



The transfer and growth of Salmonella modelled during pork processing and applied to a risk assessment for the catering sector

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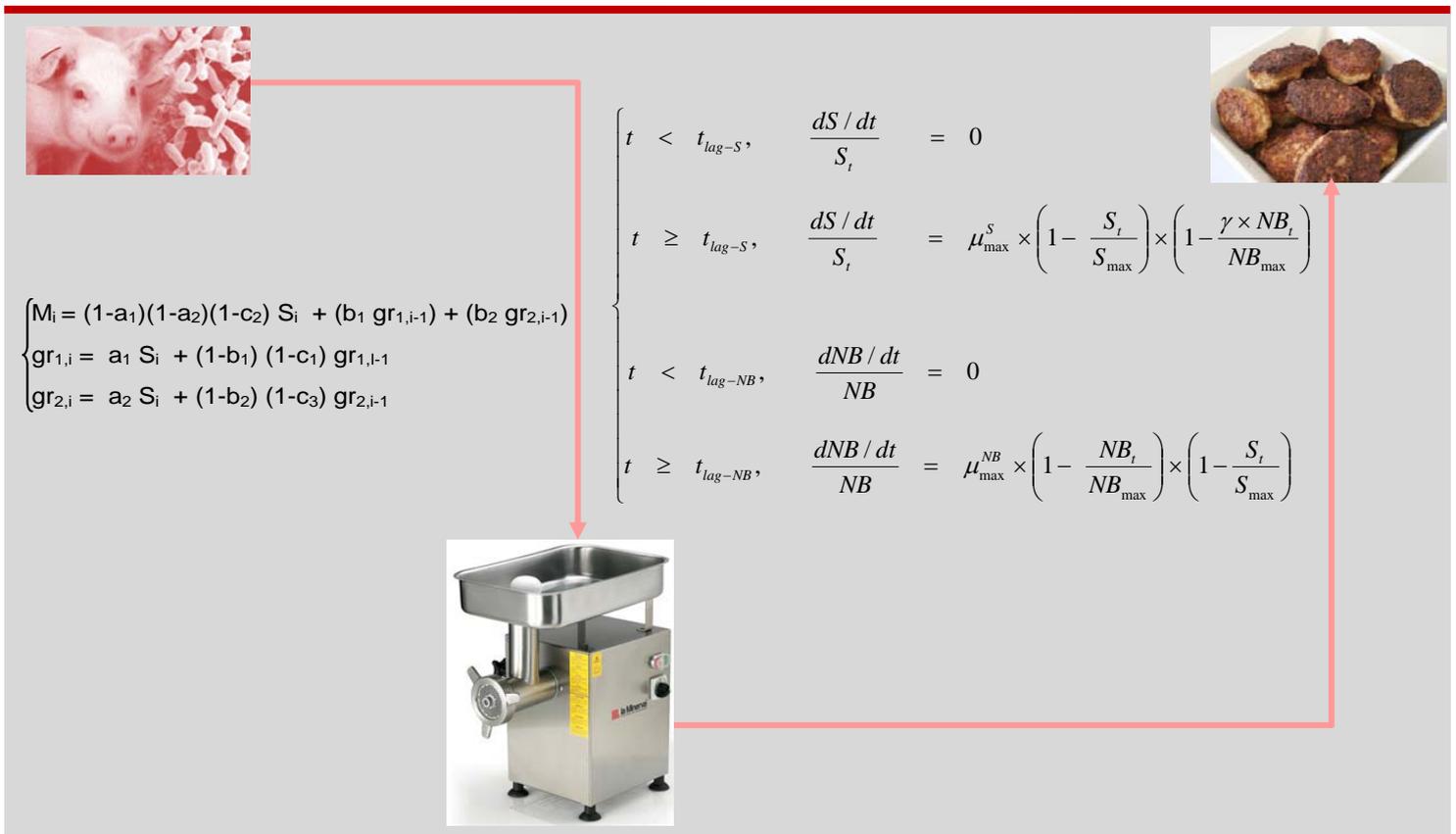
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The transfer and growth of *Salmonella* modelled during pork processing and applied to a risk assessment for the catering sector



Cleide Oliveira de Almeida Møller
 PhD thesis
 2012

**The transfer and growth of *Salmonella* modelled during
pork processing and applied to a risk assessment for
the catering sector**

PhD thesis

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Technical University of Denmark
National Food Institute
Division of Food Microbiology
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June 2012

Preface

This research project was carried out at the Microbial Food Safety Group from the Division of Food Microbiology, National Food Institute, Technical University of Denmark in collaboration with seven co-authors. The project was conducted from November 2007 until June 2012. An external research stay of three weeks at the Food Science Department, Rutgers University, New Jersey, USA was included. This project was financed by the Technical University of Denmark through the FoodDTU programme.

