns are raised about the contamination of RTE foods, such as vegetables, fruits and dairy products, as these foods do not require any further thermal treatment for their consumption. However, even raw foods may be potential vehicles of pathogens and further cause foodborne diseases, if inadequate thermal treatments are applied (Redmond et al., 2004).

Except for the improper storage temperatures, problems in the refrigerators may also be raised due to non-systematic and improper cleaning of the interior of the cooler. In particular, direct contact of foods with poorly sanitized surfaces in the refrigerator may favor the transfer of pathogens from raw to RTE foods, such as vegetables (Ravishankar et al., 2010; Jackson et al., 2007). Similarly, raw foods placed on the upper shelves may contaminate foods below them by dripping onto them, if improperly packaged. As a further step, during meal preparation, the hygienic conditions of the kitchen surfaces

and utensils must be maintained at high levels, in order to avoid any transfer of contamination. A very common mishandling of such nature is the use of the same cutting board and/or knife for meat, fish and fresh produce (van Asselt et al., 2008; Jay et al., 1999). Since common dishwashing procedures do not adequately eliminate the risk of cross contamination (de Jong et al., 2008), it is suggested that different surfaces and utensils be used for each different food commodity. Insufficiency of cleaning may gradually lead to biofilm formation, rendering more difficult the elimination of pathogens from the domestic environment with conventional sanitizers (Simões et al., 2010). In addition, a possibly unpredictable contamination transfer route is through the cleaning utensils that are used for the sanitation of the food-contact surfaces. Kitchen sponges, dish cloths, and washing brushes have been reported to be not only carriers of the most common foodborne pathogens (Mattick et al., 2003), but also to favor their growth, due to the presence of food residues and high amounts of available water. Improper personal hygiene may also favor the transfer of contamination to RTE or Ready to Cook products (Haysom and Sharp., 2005; Bloomfield, 2003). Indeed, in the survey of Gong et al. (2011) only 59.6% of the respondents cleaned their hands properly after handling raw meat, while other respondents used only water (31.4%), wiping with a towel (7.9%) or did not clean their hands (1.1%). Kohl et al. (2002) reported that inconsistent hand washing before and after handling of raw meat and foods ready for consumption increased the risk of *Salmonella* sporadic outbreaks. As a conclusion, in the domestic environment different types of foods must be stored and prepared separately, the personal hygiene must be maintained properly and kitchen equipment and supplies should be cleaned and sanitized correctly.

1.3.2.4 Thawing practices

Frozen storage is used to preserve the quality and safety of products for long-term periods, as any microbial or enzymatic activity is much slower than in refrigerator. However, contrary to refrigeration, the preparation of foods

after freezing requires thawing before cooking. This step may favor microbial proliferation if the suggested guidelines are not followed. But due to fact that the duration of this process is short, only the growth of pathogenic bacteria is of high importance. The USDA/FSIS (2010b) has issued some general guidelines for the thawing practices that should be followed by the consumers, in order to avoid any undesirable microbial growth. According to these recommendations, food should be thawed under refrigerated conditions (4°C) or under regularly changed cold water, while microwave is also allowed but only if the food is consumed immediately after defrosting. In contrast, thawing on the counter-top (environmental conditions) or under hot water is not suggested, as the microbes may be exposed to temperatures within the danger zone (4-60°C). Under all circumstances, foods should not be refrozen after thawing. The majority of the consumers though do not follow these recommendations, as they are not in accordance with their personal convenience. A summary of the thawing methods, as reported in surveys conducted in different counties are given in Table 1-2. In general, the method that is chosen by most respondents was thawing at room temperature, while refrigerator was their second choice. However, under room temperatures, the exterior of the frozen product is thawed quickly and bacteria that may likely occur at this part are exposed to elevated temperatures for a long period. Similar are the effects of thawing under hot water, while in the refrigerator or under cold water, food is not exposed to any temperatures in the danger zone. Finally, despite that the final food temperature in microwave may be high, food is thawed rapidly and, thus no microbial proliferation is allowed.

Table 1-2 Percentage (%) of consumer practices which are commonly followed for the thawing of frozen foods, as revealed from surveys in different countries

Thawing method						Country of origin	*Source*
Cold water	**Room temperature**	**Hot water**	**Refrigerator**	**Microwave**	**Cook frozen**		
n.a.[a]	50.4	12.8	16.6	11.4	8.8	Slovenia	*Jevšnik et al., 2008*
28.0	41.6	*n.a.*	12.0	18.4	*n.a.*	West Indies	*Bardie et al., 2006*
n.a.	56.0	1.0	23.0	13.0	1.0	Ireland	*Kennedy et al., 2005*
n.a.	45.2	*n.a.*	20.2	*n.a.*	*n.a.*	West Indies	*Surujlal and Badrie, 2003*
n.a.	21.0	*n.a.*	41.0	31.0	*n.a.*	USA (Arizona)	*Meer and Misner, 2000*
2.2	40.1	*n.a.*	34.4	18.5	1.2	Australia	*Jay et al., 1999*
23.8	30.9	16.6	7.0	5.8	8.1	China	*Gong et al., 2011*
4.1	46.2	*n.a.*	25.8	18.2	5.7	New Zealand	*Gilbert et al., 2007a*
2.0	66.9	2.9	14.7	2.2	9.4	Turkey	*Karabudak et al., 2008*
n.a.	61.8	*n.a.*	38.2		*n.a.*	USA (Florida)	*Trepka et al., 2007*
7.6	25.6	*n.a.*	51.4	30.6	0.9	Canada	*Nesbitt et al., 2009*
n.a.	16.0	*n.a.*	56	6	22	USA (California)	*Phang and Bruhn, 2011*

[a] not applicable

1.3.2.5 Cooking practices

Cooking is the last barrier before consumption that may eliminate the risk of a potential foodborne disease. This treatment incorporates two different variables; the cooking method and amount of cooking, which describes the target temperature at the geometrical centre of the product. The first variable is more of a personal preference issue, as it may directly affect the taste and the texture of the cooked food. Most of the studies on cooking methods have been focused on the survival of pathogenic bacteria during cooking of meat and meat products. According to relevant surveys, consumers prefer mostly outdoor grill (BBQ), broiling and frying to cook their meat, compared to other methods such boiling (McKenna et al., 2004; Lorenzen et al., 1999). However, it has been found that the way of heat transfer to the interior of the food and thus, the intensity of thermal treatment that characterizes each cooking method, may significantly affect the ability of the cooking method to ensure the safety of the cooked product (Shen et al., 2010). Despite these research findings, no specific suggestions have been issued from the food safety authorities for the safest cooking methods of meats or other products.

Conversely, the level of 'doneness' has a more immediate effect on the survival of pathogens in foods. Monitoring the internal temperature (at the most thick part or the geometrical centre) of foods with a precise thermometer is the recommended method to determine if a product is cooked and safe for consumption (USDA/FSIS, 2011a). The suggested internal temperatures depend on the type of food, and are presented in Table 1-3. Indeed, sometimes standing time is also required as an extra parameter to ensure the destruction of all pathogens (Table 1-3; Passos and Kuayre, 2002). As it can be observed in Table 1-3, higher target-temperatures are required when the contamination may be allocated to the interior of the product (e.g. ground or blade/needle tenderized meat), compared with the whole cuts where the bacteria are mostly located on the surface.

Table 1-3 Suggested minimum internal temperatures which ensure the elimination of pathogenic bacteria in different food commodities (Source: USDA/FSIS, 2011b)

Product	Type	Temperature
Beef, pork, lamb and veal	Whole cuts (steaks, roasts and chops)	62.7^{o}C (145°F) + 3 minutes standing
	Ground	71.1^{o}C (160°F)
Chicken and Turkey	Breasts	73.8^{o}C (165°F)
	Ground, stuffing, and casseroles	73.8^{o}C (165°F)
	Whole bird, legs, thighs, and wing	73.8^{o}C (165°F)
Eggs	Any type	71.1^{o}C (160°F)
Fish & Shellfish	Any type	62.7^{o}C (145°F)
Leftovers	Any type	73.8^{o}C (165°F)
Ham	Fresh or smoked (uncooked)	62.7^{o}C (145°F) + 3 minutes standing

Although, any deviation from these temperatures may likely compromise the safety of the final product, consumer's sensory preferences may lead to undercooked foods (Lopez Osornio et al., 2008) and thus, compromise their safety. In addition, the use of thermometers is not easily followed in the domestic environment (McCurdy, et al., 2005) and therefore, consumers choose other methods for this reason. The color of the interior of meat or the total cooking duration is usually used as alternative methods to determine the amount of cooking (Rhee et al., 2003). However, these criteria may be affected by many factors such as fat content, freezing history, ingredients or the cooking method (King and Whyte, 2006; Berry, 1998), and further lead to unintentional undercooking.

1.3.2.6 Handling of food leftovers

The daily meals are often not consumed entirely, and improper storage conditions or mishandling of the leftovers may favor the growth of bacteria, which survived the cooking process or, most likely, occurred during consumption (post-contamination). Therefore, it is suggested that after cooking, leftovers should be immediately cooled below 4°C, or kept above 60°C, if intended to be consumed in short period (such as in catering services). In cases where foods are exposed to the danger zone (4-60°C) for more than 2 hours (or 1 hour if temperature above 32.2^{o}C), they should be discarded (USDA/FSIS, 2012a), as these temperatures may favor not only the growth of pathogenic bacteria, but also of spoilage microorganisms, accelerating this way the deterioration of leftovers quality. In addition, well sanitized storage containers should be used, in order to avoid any cross-contamination phenomena (Beumer and Kusumaningrum 2003). Leftovers would not pose such a significant health risk, if reheating was applied promptly (Adekunle et al., 2009). Foods containing meat should be reheated up to 73.8^{o}C (165°F), sauces and soups until boiling, while slow reheating should be avoided (USDA/FSIS, 2012a).

As observed in most consumer practices, handling of leftovers is based on the personal convenience and they are usually not compatible with the issued guidelines. Gong et al. (2011) showed that 48.7% of the respondents in mainland China stored their leftovers at room temperature and only 18.1% put them in the refrigerator. In the same survey, from those who responded that they keep food out of the refrigerator, only 27.5% keep it for less than 2 hours, while there is a 14.5% of that may keep it for more than 1 day. Regarding the level of reheating, most of the consumers do not use a thermometer for this purpose, but just optical observations and sensory preferences. Even under these options, Badrie et al. (2006) reported that 34.5% of the respondents in West Indies reheat their leftovers until sufficiently warm or do not reheat at all, before consumption. The corresponding answers in New Zealand and Turkey were

16.4% (Gilbert et al., 2007b) and 37.5% (Karabaudak et al., 2008), respectively. Overall, it is derived that consumers may mistakenly perceive leftovers as low-risk foods, probably due to the prior cooking process, and therefore, the safe handling procedures for this type of food should be brought to their attention.

1.4 Modelling of microbial behaviour

1.4.1 Predictive modelling

As described in paragraph 1.2, the behaviour of spoilage or pathogenic bacteria may be affected by various environmental or food-related factors. Consequently, it is of high importance to evaluate the growth of both spoilage and pathogenic microorganisms. Specific challenge tests on artificially contaminated products or trials, where commercial products are stored under constant conditions, should be conducted in order to evaluate the safety or the shelf-life, respectively, of a product under these conditions. However, these experimental trials are too laborious and time consuming, judging from the limited information obtained (e.g. results refer only to the specific food and/or experimental conditions). Therefore, it is essential to develop means that may provide all the information needed about the microbial safety or quality of a product, by considering all the processing or environmental parameters that may inhibit or stimulate microbial growth (e.g. pH, temperature, a_w, competitive microflora). Predictive microbiology is focused on the development and validation of such tools.

Predictive modelling is a part of food microbiology which studies the use of mathematical equations and statistical approaches to predict the growth, survival or inactivation of pathogenic or spoilage bacteria in foods. In other words, predictive microbiology utilizes all the appropriate microbiological data required to quantify the outcome of the interaction between the environment and

the microorganism. Therefore, Ross and McMeekin (1995) also suggested the term "quantitative microbial ecology of foods" to describe predictive microbiology. The knowledge of the behaviour of microorganisms in food products, as predicted by the mathematical models, may assist in the easier and more rapid evaluation of the microbiological safety and quality of foods (Swinnen et al., 2004).

1.4.2 Classification of predictive models

Many suggestions have been made for the classification of predictive models. Models that describe the kinetics of a microorganism are called *"kinetic models"*, while if the probability of growth of a microorganism or the production of a specific metabolite is examined, the models are termed "probabilistic" (McMeekin and Ross, 2002). Another approach classifies the models as mechanistic or empirical based on whether they have a theoretical basis or not (Fakruddin et al., 2011). Whiting and Buchanan (1993) suggested also a three level classification; primary, secondary and tertiary models: *Primary models* are models that describe the changes (growth or death) of a microbial population with time and under a specific set of environmental conditions McKellar and Lu, 2004); *Secondary models* describe the kinetic parameters obtained from primary modeling as a function of selected environmental factors; *Tertiary models* integrate the primary and secondary models in a user-friendly interface, which further links the gap between predictive modeling and the food industry. The most commonly used primary, secondary and tertiary models are presented in Table 1-4.

Table 1-4 List of the most commonly used primary, secondary and tertiary models

Primary models	Secondary models	Tertiary models
Logistic	Square root	Pathogen Modeling Program (PMP)
Modified logistic	Ratkowsky	ComBase
Three Phase Linear	Arrhenius	Seafood Spoilage & Safety Predictor (SSSP)
Gompertz	Polynomial	Refrigeration Index
Modified Gompertz	Probability	USDA Pathogen Program
Baranyi	Surface models	Growth Predictor
		Pseudomonas Predictor

1.4.3 Development of mathematical models

The development of a mathematical model for the prediction of the microbial behaviour under specific conditions or in a food system follows usually a four-step procedure: (a) experimental design, (b) data generation, (c) model development and (d) model validation. Each of these steps is very significant for the development of a successful model.

1.4.3.1 Experimental design

The careful consideration of experimental design for the collection of microbiological data is crucial for the development of an accurate model. The first step for the development of a model is to clearly determine its objective and, then to identify the environmental factors (variables) which may affect the growth potential of the target-microorganism. Following this, the less important variables

should be excluded from the process, and the range of the remaining ones has to be determined (Davies, 1993). Such planning assists in the conduction only of the necessary trials and the avoidance of extrapolation of the model (Rash, 2004), since the ability of a model to provide accurate predictions outside its interpolation area is limited.

1.4.3.2 Data generation

Following experimental design, the development of a model requires the production of microbial data derived from experimental trials. The quantity of these data is proportional to the reliability of the developed model. In addition, the quality of the generated data has also a significant effect on the accuracy of the model, as sampling points should be evenly distributed over the entire growth period (McDonald and Sun, 1999).

For the collection of the desired data, the method that is most commonly followed is the total viable count method, which requires plating on nutrient media for the enumeration of the microbial populations. Since this method is laborious and time consuming, alternative procedures have been also suggested. The use of optical density measurements (O.D.) to simulate the growth behaviour of a microorganism is increasingly used, due to the inexpensive, simple and automated nature of this method. Despite that some concerns have been raised (Krist et. al., 1998), this method is able to provide large amounts of good quality data (Mytilinaios et al., 2012; Biesta-Peters et al., 2010; Dalgaard et al., 1994), and thus contribute to the development of reliable models. Other methods for the generation of data are also reported to be the flow cytometry and the colony size measurements (Rasch, 2004).

1.4.3.3 Model development

The general concept of the development of mathematical models is divided into two phases; firstly, the microbiological data (log *vs.* time) derived from the experiments are fitted to a primary model, in order to estimate a specific kinetic parameter (e.g. growth rate, lag time, D value). As a further step, this parameter is modelled as a function of selected environmental conditions, using a secondary model. The selection of the appropriate model is based on diagnostics, which are mathematical tests that validate the suitability of a model. (Legan and Vandeven, 2002).

1.4.3.4 Model validation

Although there are some predictive models that have been developed in foods, the majority are usually developed under controlled conditions in laboratory culture media. In culture media only the combinations of the most crucial environmental parameters are described (e.g. temperature, pH, a_w, concentration of preservatives). However, in a food system, other factors (e.g. microbial interactions) could possibly cause slight deviation between the predicted and the observed values (McDonald and Sun, 1999). Therefore, a validation process is also required, in order to ensure that the predictions obtained are also valid under realistic conditions (McMeekin et al., 1997). Validating a model could be conducted with data derived from experiments on a real food or on laboratory media, or even by comparing the predictions with already published results, with the first option considered as more accurate (Manios et al., 2013; Blackburn et al., 1997). However, in any case, these data should not have been used during the construction of the model, but they must have been derived from independent trials. In addition, it is really important that the results of the validation process show that the predictions of the model are “fail-safe”, or else, the model predicts earlier growth of the microorganisms than this under realistic conditions. The latter is crucial, especially for the safety

management of a product, but also for the determination of the remaining shelf-life (Brocklehurst, 2004).

1.4.4 Practical implications of predictive models

Predictive models constitute very quick, accurate and cost effective means for the assessment of the behavior of microorganisms under specified conditions. However, there is a gap between the development of a model by the experts and its implementation in the food industry, mainly derived from the complex scientific base that predictive models are created on. The advantages, though, of quantitative microbiology compared with the classic analytical methods have played significant role in order this gap to be bridged.

In the industrial environment, predictive microbiology has many practical implications (Table 1-5). According to Membré and Lambert (2008), they may be categorized in three groups; (a) product innovation, (b) operational support and, (c) incident support. The first category includes all the necessary actions required for the development of new products, reconsideration of the formulation of existing products or determining the optimum storage conditions, in order to produce safe and of high quality products. In the second category belong the supporting decisions that can be made, based on the predictions of the model, and which may assist the production of safe products (e.g. determination of critical control points or safe processing conditions). Finally, predictive models may be also involved and support the risk management of unexpected problems caused after the release of the products.

Regarding the shelf-life of a product, mathematical models may adequately predict the dependency of the growth potential of the specific spoilage microorganisms on the current or previous environmental conditions and/or product formulation (Manios et al., 2009; Dominguez and Schaffner, 2007; Mataragas et al., 2011, 2006; Koutsoumanis et al., 2006). Thus, the shelf-life

Table 1-5 Summary of the major practical implications of predictive microbiology in the food industry environment (from Fakruddin et al., 2011)

Application area	Examples
Hazard Analysis Critical Control Point (HACCP)	Preliminary hazard analysis Identification and establishment of critical control point(s) Corrective actions Assessment of importance of interaction between variables
Risk assessment	Estimation of changes in microbial numbers in a production chain Assessment of exposure to a particular pathogen
Microbial Shelf life studies	Prediction of the growth of specific food spoilers Prediction of growth of specific food pathogens
Product research and development	Effect of altering product composition on food safety and
	spoilage Effect of processing on food safety and spoilage
	Evaluation of effect of out-of-specification circumstances
Temperature function integration and hygiene regulatory activity	Consequence of temperature in the cold chain for safety and spoilage
Education	Education on safety, especially non-technical people
Design of experiments	Number of samples to be prepared Defining the interval between sampling

determination of newly developed products becomes easier, while the reconsideration of the remaining shelf-life of a product exposed to the fluctuating conditions along chill chain is also possible. In addition, the data derived from such models may provide useful information for the optimization of the formulation of a product, without affecting its microbial stability (Membré and Lambert, 2008). From a food safety aspect, predictive models may constitute as complimentary tools in the various steps of HACCP plans. Firstly, growth/no growth models may contribute to the identification of potential hazards of a food, by specifying the conditions that growth/survival is permitted. As a further step, these results can also be used for the identification of the process stages that may affect the safety of the final product and thus, assist to define the critical control points along the production line (McMeekin et al., 2006; Legan and Vandeven, 2002; Notermans et al., 1994). When familiar to the possible source of a hazard and the factors that may affect its growth potential, the food industry may also specify the corrective actions in case of a unexpected problem in any Critical Control Point (McMeekin and Ross, 2002).

1.5 Outline and objective of the Thesis

After manufacturing, products may likely be exposed to favourable conditions for microbial growth or transfer, such as abuse temperatures during distribution, storage in retail, transportation to the domestic environment, storage in domestic refrigerators or during thawing of frozen foods, or cross contamination issues during food preparation and insufficient cooking (Figure 1-7). Since these factors are directly associated with the microbiological quality and safety of foods, it is of high importance to determine the microbial dynamics under realistic mishandling scenarios. This Thesis is focused on the investigation of such potential

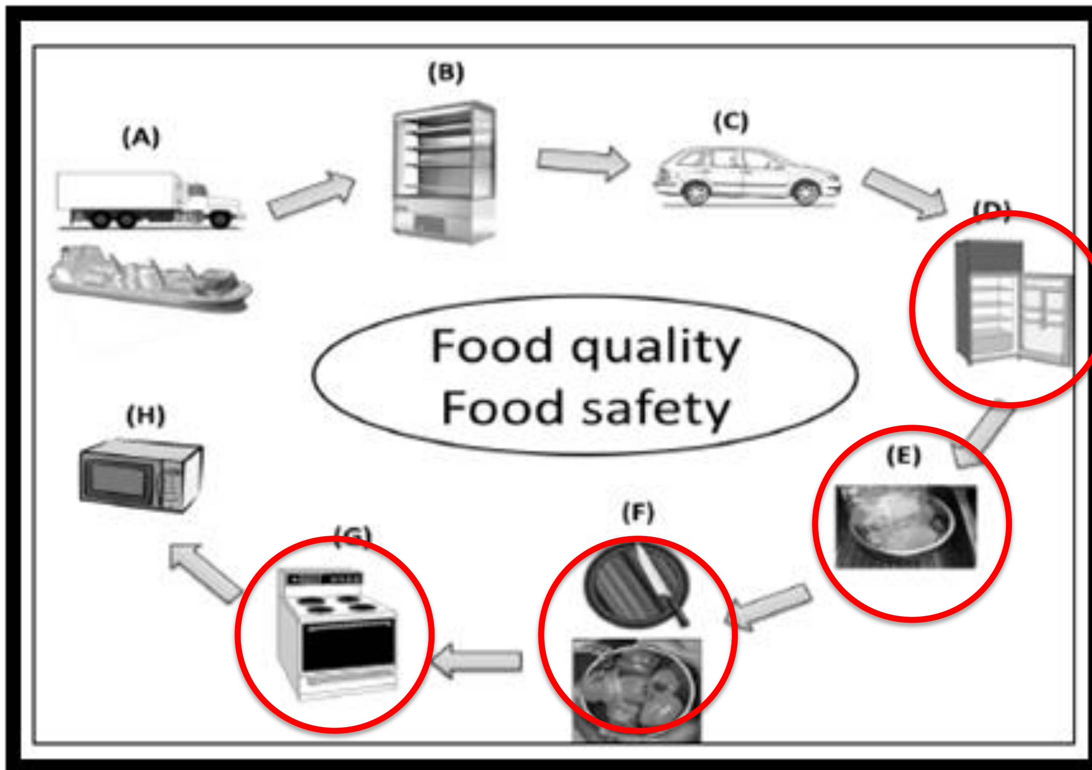


Figure 1-7 Post-manufacturing stages that may affect the quality and safety of foods, due to improper handling. (A) Transportation from processing plant to retail, (B) Retail storage, (C) Transportation from retail to home, (D) Storage in domestic refrigerators, (E) Thawing practices of frozen foods, (F) Food handling (e.g. slicing, washing), (G) Cooking, (H) Reheating. Red circles highlight the stages that have been examined in the present Thesis.

unidentified sources of risk in the domestic environment that may affect the quality and safety of RTE and RTC products.

In the first part of the Thesis, the potential of different mathematical models to predict the spoilage of RTE spreads stored in domestic refrigerators was evaluated. The development of such models requires extensive research on the identification of the specific spoilage microorganisms and their dynamics during

storage. Therefore, the spoilage pattern of the spreads was described on a microbiological, molecular and physicochemical base, before the development of the models. The validation process of the models that was followed, revealed that despite the temperature configuration of the refrigerators according to the suggested guidelines, the products could be exposed to abuse temperatures during storage or even higher during dinner. In addition, although the developed models could adequately predict the behaviour of the SSOs in the spreads under dynamic conditions, the necessity of a known initial contamination level was crucial. These findings highlighted (i) the potential of unintentional mishandlings to affect the quality of such products and (ii) the significance of the initial contamination level on the behaviour of a microorganism.

As a result of the findings of the first part, two experiments were designed on (i) the effect of common consumer-style practices (proper or improper) for handling of RTC foods in the domestic environment on the safety of the final product and, (ii) the differences on the behaviour of higher initial levels of contamination, which correspond to those used in common *in vitro* trials, and realistically low levels of contamination (1-4 cells) in a RTE food. Both studies revealed that consumer improper practices in the domestic environment may not only deteriorate the microbiological quality of the final products (study 1), but also compromise their safety.

An overview of the specific topics and their correlation is schematically given in Figure 1-8.

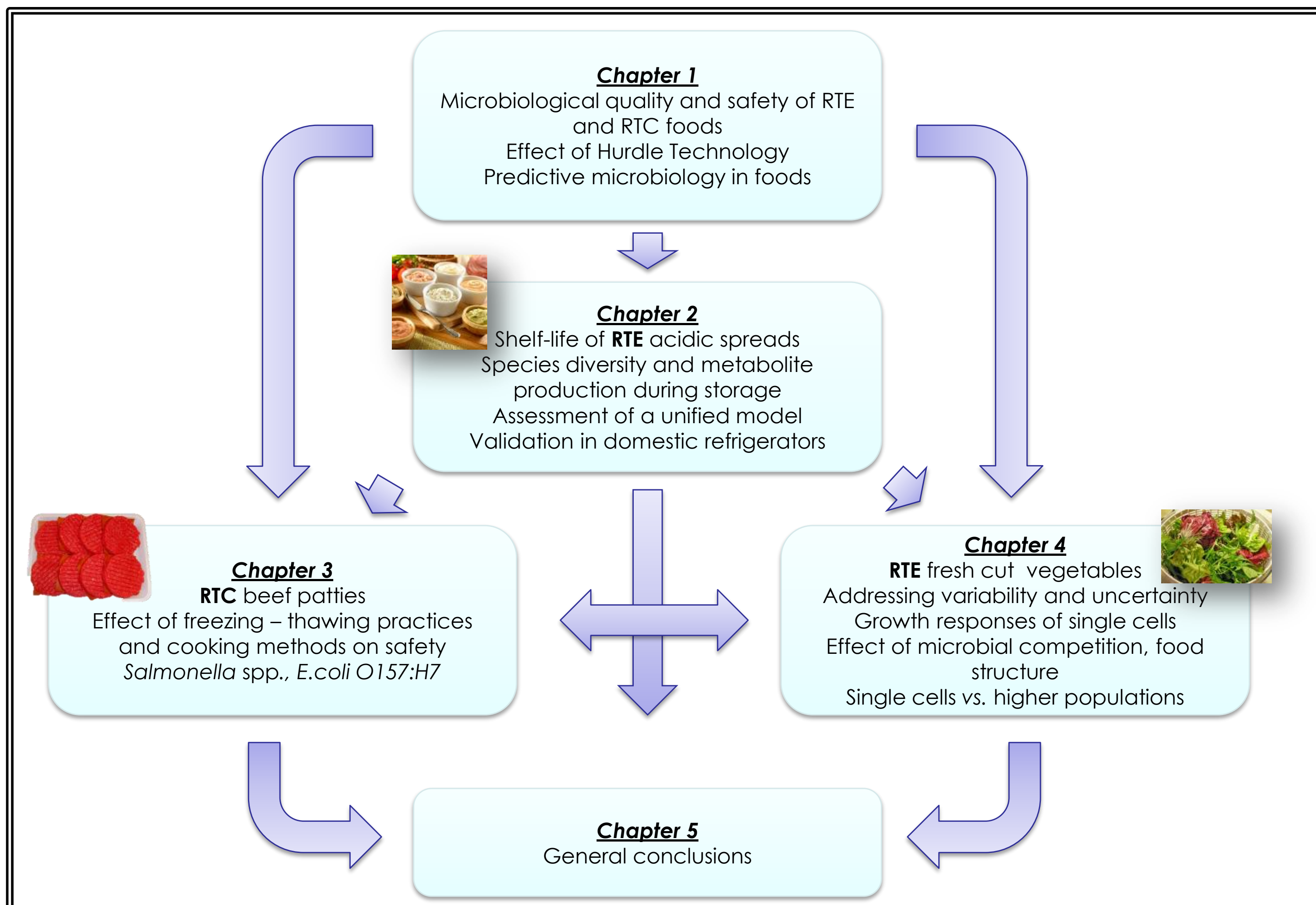


Figure 1-8 Schematic outline of the present study

<u>The objectives of each chapter are listed below</u>

Chapter 1:

(i) To identify and understand the association of food quality and safety with abiotic fluctuations encountered in the domestic environment.

(ii) To investigate the responses of microorganisms to these stresses and the correlation of these responses to food safety and quality.

(iii) To review the existing predictive modelling tools and their practical implications

Chapter 2:

(i) To correlate microbial spoilage, species diversity and physicochemical changes during storage of three acidic spreads under different temperatures

(ii) To develop product – specific predictive models for the growth of the SSOs of these products in response to temperature.

(iii) To evaluate the applicability of a unified predictive model for the growth of the SSOs based on the weak acids concentration, the initial pH and the storage temperature.

(iv) To validate the model under realistic conditions, in four domestic refrigerators

Chapter 3:

(i) To evaluate the single and synergistic effect of

a. frozen storage,
b. thawing method
c. cooking method
d. amount of cooking

of commercial-style beef patties on the survival of *Salmonella* spp. and *Escherichia coli* O157:H7, simulating common practices used by the consumers in households or restaurants.

Chapter 4:

(i) To determine the variability in the growth of 2 pathogens in lettuce and cabbage at 8°C.

(ii) To compare the response of low number of cells (1-4) with that of higher populations.

(iii) To identify the contribution of the commensal flora, temperature abuse and food structure in the above variability.

(iv) To evaluate whether broth-based growth simulations may approximate the average outgrowth of a population from single cells in foods.

2 ASSESSMENT OF A UNIFIED PREDICTIVE MODEL FOR THE SHELF-LIFE OF ACIDIC SPREADS

2.1 Introduction

Acidified sauces, salad dressings, spreads and dips are emulsified foods of animal or plant origin, which may be served as complementary dishes of most meals. Mayonnaise and mayonnaise-based dressings (e.g., ranch, blue cheese, Thousand Island, Caesar dressing), ketchup, barbeque sauce, cheddar cheese dip etc., are some well-known products of such nature. The microbial stability of these foods relies on the low pH achieved through acidulants and also weak acid preservatives, such as salts of sorbic or benzoic acid, which not only help to buffer the system but also have an intrinsic antimicrobial effect themselves (Vermeulen et al., 2007). These intrinsic factors contribute to a shelf-life of several months, even if the products are stored under ambient temperatures. Currently most food industries use the code of the Committee of the Industries of Mayonnaises and Table Sauces of the European Economic Community (CIMSCEE, 1992) in order to determine the probability of microbial growth in any acetic acid ambient-stable sauce. This probability growth/no growth model calculates a numerical value (Σ), based on the products content in undissociated acetic acid (UAC), NaCl, hexose (glucose and fructose) and disaccharides (2-1).

$$\Sigma = 15.75 \times [UAC] + 3.08 \times [NaCl] + hexose + 0{,}5 \times [disaccharides] \qquad \textbf{(2-1)}$$

If Σ value is above 63 the product is considered shelf-stable and thus, no refrigeration is required for a long-term shelf-life (e.g. ketchup and ranch). However, in some products, such as guacamole, Σ value may not exceed the determined limit and therefore, the additional hurdle of refrigeration should be implemented to ensure the maintenance of the freshness and the sensory

characteristics of the products. The guacamole-style category also includes a variety of well-known spreads and dips (i.e., tzatziki, wasabi, pepper dip etc.), which are produced based on vegetables, cheese, yoghurt and/or mayonnaise, with the addition of acetic acid as acidulant. As long as constant chill-chain conditions have been followed, the spoilage of these products is more likely related to slow physicochemical changes such as dissociation of the emulsion, oxidation, hydrolysis, and discoloration. (Waite et al., 2009). However, chill chain conditions are often not properly maintained (Nunes et al., 2009; Willocx et al., 1994), and products are often exposed to abuse temperatures, especially in retail and home refrigerators (Manios et al., 2009), which may favor the growth of undesirable bacteria, capable of compromising their quality. The acidic environment of these products usually favors the proliferation of aciduric bacteria (i.e., lactic acid bacteria) and inhibits other non-acid tolerant microorganisms such as pseudomonads or enterobacteria. The growth of these microorganisms commonly causes an increase of the acidity due to the release of acidic metabolites, swelling of the package due to excessive production of gasses, or slime formation (Vermeulen et al., 2007; Huis in't Veld, 1996). However, changes of the environmental conditions may result in significant diversity of the specific spoilage microorganisms (SSOs) and, subsequently, affect the profile of the produced metabolites that render the product organoleptically unacceptable (Ercolini et al., 2011). Considering that these products are multi-ingredient ecosystems and that they are usually stored under fluctuating temperatures, the quantification of the microbial metabolites and their correlation with the growth of the SSOs and changes in sensory characteristics may contribute to a more accurate determination of their shelf-life (Dainty, 1996).

Since the determination of food quality is affected by various factors, including the subjective consumers opinion (see section 1.1.1), the quantification of this term may only be done based on the quantitative factors, such as microbial growth or the production of microbial metabolites (Zwietering et al., 1993). In order to avoid the laborious and expensive traditional microbiological trials, predictive models may be used as means for

the rapid and inexpensive determination of the shelf-life of a fresh product or the remaining shelf-life of products exposed to chill chain conditions. The majority of the developed mathematical models refer to the growth or inactivation of pathogenic bacteria (Juneja et al., 2006, 2009; González et al., 2009), while in contrast, the developed models for the prediction of the growth of spoilage microorganisms are much fewer (Bruckner et al., 2013; Mataragas et al., 2006, 2011). This mainly relies on the fact that the successful development of a shelf-life model requires further identification of the specific spoilage microorganisms and studying of their growth potential under a range of environmental conditions (Bruckner et al., 2013). Therefore, it is essential to monitor the microbial dynamics of the SSOs directly on a product, in order to obtain more accurate predictions of its shelf-life.