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The transfer and growth of *Salmonella* modelled during pork processing and applied to a risk assessment for the catering sector

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List of abbreviations

AIC	Akaike Information criterium
approx.	Approximately
CCP	Critical control point
CDC	Center for Disease Control and Prevention
CFU	Colony forming unit
D-value	decimal reduction time or time required at a certain temperature to kill 90% of the organisms being studied
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
g	Gram
GR	Growth rate
h	Hour
HACCP	Hazard analysis and critical control points
ICMSF	International Commission on Microbiological Specifications for Foods of the International Union of Biological Societies
LPD	Lag phase duration
max.	Maximum
min	Minute
MPD	Maximum population density
MPRM	Modular process risk model
NB	Natural microbiota
QMRA	Quantitative microbiological risk assessment
R²	Symbol for the coefficient of determination of a linear regression
RLT	Relative lag time
RMSE	Root mean square error
RTK	Room temperature in the kitchen during cooling of meatballs
S.	<i>Salmonella enterica ssp. enterica</i>
T_{min}	Minimum temperature for growth
μ_{max}	Maximum growth rate
WHO	World Health Organization
z- value	is the temperature, in degrees Fahrenheit or Celsius, that is required for the thermal destruction curve to move one log cycle
%	Percent
°C	Degrees Celsius
a_w	Water activity

Summary

Salmonellosis is an important cause of foodborne human gastroenteritis in most European countries, and pork contributes significantly to the disease burden caused by *Salmonella* infections. A traditional, and very popular, pork product in Denmark is fried meatballs. Danish meatballs are typically made with ground pork as the main ingredient. They are consumed as a component in hot meals but are also widely used as filling in cold sandwiches. Because of their popularity, pork meatballs are often part of the product range in Danish catering settings. As illustrated by the fact that 55 of 77 reported outbreaks in Denmark in 2010 were associated with outside-the-home settings, such as restaurants, canteens, hotels, schools, shops, institutions and sport events (Anonymous 2011), food prepared outside the home is a significant source of foodborne illness. In the present study, Quantitative Microbiological Risk Assessment (QMRA), following the Codex Alimentarius Principles and using the modular process risk model (MPRM) methodology, was used as the tool to investigate the fate of *Salmonella* during processing of pork meatballs from the reception of whole pork cuttings, through processing, until the point of consumption in a catering unit, e.g. worksite or school canteens. Appropriate transfer, growth and inactivation models developed specifically for *Salmonella* spp. in fresh meat, preferably pork, were applied to evaluate different scenarios for a food processing line constructed from two observational studies.

Applying the proposed QMRA model (MANUSCRIPT II), risk estimates of illness from meatballs processed by the catering sector per year in Denmark were obtained (no cases applying a core temperature $>71^{\circ}\text{C}$, 27 cases applying a core temperature of 65°C , 69 cases without applying a final heat treatment in oven). When comparing with recent epidemiological data from 2011 (44 – 131 reported cases) for *Salmonella* cases in Denmark related to pork (Anonymous, 2012), these estimates are considered reasonable and realistic. Core temperatures higher than 71°C were found sufficient to inactivate *Salmonella* in meatballs, indicating that the recommendation from the Danish Food Authorities of 75°C is sufficient to eliminate this pathogen. Survival as a result of ineffective heating and growth of *Salmonella* during cooling had relevant impact on the risk estimates and, therefore, these processing steps should continue to be considered critical for meatballs safety. However, more knowledge about the critical limits was obtained, e.g. cooling of meatballs at temperatures lower than 24°C for periods shorter than three hours does not allow growth of the remaining *Salmonella* cells. In addition, prevalence and concentration of *Salmonella* had higher impact on the risk estimates, thereby justifying the efforts to keep these factors low. The flexible structure of the QMRA model allows scenario analysis, which consequently makes it possible to expand the use of the QMRA model from pathogen outbreak investigation to product development and other food safety evaluation activities, e.g. efficiency of guidelines and

establishment of critical limits.

Two of the predictive models used to build the QMRA model (MANUSCRIPT II) were also developed in this thesis:

- The transfer of *Salmonella* during grinding of pork was successfully modelled in processing of up to 110 pork slices corresponding to 21 kg meat (PAPER I). This model includes the pieces of meat that are contaminated before grinding and it gives clear explanations of all the parameters involved that combined give an overview of the dynamics of a grinding process. The structure of the model, and particularly its ability to predict the tailing phenomenon of low contaminated portions, seems relevant for different cross contamination processes.
- Effect of pork natural microbiota on growth of *Salmonella* was modelled and predicted during storage of ground pork at temperatures between 4°C and 38°C (MANUSCRIPT I). Continued growth of *Salmonella* after the natural microbiota had reached their max. population density was observed. This effect was described well by the complex Lotka-Volterra species interaction model as well as a new expanded Jameson-effect model but not by the classical Jameson-effect model. As the new expanded Jameson-effect model is more simple and practical to use, it may be preferred over the Lotka-Volterra model. Performance of both models was, however, temperature dependent, presenting good results at temperatures from 15.1 to 20.2°C.

The combination of data from observational studies, models specifically developed studying transfer and growth of *Salmonella* in pork (PAPER I and MANUSCRIPT I), and literature data related to *Salmonella* in different meat matrices resulted in a new approach that may improve the quality of estimates in risk assessments related to *Salmonella* in pork processed at the catering sector.

Sammendrag

Salmonellose er en velkendt årsag til fødevarebåren human gastroenteritis i de fleste europæiske lande, og *Salmonella* infektioner relateret til svinekød bidrager væsentligt til en forringelse af folkesundheden. Et traditionelt, og meget populært, svinekødsprodukt i Danmark er stegte frikadeller. Danske frikadeller er typisk fremstillet med hakket svinekød som hovedingrediens. De indtages oftest som en del af et varmt måltid, men er også udbredt som pålæg eller fyld i kolde sandwiches. På grund af deres popularitet, er frikadeller af svinekød ofte en del af produktsortimentet i danske storkøkkener og catering virksomheder. I 2010 blev 55 af de 77 rapporterede udbrud i Danmark forbundet med spisning uden for hjemmet, såsom restauranter, kantiner, hoteller, skoler, forretninger, institutioner og sportsarrangementer (Anonym 2011). Dette viser tydeligt, at mad, tilberedt uden for hjemmet, er en væsentlig kilde til fødevarebårne sygdomme. I denne afhandling blev kvantitativ mikrobiologisk risikovurdering (QMRA), som følger Codex Alimentarius principper, og som bygger på en modulær proces risiko modelleringsmetode (MPRM), anvendt som redskab til at undersøge, hvordan *Salmonella* spredes og overlever under forarbejdning af frikadeller lavet af svinekød. Dette blev gjort fra modtagelsen af svinekød, via forarbejdning og tilberedning, helt frem til indtagelse, f.eks. i en arbejdsplads- eller skolekantine. De bedst egnede matematiske modeller for overførsel, vækst og inaktivering udviklet specielt til *Salmonella* spp. i fersk kød, helst svinekød, blev anvendt til at vurdere forskellige scenarier for spredning, overlevelse og vækst af *Salmonella* i en sådan fødevareproduktion. De testede scenarier var udvalgt på baggrund af to observationsstudier i catering virksomheder.