Another disadvantage of spoilage predictive models, which are developed and validated on a product, is that their predictions refer only to the specific food and thus, extra trials are required for different products. However, the spoilage of closely related products is primarily affected by the same extrinsic or intrinsic parameters (e.g. temperature, pH, a_w, packaging gas composition), since other parameters such as diversity of the SSOs and structure do not differentiate significantly. Such models are termed “unified” and they may be developed to account for a group of products rather than a single product (Bruckner et al., 2013; Wilson et al., 2002). Although this “global” approach excludes significant factors for the microbial growth, such as food structure or indigenous microflora (Manios et al., 2013), it is assumed that the overall effect of these factors is represented by the impact of the variables which are taken into account. By these means, a carefully designed and validated unified model, may describe the microbial behavior in a whole food category with limited data.

The objectives of the present study were; (i) to correlate microbial spoilage, species diversity and physicochemical changes during storage of three acidic spreads under different temperatures, (ii) to develop product – specific

predictive models for the growth of the SSOs of these products in response to temperature, (iii) to evaluate the applicability of a unified predictive model for the growth of the SSOs based on the weak acids concentration, the initial pH and the storage temperature and, (iv) to validate the model under realistic conditions, in four domestic refrigerators

2.2 Materials and Methods

2.2.1 Products characteristics

Three commercial vegetable-based emulsified spreads, which were prepared using traditional Greek recipes, were kindly provided by Hellmann's (Elais-Unilever Hellas, Greece), in commercial packages of 250 g. In particular, a pepper-based spread (pH 3.90 – 4.02; consisted of 80% fine chopped sweet pepper, vinegar, extra virgin olive oil, vegetable oil, sugar, eggplant pulp, wheat fibers, salt, garlic, spices and seasonings, with 0.1% potassium sorbate as a preservative), an eggplant-based spread (pH 4.00 – 4.07; consisted of 80% eggplant pulp, sweet pepper, extra virgin olive oil, vegetable oil, vinegar, wheat fibers, salt, garlic, spices and seasonings, and 0.1% potassium sorbate as a preservative) and a fava beans-based spread (pH 4.08 - 4.15; consisted of 65% fava beans, vegetable oil, extra virgin olive oil, onion, modified starches, salt, spices and seasonings, vinegar, with 0.1% potassium sorbate as a preservative) were used in the present study. The recommended shelf-life of the products was 90 days when stored under refrigeration. All samples were freshly produced (less than 2 days) and were stored at 4°C for less than 24 hours until use.

2.2.2 Microbiological analysis

In order to evaluate the batch variability, two different batches of products were obtained and tested; the first batch was stored at 4, 10, 15 and 20°C (or 25°C for fava-beans spread) and the second at 7, 12 or 18°C. In total, 24 packages of each appetizer were stored at each temperature in high-precision incubators (MIR153, Sanyo Electric Co., Ora-gun, Gunma, Japan). Periodically, portions (10g) were aseptically removed from each sample were diluted in 90 ml of Maximum Recovery Diluent (MRD; ref. No 401691, Biolife Italiana Srl, Milan, Italy) in a sterile stomacher bag and macerated for 1 min in a stomacher (Lab Blender 400, Seward Medical, London). After sampling, the containers were discarded and two sealed packages were used for next sampling, in order to avoid any disruption of the ecosystem (intrinsic and packaging atmosphere) of the products or contamination during sampling. Serial 10-fold dilutions were made and aliquots of 1 ml or 0.1 ml of the appropriate dilution were poured or surface plated, respectively, on selective or non-selective media. Total viable counts were enumerated on tryptic glucose yeast agar (PCA; ref. No 402145, Biolife) and incubated at 30°C for 48 h. Lactic acid bacteria were enumerated on DeMan Rogosa Sharpe agar with pH adjusted to 5.8 (MRS; ref. No 401728, Biolife) following incubation at 30°C for 48 to 72 h, while yeasts and molds were enumerated on chloramphenicol glucose yeast extract agar (YGC; ref. No 401289, Biolife) at 25°C for 72 to 120 h.

2.2.3 Physicochemical analysis

Following microbiological analysis, the pH of each homogenized (10-fold) sample was measured by a digital pH meter (pH526 WTW) equipped with a glass pH electrode. In parallel, changes in total titratable acidity (TTA) of the products were monitored as follows: 10 g of each appetizer were diluted in 100 ml distilled water (40 - 45°C) and were stirred for 15 min. The mixture was centrifuged (4000 rpm, 10 min, 4°C) and the TTA of the supernatant was determined by titration with NaOH 0.1 N, in the presence of phenolphthalein.

Considering that acetic acid was the acidulant of the products while lactic acid was produced during storage (see section "*Results and Discussion*"), changes of TTA were expressed in consumption (ml) of NaOH 0.1N.

2.2.4 Sensory analysis

At regular time intervals all products were evaluated, based on their organoleptic characteristics, by a 4-6 judges panel. In particular the odor, the flavor, the color and the overall emulsion integrity of the appetizers were rated separately, from 0 to 3, where 0 represented the highest quality, 3 the worst quality and 2 the rejection point. The time that one of the examined sensory characteristics reached the rejection score, was determined as the end of the shelf-life of the product.

2.2.5 Organic acid analysis

Changes in organic acid concentration during the storage of the three appetizers were monitored by a high pressure liquid chromatography system equipped with an analytical column (Amine HPX-87H, 300 x 7.8 mm; Bio-Rad Laboratories, Richmond, California, USA) and two serially connected detectors (Refractive Index - Ultra Violet). For the preparation of the samples, in parallel with each sampling, 50 g portions of each spread were stored at -22°C until the organic acid analysis occurred. Following thawing at 4°C, each sample was homogenized with 100 ml of warm d.d.H_2O (approximately 40 - 45°C) in a blender for 2 minutes. The pulp was centrifuged (4000 rpm, 15 min, 4°C) and the supernatant was filtered through a Millipore 0.22 μm filter. The filtrate was stored at 4°C for less than 6 hours, prior analyses. Standard solutions of HPLC-grade organic acids (acetic, citric, lactic, succinic, malic, numeric, formic, tartaric and quinic acid), which have been documented in the literature to be part of the ingredients of the products, were prepared and analysed in order to identify the retention time of each acid. The

concentration of the produced acids was quantified using standard calibration curves for each acid.

2.2.6 Molecular analysis of lactic acid bacteria

A total of 374 colonies, present in the final decimal dilution of each sample, were isolated from the MRS medium at three time points uniformly dispersed during storage of the appetizers, i.e. at the beginning, in the middle and at the point of organoleptic rejection. All isolates were subcultered in MRS broth (ref. No 401729, Biolife) overnight at 30°C, streaked on PCA and MRS agar plates and tested for Gram staining and catalase reaction. The pure cultures of LAB were maintained at -22°C in MRS broth supplemented with 20% glycerol. Prior to the molecular analysis, the isolates were activated in MRS broth (24 h / 30°C) twice. The LAB isolates were characterized based on their whole cell protein imprints, with sodium dodecyl sulphate polyacrylamide gel electrophoreses (SDS-PAGE) according to Paramithiotis et al., 2000. The protein patterns of the isolates were arranged in clusters using Gel-Compare software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). For species identification, three or four representative strains from each cluster were subjected to PCR amplification according to Leisner et al. (1999) and the PCR products were analyzed with 16S rRNA gene sequencing. The results were compared with the records existing in GenBank, using the Blast program.

2.2.7 Model development

For the growth of SSOs in response to temperature, the product-specific models were developed in two stages, as it has been extensively described in Manios et al. (2009). Briefly, the populations of lactic acid bacteria (log CFU/g) which were grown in the spreads under isothermal conditions, were fitted by the Baranyi and Roberts model (Baranyi and Roberts, 1994), in order to estimate the maximum specific growth rate (μ_{max} [day^{-1}]) and the lag

time (t_λ; days) for each growth curve. The μ_{max} was further modeled as a function of storage temperature using a separate square root model (2-2) for each product.

$$\sqrt{\mu_{max}} = a \times (T - T_{min}) \tag{2-2}$$

where α is a constant, T the storage temperature and T_{min} the theoretical minimum temperature for growth of lactic acid bacteria in each product.

A typical polynomial model (2-3) was used to describe the predicted μ_{max} of LAB as a function of the storage temperature, the initial pH and the initial concentration of the undissociated acetic acid.

$$\sqrt{\mu_{max}} = a_1 + a_2 \times T + a_3 \times pH + a_4 \times UAC + a_5 \times T^2 + a_6 \times pH^2 + a_7 \times UAC^2 + a_8 \times T \times pH + a_9 \times T \times UAC + a_{10} \times pH \times UAC \tag{2-3}$$

where *UAC* is the concentration of the undissociated acid which is calculated from the concentration of the total acetic acid (*TAC*) of the products, using the Henderson-Hasselbalch equation (2-4). Constants a_1 to a_{10} were obtained from the regression analysis of the observed μ_{max} of each f (*T, UAC, pH*) and the corresponding variables of the model.

$$UAC = \frac{TAC}{1 + 10^{pH-4.76}} \tag{2-4}$$

In addition to the polynomial model, the Ratkowsky model (2-5) was also evaluated for its suitability to describe the effect of the above variables on the μ_{max}.

$$\sqrt{\mu_{max}} = a_1 \times (T - T_{min}) \times \sqrt{(1 - 10^{pH_{min} - pH})} \times \sqrt{1 - \sqrt{\frac{UAC}{UAC_{min}}}} \tag{2-5}$$

where T_{min}, pH_{min} and UAC_{min} are the theoretical minimum values of temperature, pH and undissociated acetic acid, respectively, for LAB growth.

The lag phase duration of the SSOs was incorporated into the dynamic simulation of LAB growth through the work-to-be-done parameter (h_o) (2-6).

$$h_o = t_{lag} \times \mu_{max} \tag{2-6}$$

Data collected from similar spreads (cheese-based appetizers, Manios et al., 2009; mayonnaise based, fish roe-based and garlic-based appetizers, data not shown), were also used for the development of the unified model of equations 3 and 5, in order to expand their interpolation area (Table 2-1).

2.2.8 Model validation

The developed models were validated under real (dynamic) conditions in household refrigerators. In particular, twelve packages of each appetizer were stored in four different household refrigerators which were used in a regular base. All samples were accompanied by time – temperature data loggers which monitored the changes of the surface temperature of the products. Since the products were sealed, their real internal temperature could not be measured. However, it is assumed that the nature of the products (semi-liquid) allows the fast increase of the temperature during handling. Different theoretical scenarios which may occur during handling in households, such as maintaining for 1 to 2 hours on a kitchen table, and simulating the duration of a dinner, were also implemented in order to estimate the applicability of the models under abrupt temperature fluctuations. Periodically two packages of each appetizer were transferred to the laboratory for microbiological analysis and the observed populations of lactic acid bacteria were compared with the predictions of the products-specific and the unified model.

Table 2-1 Physicochemical properties (pH and total acetic acid concentration) and storage temperature of the different types of spreads that permitted the growth of lactic acid bacteria. The kinetic parameters (μ_{max}, lag time), which derived during fitting the LAB populations (log CFU/g) of each combination by the Baranyi model, were further used for the development of the unified models.

Type of product	pH	Storage temperature (°C)	Total acetic acid (% v/w)
Pepper spread [a]	3.95	4	3.8
Pepper spread [a]	3.95	7	3.8
Pepper spread [a]	3.95	10	3.8
Pepper spread [a]	3.95	12	3.8
Pepper spread [a]	3.95	15	3.8
Pepper spread [a]	3.95	18	3.8
Pepper spread [a]	3.95	20	3.8
Eggplant spread [a]	4.03	4	2.7
Eggplant spread [a]	4.03	10	2.7
Eggplant spread [a]	4.03	15	2.7
Eggplant spread [a]	4.03	20	2.7
Fava beans spread [a]	4.15	4	2.0
Fava beans spread [a]	4.15	7	2.0
Fava beans spread [a]	4.15	10	2.0
Fava beans spread [a]	4.15	12	2.0
Fava beans spread [a]	4.15	15	2.0
Fava beans spread [a]	4.20	25	1.9
Feta-cheese spread [b]	4.25	5	3.6
Feta-cheese spread [b]	4.25	10	3.6
Feta-cheese spread [b]	4.25	15	3.6
Feta-cheese spread [b]	4.25	25	3.6
Feta-cheese with spicy pepper spread [b]	4.16	5	3.7
Feta-cheese with spicy pepper spread [b]	4.16	10	3.7
Feta-cheese with spicy pepper spread [b]	4.16	15	3.7

(Table continues)

Feta-cheese with spicy pepper spread [b]	4.16	25	3.7
Russian spread [c]	4.20	5	3.0
Russian spread [c]	4.20	10	3.0
Russian spread [c]	4.20	15	3.0
Russian spread [c]	4.20	25	3.0
Fish roe spread [c]	3.61	5	4.0
Fish roe spread [c]	3.61	10	4.0
Fish roe spread	3.61	15	4.0
Fish roe spread	3.61	25	4.0

[a] Data collected in the present study

[b] Data collected by Manios et al., 2009

[c] Unpublished data

2.3 Results and Discussion

2.3.1 Spoilage pattern

Comparing the microbial stability of the three products, fava beans-based spread (*FS*) was found to be less stable than pepper-based (*PS*) or eggplant-based (*ES*) spread, probably due to the higher pH and the lower undissociated acetic acid concentration in this product (Table 2-2). In *PS* and *ES* spread the high content of total acetic acid (3.8% and 2.7% v/w, respectively) did not allow any microbial growth at 4-10°C (Table 2-2). In contrast, in *FS*, where the concentration of acetic acid was lower (2% v/w) compared with *PS* and *ES*, the temperature threshold for initiation of microbial growth was 7°C (Table 2-2). Below these temperatures, the organoleptic rejection of the products was caused due to changes in the structure and/or texture of the products (e.g., separation of the emulsion and discoloration). In contrast with *PS* and *FS*, the different batches of *ES* affected significantly different the growth potential of the SSOs (Table 2-2). In

particular, samples which were stored at 15°C or 20°C (batch 1) favored the growth of LAB, while no growth was observed after storage of samples at18^{o}C (batch 2).

Further examination of the manufacturing process of each batch revealed that, in batch 2, canned eggplant dipped in lactic acid was used, in contrast with batch 1 in which fresh eggplant was added. This processing difference, however, did not affect the initial pH of each batch. When *PS* and *FS* samples were stored at 12°C or 7°C, respectively, the end of shelf-life of the products was also attributed to physicochemical changes, although slow microbial growth was also observed (Table 2-2). In cases where the storage conditions allowed microbial growth, lactic acid bacteria (LAB) were the predominant spoilage microorganisms of all spreads, while no or negligible growth of yeasts or molds (<1 log CFU/g; data not shown) was evident.

Table 2-2 Growth parameters (lag time, growth rate) of specific spoilage microorganisms (lactic acid bacteria) in two batches of pepper-based spread, fava beans-based spread and eggplant-based spread and final product shelf-life during storage under constant temperature conditions

Storage T (°C)	Pepper-based salad			Fava beans-based salad			Eggplant-based salad		
	Lag time (days)	Growth rate (day^{-1})	Shelf-life (days)	Lag time (days)	Growth rate (day^{-1})	Shelf-life (days)	Lag time (days)	Growth rate (day^{-1})	Shelf-life (days)
4	NG[a]	NG	>120[c]	NG	NG	>120	NG	NG	>120
7	NG	NG	>120	55.7	0.02	100[d]	NG	NG	>120
10	NG	NG	>120	19.8	0.11	86	NG	NG	>120
12	44.0	0.05	112[d]	10.7	0.13	61	NG	NG	>120
15	3.1	0.14	78	5.7	0.25	47	3.1	0.15	78
18	2.1	0.44	24	-	-	-	NG	NG	>120
20	1.8	0.84	15	-	-	-	1.7	0.44	18
25	-[b]	-	-	1.5	1.64	5	-	-	-

[a] *No growth*

[b] *Experiment not conducted*

[c] *In cases of no growth, spoilage occurred due to physicochemical changes, but the shelf-life was not precisely determined*

[d] *Enzymatic spoilage caused earlier rejection of the product, although slow growth of LAB was evident.*

The initial populations of LAB ranged from 0.8 to 3.1 log CFU/g in all products, depending on the batch. By increasing the storage temperature, a significant increase of the growth rate and decrease of the lag time of LAB was observed (Table 2-2). The end of shelf-life of the products under isothermal conditions was defined as the time that organoleptic rejection occurred. At that point, the maximum population levels of LAB ranged from 7.2 – 8.0 log CFU/g in *PS*, 7.1 – 8.8 log CFU/g in *FS* and 7.9 – 8.1 log CFU/g in *ES*, depending on the storage temperature (Table 2-3**).**

In the present study, the spoilage of the spreads was caused either by physicochemical changes or due to the microbial activity of LAB, depending on the intrinsic parameters and the environmental conditions. In general, the quality deterioration of foods is the result of multiple complex activities of microbiological and/or biochemical nature (Huis in't Veld, 1996; Jacxsens et al., 2003). The intrinsic factors of the products (i.e., pH, a_w, NaCl, organic acids, chemical preservatives) in association with the environmental conditions (i.e., storage temperature, gas composition, light) may determine the type of spoilage (microbial, biochemical or both) that may occur, by affecting the growth/no growth boundaries of the specific spoilage microorganisms. Indeed, Le Marc et al. (2002) studied the interactions of storage temperature and pH and their effect on the growth/no growth interface of *L. innocua* and reported that when storage temperature decreased from 15°C to 5°C, the corresponding pH threshold moved from 4.50 to 5.05. At lower temperatures and pH values, where microbial activity is prohibited, physical, chemical or enzymatic activities may have a slow but adverse impact on the product quality, such as separation of emulsion, oxidation of lipids of olive oil and discoloration of the emulsion (Huis in't Veld, 1996).

Table 2-3 Changes of pH (standard deviation) and titratable acidity, and LAB populations at the end of shelf-life of pepper-based salad, fava beans-salad and eggplant salad after storage under isothermal conditions

Storage T (^oC)	Pepper-based salad					Fava beans-based salad					Eggplant-based salad				
	pH		Titratable acidity (ml NaOH 0.1N)		LAB populations at rejection point (log CFU/g)	pH		Titratable acidity (ml NaOH 0.1N)		LAB populations at rejection point (log CFU/g)	pH		Titratable acidity (ml NaOH 0.1N)		LAB populations at rejection point (log CFU/g)
	Fresh product	End of shelf-life	Fresh product	End of shelf-life		Fresh product	End of shelf-life	Fresh product	End of shelf-life		Fresh product	End of shelf-life	Fresh product	End of shelf-life	
4	3.95 (0.03)	4.00 (0.02)	2.2 (0.1)	2.3 (0.1)	1.4 (0.6)	4.11 (0.07)	4.09 (0.01)	2.3 (0.2)	2.4 (0.1)	2.1 (0.2)	4.04 (0.07)	4.06 (0.01)	2.1 (0.1)	2.1 (0.1)	1.7 (0.2)
7		3.98 (0.01)		2.4 (0.0)	1.7 (0.1)		4.06 (0.00)		2.5 (0.1)	3.2 (0.1)		3.96 (0.05)		2.2 (0.1)	1.0 (0.0)
10		4.05 (0.01)		2.3 (0.1)	1.2 (0.3)		3.76 (0.03)		3.3 (0.2)	7.7 (0.2)		4.04 (0.01)		2.3 (0.1)	1.5 (0.2)
12		3.89 (0.03)		3.1 (0.2)	5.5 (0.1)		3.89 (0.02)		2.8 (0.1)	7.4 (0.2)		3.94 (0.02)		2.3 (0.1)	1.3 (0.3)
15		3.65 (0.06)		4.1 (0.1)	8.0 (0.2)		3.94 (0.01)		3.2 (0.0)	7.1 (0.1)		3.76 (0.02)		3.1 (0.1)	8.1 (0.1)
18		3.64 (0.01)		4.8 (0.2)	8.8 (0.1)		-		-	-		3.93 (0.07)		2.3 (0.2)	1.7 (0.1)
20		3.85 (0.01)		3.7 (0.1)	7.2 (0.1)		-		-	-		3.89 (0.04)		3.0 (0.1)	7.9 (0.1)
25		-		-	-		3.82 (0.01)		3.6 (0.1)	8.8 (0.1)		-		-	-

The metabolic activity of LAB caused significant ($p<0.05$) decrease in the pH of all products during storage under isothermal conditions, with pH values below 3.9 signaling the end of their shelf-life. Likewise, the total titratable acidity of the products showed significant ($p<0.05$) increase and exceeded the level of 3ml consumption of NaOH 0.1N at the point of organoleptic rejection (Table 2-3). On the contrary, no remarkable changes in the pH or the total titratable acidity were evident, when growth of LAB was not evident (Table 2-3). The analysis of samples with the HPLC revealed that the responsible factor for the increase in the acidity of the products was primarily the production of lactic acid. In all cases where growth of LAB was observed, lactic acid concentration showed significant ($p<0.05$) increase, especially in *PS* where pH of the product reached lower values at the rejection point compared with *FS* or *ES* (Figure 2-1). In particular, the total increase of lactic acid concentration at the rejection point of *PS* ranged from 80 – 100 mg/ 100ml, while the corresponding increase in *FS* and *ES* was approximately 60 to 80 mg/100 ml. On the contrary, increase in acetic acid levels was sporadically observed under some conditions which supported microbial growth (*PS* stored at 12, 15, 18°C, *FS* stored at 12, 15°C and *FS* stored at 15, 18°C) (Figure 2-1). Other organic acids such as succinic, malic and quinic acid were also observed in the fresh products and displayed slight reductions during storage, while citric acid concentrations remained almost unchanged (data not shown).

The significant increase in the acidity (decrease of pH) of the products during storage at elevated temperatures (i.e., 10 – 20°C for *PS* and *ES*, 7 - 25°C for *FS*) is a result of the production of lactic acid by the SSOs (LAB). In addition, the fact that acetic acid production was sporadically present may be associated with the dominance of the heterofermentative *L. brevis*. In cases, however, where no acetic acid production was observed, the microbial association was dominated by *L. plantarum*, which is a facultative heterofermentative species (Stiles and Holzapfel, 1997). Due to the low pH of

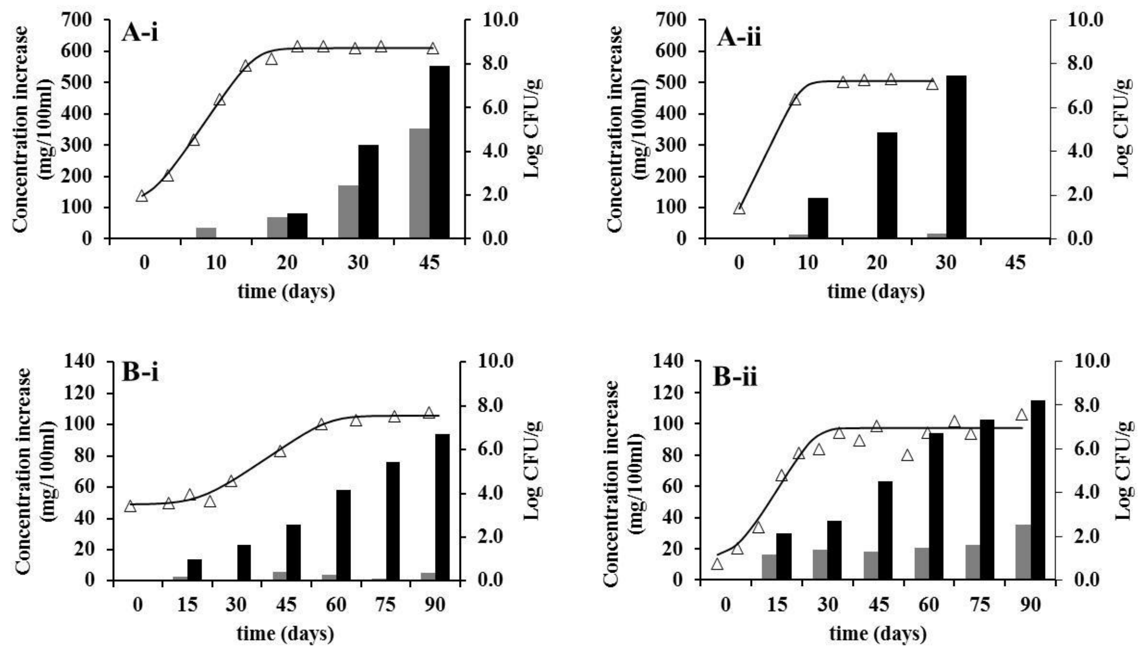


Figure 2-1 Changes of lactic acid (■) or acetic acid (■) concentration, in association with lactic acid bacteria growth (△), during storage of pepper-based salad (A) at 18°C (i) and 20°C (ii) or fava beans-based salad (B) at 10°C (i) and 15°C (ii).

the products, aciduric bacteria are generally recognized as the predominant spoilage microorganisms in emulsified foods, such as mayonnaise, mayonnaise-based salads or ketchup (Smittle, 2000; Erickson et al., 1993; Smittle and Flowers, 1982; Björkroth and Korkeala, 1997; Dakin and Radwell, 1971). Species of the genus *Lactobacillus,* including *Lb. fructivorans*, *Lb. plantarum*, *Lb. brevis*, *Lb. casei* or *Lb. buchneri* have been involved in the spoilage of various acetic acid acidified foods (Waite et al., 2009; Fiavolá et al., 2008; Leuschner and Hammes, 1999; Brocklehurst, 1992; Kurtzman et al., 1971). Occasionally, yeasts such as *Zygosaccharomyces baiili*, *Saccharomyces cerevisiae*, *Pichia membranifaciens*, *Candida inconspicua*, *Candida stellata* (Waite et al., 2009; James and Stratford, 2003; Tornai-Lechoczki et al., 2003; Hunter et al., 1994; Smittle, 1977), or, more rarely, moulds of the genus *Aspergillus* or *Penicillium* (Hunter et al., 1994) have

been also been associated with spoilage of such foods. The microbial activity of LAB usually leads to the production of organic metabolites, which have an immediate effect on the sensory characteristics of the product (Jacxsens et al., 2003). In particular, during proliferation of LAB, the energy required for their entire metabolism is obtained from the oxidation of chemical compounds, with the oxidation of sugars constituting the principle energy producing pathway. The predominant end-product of sugar fermentation is lactic acid, whereas the production of acetic acid is also possible when the spoilage association is dominated by heterofermentative species of LAB (Stiles and Holzapfel, 1997; Kandler, 1983).

LAB strains were isolated from fresh products and were grouped with SDS-PAGE, based on the whole protein profile of the isolates. Results showed that the initial microbial association of LAB in all products consisted of 6 different spoilage species (6 protein profiles). Further identification of characteristic isolates of each group revealed that strains of *Lactobacillus plantarum*, *L. brevis,* and *L. buchneri* comprised the spoilage microbiota of the spreads (Figure 2-2). Strains of <1% overall presence were not further identified, as they were considered that they did not contribute significantly to the deterioration of the product quality. In the middle of storage, only *L. plantarum* and *L. brevis* were isolated from all products (Figure 2-2), while depending on the type of the spread and the storage temperature, only one of these two Lactobacillus species were detected at the end of shelf-life (Table 2-4). The microbial diversity of the spreads at the point of organoleptic rejection was not significantly affected by the different batches of products which were used for the experiment, as different species of LAB dominated the same batch of product when it was stored under different temperatures (Table 2-4).

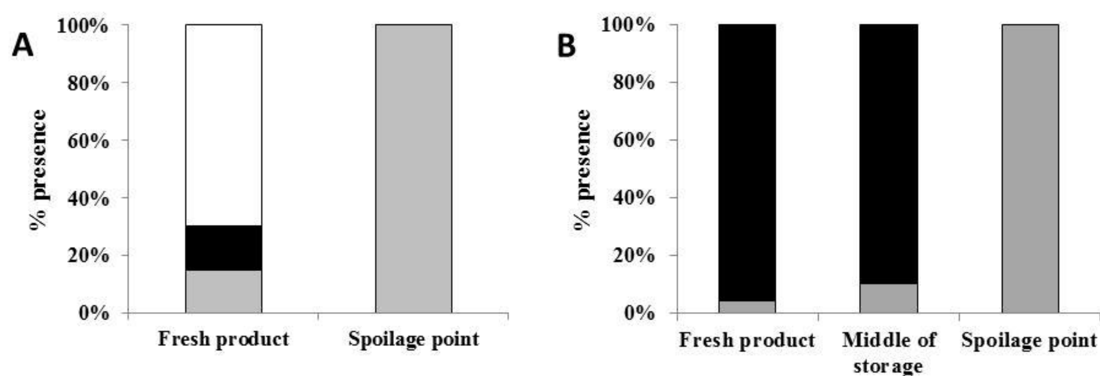


Figure 2-2 Diversity of *Lactobacillus* species during storage in pepper-based salad (A) or fava-beans salad (B) during storage at 12°C. (■) *L. brevis*, (■) *L. plantarum*, (□) *L. buchneri*

Table 2-4 Species of lactic acid bacteria that dominated the microbial association of pepper-based salad, fava beans-based salad and eggplant-based salad at the point of product rejection, after storage under different isothermal conditions

Storage T (°C)	Pepper-based spread	Fava beans-based spread	Eggplant-based spread
4	NG [aA]	NG [A]	NG [A]
7	NG [B]	*L. plantarum* [B]	NG [B]
10	NG [A]	*L. plantarum* [A]	NG [A]
12	*L. brevis*	*L. brevis* [B]	NG [B]
15	*L. brevis* [A]	*L. brevis* [A]	*L. brevis* [A]
18	*L. brevis* [B]	-	NG [B]
20	*L. plantarum* [A]	-	*L. brevis* [A]
25	- [b]	*L. plantarum* [A]	-

[a] *No growth*

[b] *Experiment not conducted*

[A] *Batch A,*

[B] *Batch B*

Although the initial microbial load of each product comprised of a significant number of LAB species, the quality deterioration seemed to be the result of only one, depending on the storage temperature and the type of spread. The contamination of these products with spoilage microorganisms may originate from the ingredients (i.e., vegetables, yoghurt, spices) which are used during the manufacturing process. During food storage, microorganisms encounter various stresses and the microorganism which adapts to these environmental hurdles will have the advantage to outgrow the other microbiota and finally dominate the microbial association at the organoleptic rejection point (Olofsson and Molin, 2007). As a subsequent result of this selectivity, the diversity of the spoilage association decreases with the storage time of foods (Li et al., 2006).

The microbial selectivity and diversity during spoilage or fermentation of products, as affected by the existing or the dynamically changing intrinsic and extrinsic parameters, has been widely studied. Jay et al. (2000) reported that heterofermentative association of LAB at the initial stage was gradually substituted by homofermentative microbiota at the end-point of meat storage under modified atmosphere packaging. In addition, Vasilopoulos et al. (2008) showed that the initial spoilage association of cooked ham comprised of a significant number of microorganisms (*Brohotrix thermosphacta*, lactic acid bacteria, enterococci, enterobacteria), but only *Leuconostoc carnosum* or *Enterococcus faecalis* dominated at the organoleptic rejection point, depending on the storage temperature. Similarly, Paramithiotis et al. (2010) stated that strains of *L. plantarum* dominated the fermentation of cauliflower, although the initial stage was characterized by heterofermentative species of LAB (i.e., *Leuconostoc mesenteroides*).

Overall, the spoilage pattern of the studied products was significantly affected by the storage conditions and the intrinsic parameters of the products. These factors affected also the population dynamics of the SSOs and subsequently the physicochemical changes that occurred in the environment of the products during storage. Although the phenomenon of food spoilage may be

easily monitored by low cost sensory evaluations, using such subjective and theoretical data for the determination of the shelf-life of products may cause their earlier rejection. In contrast, specifying the metabolic activity of the SSOs under dynamic conditions may assist in monitoring the spoilage potential of a food (Samelis et al., 2000) and further, to accurately determine the shelf-life of the products.

2.3.2 Development and validation of models

In *PS* and *FS*, the dependency of the maximum specific growth rates of LAB on storage temperature was successfully described by a square root model, showing R^2 values of 0.987 and 0.969, respectively (Figure 2-3), as previously documented (Manios et al., 2009). Conversely, the growth of LAB in *ES* varied a lot with the type of eggplant (canned or fresh) used as ingredient (Table 2-2), with *ES* made of canned vegetables not supporting growth of LAB and *ES* made of fresh eggplant allowing growth of LAB only at high temperatures. Thus, the dependency of LAB μ_{max} on temperature could not be adequately described. For the validation process of the product-specific models, the work-to-be-done parameter (h_o; Baranyi and Roberts, 1994) was used to account for the lag time completion under dynamic temperature conditions. The h_o values estimated for each spread were independent of temperature and product, as determined by t-test ($p>0.05$). Thus, an average value of 1.25 ± 0.25 and 1.64 ± 0.35 for PS and FS, respectively, was used. The predicted populations of LAB showed high correlation with the populations that grew in the examined products during storage in 4 household refrigerators (Figure 2-4; Figure 2-5). In general, the models performed well with slight under-prediction of the LAB growth in *FS*.

Based on the facts that: (i) the growth of LAB was mainly affected by the product pH and storage temperature and (ii) the diversity of LAB species was independent of product and storage temperature, two “unified” (generic) models, based on polynomial and Ratkowsky equations were developed for the growth of LAB in various acetic acid-acidified spreads as a function of

initial pH, the concentration of undissociated acetic acid and storage temperature (Table 2-5). Other parameters that may influence the microbial behaviour in the spreads such as the water activity (a_w) and lactate or benzoic acid concentration could also be incorporated as a variable of the unified models. However, these factors did not change significantly among the different products of this category and therefore, it was assumed that their effect was similar in all spreads.

Similarly to the validation process of the product-specific models, the h_o parameter was calculated for all products that were used for fitting the unified models. The linear correlation ($p<0.05$) of lag times with the reciprocal of μ_{max} of LAB, suggested that h_o is independent of the aforementioned factors controlling microbial growth. Thus, the simulation of LAB growth by the unified models under dynamic chill-chain conditions were based on the average h_o value of all products which equaled to 1.86 ± 0.76. Although both models adequately described the trend of LAB growth in the different acetic acid-based products tested, the performance indices of polynomial equation were higher than those of Ratkowsky equation and so was the visible agreement of model predictions with data (Figure 2-4; Figure 2-5).