Ved anvendelse af den udarbejdede QMRA model (MANUSKRIFT II), blev risikoen for sygdom, forårsaget af den årlige produktion af frikadeller i cateringsektoren i Danmark, estimeret til i) ingen tilfælde hvis frikadellernes kernetemperatur $>71^{\circ}\text{C}$; ii) 27 tilfælde hvis kernetemperaturen var 65°C ; og iii) 69 tilfælde hvis den afsluttende varmebehandling i ovn blev udeladt. Ved sammenligning med de aktuelle epidemiologiske data fra 2011, 44 – 131 indberettede tilfælde for svinekødsrelaterede salmonellose i Danmark (Anonym, 2012), vurderes disse estimater som rimelige og realistiske. Kernetemperaturer $>71^{\circ}\text{C}$ blev fundet tilstrækkelige til at inaktivere *Salmonella* i frikadellerne, hvilket indikerer, at anbefalingen fra de danske fødevaremyndigheder på 75°C er fuldt ud tilstrækkelig til at eliminere denne patogen. Overlevelse af *Salmonella* som et resultat af ineffektiv opvarmning og vækst under afkøling er relevant for risikovurderingen. Disse procestrin bør derfor fortsat anses som værende kritiske for fødevarerisikoen af frikadeller. F.eks. kunne det udledes, at afkølingen af frikadeller ved køkkentemperaturer $<24^{\circ}\text{C}$ i maks. 3 timer ikke tillader vækst af de tilstedeværende *Salmonella*. Derudover viste det sig, at forekomst og koncentration af *Salmonella* havde stor indflydelse på risikoestimererne, hvilket berettiger bestræbelserne på at holde disse faktorer lave. Den fleksible struktur af QMRA modellen tillader

analyse af scenarier, som gør det muligt at udvide anvendelsen af QMRA modellen f.eks. til hypotesegenerering i forbindelse med udbrudseftersporing, produktudvikling og andre fødevarsikkerhedsmæssige evalueringsaktiviteter, f.eks. effektiviteten af retningslinjer og fastsættelse af kritiske grænser.

To af de prædiktive mikrobiologiske modeller, der blev anvendt til at opbygge QMRA modellen (MANUSKRIFT II) blev også udviklet i denne afhandling:

- Overførslen af *Salmonella* fra kontaminerede til ikke-kontaminerede kød under hakning af svinekød blev med succes modelleret i forsøg hvor op til 110 skiver svinekød, svarende til 21 kg kød (PAPER I), blev hakket. Den opstillede model omfatter kødstykker, der er kontamineret før hakning og giver entydige forklaringer af de parametre, der samlet giver et overblik over dynamikken i en hakkeproces. Strukturen af modellen, og især dens evne til at forudsige fænomenet, hvor en langstrakt fase med lavt forurenede prøver observeres, synes at være relevant for forskellige krydskontamineringsprocesser.
- Effekten af svinekøds naturlige mikrobiota på vækst af *Salmonella* i kødet blev modelleret og forudsagt under opbevaring af hakket svinekød ved temperaturer mellem 9°C og 24°C (MANUSKRIFT I). Væksten af *Salmonella* fortsatte efter at den naturlige mikrobiota havde nået sit maximale niveau. Denne effekt blev beskrevet godt af den komplekse Lotka-Volterra species interaktions model samt en ny udvidet Jameson-effekt model, men ikke af den klassiske Jameson-effekt model. Da den nye udvidede Jameson-effekt model er mere enkel og praktisk at anvende, foretrækkes den frem for Lotka-Volterra modellen. Præstationen af begge modeller viste sig dog at være temperaturafhængig, og begge modeller gav de bedste resultater ved temperaturer fra 15,1 til 20,2°C.

Kombinationen af data fra observationsstudier, modeller specielt udviklet ved studier af overførsel og vækst af *Salmonella* i svinekød (PAPER I og MANUSKRIFT I) samt litteratur data vedrørende *Salmonella* i forskellige kødmatricer resulterede i en ny indfaldsvinkel til QMRA. Denne indfaldsvinkel kan være med til at forbedre kvaliteten af risikoestimerer relateret til *Salmonella* i svinekød som forarbejdes i cateringsektoren.

Resumo

A salmonelose é uma causa importante de gastroenterite de origem alimentar em humanos, na maioria dos países europeus, e a carne suína contribui significativamente para problemas de saúde pública relacionados às infecções por *Salmonella*. Um produto muito tradicional e popular na Dinamarca é a almôndega frita ("frikadeller"), que tem como ingrediente principal a carne suína moída. É consumida em refeições quentes, mas também amplamente utilizada como recheio em sanduíches frios. Devido a sua popularidade, almôndegas de suíno são muitas vezes parte da gama de produtos em serviços de alimentação dinamarqueses. Dos 77 surtos notificados na Dinamarca em 2010, 55 (71%) foram associados a refeições consumidas fora de casa, como em restaurantes, cantinas, hotéis, escolas, lojas, instituições e eventos desportivos (Anonymous, 2011). Refeições preparadas fora de casa representam fonte importante de doenças transmitidas por alimentos. No presente estudo foi realizada avaliação quantitativa de risco microbiológico (QMRA), seguindo os princípios do Codex Alimentarius e usando a metodologia do modelo de risco modular de processo (MPRM), para investigar o comportamento de *Salmonella* durante o processamento de almôndegas, desde a recepção dos cortes da carne suína, passando pelo processamento, até o ponto de consumo em unidades de serviço de alimentação como cantinas, em locais de trabalho ou em escolas. Modelos de contaminação cruzada, crescimento e inativação, desenvolvidos especificamente para o estudo de *Salmonella* spp. em carne fresca, preferencialmente suína, foram aplicados para avaliar diferentes cenários em linha de processamento de alimentos, construída a partir de dois estudos observacionais.