Similarly, both models were able to accurately predict the growth of LAB in feta cheese-spread (Manios et al., 2009) without the addition of the spicy green pepper, with the polynomial model, however, showing lower B_f and A_f compared to the Ratkowsky model. In contrast, both models significantly over-predicted the growth of LAB in the feta cheese spread with the spicy pepper, while the corresponding product-specific model showed overall better performance (Table 2-6). This may attributed to the antimicrobial effect of the capsaicin (Cowan, 1999) present in the spicy green pepper, which may suppress the growth of the SSOs. The potential variance in LAB growth introduced by the inhibitory effect of capsaicin in the above dairy spread ecosystem requires further experimentation in order to be accounted for by the “unified” model. Polynomial equations may fit a variety of datasets due to its flexibility. However, in imbalanced datasets, which may be comprised of

areas with limited data next to areas with abundant data, the risk of over-fitting is high (Geeraerd et al., 2004; Diels et al., 2007). Thus, despite the better performance of the polynomial over Ratkowsky equation, the added value of the latter relies on its biological meaningful parameters and the consistent description of the dependency of μ_{max} on the predictor variables, avoiding over-fitting.

Table 2-5 Significant (p<0.05) parameter estimates (± 95 % confidence intervals) and goodness-of-fit criteria of polynomial and Ratkowsky equations fitted as two alternative "unified" models to collective data (log CFU/g) for the growth of LAB in various acetic acid-based spreads.

Equation	Parameters	Value	-95% CI	+95% CI
Polynomial	Intercept	2.035	1.260	2.810
	T	0.818	0.549	1.086
	UAC	-6.917	-8.929	-4.905
	T^2	0.0009	0.0002	0.0016
	UAC^2	0.358	0.214	0.503
	T x pH	-0.196	-0.261	-0.131
	pH x UAC	1.259	0.874	1.644
	R^2	0.893		
	F	0.130		
	RMSE[a]	0.075		
Ratkowsky	Intercept	0.046	-2.371	1.108
	T_{min}	-5.260	0.021	0.033
	UAC_{min}	7.590	-0.054	0.030
	pH_{min}	3.793	-0.249	0.603
	R^2	0.800		
	F	0.110		
	RMSE[a]	0.093		

Over-fitting was the primary reason for the disagreement between the polynomial equation-based growth simulations and the actual growth of LAB in other products of this category, such as Russian salad or beetroot spread, as opposed to the good agreement of Ratkowsky equation-based simulations (results no shown).

The description and quantification of microbial growth dynamics in different environments and foods *via* mathematical equations has been widely studied in the literature. However, except for the CMSICEE model and a model developed by Vermeulen et al. (2007), which are growth probability models, there are limited models for the shelf-life determination of emulsified RTE spreads or dressings of low pH. Manios et al. (2009) primarily investigated the potential of a simple square root model to predict the growth of LAB in two cheese-based spreads under real chill chain conditions. Similarly to the present study, the predictions of the product-specific models of that study were in good agreement with the observed populations of LAB during

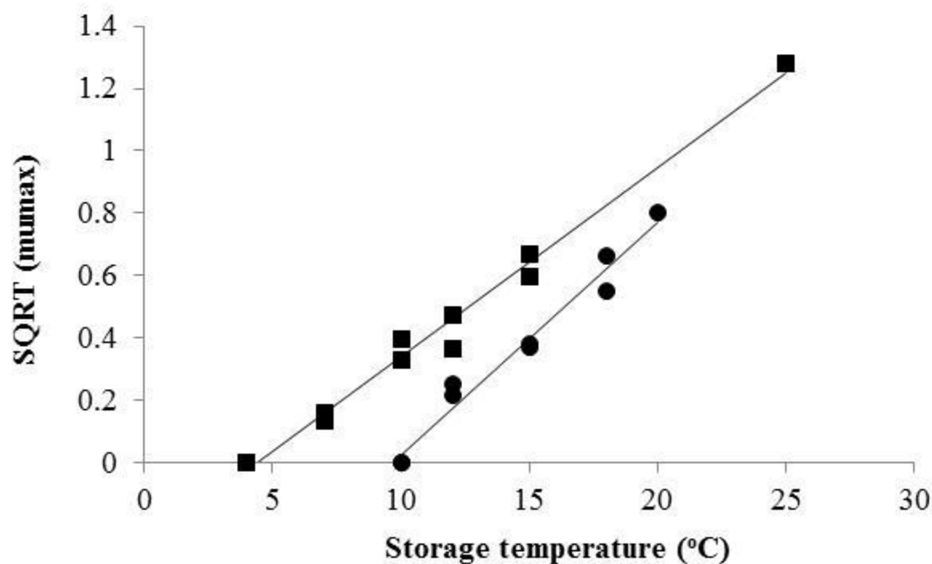


Figure 2-3 Linear regression of the maximum specific growth rates of LAB and storage temperatures in pepper-based (●) and fava beans-based spread (■).

distribution and household storage. Although in both studies the development of such models requires extensive microbiological research and thus, reduces their usefulness by food industries, they constitute the basis for the development of mathematical models for broad food categories. In particular, starting from basic models, which study the effect of individual factors on the microbial growth, the new trend of predictive models is to gradually incorporate as much influencing factors as possible (Bernaerts et al., 2004). In this way, the accuracy of the predictions of the model increases, while the necessity of time-consuming analysis decreases. Despite the proper

Table 2-6 Bias (B_f) and accuracy (A_f) factors of the models predicting the growth of LAB in pepper spread and fava beans spread, after exposure to fluctuating time-temperature profiles

Product	Model	Time-temperature profiles	B_f	A_f
Feta cheese-based spread	Square root model	A	1.09	1.09
		B	1.16	1.16
	Polynomial model	A	1.01	1.02
		B	1.11	1.11
	Ratkowsky model	A	1.06	1.06
		B	1.25	1.25
Feta cheese-based spread with spicy green pepper	Square root model	A	1.03	1.05
		B	0.85	1.17
	Polynomial model	A	1.44	1.44
		B	1.52	1.52
	Ratkowsky model	A	1.38	1.38
		B	1.47	1.47

temperature configurations (4°C) set by the consumers in household refrigerators, the actual storage temperature in the validation trials of the present study ranged from -1.2 to 13.9°C, which include values outside the temperature interpolation area of the models. Likewise, handling of some products during validation experiments also resulted in short exposure to temperatures up to 33.7°C (Figure 2-4; Figure 2-5). These abrupt fluctuations could induce an additional lag time. The high performance of the "unified" models despite these issues addresses the robustness of the present generic approach.

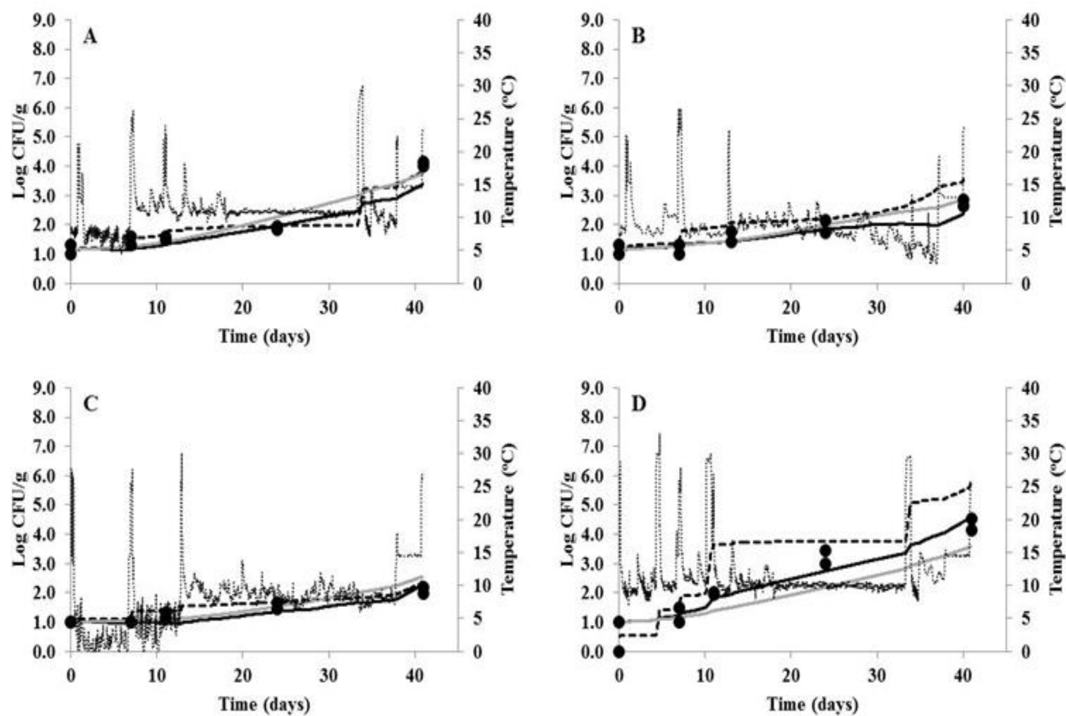


Figure 2-4 Growth of lactic acid bacteria in pepper spread under four (A-D) time-temperature profiles (----) in four household refrigerators (A-D), as predicted by the polynomial model (▬), the Ratkowski model (▬), or the product-specific model (---) in conjunction with the observed populations (●).

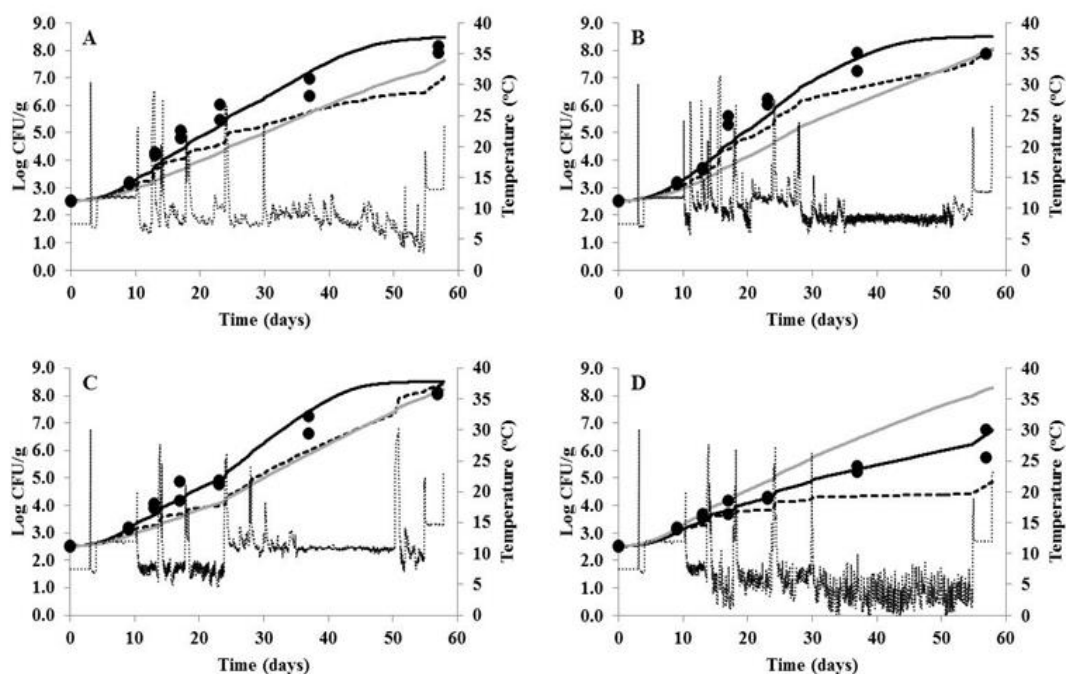


Figure 2-5 Growth of lactic acid bacteria in fava beans spread under four (A-D) time-temperature profiles (----) in four household refrigerators (A-D), as predicted by the polynomial model (—), the Ratkowsky model (—), or the product-specific model (---) in conjunction with the observed populations (●).

2.4 Conclusions

Overall, the present study contributed to the detailed description of the spoilage of a category of acetic acid acidified spreads which require refrigeration for a long-term storage. Although the ingredients of the products differed significantly, their spoilage patterns posed small deviations. In addition, the product-specific models which were developed may give accurate information for the shelf-life of the examined spreads, but such models may not be applied to other products. Therefore, the unified models which has been developed in the present study and validated under real chill chain conditions may be a significant tool for food industries in order to determine the shelf-life of existing or newly designed products.

However, in any case, the initial contamination level is essential to accurately predict the growth kinetics of the SSOs and further enable the models to determine the shelf-life of the spreads. This disadvantage is common for all spoilage predictive models but, in parallel, highlights the importance of the initial microbial load on the deriving quality of a product. The significance of the contamination level is also extrapolated to a food safety level, as the potential and dynamics of a pathogen may differ when initiating from low or high populations. Therefore, it is crucial to mimic the realistically low contamination levels of foods when conducting *in vitro* trials. These concerns have been extensively examined in chapter 4.

3 EFFECT OF REALISTIC SCENARIOS OF FREEZING, THAWING AND COOKING ON THE SURVIVAL OF *SALMONELLA* SPP. AND *ESCHERICHIA COLI* O157:H7 IN BEEF PATTIES

3.1 Introduction

Beef patties constitute conventional Ready-To-Cook (RTC) meat products, ranked among the top most frequently consumed meat products worldwide. These products may be prepared in-house from ground beef with or without the addition of other ingredients and seasonings, or they may also be found available in the form of pre-shaped frozen patties, which is highly convenient for Quick Service Restaurants. In recent years ground beef has been linked with several outbreaks of foodborne diseases caused by various serotypes of *Salmonella* spp. or shiga-toxin producing *Escherichia coli* (CDC, 2012b; 2012c; 2011c; 2010b; 2002; Schneider et al., 2011; McLaughlin et al, 2006). The causative agent of all these outbreaks originates from poor hygienic conditions during production and further improper handlings by the consumers, either in the domestic environment or in restaurants. Therefore, Food Safety authorities have issued a series of specific guidelines for safe practices during storage and preparation of ground beef. Through these guidelines it is recommended that ground beef should be stored in refrigerators for 1-2 days or in a freezer for up to 4 months. Before cooking, the necessary portion should be thawed in refrigerator, in the microwave or under regularly changed cold water, while thawing in hot water or at room temperature on the countertop is not recommended. Finally, a minimum internal temperature of 160°F (71.1^{o}C) should be targeted when cooking beef burgers or patties by using a precise thermometer, in order to eliminate the risk of surviving pathogenic bacteria (USDA-FSIS, 2012b). Although the type of cooking method has been shown to have different impacts on the survival of pathogens (Shen et al., 2011b; Rhee et al., 2003; D'Sa et al., 2000), no specific references have been made in the issued guidelines. Despite these

recommendations, the storage and cooking practices being applied by the consumers, either in households or in catering services and restaurants, are generally based on personal preferences and convenience for handling and consuming foods (Jevšnik et al., 2008; Karabudak et al., 2008; Gilbert et al., 2007a; Badrie et al., 2006). Phang and Brun (2011) reported that 16% of the consumers are thawing ground beef patties on the counter top, while 87% did not know the proper cooking temperature of ground beef. In the same study, 51% of the volunteers reported that they use the color of patties as a criterion of doneness, with 23% preferring a pink interior. However, the color in the center of cooked ground meat products may be affected by many factors others than temperature, giving false indications for the safety of the final product (King (née Turner) and Whyte, 2006).

In the existing literature, many studies have been focused separately on the effect of frozen storage (Byrne et al., 2002; Ansay et al., 1999), different thawing (Lianou and Koutsoumanis, 2009; Sage and Ingham, 1998) and cooking (Rhee et al., 2003; D'Sa et al., 2000) methods or levels of doneness (Gill et al., 2009; Passos and Kuaye, 2002) on the survival or heat tolerance response of pathogenic bacteria. However, during frozen storage and thawing of beef patties, pathogens encounter various stresses, mainly associated with the temperature accepted by the cells. The exposure of microorganisms to such stresses has been found to affect their heat tolerance (Abee and Wouters, 1999). Therefore, it is essential to simulate the combined effect of all the consumer-style practices that are being used during handling of ground beef, on the potential of pathogens to survive the heat process during cooking.

According to the above, we aimed to evaluate the single and synergistic effect of frozen storage, different thawing and cooking methods and amount of cooking of commercial-style beef patties on the survival of *Salmonella* spp. and *E. coli* O157:H7, simulating common practices used by the consumers in households or restaurants.

3.2 Materials and Methods

3.2.1 Bacterial cultures and preparation of inocula

A five-strain mixed culture of *Salmonella* spp. consisted of two strains of *S.* Typhimurium (calf bowel and epidemic isolates), *S.* Agona, *S.* Infantis, *S.* Reading (animal feeds isolates) and a three-strain mixed culture of *Escherichia coli* O157:H7 (NCTC 12079, NCTC 13125, NCTC 13127) were used in the present study. These strains were selected as they may directly (meat isolates) or indirectly (human isolates) occur in meat products. All microorganisms were maintained on tryptone soy agar (TSA; LAB M, LAB011, Lancashire, UK) slants slopes at 4°C, which were replaced every 30 days. Each strain was cultured separately by transferring a single colony from the slants into 10 ml tryptic soy broth (TSB; LAB M, LAB004) for 24 hours at 37°C, followed by a second activation step in TSB (37°C, 18 hours). Prior to inoculation, the strains were washed twice in 10 ml maximum recovery diluent (MRD; LAB M, LAB103). The washed strains were mixed equally in a sterile 100 ml – container and the strain composite of each microorganism were used for the inoculation of the samples.

3.2.2 Inoculation of ground beef

Freshly produced ground beef, which contained approximately 20% fat was obtained from a local meat processing industry on the day of each experimental trial and was kept at 4°C before inoculation. Portion (400 g) of ground beef was placed into a sterile stomacher bag and were inoculated with 5 ml of the strain composite of *Salmonella* spp. or *E. coli* O157:H7 in order to yield an initial inoculation level of approximately 6.5 – 7.0 log CFU/g. The inoculated samples were mixed thoroughly by hand-massage of the bag for 5 minutes and were stored at 4°C for 30 minutes for the attachment of the inoculum. To avoid any differences in temperature during freezing (Figure 3-1) or thawing (Figure 3-4), a constant shape (14 x 9 x 3.5 cm) was given to each inoculated block of 400 g. In parallel, beef patties of commercial

dimensions (90g; diameter 9 cm x 1.5 ± 0.2 cm) were prepared and they were designated for cooking without prior thawing. All samples were stored at -22°C for 5 or 75 days, representing a short- or a long-term storage under freezing that may take place in households or catering services. Since the freezing rate may affect the viability and potential injury of the cells, except for shaping the ground beef in identical forms, the blocks or patties were always placed at the same point in the freezer (without placing two blocks on each other). In this manner, the deviation of the time-temperature profiles during freezing of different samples was low (Figure 3-1).

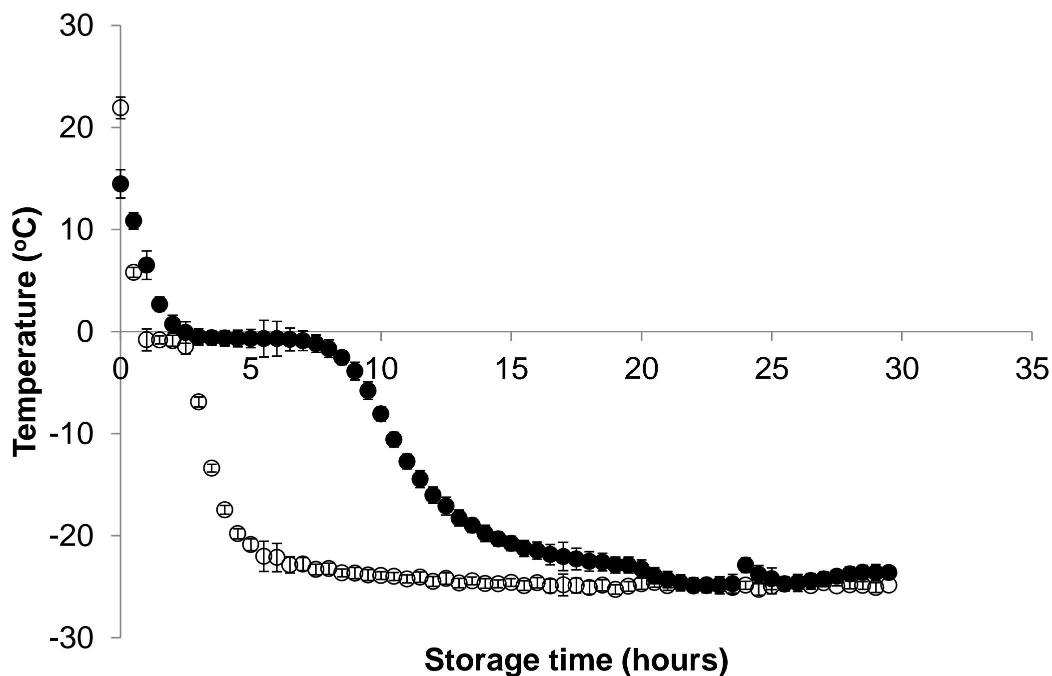


Figure 3-1 Time temperature profile during freezing of ground beef blocks of 400 g (●) or 90 g beef patties (○).

3.2.3 Thawing and cooking of beef patties

Two different common scenarios of beef patties handling were simulated; cooking after thawing and direct cooking without prior thawing. In the first case, the samples of ground beef were thawed using three different methods,

simulating realistic scenarios that are regularly being followed by the consumers. In particular, samples were left (i) at 4°C for 16 hours, simulating thawing in a refrigerator, (ii) at 20°C for 12 hours, simulating thawing on the kitchen counter overnight or (iii) they were thawed in a commercial microwave (defrosting mode) for 22-24 minutes. After thawing, ground beef was shaped in patties (90g; diameter 9 cm x 1.5 ± 0.2 cm) using sterile petri dishes to standardize their shape, and they were held at the temperature reached after thawing (i.e., 4°C for refrigerator, 20°C for kitchen counter and microwave) for less than 30 minutes before cooking.

Beef patties were cooked in a commercial oven-broiler with the heating source placed on top (Davoline 4503 Futura, Thessaloniki, Greece) preheated at 200°C, or in a pan-grill at maximum power (1500 Watts), representing two conventional methods of cooking. Thermal application was halted when the temperature at the geometrical center of the samples reached 60°C, simulating undercooking of patties, or 71°C, as suggested by the Food Safety authorities (USDA/FSIS, 2012c). Although undercooking should be avoided, some consumers prefer the patties “pink inside”. During cooking, samples were turned once when the internal temperature reached 35°C, in order to avoid extensive browning of the patties. This temperature was chosen based on preliminary experiments, where thermocouples were fitted on the top, the middle and the bottom side of the patties. Results from these trials showed that the rate of temperature increase during cooking was much higher at the lower side compared to the upper side of the patties and therefore, the final temperature at the two sides differed approximately 20°C when the internal temperature was 60 or 71°C. By turning the patties once, this difference was limited at approximately 3 - 5°C. For the same reason, beef patties cooked directly from the frozen form were turned twice (at 0°C and 35°C). The analytical flowing diagram of the procedure followed is illustrated in Figure

Changes in temperature during freezing, thawing or cooking of samples were monitored using a time-temperature data logger (Pico-PP22, TC-08

Thermocouple Data Logger, Pico Technology, Cambridgeshire, UK) equipped with Type K thermocouples. In particular, each thermocouple was fitted from one side to the geometrical center of the ground beef block or patties before freezing, to monitor the temperature changes during freezing and thawing. The same procedure was followed for the beef patties which were formed after thawing and were supposed for cooking. Due to the small thickness of the patties, small deviations of the thermocouple from the geometrical center could significantly affect the logging of the internal temperature. Therefore, a sterile metallic needle was used to fully penetrate the patties while they remained in the petri dishes, from one precisely measured spot on the one side of the petri dish to another spot at the other side. The petri dishes were broken to remove the patties without changing the form of the patties or the position of the thermocouple. The obtained data in all cases were recorded and analyzed using the PicoLog software (ver. 5.21.9).

The *F*-values of each treatment were calculated according to Murphy et al. (2004), using the equation:

$$F = \int_0^t 10^{(T(t) - T(ref)/z)} \quad \textbf{(3-1)}$$

with *T* the temperature in the center of the beef patties at t=0 to $t=t_i$ and *T(ref)* the reference temperature, which is a theoretical temperature at which the *D* value is known. In the present study, z values calculated for *Salmonella* and *E. coli* O157:H7 by Murhy et al. (2004) were used, while T(ref) was determined at 55°C. The final *F*-value for each treatment was calculated as the mean of four values derived from four independent samples.

3.2.4 Microbiological analysis

Microbiological analyses of the samples were conducted at four stages during the experimental procedure; (i) after inoculation, to determine the inoculation level, (ii) after freezing and before thawing, to evaluate the effect

of freezing, (iii) after thawing, to evaluate the effect of thawing method and, (iv) after cooking, to evaluate the effect of cooking method and amount of cooking on the survival of the pathogens. In particular, at the first three stages of sampling, 25 g of ground beef was mixed with 225 ml MRD in a stomacher for 2 min. After cooking, beef patties were immediately transferred to a sterile stomacher bag containing 200 ml MRD for immediate drop of the temperature, while MRD was added to the bag to obtain 1:5 initial dilution. Following decimal dilutions, *Salmonella* spp. populations were enumerated on xylose lysine deoxycholate agar (XLD; LAB M, LAB032) and TSA + 0.6% pyruvate after incubation at 37°C for 24 hours. Similarly, *E. coli* O157:H7 populations were inoculated on sorbitol McConkey agar (LAB M, LAB161) supplemented with cefixime tellurite (CT-SMAC; LAB M, X161) and TSA + 1% Yeast Extract (YE; LAB M, MC001) and were incubated at 37°C for 24 hours. In parallel, the pH of the first decimal dilution (stomacher bag) and the weight of patties before and after cooking were measured, in order to monitor the effect of each specific treatment on the acidity and the total weight loses of the samples. In addition, these parameters served also as a confirmation mean for the repeatability of the process.

3.2.5 Statistical analysis

The experiment was performed twice with two independent samples analyzed per repetition (n=4). Microbial populations of the two pathogens were converted to log CFU/g before statistical analysis. Each repetition was considered as a blocking factor in a randomized block design. The statistical analysis of mean populations of pathogens, pH changes, weight losses and *F* values were conducted with the Tukey honest significant difference (HSD) using the STATISTICA (StatSoft, ver.7.0, USA) statistical analysis software. In all cases, p values less than 0.05 ($p<0.05$) were considered statistically significant.

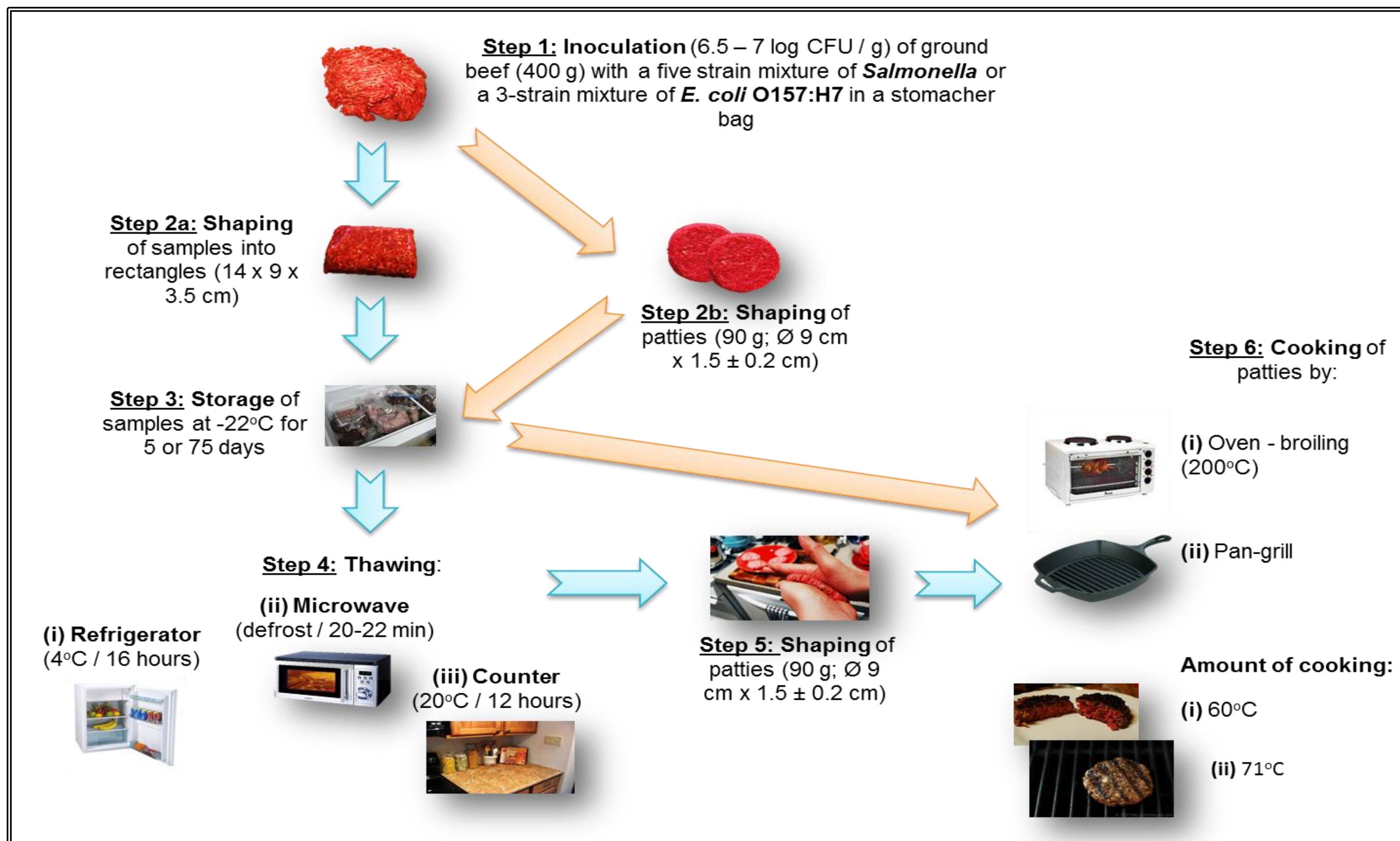


Figure 3-2 Flow diagram of the experimental procedure that was followed.

3.3 Results and Discussion

3.3.1 Effect of freezing and thawing

The levels of the total indigenous microflora of the freshly produced ground beef ranged from 4.4 to 4.8 log CFU/g among the different batches which were used in the present study. Given that the samples were inoculated at higher levels (6.5-7.0 log CFU/g), populations enumerated on the non-selective media may be only attributed to the presence of the pathogens. Following inoculation of ground beef and prior to freezing, *Salmonella* populations were 6.8 to 7.0 log CFU/g, while *E. coli* O157:H7 inoculation level ranged from 6.6 to 6.8 log CFU/g (Table 3-1). Further frozen storage (-22°C) of samples for 5 days caused slight but statistically significant ($p<0.05$) reductions of both pathogens, as *Salmonella* and *E. coli* O157:H7 populations showed decrease of 0.7 log CFU/g (Table 3-1). However further storage at -22°C up to 75 days did not cause any further reductions of the populations of the two pathogens. With regards to the effect of the thawing method, the populations of the pathogens did not show any significant ($p > 0.05$) changes when ground beef was thawed in refrigerator or in microwave, compared with the populations obtained immediately before thawing. In contrast, thawing of samples under conditions which simulate the thawing procedure on kitchen counter, resulted in increased populations ($p<0.05$) of *Salmonella* and *E. coli* O157:H7 by 0.7 - 0.9 log CFU/g (Table 3-1).

Although freezing is not considered as a means to reduce bacterial contamination of foods but only to halt the potential microbial proliferation (Simpson Beauchamp et al., 2010), cells are exposed to stresses which may lead to reversible or non-reversible mechanical injuries. Indeed, the slight

Table 3-1 Means (Log CFU/g ± standard deviation) of *Salmonella* spp. and *E. coli* O157:H7 populations after storage at -22°C for 5 or 75 days and subsequent thawing in a household refrigerator, a microwave or on kitchen counter.

			5 days at -22°C		75 days at -22°C	
Pathogen	**Thawing method**	**Inoculation level**	**Before Thawing**	**After thawing**	**Before thawing**	**After thawing**
***Salmonella* spp.**	**Counter**	7.0 ± 0.2 A	6.3 ± 0.3 B	7.2 ± 0.1 A	6.3 ± 0.1 B	7.0 ± 0.2 A
	Refrigerator	6.8 ± 0.3 A	6.6 ± 0.3 A	6.9 ± 0.2 A	6.3 ± 0.1 B	6.6 ± 0.2 AB
	Microwave	7.0 ± 0.2 A	6.3 ± 0.3 B	6.6 ± 0.1 B	6.3 ± 0.1 B	6.7 ± 0.1 A
***E. coli* O157:H7**	**Counter**	6.8 ± 0.1 A	6.1 ± 0.2 B	7.0 ± 0.1 A	6.1 ± 0.1 B	6.8 ± 0.4 A
	Refrigerator	6.6 ± 0.3 A	5.9 ± 0.2 B	6.3 ± 0.2 A	6.2 ± 0.0 B	6.5 ± 0.2 A
	Microwave	6.8 ± 0.1 A	6.1 ± 0.2 B	6.3 ± 0.2 B	6.1 ± 0.1 B	6.1 ± 0.1 B

Different letters within a row indicate statistically significant differences ($p<0.05$) between populations of the two pathogens at the inoculation level and before or after thawing.

differences (0.5 – 0.7 log CFU/g) between counts of the two pathogens recovered on the non-selective and selective media after frozen storage for 5 or 75 days, confirm the likelihood of injury that may occurred to the cells (Figure 3-3). Dominguez and Schaffner (2009) also observed significant differences on the populations of *Salmonella* on XLT4 and PCA, during frozen storage of chicken products for 16 weeks, indicating potential injury of the pathogen.