Aplicando o modelo de QMRA proposto (MANUSCRIPT II), as estimativas de risco de salmonelose, pelo consumo de almôndegas processadas anualmente pelos serviços de alimentação na Dinamarca, foram obtidas e consideradas razoáveis (nenhum caso observado quando a temperatura no centro da almôndega atingiu mais que 71°C; 27 casos foram observados à temperatura de 65°C e 69 casos observados sem tratamento térmico em forno), quando comparado com os dados epidemiológicos de 2011 para os casos de *Salmonella* (44-131 casos relatados) na Dinamarca relacionados com a carne suína (Anonymous, 2012). Temperaturas superiores a 71°C foram suficientes para inativar *Salmonella* em almôndegas, indicando que a recomendação (de 75°C) das autoridades de vigilância sanitária dinamarquesa é muito severa, em casos onde apenas esse patógeno é considerado. A sobrevivência devido ao tratamento térmico ineficaz e crescimento de *Salmonella* durante a etapa de resfriamento teve impacto relevante sobre as estimativas de risco e, portanto, tais etapas de processamento devem continuar a ser consideradas pontos críticos durante o preparo de almôndegas. No entanto, novos conhecimento sobre os limites críticos foi obtido, por exemplo resfriamento de almôndegas abaixo de 24°C, por períodos inferiores a três horas, não permite o crescimento das células

remanescentes de *Salmonella*. Além disso, a prevalência e a concentração de *Salmonella* representaram os fatores de maior impacto nas estimativas de risco, o que justifica os esforços para mantê-los baixos. A estrutura flexível do modelo de QMRA permite a análise de diversos cenários o que, conseqüentemente, torna possível expandir a utilização desse modelo, nas investigações de surtos alimentares, desenvolvimento de produto e até outras atividades relacionadas à segurança dos alimentos, como verificar a eficiência das recomendações das autoridades e estabelecer limites críticos.

Dois dos modelos preditivos utilizados para construir o modelo de QMRA (MANUSCRIPT II) também foram desenvolvidos nesta tese:

- A contaminação cruzada de *Salmonella* durante a moagem da carne suína foi modelada com êxito no processamento de até 110 fatias de carne, correspondentes a 21kg (PAPER I). Este modelo inclui fatias contaminadas antes da moagem e dá explicações claras sobre todos os parâmetros envolvidos, fornecendo uma visão geral da dinâmica no processo de moagem. A estrutura do modelo e, particularmente, a sua capacidade para prever o fenômeno caudal representado pelas porções com baixo nível de contaminação, parece ser relevante para diferentes processos de contaminação cruzada.
- A interferência da microbiota natural de suíno sobre o crescimento de *Salmonella* foi modelada e prevista durante o armazenamento de carne moída, em temperaturas entre 4°C e 38°C (MANUSCRIPT I). Foi observado o contínuo crescimento da *Salmonella*, após a microbiota natural ter atingido a sua densidade populacional máxima. Este efeito foi descrito com sucesso pelo complexo modelo de interação de espécies Lotka-Volterra e a nova versão expandida do modelo do efeito de Jameson, mas não pela versão clássica do modelo do efeito de Jameson. A nova versão expandida do modelo do efeito de Jameson é mais simples e mais prática para ser utilizada e, portanto, preferível em relação ao de Lotka-Volterra. O desempenho destes dois modelos é dependente da faixa de temperatura, apresentando boa performance entre 15,1°C e 20,2°C.