During freezing, the microbial cells are struggling to maintain the chemical potential of the intracellular water phase in equilibrium with the water outside the cell, by discarding water, in order to avoid internal freezing. However, if the rate of temperature decrease (T_d) is very high, the cells are not able to

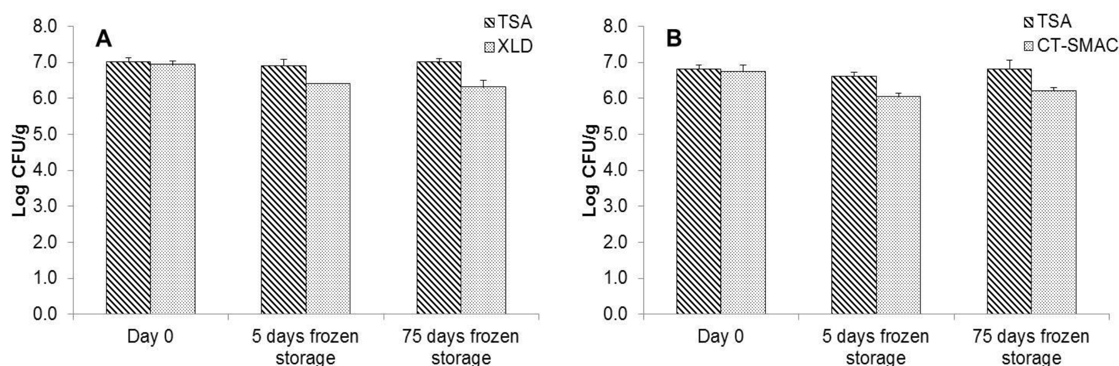


Figure 3-3 Populations of *Salmonella* (A) and *E. coli* O157:H7 (B) recovered on non-selective (TSA) or selective (XLD, CT-SMAC) media immediately after inoculation (day 0) and after 5 or 75 days of storage at -22°C and before thawing. Each bar represents the mean (± standard deviation) of 8 independent samples.

maintain the equilibrium and thus, the ice crystals that are being formed inside the cell may cause significant damages (Mazur, 1984; Elkest and Marth, 1992). There are studies in the literature stating that microbial populations may either decrease (Simpson Beauchamp et al., 2010; Dominguez and Schaffner, 2009; Niemira et al., 2003; Yamamoto and Harris, 2001; Semanchek and Golden, 1998) or remain unchanged (Lianou and Koutsoumanis, 2009; Dykes and Moorhead, 2000) during frozen storage. These differences may be attributed to the fact that this phenomenon is highly affected by various factors such as the type of strain, the inoculum history, the type of food matrix, the freezing temperature, the T_d etc. (Dykes and Moorhead, 2000). The findings of the present study are also in accordance with those of Sampers et al. (2010) who reported a 1-log reduction of Campylobacter spp. after storage of poultry meat at -22°C for 1 day, while no further decline was observed during long-term frozen storage. However, despite that the decrease of the two pathogens during freezing was statistically significant, in a biological manner it cannot be concluded that freezing is a sufficient tool for the destruction of pathogens.

With regards to the microbial increase which was observed during thawing of ground beef on kitchen counter (20°C for 12 hours), time-temperature profiles revealed that the samples remained above 5°C for approximately 7 hours (Figure 3-4). This extensive exposure above the lower limit of the danger zone (5 – 60°C) may promote the proliferation of the pathogens. Therefore, such handling of ground beef contradicts with the issued guidelines of USDA (USDA-FSIS, 2010b) and other national Food Safety authorities. Such phenomenon was not evident during thawing at 4°C, as the samples were not exposed to abuse temperatures or in microwave because the defrosting cycle was completed within 22 – 24 minutes (Figure 3-4). In particular, during thawing under refrigerated conditions, the internal temperature of the samples increased from -22°C to approximately -2°C within four hours. At that temperature increase rate (T_i) decreased significantly, due to the effect of the latent heat of fusion and remained at low levels for approximately 8 - 9 hours before temperature exceed 0°C and reached the final 4°C. Similar were the observations during thawing in microwave with the samples remaining close to 0°C approximately for 12 minutes, before reaching 20°C (Figure 3-4). In contrast, during 12-hour thawing on counter samples remained at temperatures ≤ 5°C for only 4.5 hours and then reached 20°C within 3 hours.

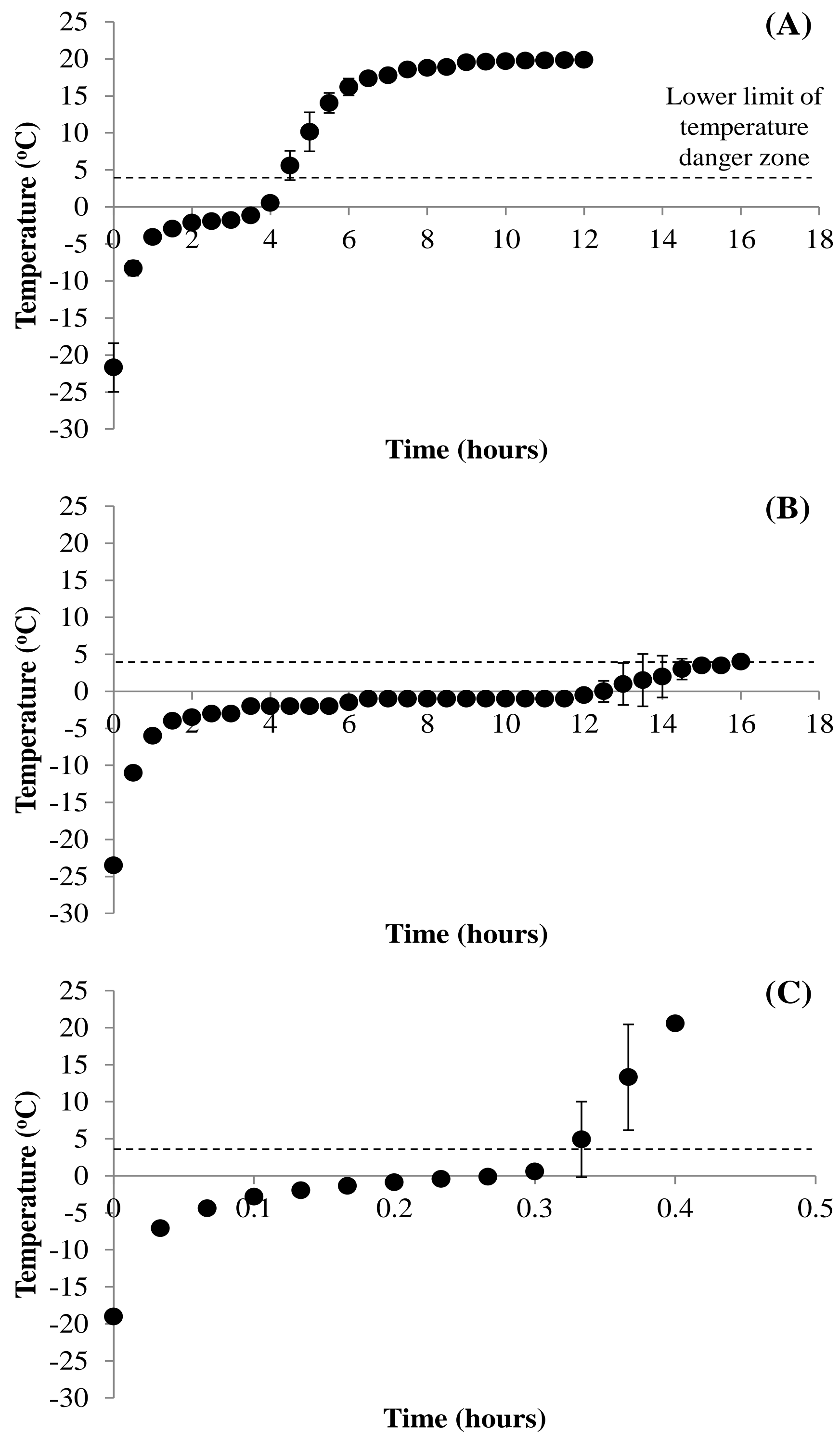


Figure 3-4 Changes of temperature during thawing of ground beef blocks (400 g) on counter (A; 20°C for 12 hours), in refrigerator (B; 4°C for 16 hours) or in microwave (C; defrosting mode for 22-24 minutes). Each point represents the mean (± standard deviation) of 8 independent samples.

3.3.2 Effect of cooking method and amount of cooking

The mean temperature of the oven-broiler during cooking was 189 ± 17^oC, while the corresponding temperature in pan-grill was 381 ± 9^oC. Total cooking duration varied significantly with the thawing method and the cooking practice that was followed, as well as the amount of cooking of patties (Table 3-2). In particular, cooking directly from the frozen state required significantly higher cooking times compared with samples which were cooked after thawing (Berry, 1998). For instance, cooking of samples without prior thawing (internal temperature before cooking ca. -20^oC) required up to 16.34 ± 1.43 minutes while cooking of samples under the same conditions but after thawing in microwave (internal temperature ca. 20^oC), required 8.64 ± 0.91 minutes. The effectiveness of the cooking method in association with the amount of cooking on the elimination of both pathogens followed the order: oven-broiling – 71^oC > pan-grill – 71^oC > oven-broiling – 60^oC, pan-grill – 60^oC. Cooking of beef patties in the oven-broiler resulted in higher reductions of the two pathogens compared with cooking in pan-grill (Figure 3-5; Figure 3-6), regardless of the duration of frozen storage or the thawing method. These differences were more evident when samples were cooked at an internal temperature of 71^oC, as the populations of both pathogens reduced

Table 3-2 Total duration (minutes) of thermal process during cooking of 90 g beef patties in the oven-broiler or in pan-grill, up to two levels of doneness, and after thawing with four different methods.

Thawing practice	Cooking time (min)			
	oven-broiler – 71°C	pan-grill – 71°C	oven-broiler – 60°C	pan-grill – 60°C
Counter	9.43 ± 0.67	6.08 ± 0.99	7.29 ± 0.56	3.89 ± 0.75
Refrigerator	10.29 ± 0.85	7.19 ± 0.62	8.36 ± 0.89	5.73 ± 0.95
Microwave	8.64 ± 0.91	5.18 ± 1.36	6.32 ± 0.40	3.32 ± 0.63
No thawing	16.34 ± 1.43	9.7 ± 0.21	13.93 ± 1.02	8.26 ± 0.33

below the detection limit (0.7 log CFU/g) when cooked in the oven-broiler, while total logarithmic reductions after cooking in pan-grill ranged from 1.2 to 4.1 log CFU/g and 1.7 to 3.1 log CFU/g for *Salmonella* and *E. coli* O157:H7, respectively. In contrast, undercooking (final internal temperature 60°C) of beef patties, which is often followed by consumers, resulted in significantly lower decrease (0.5 – 2.7 log CFU/g) of the populations of the two pathogens, regardless of the cooking method (Figure 3-5; Figure 3-6). The higher capability of cooking in oven-broiler to destroy the pathogens in comparison with pan-grill was also confirmed by the calculated thermal process lethality (*F value*; Table 3-3, Table 3-4). Indeed, *F-values* after cooking in oven-broiler at 71°C were significantly higher compared with cooking in pan-grill at 71°C, while cooking at an internal target temperature of 60°C showed the lowest *F-values* regardless of the cooking method. Contrary, the method which was followed for the thawing of beef patties did not have any significant ($p>0.05$) impact on the final thermal process lethality (Table 3-3, Table 3-4).

The correlation of various cooking methods with the survival of pathogens has been widely studied in the literature (Shen et al., 2011b; Rhee et al., 2003; D'Sa et al., 2000). Data derived from the present study are in accordance with the findings of Shen et al. (2010) who reported that roasting (which is close to the oven-broiling method) of non- intact beefsteaks caused higher reductions of *E. coli* O157:H7 compared with pan-broiling. The effectiveness of each cooking method and amount of cooking could be possibly explained by the total duration of each cooking process. Cooking in pan-grill was more intense and rapid procedure and therefore, the total cooking time was shorter. Contrary, a more smooth and slow cooking process was observed in the oven-broiler, resulting in longer cooking times. Challenge tests on the thermotolerance of pathogens, which are based on broth culture media, have shown that their survival is associated with the duration of the total exposure to lethal temperatures (Skandamis et al., 2008). However, cooking processes incorporate a wide range of gradually

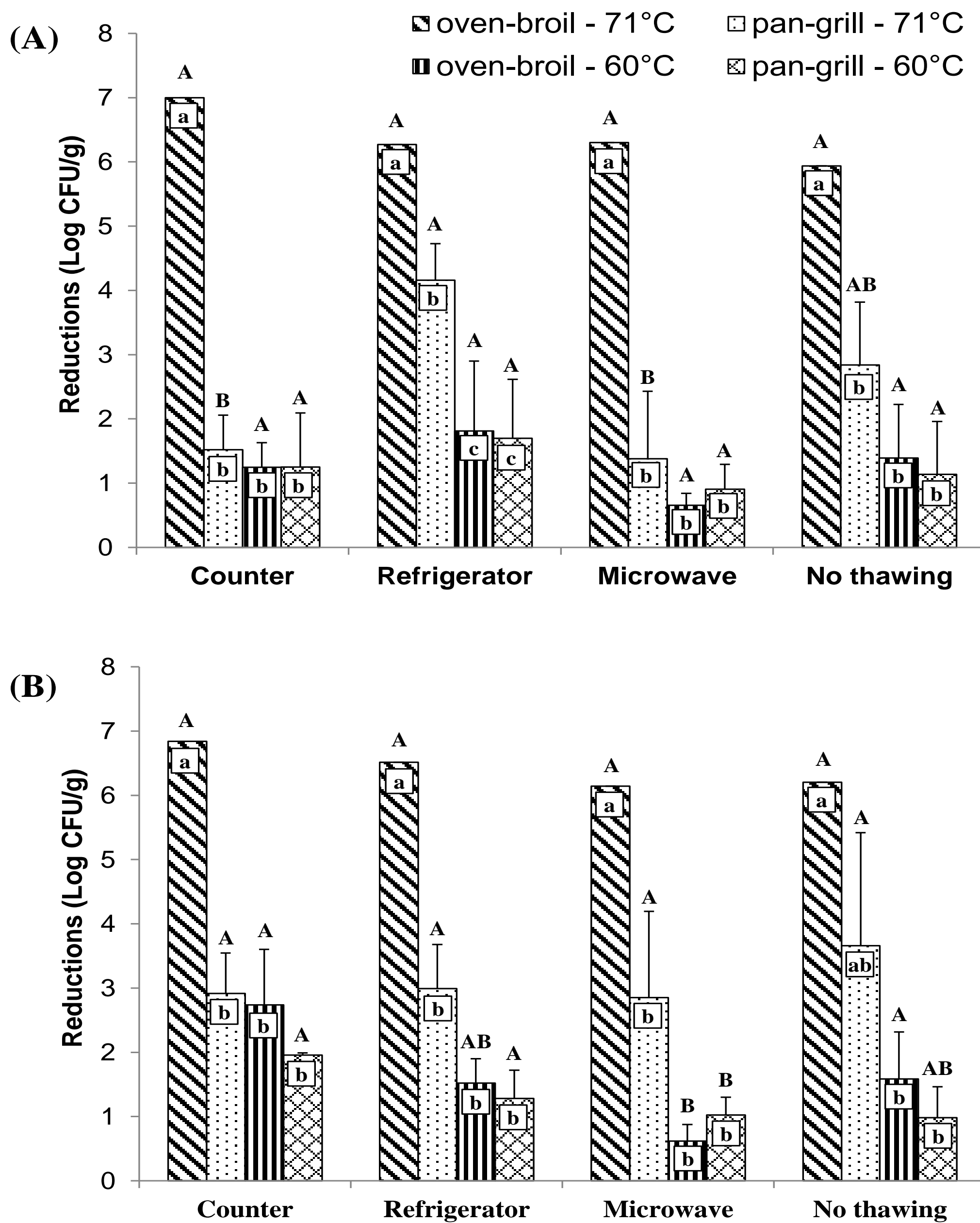


Figure 3-5 Logarithmic reductions of *Salmonella* spp. in beef patties after storage at -22°C for 5 (A) or 75 days (B), thawing with four different methods (counter, refrigerator, microwave, no thawing) and cooking in a kitchen oven-broiler or in a pan-grill until the internal temperature of the patties reached 60 or 71°C. Similar lower case letters for a particular thawing method indicate non-significant differences among the two different cooking methods and amount of cooking. Similar upper case letters indicate no significant effect of the different thawing methods on the same cooking method and amount of cooking.

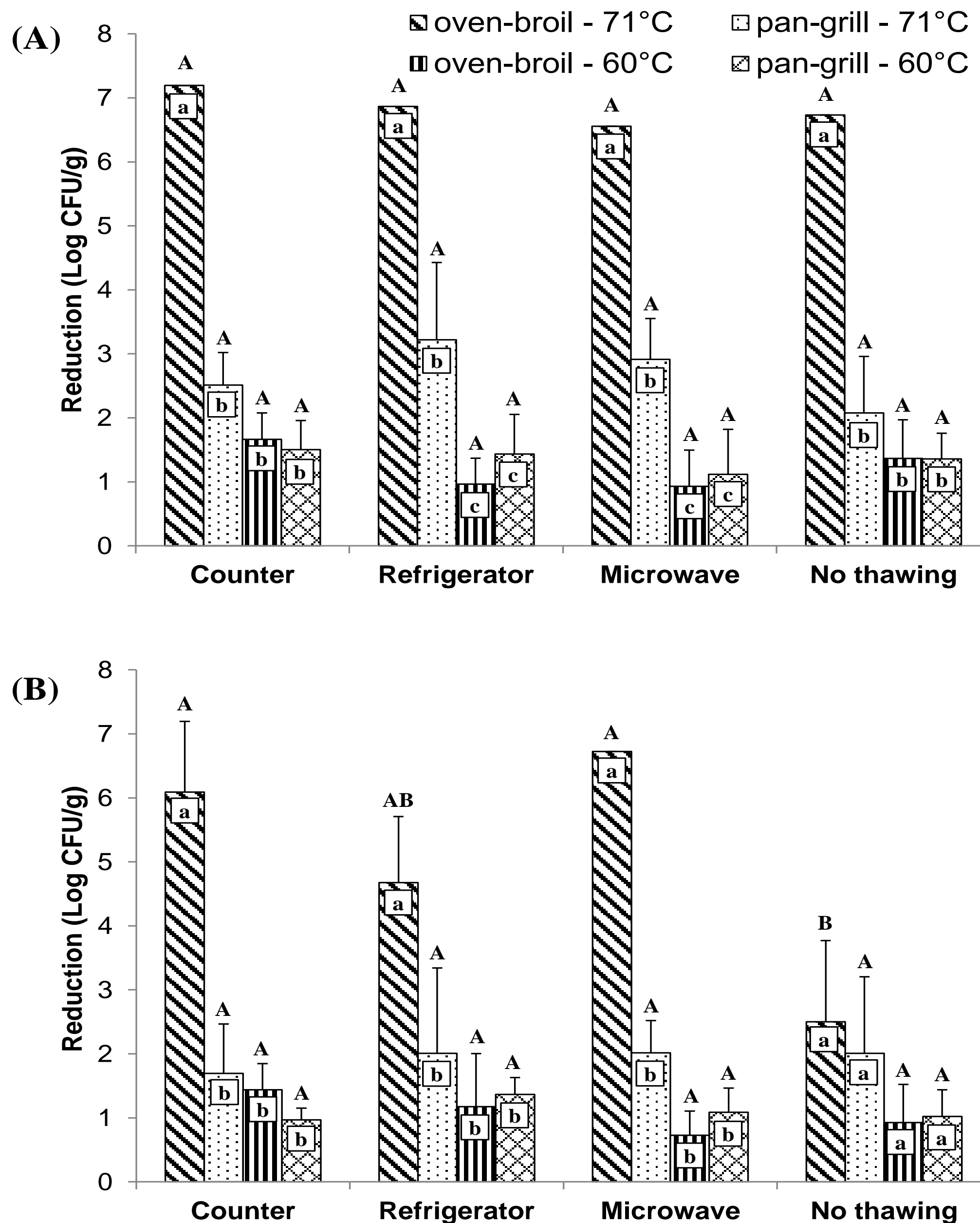


Figure 3-6 Logarithmic reductions of *Escherichia coli* O157:H7 in beef patties after storage at -22°C for 5 (A) or 75 days (B), thawing with four different methods (counter, refrigerator, microwave, no thawing) and cooking in a kitchen oven-broiler or in a pan-grill until the internal temperature of the patties reached 60 or 71°C. Similar lower case letters for a particular thawing method indicate non-significant differences among the two different cooking methods and levels of doneness. Similar upper case letters indicate no significant effect of the different thawing methods on the same cooking method and amount of cooking.

Table 3-3 Mean values of thermal process lethality (F value) of *Escherichia coli* O157:H7 in beef patties which were cooked in oven-broiler or in pan-grill at 60°C or 71°C, after thawing with four different practices.

Thawing method	Cooking method – amount of cooking			
	oven-broiler (71°C)	pan-grill (71°C)	oven-broiler (60°C)	pan-grill (60°C)
Counter	491.5 ± 58.3^{Aa}	303.7 ± 55.74^{Ab}	4.0 ± 0.6^{Ac}	2.4 ± 0.7^{Ac}
Refrigerator	497.9 ± 105.7^{Aa}	312.2 ± 137.2^{Ab}	4.1 ± 0.5^{Ac}	3.4 ± 0.1^{Ac}
Microwave	505.7 ± 69.2^{Aa}	381.5 ± 151.5^{Ab}	3.6 ± 0.7^{Ac}	2.4 ± 1.0^{Ac}
No thawing	566.6 ± 120.0^{Aa}	339.8 ± 153.4^{Ab}	3.2 ± 1.0^{Ac}	1.5 ± 0.6^{Ac}

Mean values with different upper case letters within a column are statistically different (p<0.05). Mean values with different lower case letters within a row are statistically different (p<0.05).

Table 3-4 Mean values of thermal process lethality (F value) of *Salmonella* spp. in beef patties which were cooked in oven-broiler or in pan-grill at 60°C or 71°C, after thawing with four different practices.

Thawing method	Cooking method – amount of cooking			
	oven-broiler (71°C)	pan-grill (71°C)	oven-broiler (60°C)	pan-grill (60°C)
Counter	313.4 ± 37.4^{Aa}	169.4 ± 60.4^{Ab}	3.6 ± 0.4^{Ac}	1.7 ± 0.4^{Ac}
Refrigerator	296.4 ± 70.9^{Aa}	174.7 ± 93.0^{Ab}	3.5 ± 0.7^{Ac}	2.8 ± 1.2^{Ac}
Microwave	289.4 ± 56.5^{Aa}	213.9 ± 48.7^{Ab}	3.8 ± 0.3^{Ac}	2.3 ± 0.4^{Ac}
No thawing	294.5 ± 54.9^{Aa}	116.9 ± 79.9^{Ab}	3.3 ± 0.9^{Ac}	1.5 ± 0.9^{Ac}

Mean values with different upper case letters within a column are statistically different (p<0.05). Mean values with different lower case letters within a row are statistically different (p<0.05).

increasing temperatures, including non-lethal and lethal values. In addition, if only cooking time was responsible for the destruction of the pathogens, then higher reductions should also be expected during the cooking of frozen samples compared with the thawed samples. Thus, the rate of heat transfer in the geometrical center of samples, as well as the way that heat was transferred to the internal part of patties during cooking may likely explain the higher capability of cooking in oven-broiler to reduce the pathogens, compared with cooking in pan-grill (Shen et al., 2010). The time-temperature profiles showed that the heat transfer rate during cooking in oven-broiler was slower compared with cooking in pan-grill (Figure 3-7). In this way, the heat was more evenly distributed in beef patties and thus, the pathogens were consistently exposed to lethal temperatures for a longer time. In addition,

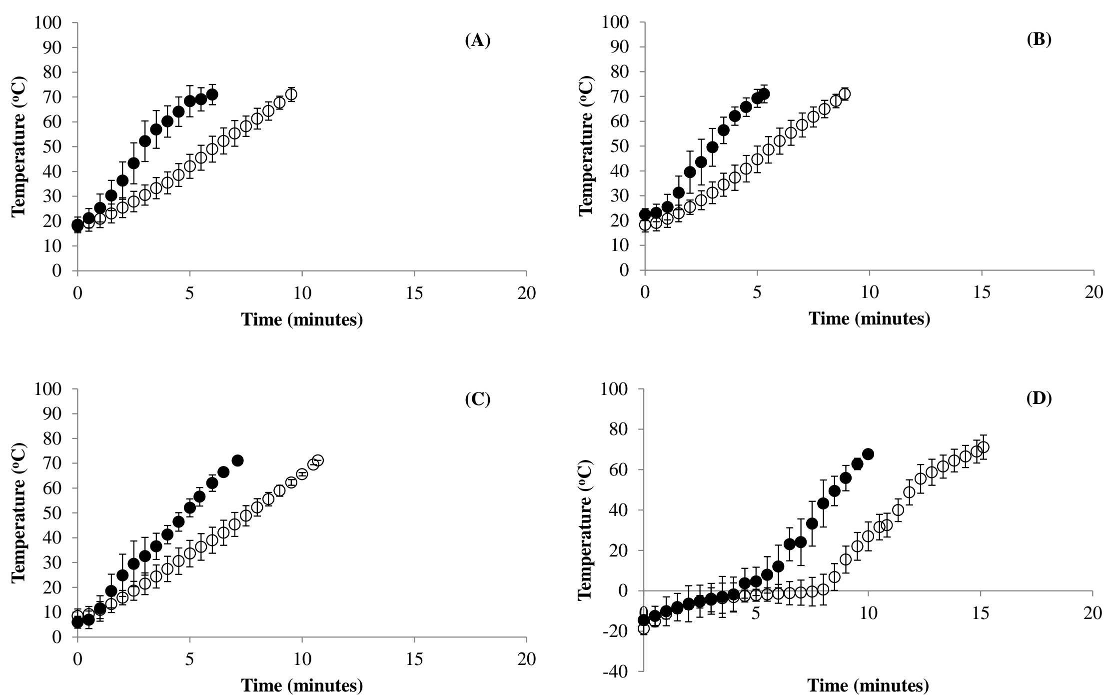


Figure 3-7 Time-temperature profiles during cooking of beef patties in oven-broiler (open symbols) and pan-grill (closed symbols) until 71°C and after thawing on countertop (A), in microwave (B), in refrigerator (C) or without prior thawing (D). Each point represents the mean (± standard deviation) of 16 independent samples.

during cooking in pan-grill, heat is transferred only by conduction, while in oven-broiler the heat transfer takes place both by conduction (due to the direct conduct with the heated cooking utensil) and convection. Of high importance was also the fact that cooking in pan-grill was not able to eliminate the pathogens, as it was observed in oven-broiler, even when beef patties were cooked until 71°C. These findings contradict with the issued guidelines, where it is stated that cooking of ground beef at an internal temperature of 160°F or 71.1^{o}C may sufficiently ensure the safety of the product (USDA-FSIS, 2012c). The results of the present study highlight the differences in the effectiveness of common cooking methods to ensure the safety of beef patties and therefore, the suggested cooking temperatures should be reconsidered in accordance with this variable.

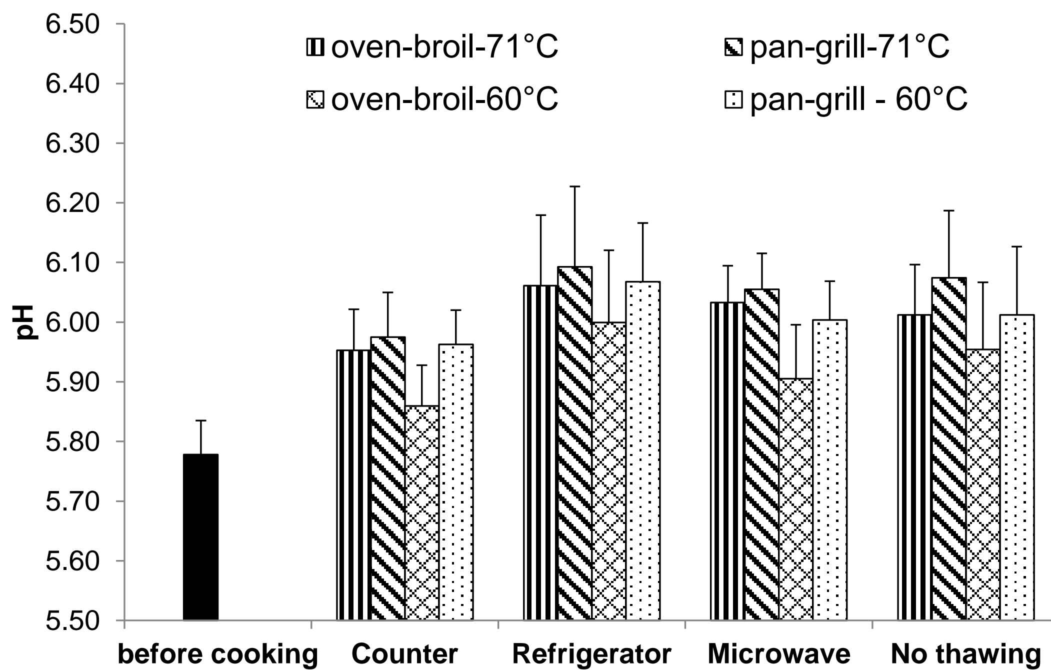


Figure 3-8 pH changes during cooking of beef patties in oven-broiler or in pan-grill up to two levels of doneness (60°C and 71°C). Columns with asterisk indicate no significant differences ($p>0.05$) of the pH of the treatment in comparison with that before cooking of the samples.

The different cooking practices and amounts of cooking that were tested in the present study also had an immediate impact on the pH of the cooked beef patties as well as the percentage of weight loses during cooking (Figure 3-8). Except for cooking in oven-broiler at 60°C after thawing on counter or in microwave, all thawing/cooking practices resulted in statistically significant ($p<0.05$) increase of the pH of the final products. The initial pH of fresh samples was 5.78 ± 0.06 and, depending on the thawing method, it ranged from 5.95 – 6.06, 5.98 – 6.09, 5.86 – 6.00 and 5.96 – 6.07 after cooking in oven-broiler at 71°C, pan-grill at 71°C, oven-broiler at 60°C and pan-grill at 60°C, respectively. These results are in agreement with previous studies, where significant increase of pH during cooking of intact or non-intact beef has been observed (Shen et al., 2011a; 2011b; Mukherjee et al., 2009). The increase of beef patties pH during cooking may likely be associated with the loss of acidic compounds (Vasanthi et al., 2007). Such increase of pH may not have any significant effect on the sensory characteristics of the final product, however, this parameter was primarily used to confirm the reproducibility of the cooking method and amount of cooking that was followed among different samples.

Despite that cooking in the pan-grill caused slightly higher increase of pH in beef patties comparing with cooking in oven-broiler, but these differences were not statistically significant ($p>0.05$). In contrast, for each specific cooking method and amount of cooking, the samples had higher pH values after thawing in refrigerator or after cooking without prior thawing (Figure 3-8). As long as these two thawing practices required more cooking time compared with those of microwave and counter, it is speculated that the longer exposure to heat caused greater release of acidic compounds and, thus increased the final pH of the product, at least locally at the outer part of the patties. In addition to pH increase, an expected weight loss of samples was also observed during cooking (Figure 3-9). Although the thawing practice which was followed did not affect significantly ($p < 0.05$) the weight loses of samples, the cooking method and the amount of cooking played significant ($p < 0.05$) role; beef patties cooked in pan-grill at 71°C lost approximately 25.7

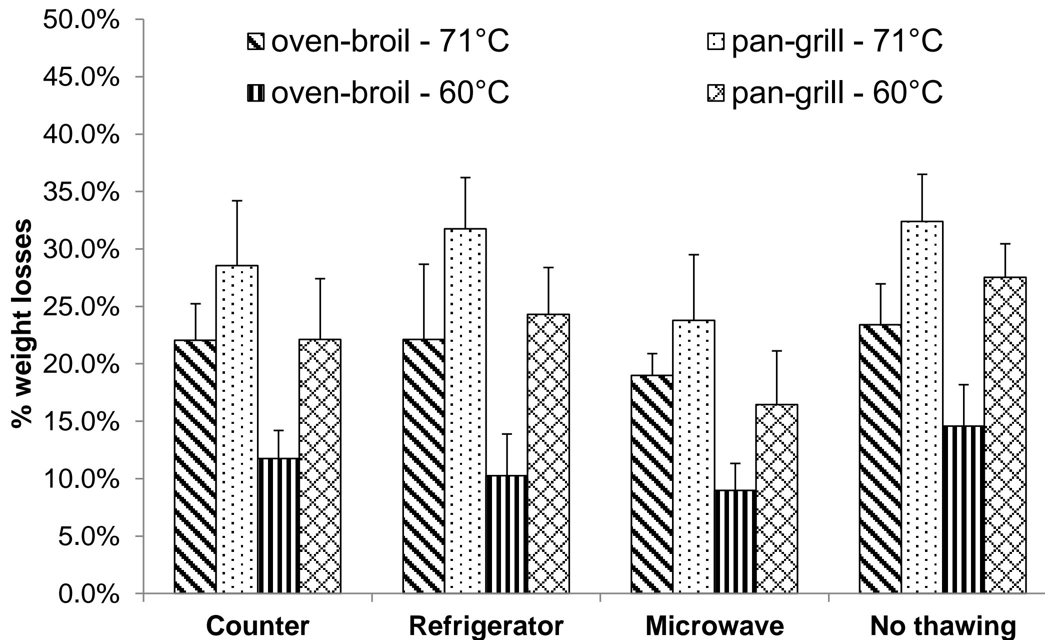


Figure 3-9 Weight loses (%) of beef patties during cooking in oven-broiler or in pan-grill up to two levels of doneness (60°C and 71°C). Within a thawing method, different letters indicate statistically significant differences ($p<0.05$).

±3.8 g, while the corresponding weight loses after cooking in oven-broiler at 71°C, pan-grill at 60°C and oven-broiler at 60°C were 19.2 ± 1.6 g, 10.9 ± 2.1 g and 20.5 ± 4.3 g, respectively. These differences may be attributed to the intensity of each cooking procedure rather than the duration of cooking time. In general, losses of weight during cooking is the result of extensive release of water, which was held within the myofibrils of meat (Barbera and Tassone, 2006), as well as the melted fat.