A combinação de dados obtidos dos estudos observacionais, nos modelos especificamente desenvolvidos para estudar a contaminação cruzada e o crescimento de *Salmonella* em carne suína (PAPER I e MANUSCRIPT I), bem como os dados da literatura referentes ao comportamento de *Salmonella* em tipos diferentes de carne, resultou em nova abordagem, que pode melhorar a qualidade das estimativas nas avaliações de risco, relacionadas à *Salmonella* na carne suína processada no setor de serviços de alimentação.

List of publications

Papers in peer-reviewed journals included in the thesis:

1. **C.O.A. Møller**, M.J. Nauta, B.B. Christensen, P. Dalgaard, T. B. Hansen (2012) Modelling transfer of *Salmonella* Typhimurium DT104 during simulation of grinding of pork. *Journal of Applied Microbiology* **112**, 90-98.
2. **Møller C.O.A.**, Ilg Y., Aabo S., Dalgaard P., Christensen B.B., Hansen T. B., **(submitted)**. Effect of natural microbiota on growth of *Salmonella* spp. in fresh pork – a predictive microbiology approach. *Journal of Food Microbiology*.
3. **Møller C.O.A.**, Nauta M.J., Schaffner D.W., Christensen B.B., Dalgaard P., Hansen T. B., **(in preparation)**. Risk assessment of *Salmonella* spp. in Danish meatballs produced at the catering sector.

Notes of unpublished studies

1. **Møller C.O.A.** Using a two species interaction model for quantifying levels of *Salmonella* Typhimurium DT104 under detection limit in minced pork by cold-enrichment.
2. **Møller C.O.A.**, Facing safety against quality in fresh minced pork by quantifying the potential for *Salmonella* growth within the shelf-life period

Conference presentations (poster or oral):

1. **Møller C.O.A.**, Kampmann Y., Aabo S., Christensen B.B., Dalgaard P., Hansen T. B. “Predicting growth of *Salmonella* in fresh pork products”. Coldchain management - 3rd International Workshop, Bonn, Germany, June 2^{sd}-3rd, 2008. Oral presentation.
2. **Møller C.O.A.**, Kampmann Y., Aabo S., Christensen B.B., Dalgaard P., Hansen T. B. “Predicting growth of *Salmonella* in fresh pork products”. 6th LMC Symposium in Food Microbiology, Helsingør, Denmark, May 7th-8th, 2008. Poster presentation.
3. **Møller C.O.A.**, Hansen T.B., Andersen J.K. “Using ComBase Predictor and Pathogen Modeling Program as support tools in outbreak investigation: an example from Denmark” 6th International Conference on Predictive Modeling in Foods, Washington DC, USA, September 8th-12th, 2009. Poster presentation.
4. **Møller C.O.A.**, Dalgaard P., Christensen B.B., Hansen T. B. “Effect of natural microbiota on growth of *Salmonella* in fresh pork meat – a predictive microbiology approach”. 22nd International ICFMH Symposium – Food Micro. Copenhagen, Denmark, August 30th-September 3th, 2010. Poster presentation.
5. **Møller C.O.A.**, Ilg, Y., Aabo, S., Dalgaard P., Christensen B.B., Hansen, T.B., “Quantifying the effect of natural microbiota on growth of *Salmonella* Typhimurium DT104 and *Salmonella* Derby in fresh pork”. 9th International Conference on the Epidemiology and Control of biological, chemical and physical hazards in pigs and pork – SafePork. Maastricht, The Netherlands, June 19th-22^{sd} 2011. Poster presentation

6. **Møller C.O.A.**, Nauta M.J., Christensen B.B., Dalgaard P., Hansen T. B. “Modelling transfer of *Salmonella* during simulation of grinding of pork with natural microbiota”. 7th International Conference on Predictive Modelling in Foods. Dublin, Ireland, September 12-15, 2011. Oral presentation.

Chapter 1

Introduction

1 Introduction

Salmonella has been reported as one of the most critical pathogens causing human foodborne diseases in industrialized countries (CDC, 2011; EFSA, 2010a). This pathogen is generally transmitted via the food chain through foods of animal origin, such as eggs, chicken, pork or beef (Pires et al., 2010). The application of elaborated intervention strategies in the primary sector ranging from feed control to vaccination has reduced human salmonellosis in Europe since 2003. In Denmark, bacteriological testing has indicated that the herd infection level was reduced by 50 % (from 14.7 % to 7.2 % in small herds and from 22.2 % to 10.4 % in large herds) in the time period between 1993 (when the programme was implemented) and 1998. In the same period, the level of *Salmonella* contamination in pork products, as determined by the routine monitoring programme, was reduced from 3 % to <1 % (EFSA, 2010a; Wegener et al., 2003). Nevertheless, pork still contributes significantly to the public health disease burden caused by *Salmonella* infections in European countries (van Hoek et al., 2012).

In addition, the World Health Organization - WHO (2007) estimated that up to 30 % of individuals in developed countries become ill from contaminated food or water each year. Up to 70 % of these illnesses are estimated to be linked to food prepared by food service establishments (Filion and Powell, 2011; Hensen et al., 2006; Jones et al., 2004; Lee and Middleton, 2003) pointing at food prepared outside of home as a significant source of foodborne illnesses (Jones and Angulo, 2006). Considering the increasing number of meals served outside the home every year, food safety becomes crucial in the catering sector (Poumeyrol et al., 2012). In many countries food safety guidelines have been established specifically for food service operations, e.g. time/temperature guidelines for heating, hot-holding, cooling and chilled storage. These guidelines are enforced through self-inspection systems as well as routine inspections in order to prevent catering-associated foodborne disease outbreaks (Filion and Powell, 2011). Nevertheless, outbreaks occur and, e.g. in Denmark, 55 of 77 reported outbreaks in 2010 were associated with outside-the-home settings such as restaurants, canteens, hotels, schools, shops, institutions and sport events (anonymous, 2011).

The present thesis was undertaken to propose models to evaluate the safety of pork products in food service sector and suggests tools for food producers and risk assessors to obtain more accurate risk estimates related to *Salmonella* through pork processing in food service establishments.

1.1 Objectives of the study

In catering processing, as for food production lines in general, product safety is challenged as results of i) spread, ii) growth and iii) survival of pathogens. In the present thesis the experimental work was therefore related to these aspects.

The general aim was to develop modelling tools that make it possible to quantify and assess the changes in concentration of *Salmonella*, from a selected raw pork product through catering process to a final meal, and based on scenario analysis to evaluate existing practices related to food safety of the final meals.

In relation to spread and growth of *Salmonella* during pork processing in catering, the specific objectives of this study were:

- To understand, model and predict the dynamics involved in transfer of *Salmonella* through pork grinding.
- To model and predict the effect of the pork natural microbiota on growth of *Salmonella* during storage of ground pork at different conditions of temperature abuse.

In relation to survival of *Salmonella* during pork processing in catering, the objective was:

- To apply the developed transfer and growth models in a Quantitative Microbiological Risk Assessment (QMRA), in order to evaluate the risk of consuming 'frikadeller', the popular Danish version of meatballs with ground pork, processed by the catering sector.
- To apply the developed QMRA in order to challenge the efficiency of different Food Safety Authorities recommendations related to heat treatment for inactivation of *Salmonella*.

1.2 Outline of performed work

The work was started by investigating and modelling the *Salmonella* transfer during mimicking of a semi-industrial grinding process. The transfer was measured from one piece (approximately 1.000 g) of pork (cut in five 200-g-slices) inoculated with *S. Typhimurium* DT104 to the following up to 20 *Salmonella* free pieces (cut in 100 slices). In addition, the effect of natural microbiota from ground pork on growth of *Salmonella* cocktail (*S. Typhimurium* DT104, *S. Typhimurium* DT12 and *S. Derby*) was examined during storage at different temperatures from 9.4 to 24.1°C and then modelled by two species interaction models. Finally, observational studies were performed in two units from the catering sector to ensure a detailed understanding of the flow diagram of production

of the selected pork product, Danish meatballs, and for collection of time and temperature profiles from each stage of the process.

The data obtained in these studies was finally applied to a risk assessment modelling in order to describe how predictive models on transfer, growth and survival of *Salmonella* can be functional tools in risk assessment and, subsequently, be used to assess the risk associated with the presence of *Salmonella* in the post-processing environment of the catering sector. Figure 1 shows a diagram of the experimental and modelling activities performed in this thesis.