The heat resistance of *E. coli* O157:H7 during cooking was significantly affected by the combined effect of frozen storage duration and the thawing practice which was followed. In particular, the populations of the pathogen in samples which were stored at -22°C for 5 days and were cooked in oven-broiler up to 71°C without prior thawing declined by 6.7 ± 0.0 log CFU/g, while the corresponding reduction after 75 days of frozen storage was 2.5 ±

1.1 log CFU/g. Similarly but on a lower extent, populations of *E. coli* O157:H7 reduced by 4.7 ± 1.0 log CFU/g after long-term frozen storage and thawing under refrigerated conditions, following cooking at 71°C in the oven-broiler, compared with the reductions (6.9 ± 0.0 log CFU/g) caused after short-term storage at -22°C and thawing/cooking under the same conditions. Following cooking in the pan-grill at 71°C or undercooking (60°C) of samples, such phenomenon was not evident probably due to the low capability of these cooking practices to decrease the populations of the pathogen, as shown above (Figure 3-6). Contrary to *E. coli* O157:H7, *Salmonella* spp. did not pose any induced heat tolerance regardless of the frozen storage duration, the thawing method or the cooking practice which was followed (Figure 3-5).

To the author's knowledge, there is lack of studies in the literature reporting induced heat tolerance of pathogens after frozen storage. In contrast, it is generally recognized that cold shock sensitizes pathogenic bacteria against further thermal processes (Shen et al., 2011a; Novak and Juneja, 2003; Byrne et al., 2002), by prohibiting the coding of thermal-protective proteins (Miller et al., 2000) or due to the mechanical injuries that may occur during freezing (Dominguez and Schaffner, 2009). However, in these studies the duration of frozen storage was limited up to a few hours or days and thus, the effect of long term frozen storage has not been determined. Indeed, Jackson et al. (1995) demonstrated higher survival of *E. coli* O157:H7 after thermal treatment at 55°C in nutrient broth, which was pre-stored at -18°C for 8 or 15 days, compared with cultures that remained frozen for 1 day.

The increased survived population of *E. coli* O157:H7 after cooking could be possibly attributed to the "tailing effect" that is regularly observed during thermal inactivation treatments. This phenomenon may be the result of various factors including the residual of a heat resistant population within a homogeneous microbial population, potential gradual mutation of the pathogen during the heat process or even the heterogeneity of the heat treatment in the whole product surface (Miller et al., 2009; Cerf, 1977). However, in the present study, the increased survival of *E. coli* O157:H7 was

observed only after thawing in refrigerator or after direct cooking of frozen patties. Since no such observations were made after thawing in microwave or on the counter, the survival of the pathogen may not be the result of tailing effect during cooking. In contrast, it is speculated that this induced heat tolerance of *E. coli* O157:H7 may be attributed to mechanisms used by the cells to survive long-term frozen storage (i.e. specific protein synthesis) or to the structural properties of long-term stored patties compared with patties which are stored under frozen conditions for a shorter period (i.e. size of formed ice crystals). However, further investigation on molecular and/or mechanical basis is essential to provide more specific explanation of this phenomenon. In addition, the increased heat tolerance of the pathogen was observed primarily in frozen samples and in samples which were thawed in refrigerator, compared with samples thawed on counter or in microwave. The metabolic activity of the pathogen during thawing on counter or during holding and preparing the samples after thawing in microwave (approximately 30 minutes) could possibly lead to the loss of the protective effect of freezing (Jackson et al., 1995).

3.4 Conclusions

An attempt to simulate the most common consumer-style practices during storage of ground beef and preparation of beef patties was made, even if these practices are not in accordance to the issued guidelines, in order to evaluate their effect of the survival of two major pathogens. Overall, it was found that the combined effect of sequentially applied practices such as freezing, thawing and cooking may have significantly different impact on the heat resistance of pathogens, compared with the application of a single treatment. In particular, long-term frozen storage, in association with direct cooking without prior thawing, may induce the thermo-tolerance of *E. coli* O157:H7. Furthermore, cooking in the pan-grill may not ensure the final safety of beef patties, even if the internal cooking temperature is in accordance with the recommendations. Thus, the significance to incorporate

the frozen storage duration and the cooking method as a potential variable to the issued guidelines is highlighted. In parallel, the results may be used to instruct the consumers and catering services for the appropriate defrosting or cooking practices of beef patties, taking into account the variability that might occur for practical purposes in different establishments (i.e. households, food caterings, and restaurants).

4 DYNAMICS OF LOW (1-4 CELLS) *vs.* HIGH POPULATIONS OF *LISTERIA MONOCYTOGENES* AND *SALMONELLA* TYPHIMURIUM IN FRESH-CUT SALADS

4.1 Introduction

Numerous foodborne disease outbreaks associated with fresh produce or minimally processed vegetable salads have been reported worldwide over the past decade (CDC, 2011b; 2008; 2006; Lienemann et al. 2008; Horby et al., 2003). *Listeria monocytogenes* and various serotypes of *Salmonella* (e.g., Typhimurium, Newport, Reading and Saintpaul) or *Escherichia coli* (e.g., O157:H7, O145 and O104), are the most common causative agents. Contamination of fresh-cut salads with pathogens may occur during any point of cultivation and harvesting of the vegetables or during preparation of the cut-salads in the establishments (Brackett, 1999; Burnett and Beuchat, 2001; Doyle and Eriksson, 2008; Koseki, et al., 2011). The impermeability of the intact plant tissue results in a lack of available nutrients on the leaf surface. This, in conjunction with the exposure of plants to UV irradiation in the field and the competition of the immigrants with the epiphytic flora for nutrients, may inhibit the survival of pathogenic bacteria (Erickson et al., 2010). However, Leveau and Lindow (2001) suggested the existence of scattered sites with limited amounts of sugars (i.e. glucose, fructose) and moisture on the leaf surface, where growth of pathogens may occur. Further processing of vegetables such as cutting, slicing or shredding, in order to prepare pre-bagged Ready-To Eat salads, causes release of the nutritious juices, which provide appropriate substances for survival and/or microbial growth. Thus, evaluating the extent of pathogens proliferation in various fresh-cut salads is imperative in order to assess the safety risks originating from these foods.

The activity of the epiphytic flora, the structure of the micro-environment where microbial growth occurs, the availability of nutrients, as well as the

storage temperature constitute stressful factors which may introduce high variability in the growth potential of foodborne pathogens on fresh-cut salads (Schuenzel and Harrison, 2002; Ongeng et al., 2007, Leveau and Lindow, 2001; Franz et al., 2010). This variability markedly impacts especially the dynamics (i.e. lag time) of low populations, such as 1-10 cells, and increases with the intensity of the stress (Francois, et al., 2006a). The majority, though, of challenge tests are performed with high inoculum levels (>10^3 cells), in order to monitor the average behavior of the population derived from the fastest growing cells and hence, the worst case scenario for risk assessment. However, high inocula differ from the realistic scenario of low contamination, and results may also include uncertainty due to the potential interaction between cells of the inoculum (i.e. quorum sense). Thus, in order to extrapolate the results to realistic conditions of low contamination, the variability of population numbers due to the variance of individual cell lag times needs to be evaluated.

A significant number of predictive models have been developed to determine the kinetic parameters of individual cells under various environmental conditions (Métris, et al., 2005; Guillier and Augustin, 2006). The majority of these models are based on experimental data derived from laboratory media, contrary to the limited information available for the assessment of individual cell variability in foods (Francois, et al., 2006b; Manios, et al., 2011). In such *"static models"*, however, the effect of the activity of the epiphytic flora, the food structure or the diffusion of nutrients is difficult to describe. In contrast *"dynamic models"* are mathematical equations which are able to incorporate various influencing factors and provide more accurate predictions than *"static models"*. Development of such models, however, requires multiple specific measurements and may be proved rather complex for non-experienced food scientists, increasing the risk of unsafe predictions (Bernaerts et al., 2004). Thus, challenge tests with realistically low inocula on real food matrices should be conducted, to assess the risk of a pathogen with less uncertainty.

The objectives of the study were (i) to determine the variability in the growth of 2 pathogens in lettuce and cabbage at 8°C, (ii) to compare the response of low number of cells (1-4) with that of higher populations, (iii) to identify the contribution of the commensal flora, temperature abuse and food structure in the above variability and, (iv) to evaluate whether broth-based growth simulations may approximate the average outgrowth of a population from single cells in foods.

4.2 Materials and Methods

4.2.1 Bacteria and culture conditions

Listeria monocytogenes Scott A (serotype 4b; epidemic strain) and *Salmonella* enterica subsp. enterica Le Minor and Poppof serovar Typhimurium (calf bowel isolate) were used in the present study. Cultures of both strains were maintained on tryptone soy agar (TSA; LAB M, LAB011, Lancashire, UK) slants at 4°C. The microorganisms were grown twice in 10 ml tryptic soy broth (TSB; LAB M, LAB004) for 24 hours at 30 or 37°C, for *L. monocytogenes* and *S.* Typhimurium, respectively. Prior to inoculation, the activated strains were washed twice and resuspended in 10 ml maximum recovery diluent (MRD; LAB M, LAB103).

4.2.2 Isolation of low number of cells

The isolation of a few cells was conducted according to a modified protocol by Francois et al. (2003). Briefly, the cell density of the activated and washed cultures was standardized at 10^8 CFU/ml, using an optical density (O.D.) – log CFU/ml calibration curve. Following decimal dilutions, 200 μl of the 10^3

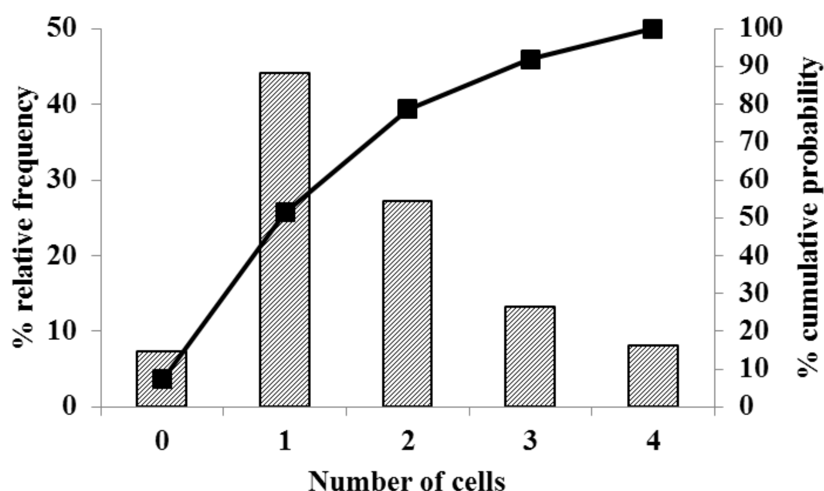


Figure 4-1 Percentage (%) relative frequency (bars) and cumulative probability (line) of cells contained in a microplate well (in 200 μl total volume per well) and used to inoculate samples of lettuce or cabbage salad or the corresponding extracts. Counts were estimated by plating the whole content of an additional microplate column.

CFU/ml solution were transferred to each well of the first column of a 8 x 12 microplate, which was prefilled with 200 μl of MRD. Half-fold dilutions were made across the microplate for the determination of the column which contained at least one cell and maximum four cells (column – target; *CT*). The latter decision was made to avoid the inoculation of samples with no cells. The whole content of the wells of the *CT* was used for the inoculation of samples. In parallel, the distribution of cells in the wells of the *CT* was checked at each experimental trial, by plating the whole content of the *CT* of an independent microplate on TSA, which served as control. Results showed that 92.6% of the samples were inoculated with 1-4 cells, whereas 7.4% of samples were possibly inoculated with no cells (Figure 4-1).

4.2.3 Preparation, inoculation and microbial analysis of vegetables

Fresh whole heads of Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) and white cabbage (*Brassica oleracea*) were purchased from local supermarkets on the same day of each experimental setup. The 4-5 outer leaves and the core of each lettuce were discarded and the remaining leaves were thoroughly washed with tap water to remove any organic material. In a further step, the washed leaves were dipped for 5 minutes in plastic containers containing a fresh solution of chlorine bleach (200 ppm sodium hypochlorite), simulating practices that are being commonly followed in catering services or households to reduce the microbial load of fresh produce. In contrast with lettuce, the heads of cabbage were cut in four quarters before dipping in the sanitizer. Following treatment with chlorine, the vegetables were washed thoroughly with sterile water for 15 min to remove the sanitizer. Both vegetables were allowed to dry for 30 min in a laminar flow cabinet before use.

Lettuce leaves were evenly cut into strips (*ca.* 1 cm width) using a sterile pair of kitchen scissors, while cabbage was cut with a household cabbage shredder, representing the commercially prepared, fresh-cut salads of leafy vegetables. Ten grams of each vegetable were transferred to sterile plastic containers (100 ml; 4.5 cm diameter x 7 cm height) and spot-inoculated (5-6 spots per sample) at a low (1-4 cells per sample or -1 to -0.4 log CFU/g) or a high (1000 cells per sample or 2 log CFU/g) level with *L. monocytogenes* or *S.* Typhimurium. The inoculated samples of both vegetables were stored at 8°C, which is an average temperature that RTE salads may encounter in retail (Franz, et al., 2010; Tromp et al., 2010) or domestic refrigerators (Marklinder et al., 2004). Absence of *L. monocytogenes* and *Salmonella* spp. in 25 g non-inoculated portions of both salads was confirmed *via* the methodology described by ISO standards 11290-1:1996/1:2004(E) and 6579:2002, respectively.

At regular time intervals, 40 ml of MRD were added to each sample and hand-shaken for 30 seconds in a standardized manner. Appropriate decimal dilutions were prepared in 9 ml MRD and plated on selective or non-selective media to monitor the growth kinetics of the inoculated pathogens and the indigenous microflora, respectively, as follows: total viable counts were determined by surface spreading on TSA plates and after incubation for 48 hours at 30°C. *Pseudomonas* species were enumerated by spread plating on pseudomonas agar base (LAB M, LAB108) plates supplemented with C.F.C. (LAB M, X108), following incubation at 25°C for 48 hours. Enterobacteria were enumerated by pour plating in violet red bile glucose agar (VRBG; LAB M, LAB088) incubated at 37°C for 24 hours. *L. monocytogenes* was spread plated on agar *Listeria* Ottaviani Agosti (ALOA, Biolife, 401605, Milan, Italy) plates with ALOA enrichment-selective supplements (Biolife; 423501) and incubated at 37°C for 48 hours. *Salmonella* counts were determined by surface spreading on xylose lysine deoxycholate agar (XLD; LAB M, LAB032) after incubation at 37°C for 24 hours. To decrease the detection limit of the sampling method, especially for the enumeration of the low inoculum of the pathogens (1-4 cells/sample), aliquots (1 ml) of the vegetable – MRD homogenate was equally distributed on the surface of three plates of each selective medium (i.e. XLD, ALOA). The detection limit of the plating method was 0.7 log CFU/g.

4.2.4 Preparation, inoculation and microbial analysis of vegetable sterile extracts

Considering that the microbial growth on vegetables may occur either on the vegetable surface or in the liquid micro-environment formed by the release of juices at wounds or the edge of cut surfaces, liquid and solidified extracts of lettuce and cabbage were prepared. These extracts were further sterilized in order to investigate the behavior of *L. monocytogenes* and *S.* Typhimurium in the absence of the indigenous microflora and compare it with that on salads. A modified protocol of Allende et al. (2007) was followed for the preparation

of extracts. Fresh heads of lettuce and cabbage were obtained, washed and treated with chlorine bleach as previously described. The dry leaves of the vegetables were blended (Waring Pro, HGB50E1, Torrington, CT, USA) in 1:1 ratio with warm distilled water (45°C) for 1 min at high speed. The derived pulp was heated at 80°C for 2 hours to denature the enzymes and proteins of the vegetables. The homogenate was filtered through Whatman paper and autoclaved at 121°C for 15 min either in 5 ml tubes or in 500 ml flasks with 1.5% w/v agar (Agar Bios Special LL; Biolife, 4110302). Equal volumes (15 ml) of the sterile extract supplemented with agar were poured into petri dishes. The pH of the each medium was measured (WTW pH526 Metrohm Ltd, Switzerland) before and after autoclaving and was 6.17 ± 0.06, 6.05 ± 0.09 for lettuce and 5.99 ± 0.09, 5.84 ± 0.13 for cabbage, respectively. Both solidified and liquid media were kept at 4°C for less than 24 hours before use.

Each sample of solidified (15 g per sample) or liquid (5 ml per sample) extracts of lettuce and cabbage was inoculated with 1-4 cells (-1.2 to -0.6 log CFU/g and -0.7 to -0.1 log CFU/ml for solidified and liquid extracts respectively) or 1000 cells (1.8 log CFU/g or 2.3 log CFU/ml for solidified and liquid extracts respectively) of *L. monocytogenes* or *S.* Typhimurium. All samples were stored at 8°C and 10°C, in order to determine the impact of marginal changes in the storage temperature, close to the growth/no growth boundaries of the microorganisms, on the variability of microbial growth, especially of low inocula. Prior to incubation, the plates containing the solidified extracts were securely sealed with parafilm to avoid moisture losses. For the enumeration of the microorganisms in liquid extracts, 1ml of each liquid medium was surface plated directly on three TSA plates. Similarly, the whole content (inoculated vegetable extract agar) of plates with the solidified extracts was homogenized with 30 ml MRD (1:3 dilution) in a stomacher bag for 1 min and 1 ml of the homogenate was plated on TSA. *L. monocytogenes* and *S.* Typhimurium populations were enumerated following incubation at 30°C for 48 hours or 37°C for 24 hours, respectively. The

detection limit of the plating method was 0.5 log CFU/g for solidified extracts and 0.7 log CFU/ml for liquid extracts.

4.2.5 Monte Carlo simulation

To assess the ability of broth-based models to simulate the growth of a few (1-4) *L. monocytogenes* cells on lettuce or cabbage, data collected from the present study were compared to simulation results derived from the published data of Francois et al. (2006a), where the mathematical expression of the effect of pH, a_w and storage temperature on the individual cell lag time and the generation time of *L. monocytogenes* was described. To the authors' knowledge, such data are not available for *Salmonella* in the literature, and hence, simulations were conducted only for *L. monocytogenes*. Considering the intrinsic parameters of the salads tested and storage temperature, as shown in Table 4-1, the Weibull distributions of broth-based individual lag times (Francois et al. 2006a) were used to simulate the growth (log increase) of *L. monocytogenes* in salads, initiating from a few cells and after 12 days at 8°C. We chose the particular lag time distributions because they refer to experimental conditions of pH, a_w and temperature that are close to the conditions of the fresh-cut salads of the present study. The model used for the simulation of *L. monocytogenes* growth is based on the three phase linear model of Buchanan et al. (1997), and is given by equations (4-1) and (4-2). The μ_{max} was estimated by the growth of the high inoculum of *L. monocytogenes* in each salad, using the model of Baranyi, and was fixed at 0.016 and 0.011 day^{-1} for growth in lettuce and cabbage salad, respectively.

$$N_t = N_0 \quad t \leq t_{lag} \qquad \textbf{(4-1)}$$

$$Log(N_t) = Log(N_0) + \mu_{max} \times (t - t_{lag}) \quad t > t_{lag} \qquad \textbf{(4-2)}$$

where t is time and t_{lag} a value of lag time from the distributions of Francois et al. (2006a).

Table 4-1 Approximation of experimental conditions in salads by the broth-based study of Francois et al. (2006a) and corresponding parameters of Weibull distributions of individual lag times, used for the simulation of *Listeria monocytogenes* growth on lettuce or cabbage salad, starting from 1-4 cells/sample.

Salad	**Conditions in salads**			**Modelling conditions**					
				Conditions in broth (Francois et al. 2006a)			**Parameters of individual lag time distribution**		
	pH	Water activity (a_w)	T (^oC)	pH	Water activity (a_w)	T(^oC)	a	b	shift
Lettuce	6.0 ± 0.2	0.978 ± 0.002	8	6.0	0.974	7	2.878	94.24	-
Cabbage	5.8 ± 0.2	0.970 ± 0.002	8	5.5	0.970	7	2.845	230.2	24.14

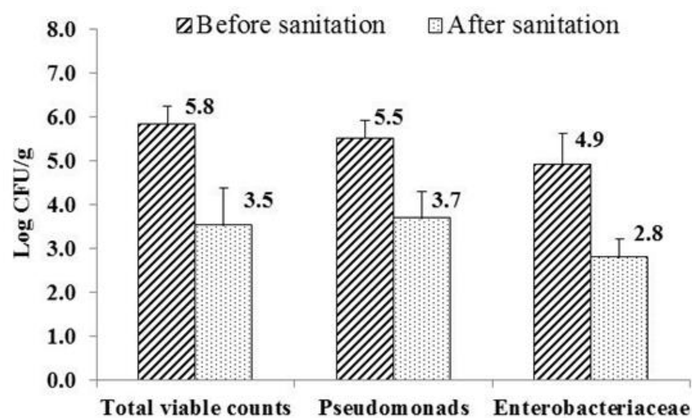
4.2.6 Statistical analysis

The experiments on fresh-cut salads were conducted in two replicates, using an independent batch of vegetable for each replicate. At each sampling day, two samples of each salad inoculated with 1000 cells/sample (n=4), and 10 samples inoculated with 1-4 cells/sample (n= 20) of the pathogens, were analyzed. For the preparation of the sterile extracts, four different batches of vegetables were used in four independent replicates. Similarly to the experiment on the vegetables, 10 samples of liquid or solidified extracts (n=40), which were inoculated with 1-4 cells/sample, and 2 samples inoculated with 1000 cells/sample (n=8) of the pathogens were analyzed at each sampling point. Populations of pathogens, which were enumerated on selective media were then transformed to log CFU/g in case of salads and solidified extracts and in log CFU/ml in case of liquid extracts. The average and standard deviation (SD) of log CFU/g or CFU/ml was calculated for samples inoculated with 1000 cells/sample. Populations derived from 1-4 cells/sample are presented individually for each sample, in order to emphasize the observed variability.

4.3 Results and Discussion

4.3.1 Growth kinetics on vegetables

Changes in the levels of indigenous microbial association of lettuce and cabbage salads were monitored at 8°C. *Pseudomonas* spp. (4.8-5.5 log CFU/g) and Enterobacteriaceae (4.9-5.8 log CFU/g) comprised the microbial flora of both salads (Figure 4-2). Yeasts and lactic acid bacteria were also present but at the lower levels of <2.3-2.6 and <1.8-2.0 log CFU/g, respectively (results not shown). Following treatment of vegetables with 200 ppm sodium hypochlorite, the reductions of pseudomonads ranged from 1.8



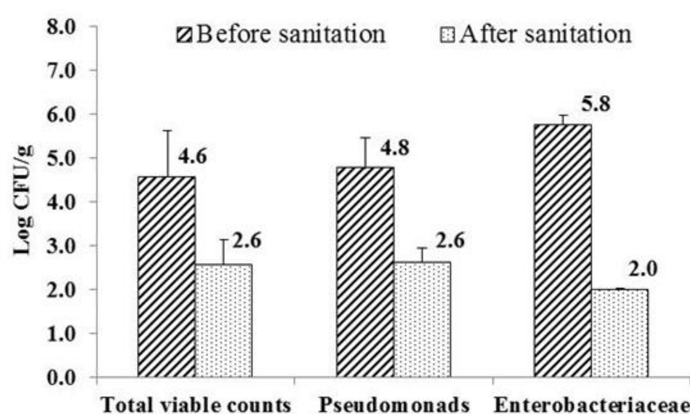


Figure 4-2 Populations (log CFU/g) of the indigenous microflora of lettuce (top) and cabbage (bottom) before and after treatment with 200 ppm sodium hypochlorite for 5 min and washing with sterile water for 15 min.

to 2.2 log CFU/g and that of Enterobacteriaceae from 2.9 to 3.8 log CFU/g (Figure 4-2). Both products were organoleptically unacceptable after 10-12 days, when the specific spoilage microorganisms (pseudomonads) had reached their maximum level of 9.4 ± 0.2 or 8.1 ± 0.3 log CFU/g in lettuce and cabbage salad, respectively (Figure 4-3 and Figure 4-4).

The microbial load of minimally processed salads usually consists of microorganisms related to soil or humans, such as pseudomonads, enterobacteria, lactic acid bacteria and yeasts (Randazzo, et al., 2009; Abadias, et al., 2008). Although disinfection of fresh produce aims to reduce

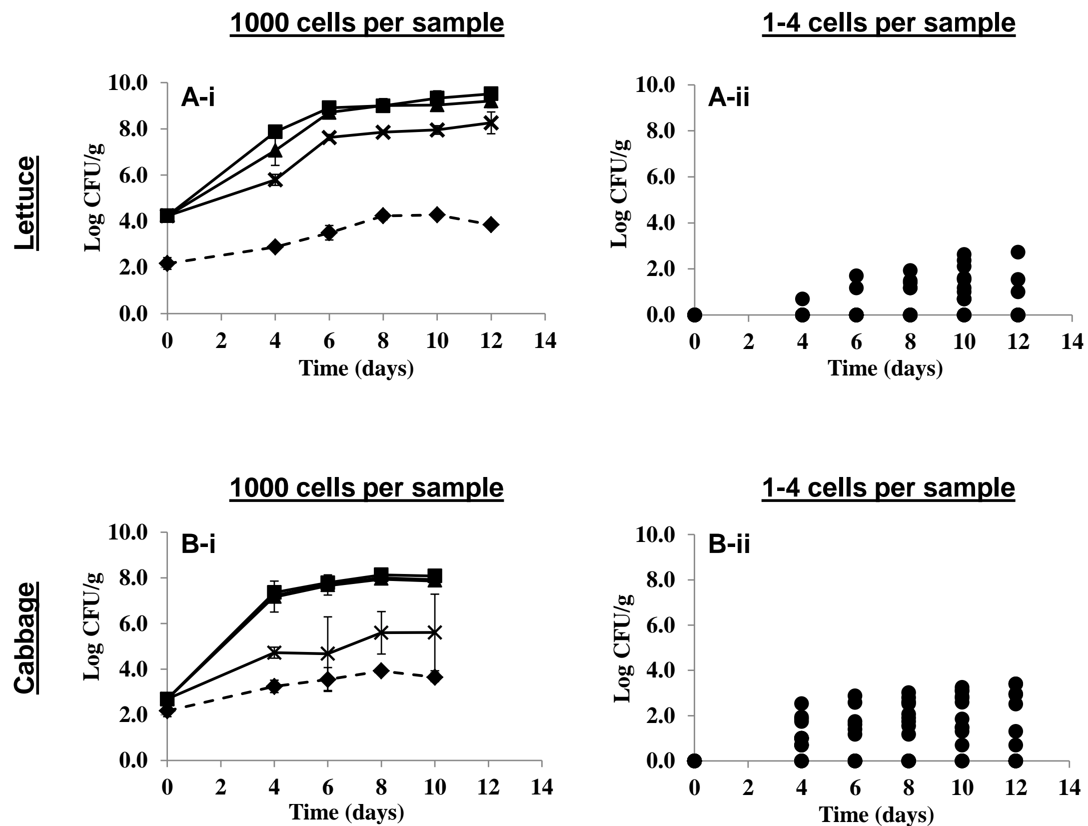


Figure 4-3 Growth of 1000 (i) or 1-4 cells (ii) of *Listeria monocytogenes* per sample of lettuce (A) or cabbage (B) salad at 8°C. (◆) 1000 cells of *L. monocytogenes*, (■) total viable counts, (▲) pseudomonads, (×) Enterobacteriaceae, (●) 1-4 cells of *L. monocytogenes*. Counts of the indigenous microflora (A-i, B-i) also refer to (A-ii, B-ii). The initial levels of *L. monocytogenes* in samples inoculated with 1-4 cells (-1 to -0.4 log CFU/g; graph A-ii, B-ii), are rounded to 0 log CFU/g, with detection limit of 0.7 log CFU/g.

the epiphytic flora and prolong the shelf-life of the final products (Delaquis, et al., 2004; Koseki and Itoh, 2001), reducing the epiphytes may also weaken their antagonistic activity against pathogens that survive the intervention or potentially contaminate the products during further processing.

With regards to the growth of pathogens, *L. monocytogenes* cells were able to grow on both salads, regardless of the inoculum level. Increase of *L.*

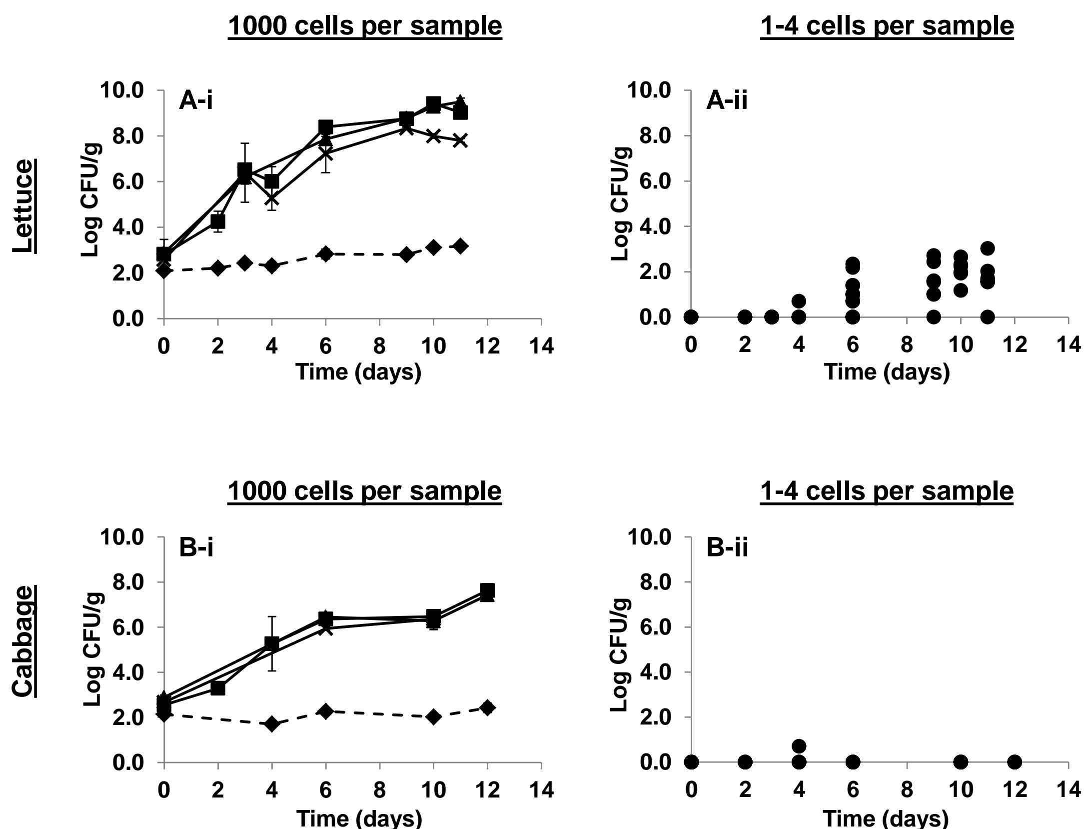


Figure 4-4 Growth of 1000 (i) or 1-4 cells (ii) of *Salmonella* Typhimurium on lettuce (A) and cabbage (B) salad at 8°C. (◆) 1000 cells of *S.* Typhimurium, (■) total viable counts, (▲) Pseudomonadaceae, (×) Enterobacteriaceae, (●) 1-4 cells of S. Typhimurium. Counts of the indigenous microflora (A-I, B-i) also refer to (A-ii, B-ii). The initial levels of *S.* Typhimurium in samples inoculated with a 1-4 cells (-1 to -0.4 log CFU/g; graph A-ii, B-ii), are rounded to 0 log CFU/g.

monocytogenes starting from 1000 cells/sample occurred with limited variation (standard deviation <0.5 log CFU/g), while growth initiating from a few (1-4) cells/sample ranged from <0.7 log CFU/g (detection limit) to 3.4 log CFU/g (Figure 4-3). Similar observations were made on the growth variability of the high and low inocula of *S.* Typhimurium on lettuce, whereas no growth of this microorganism was obtained on cabbage (Figure 4-4). It is notable however, that the total log-increase (LI) from 1-4 cells/sample of both pathogens was higher compared with that of 1000 cells/sample. More specifically, a few cells of *L. monocytogenes* exhibited LI $_{(t=12\ days)}$ = 2.7 and

3.4 log CFU/g on lettuce and cabbage salad, respectively, while the corresponding increase from 1000 cells was LI $_{(t=12\ days)}$ = 2.1 and 1.8 log CFU/g (Figure 4-3). Similarly, 1-4 cells/sample of *S.* Typhimurium showed LI $_{(t=12\ days)}$ = 3.0 log CFU/g on lettuce salad, whereas only LI $_{(t=12\ days)}$ = 1.1 log CFU/g was observed when starting from 1000 cells/sample (Figure 4-4). In all cases, the growth of pathogens ceased when the epiphytic microflora reached its maximum population density.

A typical fresh-cut salad sample consists of various micro-environments with different micro-architectures such as liquid phase (juices), solid phase (tissue) or combination of those. The diffusion, the variety and the intensity of potentially stressful factors among these micro-environments may vary significantly (Wilson et al., 2002; Leveau and Lindow, 2001), along with the physiology of individual cells (Dupont and Augustin, 2009; Koutsoumanis, 2008). During random localization of individual cells on vegetable samples, the probability of two cells, which have been inoculated on two independent samples, to occur in a similar micro-environment is decreased, and in combination with their individuality, significantly different growth potential for each cell could be expected. Conversely, distributing higher inocula on different spots of independent samples increases the similarities in spatiotemporal occurrence of cells at optimum (i.e., highest potential to grow in adverse conditions) or similar physiological state. Thus, the growth variability observed between such samples is lower.

An important observation with regards to food safety was the higher LI that a few cells of the pathogens exhibited during storage on both vegetables, compared to that of the higher initial inoculum (i.e., 1000 cells/sample). For instance, the percentage of lettuce or cabbage samples, inoculated with 1-4 cells/sample of *L. monocytogenes* and exceeding the microbiological criterion of 100 CFU/g, varied from 5 to 70%, depending on storage time. Conversely, the inoculum of 1000 cells/sample showed total LI equal or lower than this criterion (i.e., ≤ 2 log CFU/g). A critical factor affecting the capacity for growth of various initial pathogen levels on a food surface is the interaction with the

epiphytic flora. The observation that the growth of *L. monocytogenes* and *S.* Typhimurium ceased when the background flora of salads reached the maximum population density (Figure 4-3and Figure 4-4), may be associated with the so-called “Jameson effect” (Mellefont, et al., 2008), due to the competition for nutrients, or the production of microbial metabolites by the indigenous flora. In contrast, as detailed in *section 4.3.2*, no such inhibition of the pathogens was observed in the sterile extracts, apparently due to the absence of the background flora. However, given that the epiphytes reached their maximum population density at the same storage time in all batches of each vegetable, (i.e., 10 and 8 days for lettuce and cabbage, respectively), the growth of the pathogens ceased simultaneously, regardless of the initial inoculation level. Thus, the differences in total log increase between samples inoculated with high and those with low inocula could be attributed to differences in both the growth rate and/or lag time. According to previous reports, the growth rate is considered independent of the initial inoculum size (Robinson et al., 2001). Therefore, the aforementioned differences in LI of different inocula should only be due to the variability in individual lag times. It needs to be stressed however that the majority of studies on the effect of the inoculum size on the growth kinetics of pathogens has been performed on liquid media, where after each bacterial division, the daughter cells drift away from the mother cell. Thus, the possibility of “crowding effect”, i.e., the growth deceleration due to interactions of closely located cells is limited, and this may explain why the growth rate of planktonic cultures is not affected by their initial inoculum size. On the contrary, when cells are immobilized on a solid matrix, as is the case of fresh-cut salads, cells grow as colonies and hence, interactions may occur between cells within a colony (especially among cells in the outer and inner part of colonies) or between adjacent colonies. Such interactions are thought to increase with the proximity of colonies, i.e., with the population density, because the higher the population the lower the distance between colonies. This most likely explains the slower growth rate of 1000 cells/sample compared to that of a few cells/sample. Likewise, Thomas et al. (1997) reported that increasing the distance between adjacent

colonies of *L. monocytogenes* from 100 μm to 3000 μm, increased the cell density in the colonies from approximately 1.5 to 7 log CFU per colony, respectively. The above results imply that if log increase of pathogens on a food was judged only by challenge tests with unrealistically high inocula, as the worst case scenario, the actual risk would have been underestimated. Overall, although the risk posed by a few *L. monocytogenes* or *S.* Typhimurium cells on vegetable salads may be characterized by high variability, such assessments are more realistic and representative of a low contamination scenario. Considering also that the growth kinetics of the pathogens may differ significantly, depending on the initial inoculation level, challenge tests initiated from low number of cells (<5-10 cells) should be considered as a more reliable method to evaluate the real risk.

4.3.2 Growth kinetics on sterile extracts of vegetables

Remarkable variation in the growth potential of the pathogens was observed between different batches of vegetables used for the preparation of the sterile extracts (Figure 4-5; Figure 4-6). This variability could even cause the transition of the pathogens from a no growth to a growth state. For example, in batch A of lettuce liquid extract (lettuce extract broth; LEB) no growth was observed for 1000 cells/sample of *S.* Typhimurium at 8°C, in contrast to batch B, where clear increase was obtained (Figure 4-6). Similar results were observed when a few cells (1-4/sample) of *S.* Typhimurium were inoculated on cabbage solidified extract (cabbage extract agar; CEA) and stored at 8°C (Figure 4-6), as well as when a few cells (1-4/sample) of *L. monocytogenes* were inoculated on lettuce solidified extract (lettuce extract agar; LEA) and stored at the same temperature (Figure 4-5). On CEA or CEB no growth of *L. monocytogenes* was permitted in none of the studied batches (Figure 4-5).

Given that the methodology of sterile extracts preparation was standard, it is assumed that the variability in growth of the microorganisms between different batches may be attributed to the nutritional composition of each

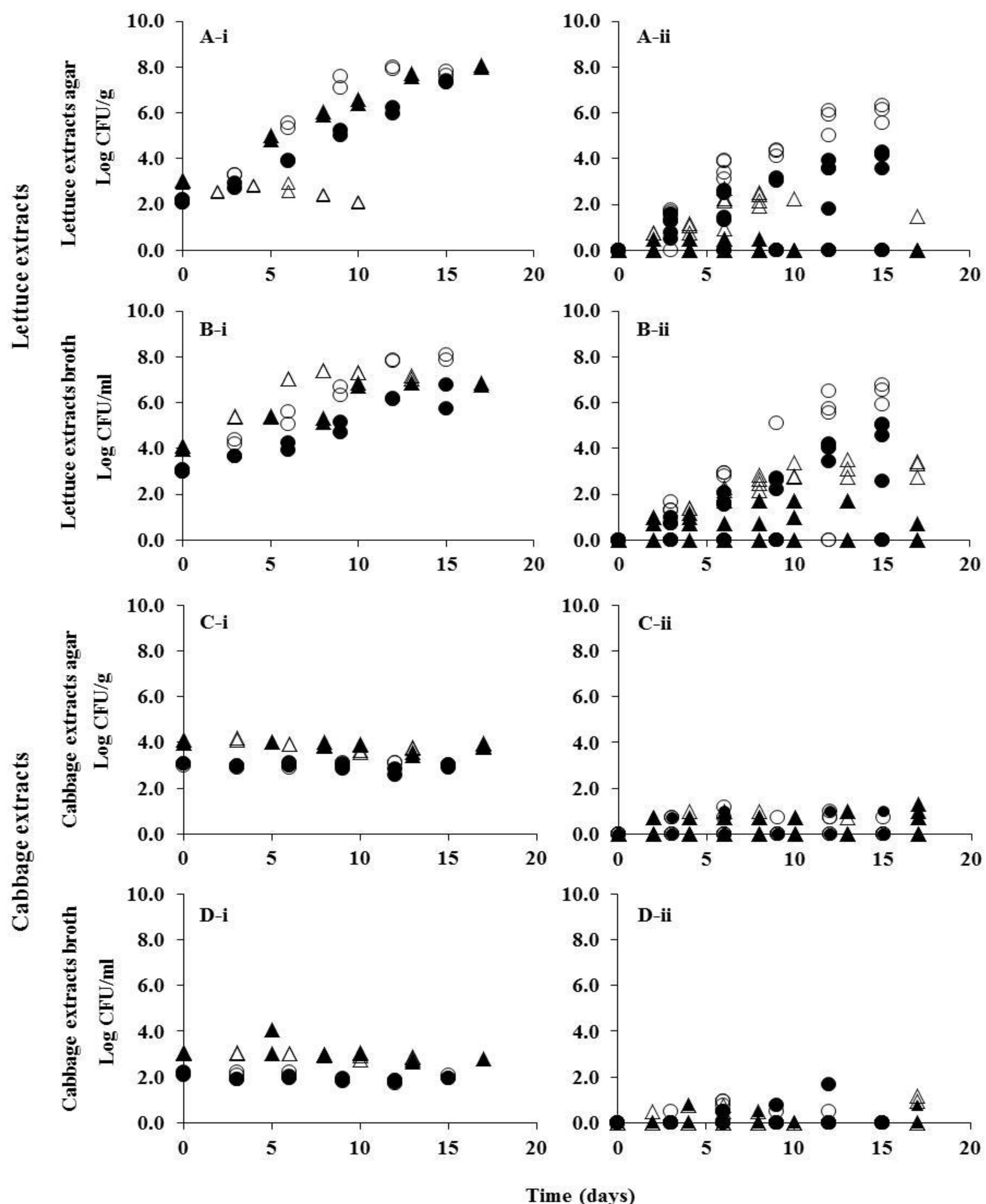


Figure 4-5 Growth of 1000 (i) or 1-4 cells (ii) of *Listeria monocytogenes* on two different batches (batch A, ●,○; batch B, ▲,△) of LEA (A), LEB (B), CEA (C) and CEB (D) stored at 8°C (closed symbols) or 10°C (open symbols). For readability reasons, results of two representative batches are presented. The initial levels of L. monocytogenes in samples inoculated with 1-4 cells/sample (-1.2 to -0.6 log CFU/g and -0.7 to -0.1 log CFU/g for solidified and liquid extracts, respectively) are rounded to 0 log CFU/g or ml.

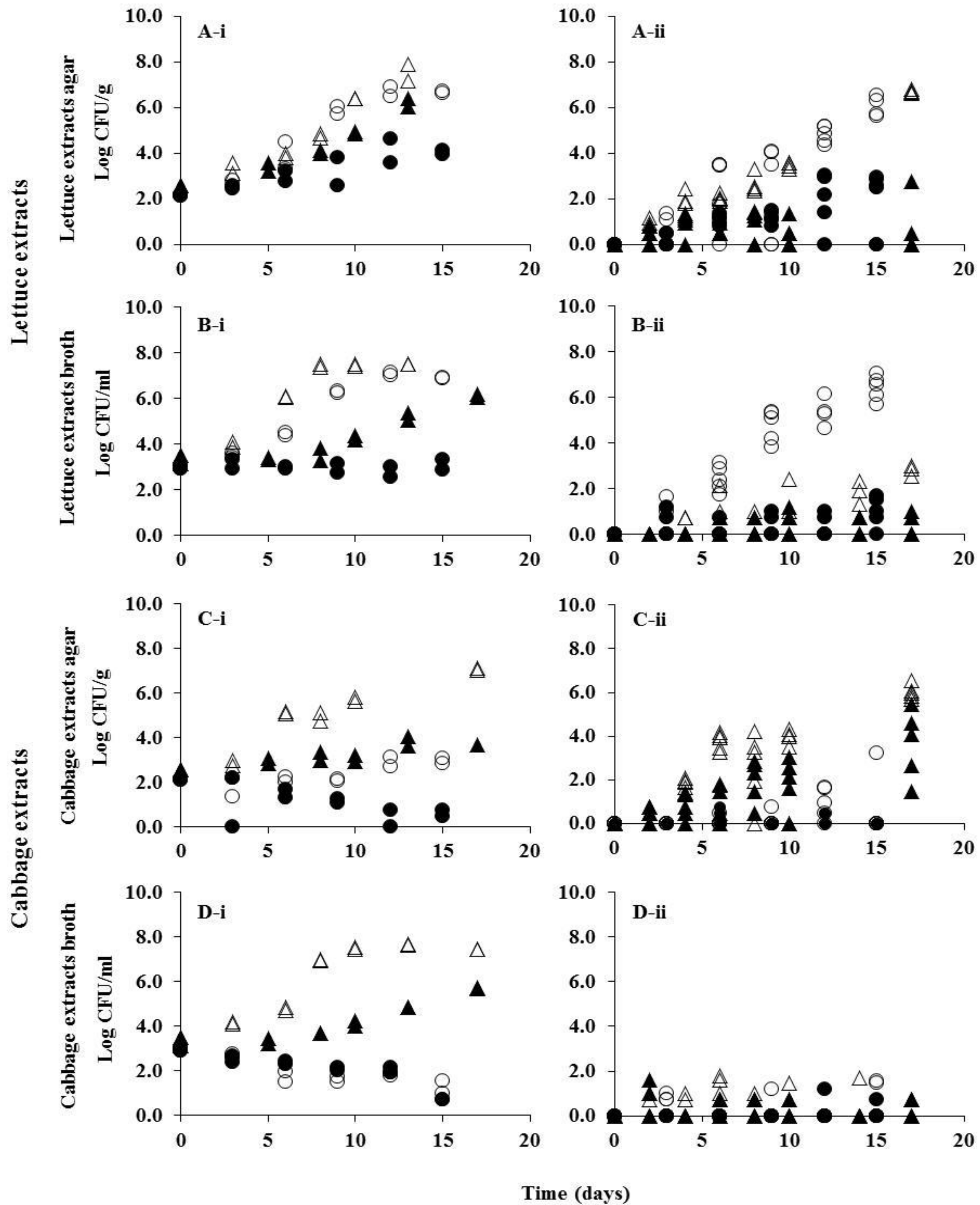


Figure 4-6 Growth of 1000 (i) or 1-4 cells (ii) of *Salmonella* Typhimurium on two different batches (batch A, ●,○; batch B, ▲,△) of LEA (A), LEB (B), CEA (C) and CEB (D) stored at 8°C (closed symbols) or 10°C (open symbols). For readability reasons, results of two representative batches are presented. The initial levels of *S.* Typhimurium in samples inoculated with 1-4 cells/sample (-1.2 to -0.6 log CFU/g and -0.7 to -0.1 log CFU/g for solidified and liquid extracts, respectively) are rounded to 0 log CFU/g or ml.

batch. In the present study, 4 different batches of lettuce or cabbage were obtained within 3 months and from different retailers, and thus, variability in the composition of each batch should be expected. Notably, the batch-variability was less evident between different batches of vegetable salads; it is assumed that diluting or autoclaving the vegetable nutrients during preparation of the extracts may also have affected the dynamics of the pathogens. Ongeng et al. (2007), who followed a similar protocol for the preparation of cabbage extract, stated that processing of cabbage juice by autoclaving might have significant impact on μ_{max} of *L. monocytogenes*.

The comparison between growth of pathogens in sterile extracts and salads suggests that the role of the epiphytic flora on pathogen behavior is crucial. Although *S.* Typhimurium did not grow on cabbage salad, remarkable growth from 1-4 cells/sample was observed on CEA (Figure 4-4, Figure 4-6), indicating a potential competitive effect of the epiphytic flora of cabbage on the pathogen. In contrast, growth of a few or 1000 cells/sample of *L. monocytogenes* occurred only on cabbage, contrary to the complete inhibition of the bacterium on CEA or in CEB (Figure 4-3 and Figure 4-5). Similarly, a few cells/sample of *S.* Typhimurium grew on lettuce but not on LEB at 8°C (Figure 4-4, Figure 4-6), suggesting that the increase in levels of epiphytic flora on the surface of cut tissue occasionally enhanced the growth of the pathogens.

Although the exact mechanisms have not been fully elucidated, the inhibitory interference of the spoilage flora against pathogens may be attributed to the competition for the available nutrients, the production of antimicrobial substances (i.e. lactic acid, bacteriocins) and/or the alteration of the physicochemical properties (i.e. pH, redox potential) of the micro-environment in which the growth of pathogens may occur (Huis in't Veld, 1996; Jay, et al., 2005; Al-Zeyara, et al., 2011).

Contrary to the competitive behavior, the stimulatory activity of the background flora that was observed may be a potential result of *metabiosis* (Marshal and Schmidt, 1991). In particular, the conversion of macro-

molecules by psychrotrophs to readily available nutrients, may promote the growth of the pathogens (Huis in't Veld, 1996). Enhanced survival of *E. coli* O157:H7 was observed on lettuce phyloplane in the presence of *Wausteria paucula* (Cooley, et al., 2006). In addition, Fatica and Schneider (2011) have recently reported that biofilms formed by the indigenous microflora on the surface of the vegetables may impart protection to the pathogenic cells against environmental stressful factors. Although Ongeng et al. (2007) reported no interference of the commensal flora of cabbage with *L. monocytogenes* at 4^oC or 30^oC, the spoilage flora tested was activated in broth and artificially inoculated on cabbage. It is likely, therefore, that the distribution and adaptation of native flora in the cabbage environment in our study may explain the difference of the present results to the results of that study. Such findings indicate that the stimulatory or competitive effect of epiphytes of lettuce or cabbage on pathogens is uncertain and may be inadequately described by broth-based experiments.

The structure (i.e., solid vs. liquid) of product extracts also influenced the growth of pathogens. A few (1-4) cells/sample of *S.* Typhimurium could initiate growth on the surface of CEA, but not in the corresponding broth, in which only the high inoculum of 1000 cells/sample was capable of growth initiation (Figure 4-6). Although it would be expected that the nutrients of the vegetables would be more readily available in broths (Stecchini, et al., 1998), liquid extracts were on average more inhibitory than solid ones.

Many researchers have investigated the effect of the micro-structure on the growth kinetics of pathogens (Ongeng et al., 2007; Noriega, et al., 2010a; Theys, et al., 2009). It is likely that the intensity of stress factors, e.g., non-fermentable nutrients, or phenolic and acid compounds, possibly extracted from the plant tissues, is perceived by bacteria more in juices than on agar surfaces (Skandamis, et al., 2000). Delaquis et al. (2006) reported the presence of a substance, probably of phenolic nature, which was obtained during shredding of iceberg lettuce and caused the inhibition of *L. monocytogenes*. In addition, Noriega et al. (2010b) documented that

microorganisms immobilized on a food or solid medium surface may adapt to their acidic metabolites, as the diffusion is limited compared with vegetable juices or broth media, and subsequently exhibit increased tolerance to inhibitory antimicrobial compounds. The presumable presence of antimicrobial substances in the extracts of the cabbage is being also potentially confirmed by the death of *S.* Typhimurium which occasionally occurred and was more evident in 1000 cells/sample inoculum than in the inoculum of a few cells/sample (Figure 4-6). In addition, the inhibition of the pathogens in the liquid extracts may be also attributed to the lower levels of available oxygen in the interior of the broths, compared with the surface of solidified extracts. Indeed, the diffusion rate of oxygen is much slower in broth media, especially at the lower levels, and this may affect the microbial growth rate or even growth initiation (Theys et al., 2008; Koutsoumanis et al., 2004). Coleman et al. (2003) and Yoon et al. (2003) reported slower growth rate of *E. coli* O157:H7 and *L. monocytogenes*, respectively, in broth media during incubation without agitation, compared with cultures incubated under continuous agitation. However, since this phenomenon was observed only occasionally in the present study, it is assumed that oxygen diffusion was not the major limiting factor for growth in broth extracts. The results of the present study are based on the assumption that the solidified extracts do not represent precisely the surface of the vegetables but they simulate the slower diffusion rate of nutrients or inhibitory substances compared with juices released in wounds or cut surfaces. Thus, it may be concluded that the growth dynamics of pathogenic bacteria on a real food system may differ significantly and the structure effect should be taken under consideration for the development of simulation models.

Increase in storage temperature of the sterile extracts from 8 to 10°C had such a pronounced effect on pathogen growth; cells were shifted from a no growth state to a growth status. *S.* Typhimurium showed significant increase on LEB at 10°C, regardless the initial inoculum level, while no growth was observed when the same batch of extracts was stored at 8°C (

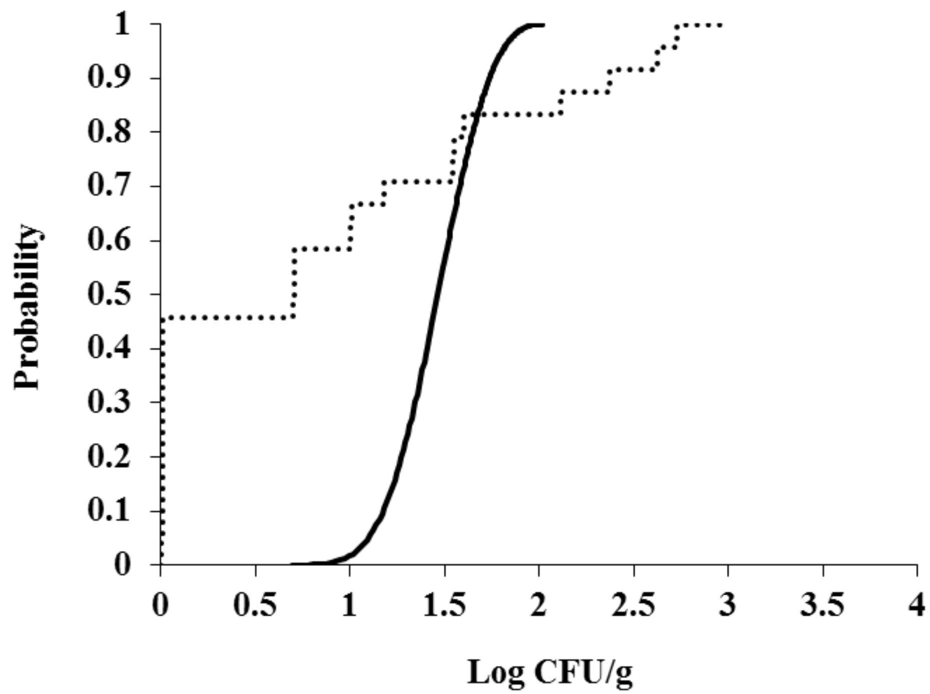
Figure 4-6). In contrast, *L. monocytogenes* exhibited growth under both storage temperatures on LEA and LEB (Figure 4-5). This was more evident for *Salmonella*, due to the range of 8-10°C being close to the growth boundaries of this microorganism.

Time-temperature profiles of fresh-cut salads during storage in a salad bar have shown that the storage temperature may often exceed 10°C, resulting in 17%, 15% and 194% increase of *E. coli* O157:H7, *Salmonella* and *L. monocytogenes*, respectively (Franz et al., 2010). Concerns are also raised for the safety of vegetable salads in the domestic environment. Leftovers of leafy salads may be packed in kitchen containers (air packaging) and stored in household refrigerators, in which the average temperature may exceed 8°C (Marklinder et al, 2004). Thus it is concluded that marginal changes of temperature, which are commonly encountered during distribution or storage of freshly-cut salads, may affect the growth dynamics of pathogenic cells and render the final product unsafe.

4.3.3 Monte Carlo simulation

The growth of single cells of *L. monocytogenes* on lettuce or cabbage was simulated through a stochastic Monte Carlo approach and using the distributions of individual cell lag times observed by Francois et al. (2006a). Results showed that the Monte Carlo simulation slightly over-predicted the growth of single cells of *L. monocytogenes* after 12 days on lettuce. For instance, the model predicted that there is 60% likelihood a single cell of the pathogen to reach 1.5 log CFU/g, while the observed growth under the same probability was 1 log CFU/g (Figure 4-7). In contrast, remarkable under-estimation of the observed growth in cabbage was recorded, as the predictions showed that 60% of the individual cells could grow at 0.5 log CFU/g, whereas the observed growth was 2.6 log CFU/g. There are a limited number of studies in the literature where simulated growth of single cells is compared with observations from growth of single cells on foods. Francois et al. (2006b) simulated the growth of single cells of *L. monocytogenes* on liver

pâté and cooked ham. The results of that study showed that the broth-based simulations predicted slightly faster growth compared to that occurred in liver pâté, while this over-prediction was more evident on cooked ham. This deviation of the broth-based predictions from the observed growth suggests poor transferability of broth-based data to foods, because such models do not adequately encompass the effect of the epiphytic flora, the micro-structure or the scattered availability of nutrients. Thus, extrapolating broth based predictions of microbial growth from single cells to lettuce, may lead to either fail-safe or fail-dangerous predictions.



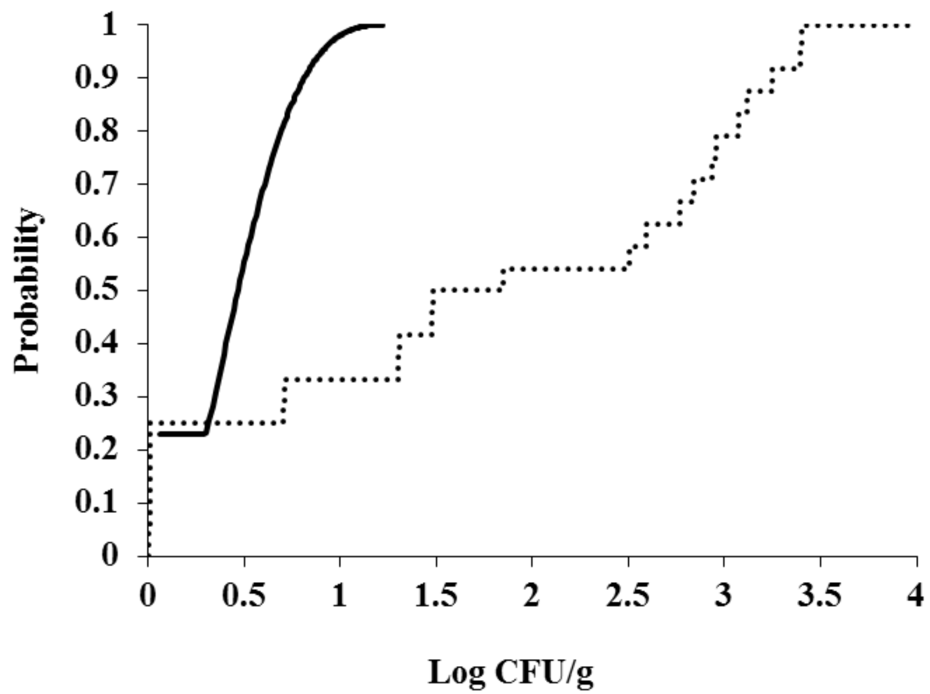


Figure 4-7 Predicted (—) and observed (···) cumulative distribution of *L. monocytogenes* on lettuce (A) and cabbage (B) after 12 days of storage.

4.4 Conclusions

The effect of epiphytic flora, the diverse structure of micro-environments in a fresh-cut salad, the distribution of available nutrients and the marginal changes of storage temperature may be inhibitory or stimulatory for the growth of very low (down to a few cells) levels of pathogens. In addition, it was shown that the ability of fresh-cut salads to support pathogen growth may be underestimated when using high inocula, underlying the need to perform challenge tests with low numbers or even single cells of target organism. Simulating the growth from 1-4 *L. monocytogenes* cells on lettuce or cabbage salad, based on data derived from laboratory media, showed deviation from the growth observed on foods. Especially in cabbage salad, significant underestimation of the actual growth was observed, leading to fail-dangerous predictions. Therefore, future predictive models need also to incorporate the variability in the growth responses of realistically low inocula, in response to inimical factors. This will ensure the development of reliable tools for risk assessment of pathogens in RTE leafy salads.

5 GENERAL DISCUSSION AND CONCLUSIONS

The objective of the present Thesis was to identify consumer mishandlings in the domestic environment that may likely deteriorate the quality or compromise the safety of RTE or RTC foods. These practices are either the result of the consumers' limited awareness for the potential introducing risks or due to their intentional ignorance, giving priority to personal preferences and convenience for food handling. Following determination of these risks, in the first and the third study we developed and evaluated the ability of mathematical models to provide valid predictions for the quantification of these risks.

In the first experimental chapter, the shelf-life of vegetable-based acidic spreads was determined, and a unified predictive model was developed for the prediction of the growth of lactic acid bacteria. Consumers' intervention in this study refers to their inability to ensure proper storage temperatures in the domestic refrigerators. Such mishandling may result in reduced shelf-life of the products compared with the suggested one. It has to be stressed, however, that the samples used in the particular study were freshly prepared and thus, this shelf-life could be further reduced, if the products were exposed to abused temperatures during distribution or in retail. Therefore, it is concluded that the determination of the shelf-life of such products should be conducted under realistic temperature profiles, and maybe change with seasonal changes of environmental temperature.

In the second part of the first chapter, product-specific models for the prediction of the growth of SSOs under dynamic temperatures were developed. Since, however, such models require extensive experimental designs, the microbiological data obtained from different products were incorporated into a unified model, in order to predict the growth of SSOs in all products of this category. In particular, this model was able to provide accurate predictions using only the initial pH, the initial concentration of the undissociated acetic acid and the storage temperature. This model constitutes a useful tool for food industries, as it may assist in monitoring of

the microbial quality of such products along the food-supply chain, based on actual growth of their spoilage organisms, as well as in the management of products distribution both in the national and international markets. Furthermore, this model may be used for the formulation design of new products of this category, or the reformulation of existing products in order to improve their stability.

The second experimental chapter of the Thesis refers to common consumers' mishandlings during preparation and cooking of beef patties that may affect the safety of final products. The results of this part revealed that the guidelines issued by Food Safety authorities may likely lack of some specific points, which may increase the risk of microbial nature in the final products. Such points include the duration of frozen storage and the method of cooking, as these factors favored the survival of pathogenic bacteria after cooking at the suggested temperatures. The results may be used to instruct the consumers and catering services for the appropriate combinations of frozen storage duration, defrosting and cooking practices of beef patties, taking into account the variability that might occur for practical purposes in different establishments (i.e. households, food caterings and restaurants).

In the third experimental chapter, a realistic scenario of low contamination of fresh cut salads was simulated, in order to determine the growth dynamics and variability of pathogenic bacteria in a complex food environment. As a further step, the observations of this part were correlated with the growth predictions of a broth-based model. High variability on the growth potential of the pathogens initiating from a few cells during growth on the salads was observed. This variability is mainly attributed to the effect of the indigenous microflora activity, the storage temperatures and the available nutrients occurring in the micro-environment of microbial growth. As a result, the broth-based model, which was not able to incorporate these parameters, provided also fail dangerous predictions for the growth of the pathogens on the salads. Thus, it is concluded that high uncertainty should be expected when extrapolating broth-based simulations from single cells in foods. In addition,

the stimulatory or competitive effect of background flora and the marginal changes of storage temperature is more pronounce and perhaps evident only at low inocula, suggesting that that challenge tests in fresh produce based on high initial bacterial numbers may underestimate the actual growth risk.

Overall, the results of the present Thesis highlight the necessity for consumers' training on food handling issues in the domestic environment. Thus, they may be used to improve the issued guidelines in order to reduce the potential risk of earlier food deterioration or food contamination with pathogens. On the other hand, predictive models are essential means to bridge the gap between basic food microbiology and the food industries, as long as all the factors affecting microbial growth kinetics and/ or potential are incorporated in to the models.

Conclusions chapter by chapter:

Shelf-life of acidic spreads (chapter 2):

(i) Although the formulations of the products differed significantly, their spoilage patterns were quite similar

(ii) The product-specific models may adequately simulate the growth of LAB in the spreads, however, these models may not be applicable to other products

(iii) The unified model, and especially the Ratkowsky equation, may be a significant tool for the relevant food industry for the determination of the shelf-life of existing or newly designed products

Effect of common consumer practices (chapter 3):

(i) Undercooking of beef patties increases significantly the risk of pathogen survival.

(ii) Under particular circumstances, the suggested cooking temperature for ground beef (71.1^{o}C) may not ensure the safety of the final product

(iii) In contrast, the “safe” cooking temperature of beef patties should be determined in association with the duration of frozen storage and the cooking method.

Growth dynamics of low number of cells (chapter 4):

(i) The probability of growth of individual pathogenic cells on leafy greens is highly affected by the indigenous microflora, the storage temperature and the available nutrients

(ii) High uncertainty should be expected when extrapolating broth-based simulations from single cells in foods. The stimulatory or competitive effect of background flora is more pronounce and perhaps evident only at low inocula, suggesting that challenge tests in fresh produce based on high initial bacterial numbers may underestimate the actual growth risk

General conclusions:

(i) Necessity for consumers’ training on food handling issues in the domestic environment.

(ii) The results may be used to improve the issued guidelines in order to reduce the potential risk of earlier food deterioration or food contamination with pathogens.

(iii) Predictive models are essential means to bridge the gap between basic food microbiology and the food industries, as long as all the factors affecting microbial growth kinetics and/ or potential are incorporated in to the models.

6 ONGOING RESEARCH

The findings of the present Thesis highlighted the possibility of unintentional consumer mishandlings to compromise the safety and deteriorate the quality of foods. All studies reported that the simulation of realistic scenarios which may occur in the domestic environment, may reveal unknown, and in some cases unexpected risks for the quality and safety of foods.

Based on these indications, an experimental procedure was designed in order to evaluate the potential risk that may be introduced in the kitchen environment by improper sanitation of kitchen sponges and further contamination transfer to pre-cooked meals through cleaned kitchen utensils, such as food containers. The scenario that simulated this hypothesis was as follows; contaminated kitchen sponges with food and detergent residuals remain in the sink overnight. These sponges are further used to clean different types of food containers, facilitating the contamination transfer to these containers. After overnight drying, the containers are used to store the leftovers of pre-cooked meals. Based on this scenario, the objectives of the study were to assess (i) the survival of *Salmonella* spp. in various food residues with or without the addition of commercial detergent, simulating the co-existence of the pathogen with food residues in an improperly sanitized kitchen sponge and (ii) the transferability of the pathogen from contaminated food containers to precooked meals. Considering that the exposure of cells to sublethal stresses during this simulation scenario (i.e. detergent, starvation on container surface, acids and fat of food residues) may likely affect the growth dynamics of the pathogen and, hence may increase or lower the risk of foodborne diseases, the growth parameters of the pathogen detached from the surfaces and subsequently exposed to a food model were also determined.

The experimental design included the preparation of five food residues of different origin (i.e., ground beef [GB], lettuce [L], mayonnaise [M], ground beef + lettuce [GB+L], ground beef + lettuce + mayonnaise [Mix]) with (+) or without (-) the addition of a commercial detergent. Maximum recovery diluent

Table 6-1 Initial and final pH of different food residues, which were inoculated with Salmonella, during storage at 25°C for 24 hours. The + or – symbols indicate the presence or absence of the commercial detergent, respectively.

Food residues	Initial pH	pH after storage at 25°C for 24h
MRD (-)	6.81 ± 0.15	6.87 ± 0.09
MRD (+)	7.71 ± 0.06	7.29 ± 0.13
Ground beef (-)	6.00 ± 0.08	5.79 ± 0.16
Ground beef (+)	6.86 ± 0.16	6.10 ± 0.53
Lettuce (-)	6.22 ± 0.07	4.78 ± 0.28
Lettuce (+)	7.72 ± 0.05	5.02 ± 0.31
Mayonnaise (-)	4.01 ± 0.09	4.00 ± 0.07
Mayonnaise (+)	4.98 ± 0.08	4.95 ± 0.06
Ground beef + Lettuce (-)	5.91 ± 0.03	4.98 ± 0.42
Ground beef + Lettuce (+)	6.94 ± 0.08	5.52 ± 0.36
Ground beef + Lettuce + Mayonnaise (-)	5.23 ± 0.11	4.63 ± 0.15
Ground beef + Lettuce + Mayonnaise (+)	6.35 ± 0.53	5.24 ± 0.57

(MRD) was used as a control of all food residues. Each residue was inoculated (6 log CFU/ml) with a 5-strain composite of *Salmonella* Typhimurium (2 strains), *S.* Agona, *S.* Reading and *S.* Enteritidis and stored at 25°C for 24h. Then, plastic, metal and glass coupons were spot-inoculated with each food residue and stored for another 24 h at 25°C. Each coupon was placed under constant pressure (50g) above three pre-cooked meals (beef patties, lasagna, tomato sauce) in order to evaluate the transfer of the pathogen to a solid, semi-solid or liquid food type. The growth parameters (μ_{max}, t_{λ}) of the cells recovered from each surface were also determined using optical density data; decimal and half-fold dilutions of the detached cells were made in a microtiter plate containing TSB of pH 5.5 and 2% NaCl. Following

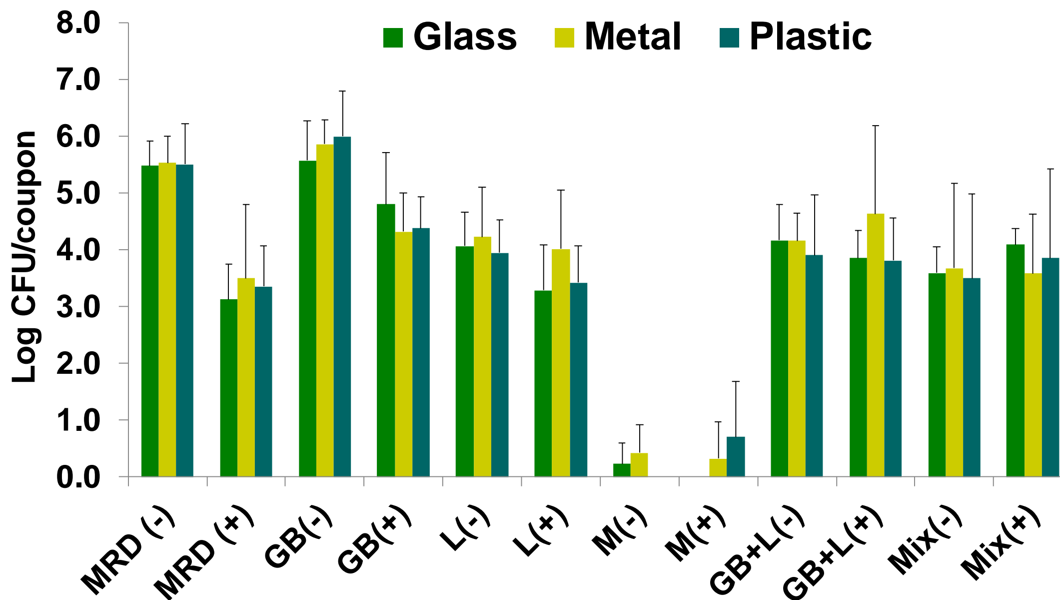


Figure 6-1 Populations of *Salmonella* spp. habituated in different food residues and recovered from different food-related surfaces with the swab method.

incubation in Bioscreen C, the Time to Detection (TTD; time for the O.D. to reach 0.2) values were used to determine the growth rate and the lag time of each inoculum.

Preliminary results showed that food residues supplemented with 1% detergent posed higher pH compared with those where no detergent was added. In addition, the pH of all food residues decreased after storage at 25°C for 24 hours, due to the metabolic activity of the pathogen and the endogenous microflora (Table 6-1). Further detachment of the pathogen from the surfaces using the swab method revealed that the type of surface did not affect the populations of Salmonella spp. recovered from the coupons using the swab method (Figure 6-1). Especially in mayonnaise residue, the presence of organic acids (acetic acid) reduced the pH at lethal values for the pathogen, and therefore no or very low populations were recovered from this residue (Figure 6-1).

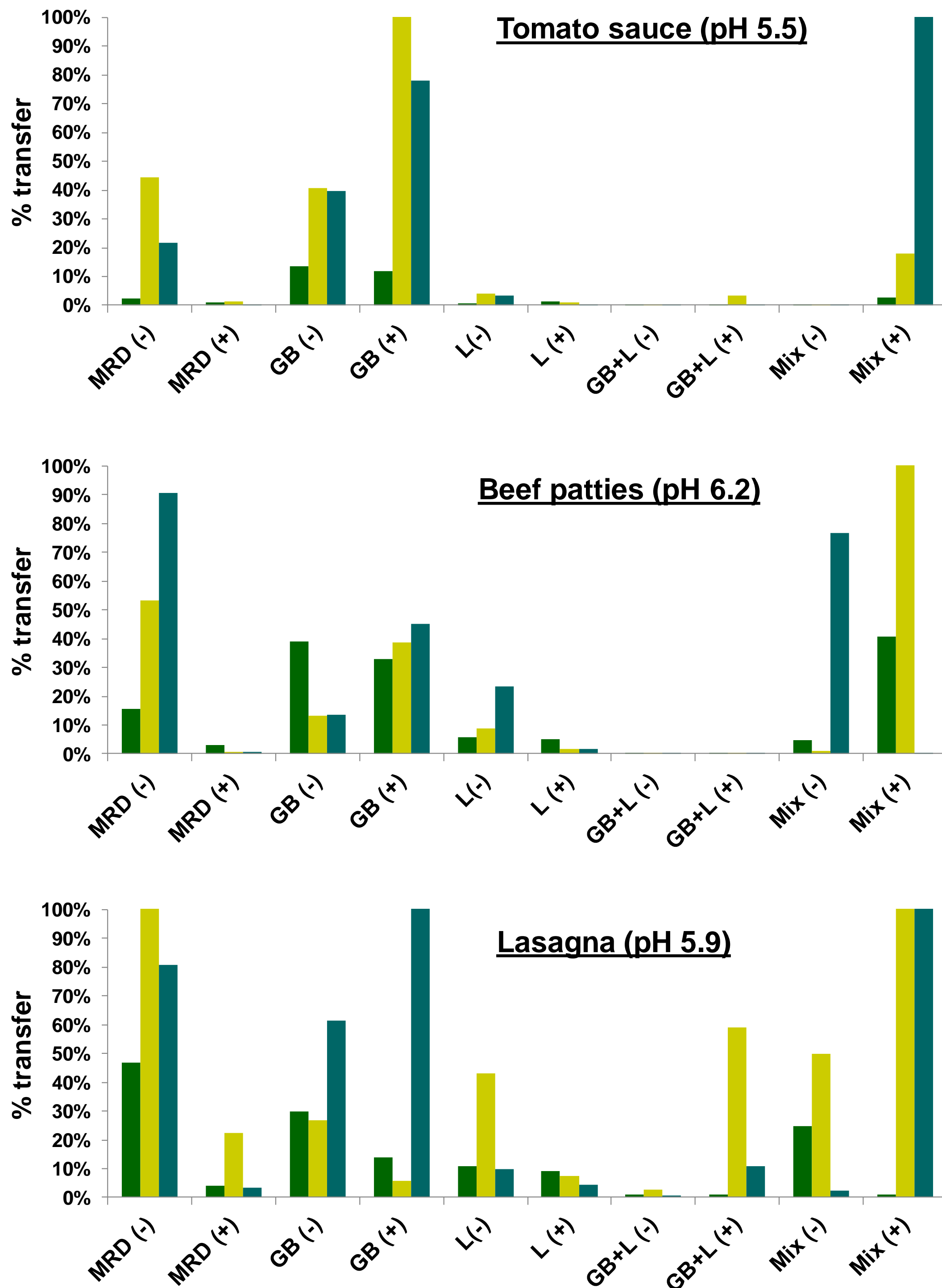


Figure 6-2 Populations of *Salmonella* spp. transferred to tomato sauce, beef patties or lasagna, after habituation in different food residues and types of surfaces for 24 h at 25°C . (■) Glass, (■) Metal, (■) Plastic

Regarding the transferability of pathogen from the inoculated surfaces to pre-cooked meals, the transferred populations were higher after habituation in MRD (without detergent) or ground beef (with or without detergent) compared to other types of food residues. Cells attachment on glass surfaces was stronger than on the plastic or metal coupons, and therefore, the populations transferred to the pre-cooked meals from glass surfaces were lower. In parallel, the combined effect of rehydration and friction in the lasagna meal resulted in higher recovered populations of the pathogen, compared with tomato sauce or beef patties, where only rehydration or friction, respectively, occurs (Figure 6-2).

The growth rate of *Salmonella* spp. was not significantly affected (0.63 – 0.86 h^{-1}) by the type of food residue or the type of surface. Cells exposed to all food residues, except for lettuce, posed shorter lag time (0.05 – 3.28 hours) compared with cells which were not exposed to any type of food residue or surface (control; 6.23 h). In lettuce (with or without detergent) residue, the potential presence of antimicrobial compounds of phenolic nature, which were transferred to the growth environment (TSB with pH 5.5 and NaCl), may prolonged the lag time of the pathogen (2.8 – 7.9 h) (Figure 6-3).

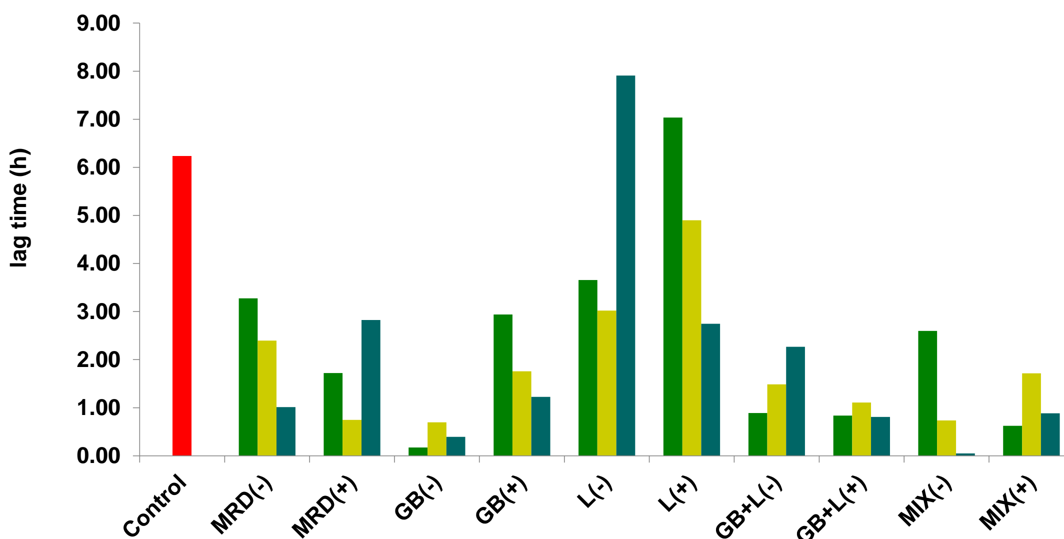


Figure 6-3 Lag time of *Salmonella* spp. cells recovered from (■) glass, (■) metal or (■) plastic surfaces after habituation to different food residues, in comparison with a freshly activated culture (control, ■)

The results of this study may be proved useful for the evaluation of risks that may derive from unexpected contamination sources (i.e., kitchen sponges), while in addition, they facilitate the quantification of the microbial transfer from kitchen utensils and surfaces to pre-cooked meals.

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APPENDIX I

- **Oral presentations in international conferences**

1. Manios, S.G., Ketsatis, S., Psomas, A., Nychas, G.-J., & Skandamis P.N. Development and validation of a tertiary simulation model for predicting the growth of lactic acid bacteria in acid pourable appetizers. *6th International Conference of Predicting Modeling in Foods*, Washington, USA, September 11-14, 2009.
2. S. G. Manios, S. A. Ketsatis, A. E. Kapetanakou, A. S. Gounadaki, & P.N. Skandamis. Assessment and modelling of the microbial spoilage of four traditional Greek appetizers. *96th IAFP meeting, Grapevine, Texas, USA,* 12-15 July 2009
3. Manios, S. G., Konstantinidis, N., Gounadaki, A. S., & Skandamis, P.N. Single cell variability and population dynamics of *Listeria monocytogenes* and *Salmonella* Typhimurium in fresh-cut salads and their sterile liquid or solidified extracts. *7th International Conference of Predicting Modeling in Foods*, Dublin, Ireland, September 12-15, 2011.

- **Oral presentations in national conferences**

1. Μανιός Σ. Γ., Κετσάτης Σ. Α., Ψωμάς Α., & Σκανδάμης Π.Ν. Σχεδιασμός τριτογενούς μοντέλου πρόβλεψης του χρόνου ζωής Έτοιμων Προς Κατανάλωση ορεκτικών σε συνδυασμό με την μικροβιακή παραλλακτικότητα. *3ο Πανελλήνιο Συνέδριο Βιοτεχνολογίας & Τεχνολογίας Τροφίμων, Ρέθυμνο* 2009.
2. Μανιός Σ. Γ., Κετσάτης Σ.Α., Ψωμάς Α. & Σκανδάμης Π.Ν. ανάπτυξη και επικύρωση τριτογενούς μοντέλου πρόβλεψης του εμπορικού χρόνου ζωής όξινων ορεκτικών. *3ο Πανελλήνιο Συνέδριο ΔΕΔΥΤ, Θεσσαλονίκη,* Ιούνιος 2010.

3. Μανιός Σ. Γ., Γιοβάνης Θ., Λαλεχού Α., & Σκανδάμης Π.Ν. Επίδραση κοινών πρακτικών απόψυξης, μεθόδων και βαθμού θερμικής επεξεργασίας στην επιβίωση των παθογόνων μικροοργανισμών *Salmonella* και *Escherichia coli* O157:H7 σε μπιφτέκια βόειου κιμά. *2o Πανελλήνιο Συνέδριο για το κρέας και τα προϊόντα του: «από το στάβλο στο πιάτο». Αθήνα,* Σεπτεμβριος 2010.
4. Μανιός Σ. Γ., & Σκανδάμης Π.Ν. Επίδραση της συντήρησης υπό κατάψυξη, της απόψυξης και διαφορετικών μεθόδων ψησίματος στη επιβίωση των παθογόνων *Escherichia coli* O157:H7 και *Salmonella* σε μπιφτέκια βόειου κιμά. *Ε.Δ.Ε. Θεσσαλονίκη,* Νοέμβριος 2011.
5. Μανιός Σ. Γ., Κωνσταντινίδης Ν., Γουναδάκη Α. Σκανδάμης Π.Ν. Δυναμικό αύξησης μεμονωμένων κυττάρων *Listeria monocytogenes* και *Salmonella* Typhimurium σε έτοιμες προς κατανάλωση σαλάτες λαχανικών και στα στερεά ή υγρά εκχυλίσματά τους. *Ε.Δ.Ε. Θεσσαλονίκη,* Νοέμβριος 2011.

- **Poster presentations in international conferences**

1. Stavros G. Manios, Panagiotis N. Skandamis. Effect of household storage, thawing and cooking practices on the survival of *Salmonella* and *Escherichia coli* O157:H7 in beef patties. 98th IAFP meeting, Milwaukee, USA, 31 July-3 August, 2011.
2. Stavros G. Manios, Nikos Konstantinidis, Panagiotis N. Skandamis. Single cell variability and population dynamics of *Listeria monocytogenes* and *Salmonella* Typhimurium in fresh-cut salads and their sterile liquid or solidified extracts. 98th IAFP meeting, Milwaukee, USA, 31 July-3 August, 2011.
3. Stavros G. Manios, Thomas Giovanis, Argiro Lalechou, Panagiotis N. Skandamis. Survival of Salmonella and E. coli during freezing, thawing and cooking of ground beef patties, simulating common household

practices. 11th International congress on engineering and food. 22-26 May, 2011.

4. Stavros G. Manios, Antonis Psomas, Panagiotis N. Skandamis. User-friendly software predicting the microbial spoilage of emulsified acid foods. 11th International congress on engineering and food. 22-26 May, 2011.

Publications

- Manios, S. G., Konstantinidis, N., Gounadaki, A. S., & Skandamis, P. N. (2013). Dynamics of low (1–4 cells) vs high populations of *Listeria monocytogenes* and *Salmonella* Typhimurium in fresh-cut salads and their sterile liquid or solidified extracts. *Food Control*, *29*(2), 318–327.

- Manios, S. G., Lambert, R. J. W., & Skandamis P.N. A generic model for spoilage of acidic emulsified foods: combining physicochemical data, diversity and levels of specific spoilage organisms. Submitted manuscript under review.

APPENDIX II - *Publications*

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Dynamics of low (1–4 cells) *vs* high populations of *Listeria monocytogenes* and *Salmonella* Typhimurium in fresh-cut salads and their sterile liquid or solidified extracts

Stavros G. Manios [a,b], Nikolaos Konstantinidis [b], Antonia S. Gounadaki [b], Panagiotis N. Skandamis [b,*]

[a] *Cranfield University, Department of Cranfield Health, Cranfield, Bedfordshire, MK43 0AL, UK*
[b] *Agricultural University of Athens, Department of Food Science & Technology, Laboratory of Food Quality Control & Hygiene, Iera Odos 75, GR-11855 Athens, Greece*

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The growth variability of low or high populations of *Listeria monocytogenes* and *Salmonella* Typhimurium in fresh-cut lettuce and cabbage was studied. The salads were inoculated with a few (1–4) or 1000 cells/sample of the above organisms and stored at 8 °C. Their liquid or solidified sterile extracts were also inoculated with the same cell numbers to evaluate the behavior of pathogens in the presence or absence of the epiphytic flora and illustrate the effect of the micro-environment structure, where growth may occur. The sterile extracts were stored at 8–10 °C simulating marginal temperature fluctuations. Inoculum of 1000 cells/sample increased with limited variation (SD < 0.5 log CFU/g) on vegetable salads, as opposed to the great variability (<0.7–3.4 log CFU/g) in the growth from 1 to 4 cells/sample. Total logarithmic increase of 1000 cells/sample of the pathogens on the salads ranged from 1.8 to 2.1 log CFU/g, contrary to 1–4 cells/sample, which exhibited higher increase (2.7–3.4 log CFU/g). The latter suggests that "fail-dangerous" implications may derive from challenge tests with unrealistic high inocula. Different batches of vegetables used for preparation of sterile extracts, introduced high variability in the growth of 1–4 cells/sample, suggesting nutrient-dependent effect on growth of pathogens. Low inoculum of *L. monocytogenes* did not increase in sterile cabbage extracts, whereas they increased from 1 to 3.5 logs in cabbage salad, probably due to the stimulatory effect of indigenous flora. In contrast, 1–4 cells/sample of *S.* Typhimurium grew only on the solidified extract of cabbage but not on the salad, indicating competitive activity of the indigenous microflora against the pathogen. *Salmonella* showed no growth at 8 °C but increased 4 logs at 10 °C, illustrating the impact of boundary storage conditions. Monte Carlo simulation of bacterial growth based on broth data overestimated growth of *L. monocytogenes* on lettuce, while remarkably underestimated the actual increase in cabbage. Significant deviation between model and data is likely when extrapolating broth-based simulations of growth from low populations in foods, due to the various factors affecting the microbial growth on a real food, which are (inevitably) ignored by broth-based models.

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1. Introduction

Numerous foodborne disease outbreaks associated with fresh produce or minimally processed vegetable salads have been reported worldwide the past decade (CDC, 2006, 2008, 2011; Horby et al., 2003; Lienemann et al., 2008). *Listeria monocytogenes* and various serotypes of *Salmonella* (e.g., Typhimurium, Newport, Reading, Saintpaul etc.) or *Escherichia coli* O157:H7, O145, O104, etc., are the most common causative agents. Contamination of fresh-cut salads with pathogens may occur during any point of cultivation and harvesting of the vegetables or during preparation of the cut-salads in the establishments (Brackett, 1999; Burnett & Beuchat, 2001; Doyle & Erickson, 2008; Koseki, Mizuno, & Yamamoto, 2011). The impermeability of the intact plant tissue results in lack of available nutrients on the leaf surface. This, in conjunction with the exposure of plants to UV irradiation at the field and the competition of the immigrants with the epiphytic flora for nutrients, may inhibit the survival of pathogenic bacteria (Erickson et al., 2010). However, Leveau and Lindow (2001) suggested the existence of scattered micro-cites with limited amounts of sugars (i.e., glucose, fructose) and moisture on the leaf surface, where growth of pathogens may occur. Further processing of vegetables such as cutting, slicing or shredding, in order to prepare

* Corresponding author. Tel.: +30 210 5294684; fax: +30 210 529 4683.
E-mail address: pskan@aua.gr (P.N. Skandamis).

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pre-bagged Ready-To-Eat salads, causes release of the nutritious juices, which provide appropriate substances for survival and/or microbial growth. Thus, evaluating the extent of pathogens proliferation in various fresh-cut salads is imperative in order to assess the safety risks originating from these foods.

The activity of the epiphytic flora, the structure of the microenvironment where microbial growth occurs, the availability of nutrients, as well as the storage temperature constitute stressful factors which may introduce high variability in the growth potential of foodborne pathogens on fresh-cut salads (Franz, Tromp, Rijgersberg, & Van der Fels-Klerx, 2010; Leveau & Lindow, 2001; Ongeng, Ryckeboer, Vermeulen, & Delvieghere, 2007; Schuenzel & Harrison, 2002). This variability markedly impacts especially the dynamics (i.e., lag time) of low populations, such as 1–10 cells, and increases with the intensity of the stress (Francois, Devlieghere, Standaert et al., 2006). The majority, though, of challenge tests are performed with high inoculum levels ($>10^3$ cells), in order to monitor the average behavior of the population derived from the fastest growing cells and hence, the worst case scenario for risk assessment. However, high inocula differ from the realistic scenario of low contamination, and results may also include uncertainty due to the potential interaction between cells of the inoculum (i.e., quorum sense). Thus, in order to extrapolate the results to realistic conditions of low contamination, the variability of population numbers due to the variance of individual cell lag times needs to be evaluated.

A significant number of predictive models have been developed to determine the kinetic parameters of individual cells under various environmental conditions (Guillier & Augustin, 2006; Métris, Le Marc, Elfwing, Ballagi, & Baranyi, 2005). The majority of these models are based on experimental data derived from laboratory media, contrary to the limited information available for the assessment of individual cell variability in foods (Francois, Devlieghere, Uyttendaele et al., 2006; Manios, Konstantinidis, Gounadaki & Skandamis, 2011). In such "*static models*", however, the effect of the activity of the epiphytic flora, the food structure or the diffusion of nutrients is difficult to be described. In contrast "*dynamic models*" are mathematical equations which are able to incorporate various influencing factors and provide more accurate predictions than "*static models*". Development of such models, however, requires multiple specific measurements and may be proved rather complex for non-experienced food scientists, increasing the risk of unsafe predictions (Bernaerts et al., 2004). Thus, challenge tests with realistically low inocula on real food matrices should be conducted, to assess the risk of a pathogen with less uncertainty. The objectives of the study were (i) to determine the variability in the growth of two pathogens at very low (single or a few cells) in comparison with a higher inoculum in lettuce and cabbage salad, (ii) to identify the contribution of the epiphytic flora, food structure and temperature abuse in the above variability, and (iii) to evaluate the ability of a broth-based growth simulation model to approximate the average outgrowth of a population from single cells in foods.

2. Materials and methods

2.1. Bacteria and culture conditions

L. monocytogenes Scott A (serotype 4b; epidemic strain) and *Salmonella enterica* subsp. enterica Le Minor and Poppof serovar Typhimurium (calf bowel isolate) were used in the present study. Cultures of both strains were maintained on tryptone soy agar (TSA; LAB M, LAB011, Lancashire, UK) slants at 4 °C. The microorganisms were grown twice in 10 ml tryptic soy broth (TSB; LAB M, LAB004) for 24 h at 30 or 37 °C, for *L. monocytogenes* and *S.* Typhimurium, respectively. Prior to inoculation, the activated strains were washed twice and resuspended in 10 ml maximum recovery diluent (MRD; LAB M, LAB103).

2.2. Isolation of low number of cells

The isolation of a few cells was conducted according to a modified protocol by Francois et al. (2003). Briefly, the cell density of the activated and washed cultures was standardized at 10^8 CFU/ml, using an optical density (O.D.) – log CFU/ml calibration curve. Following decimal dilutions, 200 μl of the 10^3 CFU/ml solution were transferred to each well of the first column of a 8 × 12 microplate, which was prefilled with 200 μl of MRD. Half-fold dilutions were made across the microplate for the determination of the column which contained at least one cell and maximum four cells (column – target; *CT*). The latter decision was made to avoid the inoculation of samples with no cells and under the assumption that the difference between the kinetics of one and four cells is not significant. The whole content of the wells of the *CT* was used for the inoculation of samples. In parallel, the distribution of cells in the wells of the *CT* was checked at each experimental trial, by plating the whole content of the *CT* of an independent microplate on TSA, which served as control. Results showed that 92.6% of the samples were inoculated with 1–4 cells, whereas 7.4% of samples were possibly inoculated with no cells (Fig. 1).

2.3. Preparation, inoculation and microbial analysis of vegetables

Fresh whole heads of Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) and white cabbage (*Brassica oleracea*) were purchased from local supermarkets on the same day of each experimental setup. The 4–5 outer leaves and the core of each lettuce were discarded and the remained leaves were thoroughly washed with tap water to remove any organic material. In a further step, the washed leaves were dipped in plastic containers with fresh solution of chlorine bleach (200 ppm sodium hypochlorite) and remained for 5 min, simulating practices that are being commonly followed in catering services or households to reduce the microbial load of fresh produce. In contrast with lettuce, the heads of cabbage were cut in four quarters before dipping in the sanitizer. Following treatment with chlorine, the vegetables were washed thoroughly with sterile water for 15 min to remove the sanitizer. Both vegetables were allowed to dry for 30 min in a laminar flow cabinet before use.

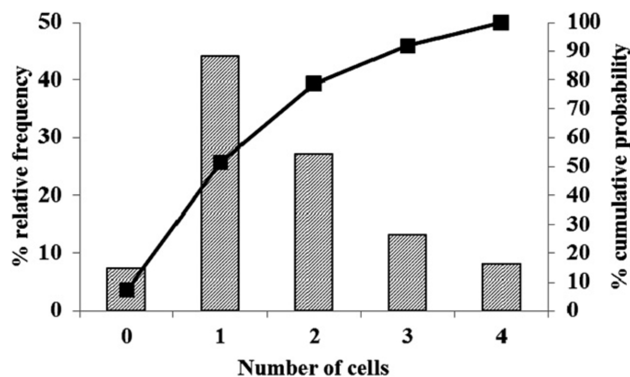

Fig. 1. Relative frequency (%: bars) and cumulative probability (line) of cells contained in a microplate well (in 200 μl total volume per well) and used to inoculate samples of lettuce or cabbage salad or the corresponding sterile extracts. Counts were estimated by plating the whole content of an additional microplate column.

Lettuce leaves were evenly cut into strips (*ca.* 1 cm width) using a sterile pair of kitchen scissors, while cabbage was cut with a household cabbage shredder, representing the commercially prepared, fresh-cut salads of leafy vegetables. Ten grams of each vegetable were transferred to sterile plastic containers (100 ml; 4.5 cm diameter × 7 cm height) and spot-inoculated onto a low (1–4 cells per sample or −1 to −0.4 log CFU/g) or a high (1000 cells per sample or 2 log CFU/g) level with *L. monocytogenes* or *S.* Typhimurium. The inoculated samples of both vegetables were stored at 8 °C, which is an average temperature that RTE salads may encounter in retail (Franz et al., 2010; Tromp, Rijgersberg, & Franz, 2010) or domestic refrigerators (Marklinder, Lindblad, Eriksson, Finnson, & Lindqvist, 2004). Absence of *L. monocytogenes* and *Salmonella* spp. in 25 g non-inoculated portions of both salads was confirmed *via* the methodology described by ISO standards 11290-1:1996/1:2004(E) and 6579:2002, respectively.

At regular time intervals, 40 ml of MRD were added to each sample and hand-shaked for 30 s in a standardized manner. Appropriate decimal dilutions were prepared in 9 ml MRD and plated on selective or non-selective media to monitor the growth kinetics of the inoculated pathogens and the indigenous microflora, respectively, as follows: total viable counts were determined by surface spreading on TSA plates and after incubation for 48 h at 30 °C. *Pseudomonas* species were enumerated by spread plating on pseudomonas agar base (LAB M, LAB108) plates supplemented with C.F.C. (LAB M, X108), following incubation at 25 °C for 48 h. Enterobacteria were enumerated by pour plating in violet red bile glucose agar (VRBG; LAB M, LAB088) incubated at 37 °C for 24 h. *L. monocytogenes* was spread plated on agar *Listeria* Ottaviani Agosti (ALOA, Biolife, 401605, Milan, Italy) plates with ALOA enrichment-selective supplements (Biolife; 423501) and incubated at 37 °C for 48 h. *Salmonella* counts were determined by surface spreading on xylose lysine deoxycholate agar (XLD; LAB M, LAB032) after incubation at 37 °C for 24 h. To decrease the detection limit of the sampling method, especially for the enumeration of the low inoculum of the pathogens (1–4 cells/sample), aliquots (1 ml) of the vegetable – MRD homogenate was equally distributed on the surface of three plates of each selective medium (i.e., XLD, ALOA). The detection limit of the plating method was 0.7 log CFU/g.

2.4. Preparation, inoculation and microbial analysis of vegetable sterile extracts

Considering that the microbial growth on vegetables may occur either on the vegetable surface or in the liquid micro-environment formed by the release of juices at wounds or the edge of cut surfaces, liquid and solidified extracts of lettuce and cabbage were prepared. These extracts were further sterilized in order to investigate the behavior of *L. monocytogenes* and *S.* Typhimurium in the absence of the indigenous microflora and compare it with that on salads. A modified protocol of Allende et al. (2007) was followed for the preparation of extracts. Fresh heads of lettuce and cabbage were obtained, washed and treated with chlorine bleach as previously described. The dry leaves of the vegetables were blended (Waring Pro, HGB50E1, Torrington, CT, USA) in 1:1 ratio with warm distilled water (45 °C) for 1 min at high speed. The derived pulp was heated at 80 °C for 2 h to denature the enzymes and proteins of the vegetables. The homogenate was filtered through Whatman paper and autoclaved at 121 °C for 15 min either in 5 ml tubes or in 500 ml flasks with 1.5% w/v agar (Agar Bios Special LL; Biolife, 4110302). Equal volumes (15 ml) of the sterile extract supplemented with agar were poured into petri dishes. The pH of the each medium was measured (WTW pH526 Metrohm Ltd, Switzerland) before and after autoclaving and was 6.17 ± 0.06, 6.05 ± 0.09 for lettuce and 5.99 ± 0.09, 5.84 ± 0.13 for cabbage, respectively. Both solidified and liquid media were kept at 4 °C for less than 24 h before use.

Each sample of solidified (15 g per sample) or liquid (5 ml per sample) extracts of lettuce and cabbage was inoculated with 1–4 cells (−1.2 to −0.6 log CFU/g and −0.7 to −0.1 log CFU/ml for solidified and liquid extracts respectively) or 1000 cells (1.8 log CFU/g or 2.3 log CFU/ml for solidified and liquid extracts respectively) of *L. monocytogenes* or *S.* Typhimurium. All samples were stored at 8 °C and 10 °C, in order to determine the impact of marginal changes in the storage temperature, close to the growth/no growth boundaries of the microorganisms, on the variability of microbial growth, especially of low inocula. Prior to incubation, the plates containing the solidified extracts were securely sealed with parafilm to avoid moisture losses. For the enumeration of the microorganisms in liquid extracts, 1 ml of each liquid medium was surface plated directly on three TSA plates. Similarly, the whole content of plates with the solidified extracts was homogenized with 30 ml MRD (1:3 dilution) in a stomacher bag for 1 min and 1 ml of the homogenate was plated on TSA. *L. monocytogenes* and *S.* Typhimurium populations were enumerated following incubation at 30 °C for 48 h or 37 °C for 24 h, respectively. The detection limit of the plating method was 0.5 log CFU/g for solidified extracts and 0.7 log CFU/ml for liquid extracts.

2.5. Monte Carlo simulation

To assess the ability of broth-based models to simulate the growth of a few (1–4) *L. monocytogenes* cells on lettuce or cabbage, data collected from the present study were compared to simulation results derived from the published data of Francois, Devlieghere, Standaert et al. (2006), where the mathematical expression of the effect of pH, a_w and storage temperature on the individual cell lag time and the generation time of *L. monocytogenes* was described. To the authors' knowledge, such data are not available for *Salmonella* in the literature, and hence, simulations were conducted only for *L. monocytogenes*. Considering the intrinsic parameters of the salads tested and storage temperature, as shown in Table 1, the Weibull distributions of broth-based individual lag times (Francois, Devlieghere, Standaert et al., 2006) were used to simulate the growth (log increase) of *L. monocytogenes* in salads, initiating from a few cells and after 12 days at 8 °C. We chose the particular lag time distributions because they refer to experimental conditions of

Table 1
Approximation of experimental conditions in salads by the broth-based study of Francois, Devlieghere, Standaert et al. (2006) and corresponding parameters of Weibull distributions of individual lag times, used for the simulation of *Listeria monocytogenes* growth on lettuce or cabbage salad, starting from 1 to 4 cells/sample.

Salad	Conditions in salads			Modelling conditions					
				Conditions in brain heart infusion broth (Francois, Devlieghere, Standaert et al., 2006)			Parameters of individual lag time distribution		
	pH	a_w	T (°C)	pH	a_w	T (°C)	a	b	Shift
Lettuce	6.0 ± 0.2	0.978 ± 0.002	8	6.0	0.974	7	2.878	94.24	–
Cabbage	5.8 ± 0.2	0.970 ± 0.002	8	5.5	0.970	7	2.845	230.2	24.14

pH, a_w and temperature that are close to the conditions of the fresh-cut salads of the present study. The model used for the simulation of *L. monocytogenes* growth is based on the three phase linear model of Buchanan, Whiting, and Damert (1997), and is given by Equations (1) and (2). The μ_{max} was estimated by the growth of the high inoculum of *L. monocytogenes* in each salad, using the model of Baranyi, and was fixed at 0.016 and 0.011 day^{-1} for growth in lettuce and cabbage salad, respectively.

$$N_t = N_0, \quad t \le t_{lag} \tag{1}$$

$$\log(N_t) = \log(N_0) + \mu_{max}\left(t - t_{lag}\right), \quad t > t_{lag} \tag{2}$$

where t is time and t_{lag} a value of lag time from the distributions of Francois, Devlieghere, Standaert et al. (2006).

2.6. Statistical analysis

The experiments on fresh-cut salads were conducted in two replicates, using an independent batch of vegetable for each replicate. At each sampling day, two samples of each salad inoculated with 1000 cells/sample ($n = 4$), and 10 samples inoculated with 1–4 cells/sample ($n = 20$) of the pathogens, were analyzed. For the preparation of the sterile extracts, four different batches of vegetables were used in four independent replicates. Similarly to the experiment on the vegetables, 10 samples of liquid or solidified extracts ($n = 40$), which were inoculated with 1–4 cells/sample, and 2 samples inoculated with 1000 cells/sample ($n = 8$) of the pathogens were analyzed at each sampling point. Populations of pathogens, which were enumerated on selective media were then transformed to log CFU/g in case of salads and solidified extracts and in log CFU/ml in case of liquid extracts. The average and standard deviation (SD) of log CFU/g or CFU/ml was calculated for samples inoculated with 1000 cells/sample. Populations derived from 1 to 4 cells/sample are presented individually for each sample, in order to emphasize the observed variability.

3. Results and discussion

3.1. Growth kinetics on vegetables

Changes in the levels of indigenous microbial association of lettuce and cabbage salads were monitored at 8 °C. *Pseudomonas* spp. (4.8–5.5 log CFU/g) and Enterobacteriaceae (4.9–5.8 log CFU/g) comprised the predominant flora of both salads (Fig. 2). Yeasts and lactic acid bacteria were also present but at the lower levels of <2.3–2.6 and <1.8–2.0 log CFU/g, respectively (results not shown). Following treatment of vegetables with 200 ppm sodium hypochlorite, the reductions of pseudomonads ranged from 1.8 to 2.2 log CFU/g and that of Enterobacteriaceae from 2.9 to 3.8 log CFU/g (Fig. 2). Both products were organoleptically unacceptable after 10–12 days, when the specific spoilage microorganisms (pseudomonads) had reached their maximum level of 9.4 ± 0.2 or 8.1 ± 0.3 log CFU/g in lettuce and cabbage salad, respectively (Figs. 3 and 4).

The microbial load of minimally processed salads usually consists of microorganisms related to soil or human, such as pseudomonads, enterobacteria, lactic acid bacteria and yeasts (Abadias, Usall, Anguera, Solsona, & Viñas, 2008; Randazzo, Scifò, Tomaselli, & Caggia, 2009). Although disinfection of fresh produce aim to reduce the epiphytic flora and prolong the shelf life of the final products (Delaquis, Fukumoto, Toivonen, & Cliff, 2004; Koseki & Itoh, 2001), reducing the epiphytes may also weaken their antagonistic activity against pathogens that survive the intervention or potentially contaminate the products during further processing.

With regards to the growth of pathogens, *L. monocytogenes* cells were able to grow on both salads, regardless of the inoculum level. Increase of *L. monocytogenes* starting from 1000 cells/sample occurred with limited variation (standard deviation < 0.5 log CFU/g), while growth initiating from a few (1–4) cells/sample ranged from <0.7 log CFU/g (detection limit) to 3.4 log CFU/g (Fig. 3). Similar observations were made on the growth variability of the high and low inocula of *S.* Typhimurium on lettuce, whereas no growth of this microorganism was obtained on cabbage (Fig. 4). It is notable however, that the total log increase (LI) from 1 to 4 cells/sample of both pathogens was higher compared with that of 1000 cells/sample. More specifically, a few cells of *L. monocytogenes* exhibited $LI_{(t = 12 \text{ days})} = 2.7$ and 3.4 log CFU/g on lettuce and cabbage salad, respectively, while the corresponding increase from 1000 cells was $LI_{(t = 12 \text{ days})} = 2.1$ and 1.8 log CFU/g (Fig. 3). Similarly, 1–4 cells/sample of *S.* Typhimurium showed $LI_{(t = 12 \text{ days})} = 3.0$ log CFU/g on lettuce salad, whereas only $LI_{(t = 12 \text{ days})} = 1.1$ log CFU/g was observed when starting from 1000 cells/sample (Fig. 4). In all cases, the growth of pathogens ceased when the epiphytic microflora reached its maximum population density.

A typical fresh-cut salad sample consists of various micro-environments with different micro-architectures such as liquid phase (juices), solid phase (tissue) or combination of those. The diffusion, the variety and the intensity of potentially stressful factors among these micro-environments may vary significantly (Leveau & Lindow, 2001; Wilson et al., 2002), along with the physiology of individual cells (Dupont & Augustin, 2009; Koutsoumanis, 2008). During random localization of individual cells on vegetable samples, the probability of two cells, which have been inoculated on two independent samples, to occur in a similar micro-environment is decreased, and in combination with their individuality, significantly different growth potential for each cell could be expected. Conversely, distributing higher inocula on

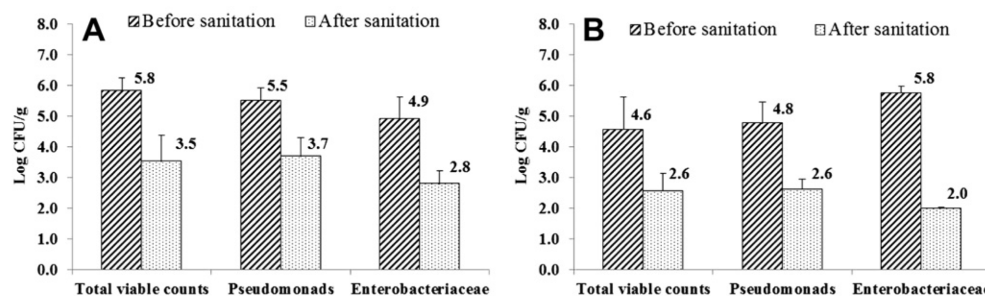

Fig. 2. Populations (log CFU/g) of the indigenous microflora of lettuce (A) and cabbage (B) before and after treatment with 200 ppm sodium hypochlorite for 5 min and washing with sterile water for 15 min.

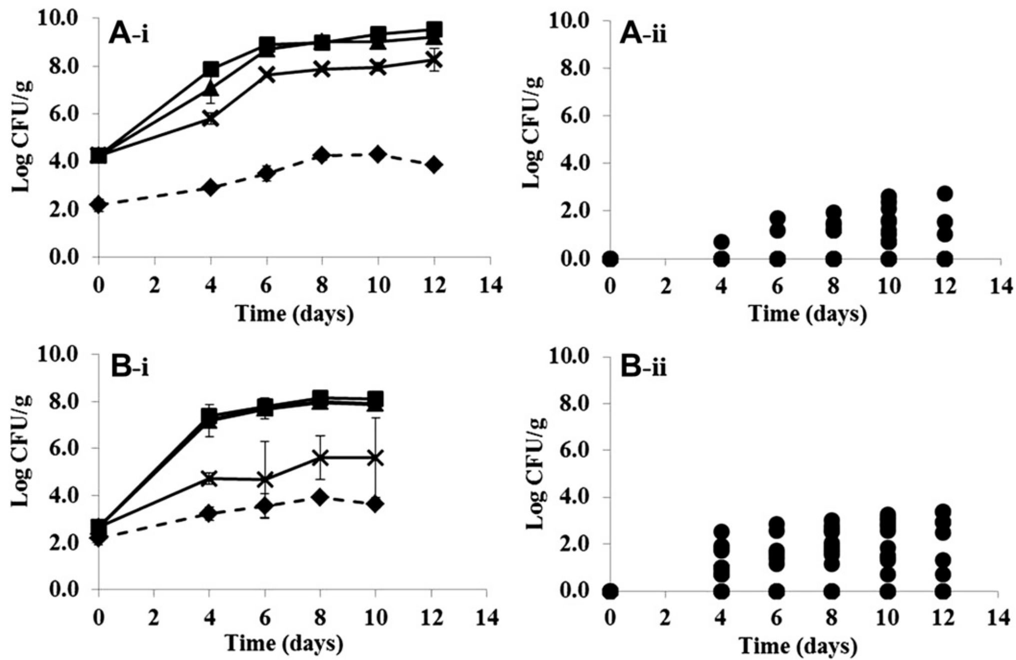

Fig. 3. Growth of 1000 (i) or 1–4 cells (ii) of *Listeria monocytogenes* per sample of lettuce (A) or cabbage (B) salad at 8 °C. (◆) 1000 cells of *L. monocytogenes*, (■) total viable counts, (▲) pseudomonads, (×) Enterobacteriaceae, (●) 1–4 cells of *L. monocytogenes*. Counts of the indigenous microflora (A-i, B-i) also refer to (A-ii, B-ii). The initial levels of *L. monocytogenes* in samples inoculated with 1–4 cells (−1 to −0.4 log CFU/g; graph A-ii, B-ii) are represented by 0 log CFU/g.

different spots of independent samples increases the similarities in spatiotemporal occurrence of cells at optimum (i.e., highest potential to grow in adverse conditions) or similar physiological state. Thus, the growth variability observed between such samples is lower.

An important observation with regards to food safety was the higher LI that a few cells of the pathogens exhibited during storage on both vegetables, compared to that of the higher initial inoculum (i.e., 1000 cells/sample). For instance, the percentage of lettuce or cabbage samples, inoculated with 1–4 cells/sample of *L. monocytogenes* and exceeding the microbiological criterion of 100 CFU/g, varied from 5 to 70%, depending on storage time. Conversely, the inoculum of 1000 cells/sample showed total LI equal or lower than this criterion (i.e., ≤2 log CFU/g). A critical factor affecting the capacity for growth of various initial pathogen levels on a food surface is the interaction with the epiphytic flora.

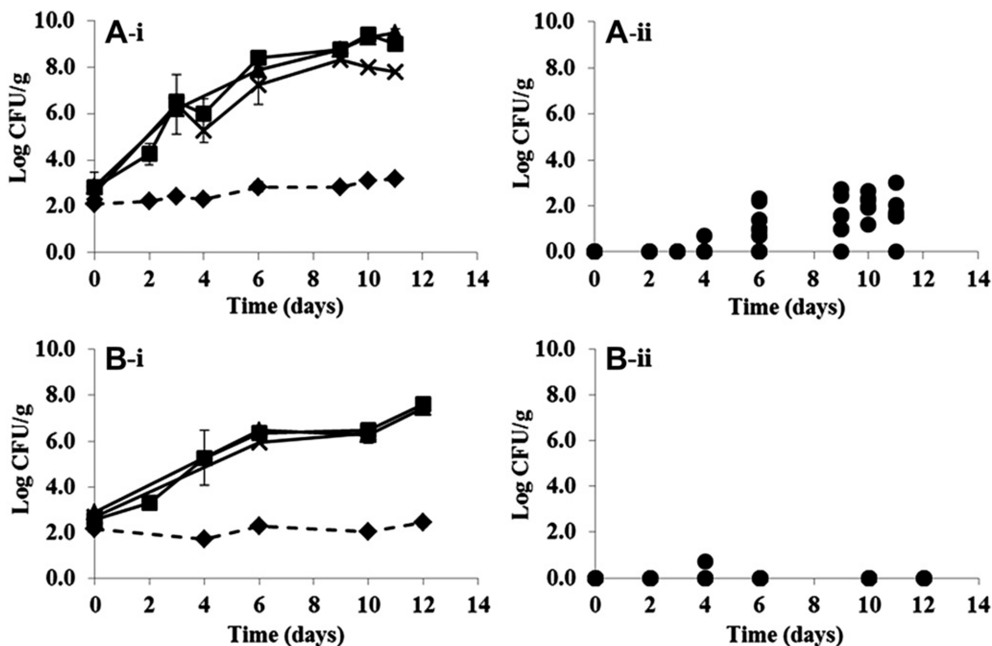

Fig. 4. Growth of 1000 (i) or 1–4 cells (ii) of *Salmonella* Typhimurium on lettuce (A) and cabbage (B) salad at 8 °C. (◆) 1000 cells of *S.* Typhimurium, (■) total viable counts, (▲) pseudomonads, (×) Enterobacteriaceae, (●) 1–4 cells of *S.* Typhimurium. Counts of the indigenous microflora (A-i, B-i) also refer to (A-ii, B-ii). The initial levels of *S.* Typhimurium in samples inoculated with a 1–4 cells (−1 to −0.4 log CFU/g; graph A-ii, B-ii) are represented by 0 log CFU/g.

The observation that the growth of *L. monocytogenes* and *S.* Typhimurium ceased when the background flora of salads reached the maximum population density (Figs. 3 and 4), may be associated with the so-called "Jameson effect" (Mellefont, McMeekin, & Ross, 2008), due to the competition for nutrients, or the production of microbial metabolites by the indigenous flora. In contrast, as detailed in Section 3.2, no such inhibition of the pathogens was observed in the sterile extracts, apparently due to the absence of the background flora. However, given that the epiphytes reached their maximum population density at the same storage time in all batches of each vegetable, (i.e., 10 and 8 days for lettuce and cabbage, respectively), the growth of the pathogens ceased simultaneously, regardless of the initial inoculation level. Thus, the differences in total log increase between samples inoculated with high and those with low inocula could be attributed to differences in both the growth rate and/or lag time. According to previous reports, the growth rate is considered independent of the initial inoculum size (Robinson et al., 2001). Therefore, the aforementioned differences in LI of different inocula should be only due to the variability in individual lag times. It needs to be stressed however that the majority of studies on the effect of the inoculum size on the growth kinetics of pathogens has been performed on liquid media, where after each bacterial division, the daughter cells are drifted away from the mother cell. Thus, the possibility of "crowding effect", i.e., the growth deceleration due to interactions of closely located cells is limited, and this may explain why the growth rate of planktonic cultures is not affected by their initial inoculum size. On the contrary, when cells are immobilized on a solid matrix, as is the case of fresh-cut salads, cells grow as colonies and hence, interactions may occur between cells within a colony (especially among cells in the outer and inner part of colonies) or between adjacent colonies. Such interactions are thought to increase with the proximity of colonies, i.e., with the population density, because the higher the population the lower the distance between colonies. This most likely explains the slower growth rate of 1000 cells/sample compared to that of a few cells/sample. Likewise, Thomas, Wimpenny, and Barker (1997) reported that increasing the distance between adjacent colonies of *L. monocytogenes* from 100 μm to 3000 μm, increased the cell density in the colonies from approximately 1.5 to 7 log CFU per colony, respectively. The above results imply that if log increase of pathogens on a food was judged only by challenge tests with unrealistically high inocula, as the worst case scenario, the actual risk would have been underestimated. Overall, although the risk posed by a few *L. monocytogenes* or *S.* Typhimurium cells on vegetable salads may be characterized by high variability, such assessments are more realistic and representable of a low contamination scenario. Considering also that the growth kinetics of the pathogens may differ significantly, depending on the initial inoculation level, challenge tests initiating from low number of cells (<5–10 cells) should be considered as a more reliable method to evaluate the real risk.

3.2. Growth kinetics on sterile extracts of vegetables

Remarkable variation in the growth potential of the pathogens was observed between different batches of vegetables used for the preparation of the sterile extracts (Figs. 5 and 6). This variability could even cause the transition of the pathogens from no growth to growth state. For example, in batch A of lettuce liquid extract (lettuce extract broth; LEB) no growth was observed for 1000 cells/sample of *S.* Typhimurium at 8 °C, in contrast to batch B, where clear increase was obtained (Fig. 6). Similar results were observed when a few cells (1–4/sample) of *S.* Typhimurium were inoculated on cabbage solidified extract (cabbage extract agar; CEA) and stored at 8 °C (Fig. 6), as well as when a few cells (1–4/sample) of *L. monocytogenes* were inoculated on lettuce solidified extract (lettuce extract agar; LEA) and stored at the same temperature (Fig. 5). On CEA or CEB no growth of *L. monocytogenes* was permitted in none of the studied batches (Fig. 5).

Given that the methodology of sterile extracts preparation was standard, it is assumed that the variability in growth of the microorganisms between different batches may be attributed to the nutritional composition of each batch. In the present study, 4 different batches of lettuce or cabbage were obtained within 3 months and from different retailers, and thus, variability in the composition of each batch should be expected. Notably, the batch-variability was less evident between different batches of vegetable salads; it is assumed that diluting or autoclaving the vegetable nutrients during preparation of the extracts may also have affected the dynamics of the pathogens. Ongeng et al. (2007), who followed a similar protocol for the preparation of cabbage extract, stated that processing of cabbage juice by autoclaving might have significant impact on $\mu_{\max}$ of *L. monocytogenes*.

The comparison between growth of pathogens in sterile extracts and salads suggests that the role of the epiphytic flora on pathogen behavior is crucial. Although *S.* Typhimurium did not grow on cabbage salad, remarkable growth from 1 to 4 cells/sample was observed on CEA (Figs. 4 and 6), indicating a potential competitive effect of the epiphytic flora of cabbage on the pathogen. In contrast, growth of a few or 1000 cells/sample of *L. monocytogenes* occurred only on cabbage, contrary to the complete inhibition of the bacterium on CEA or in CEB (Figs. 3 and 5). Similarly, a few cells/sample of *S.* Typhimurium grew on lettuce but not on LEB at 8 °C (Figs. 4 and 6), suggesting that the increase in levels of epiphytic flora on the surface of cut tissue occasionally enhanced the growth of the pathogens.

Although the exact mechanisms have not been fully elucidated, the inhibitory interference of the spoilage flora against pathogens may be attributed to the competition for the available nutrients, the production of antimicrobial substances (i.e., lactic acid, bacteriocins) and/or the alteration of the physicochemical properties (i.e., pH, redox potential) of the micro-environment in which the growth of pathogens may occur (Al-Zeyara, Javris, & Mackey, 2011; Huis in't Veld, 1996; Jay, Loessner, & Golden, 2005, chap. 13). Contrary to the competitive behavior, the stimulatory activity of the background flora that was observed may be a potential result of *metabiosis* (Marshall & Schmidt, 1991). In particular, the conversion of macromolecules by psychrotrophs to readily available nutrients, may promote the growth of the pathogens (Huis in't Veld, 1996). Enhanced survival of *E. coli* O157:H7 was observed on lettuce phyloplane in the presence of *Wausteria paucula* (Cooley, Chao, & Mandrell, 2006). In addition, Fatica and Schneider (2011) have recently reported that biofilms formed by the indigenous microflora on the surface of the vegetables may impart protection to the pathogenic cells against environmental stressful factors. Although Ongeng et al. (2007) reported no interference of the commensal flora of cabbage with *L. monocytogenes* at 4 °C or 30 °C, the spoilage flora tested was activated in broth and artificially inoculated on cabbage. It is likely, therefore, that the distribution and adaptation of native flora in the cabbage environment in our study may explain the difference of the present results to the results of that study. Such findings indicate that the stimulatory or competitive effect of epiphytes of lettuce or cabbage on pathogens is uncertain and may be inadequately described by broth-based experiments.

The structure (i.e., solid *vs* liquid) of product extracts also influenced the growth of pathogens. A few (1–4) cells/sample of *S.* Typhimurium could initiate growth on the surface of CEA, but not in the corresponding broth, in which only the high inoculum of 1000 cells/sample was capable of growth initiation (Fig. 6).

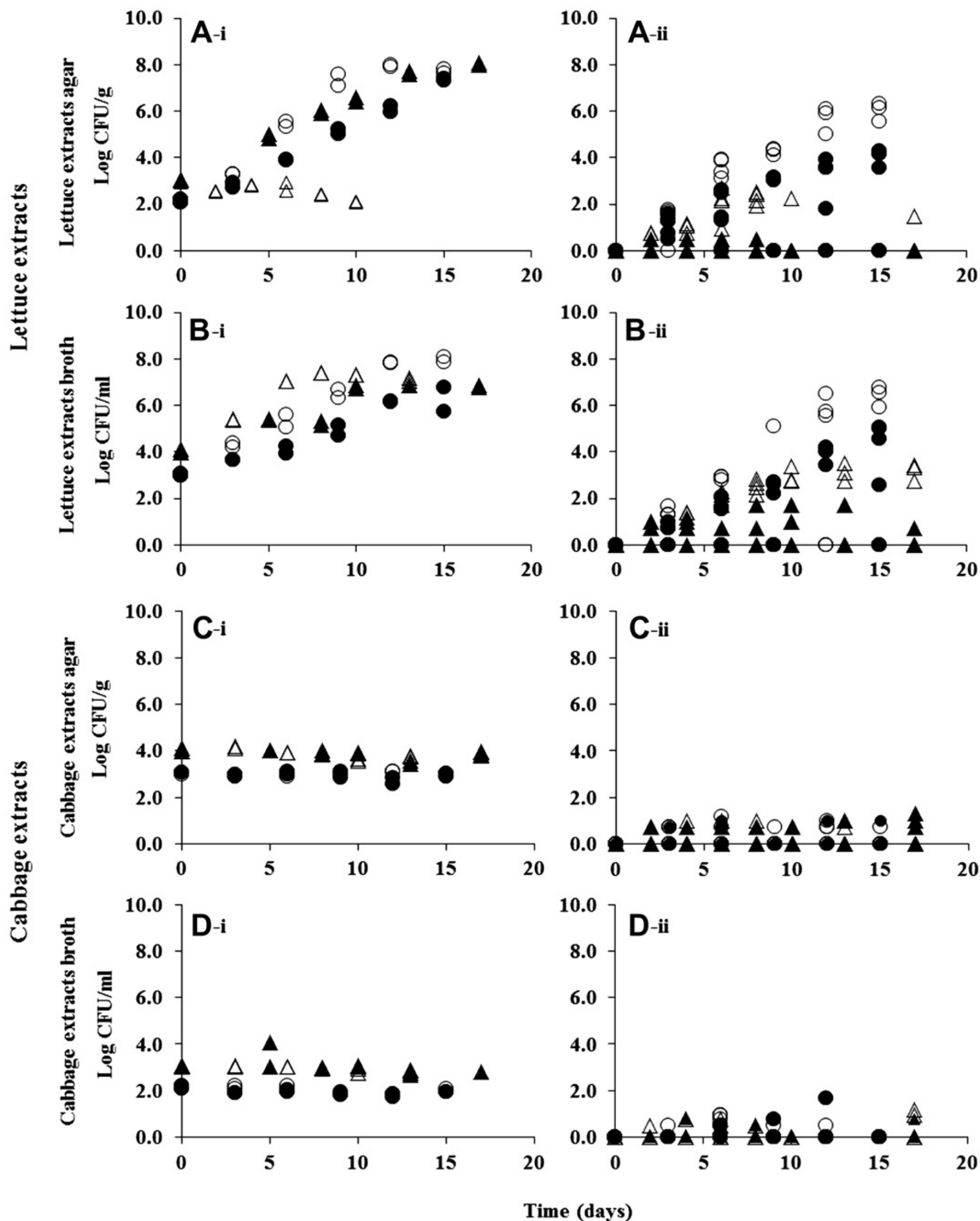

Fig. 5. Growth of 1000 (i) or 1–4 cells (ii) of *Listeria monocytogenes* on two different batches (batch A, ●,○; batch B, ▲,△) of LEA (A), LEB (B), CEA (C) and CEB (D) stored at 8 °C (closed symbols) or 10 °C (open symbols). For readability reasons, results of two representative batches are presented. The initial levels of *L. monocytogenes* in samples inoculated with 1–4 cells/sample (−1.2 to −0.6 log CFU/g and −0.7 to −0.1 log CFU/g for solidified and liquid extracts, respectively) are represented by 0 log CFU/g or ml.

Although it would be expected that the nutrients of the vegetables would be more readily available in broths (Stecchini et al., 1998), liquid extracts were on average more inhibitory than solid ones.

Many researchers have investigated the effect of the microstructure on the growth kinetics of pathogens (Noriega, Laca, & Diaz, 2010a; Ongeng et al., 2007; Theys, Geeraerd, & Van Impe, 2009). It is likely that the intensity of stress factors, e.g., non-fermentable nutrients, or phenolic and acid compounds, possibly extracted from the plant tissues, is perceived by bacteria more in juices than on agar surfaces (Skandamis, Tsigarida, & Nychas, 2000). Delaquis, Wen, Toivonen, and Stanich (2006) reported the presence of a substance, probably of phenolic nature, which derived during shredding of iceberg lettuce and caused the inhibition of *L. monocytogenes*. In addition, Noriega, Laca, and Diaz (2010b) documented that microorganisms immobilized on a food or solid medium surface may adapt to their acidic metabolites, as the diffusion is limited compared with vegetable juices or broth media, and subsequently exhibit increased tolerance to inhibitory antimicrobial compounds. The presumable presence of antimicrobial substances in the extracts of the cabbage is being also confirmed by the death of *S.* Typhimurium which occasionally occurred and was more evident in 1000 cells/sample inoculum than in the inoculum of a few cells/sample (Fig. 6). The results of the present study are based on the assumption that the solidified extracts do not represent precisely the surface of the vegetables but they simulate the slower diffusion rate of nutrients or inhibitory substances compared with juices released in wounds or cut surfaces. Thus, it may be concluded that the growth dynamics of pathogenic bacteria on a real food system may differ

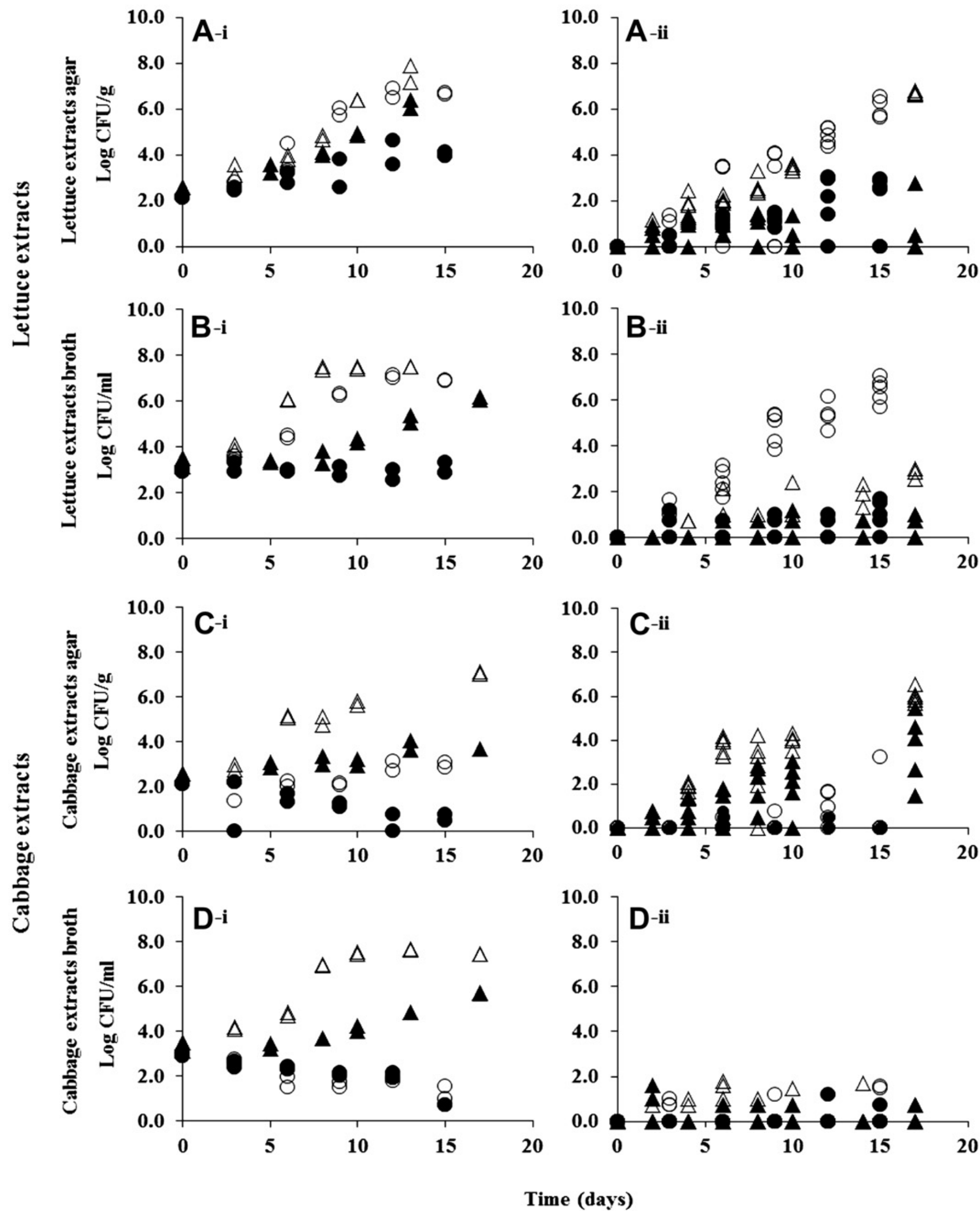

Fig. 6. Growth of 1000 (i) or 1–4 cells (ii) of *Salmonella* Typhimurium on two different batches (batch A, ●,○; batch B, ▲,△) of LEA (A), LEB (B), CEA (C) and CEB (D) stored at 8 °C (closed symbols) or 10 °C (open symbols). For readability reasons, results of two representative batches are presented. The initial levels of *S.* Typhimurium in samples inoculated with 1–4 cells/sample (−1.2 to −0.6 log CFU/g and −0.7 to −0.1 log CFU/g for solidified and liquid extracts, respectively) are represented by 0 log CFU/g or ml.

significantly and the structure effect should be taken under consideration for the development of simulation models.

Increase in storage temperature of the sterile extracts from 8 to 10 °C had such a pronounce effect on pathogen growth that could even shift cells from no growth to a growth status. *S.* Typhimurium showed significant increase on LEB at 10 °C, regardless the initial inoculum level, while no growth was observed when the same batch of extracts was stored at 8 °C (Fig. 6.). In contrast, *L. monocytogenes* exhibited growth under both storage temperatures on LEA and LEB (Fig. 5). This was more evident for *Salmonella*, due to the range of 8–10 °C being close to the growth boundaries of this microorganism.

Time–temperature profiles of fresh-cut salads during storage in a salad-bar, have shown that the storage temperature may often exceed 10 °C, resulting in 17%, 15% and 194% increase of *E. coli* O157:H7, *Salmonella* and *L. monocytogenes*, respectively (Franz et al., 2010). Concerns are also raised for the safety of vegetable salads in the domestic environment. Leftovers of leafy salads may be packed in kitchen containers (air packaging) and stored in household refrigerators, in which the average temperature may exceed 8 °C (Marklinder et al., 2004). Thus it is concluded that marginal changes of temperature, which are commonly encountered during distribution or storage of freshly-cut salads, may affect the growth dynamics of pathogenic cells and render the final product unsafe.

3.3. Monte Carlo simulation

The growth of single cells of *L. monocytogenes* on lettuce or cabbage was simulated through a stochastic Monte Carlo approach and using the distributions of individual cell lag times observed by

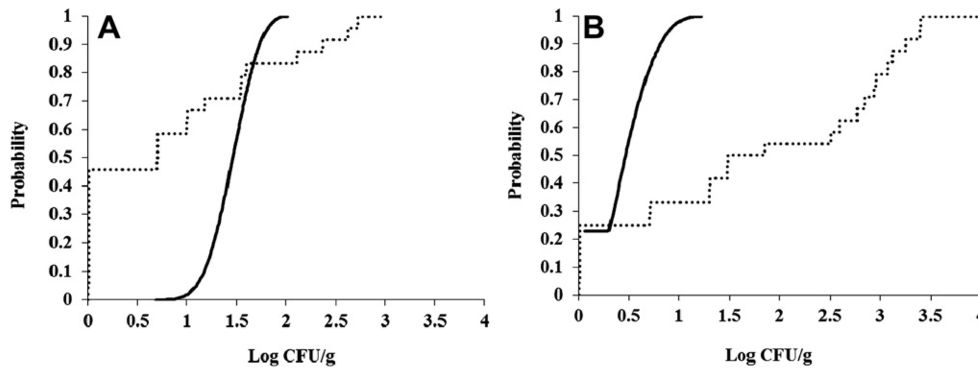

Fig. 7. Predicted (▬) and observed (···) cumulative distribution of *L. monocytogenes* on lettuce (A) and cabbage (B) after 12 days of storage.

Francois, Devlieghere, Standaert et al. (2006). Results showed that the Monte Carlo simulation slightly over-predicted the growth of single cells of *L. monocytogenes* after 12 days on lettuce. For instance, the model predicted that there is 60% likelihood a single cell of the pathogen to reach 1.5 log CFU/g, while the observed growth under the same probability was 1 log CFU/g (Fig. 7). In contrast, remarked under-estimation of the observed growth in cabbage was recorded, as the predictions showed that 60% of the individual cells could grow at 0.5 log CFU/g, whereas the observed growth was 2.6 log CFU/g. There is limited number of studies in the literature, where simulated growth of single cells is compared with observations from growth of single cells on foods. Francois, Devlieghere, Standaert et al. (2006), Francois, Devlieghere, Uyttendaele et al. (2006) simulated the growth of single cells of *L. monocytogenes* on liver pâté and cooked ham. The results of that study showed that the broth-based simulations predicted slightly faster growth compared to that occurred in liver pâté, while this over-prediction was more evident on cooked ham. This deviation of the broth-based predictions from the observed growth suggests poor transferability of broth-based data to foods, because such models do not adequately encompass the effect of the epiphytic flora, the micro-structure or the scattered availability of nutrients. Thus, extrapolating broth-based predictions of microbial growth from single cells to foods, may lead to either fail-safe or fail-dangerous predictions.

4. Conclusions

The effect of epiphytic flora, the diverse structure of micro-environments in a fresh-cut salad, the distribution of available nutrients and the marginal changes of storage temperature may be inhibitory or stimulatory for the growth of very low (down to a few cells) levels of pathogens. In addition, it was shown that the ability of fresh-cut salads to support pathogen growth may be under-estimated when using high inocula, underlying the need to perform challenge tests with low numbers or even single cells of target organism. Simulating the growth from 1 to 4 *L. monocytogenes* cells on lettuce or cabbage salad, based on data derived from laboratory media, showed deviation from the growth observed on foods. Especially in cabbage salad, significant under-estimation of the actual growth was observed, leading to fail-dangerous predictions. Therefore, future predictive models need also to incorporate the variability in the growth responses of realistically low inocula, in response to inimical factors. This will ensure the development of reliable tools for risk assessment of pathogens in RTE leafy salads.

Acknowledgments

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