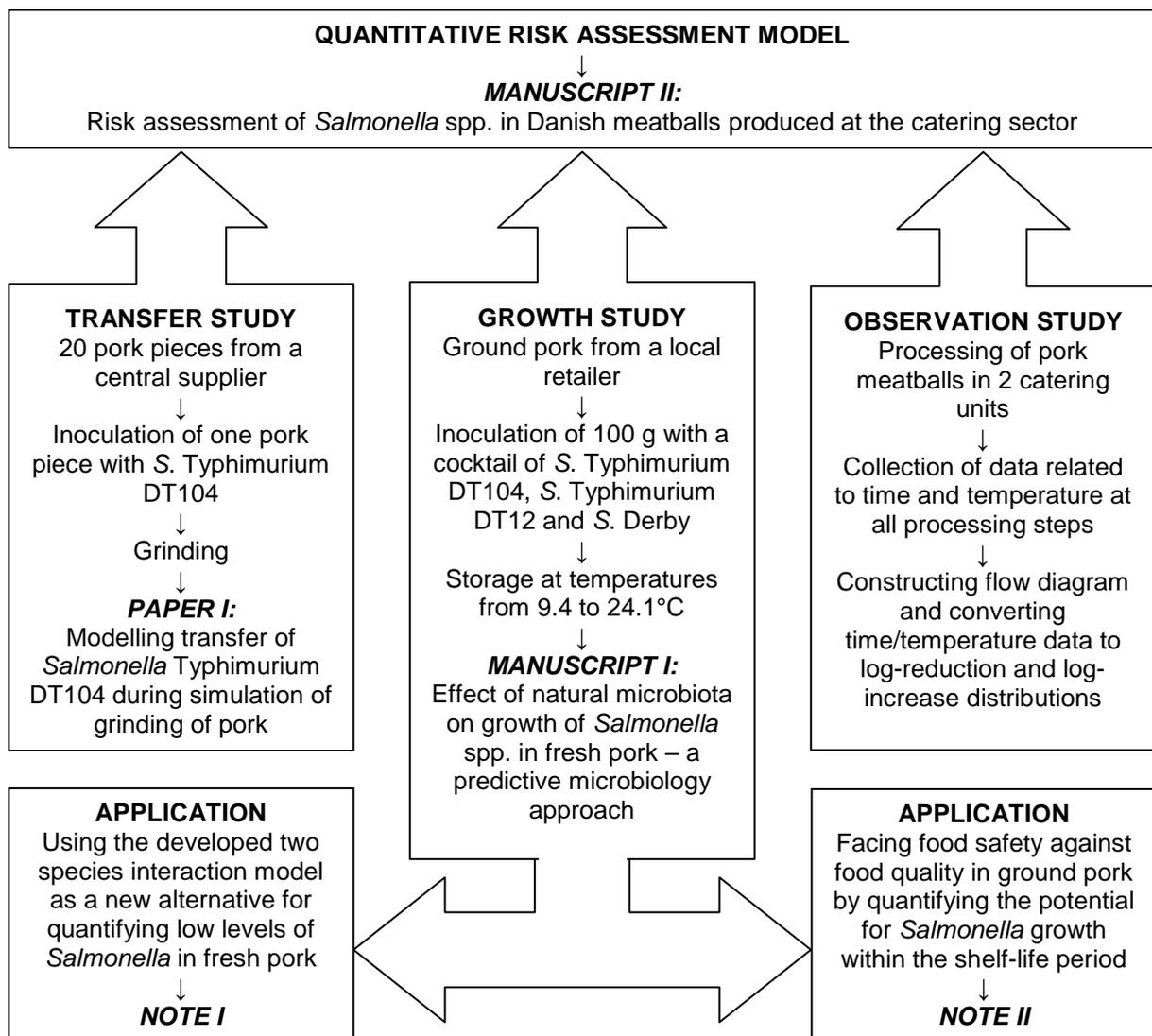


Figure 1. Outline of the experimental work and modelling activities presented in the thesis.

Chapter 2

Salmonella

2 *Salmonella*

Salmonella are rod shaped motile bacteria. They are Gram-negative, non-spore-forming and facultative anaerobic (Bergey, 1994). According to the Centers for Disease Control and Prevention (CDC) system, the genus *Salmonella* contains two species, each of which contains multiple serotypes. The two species are *S. enterica* and *S. bongori* (Brenner et al., 2000). *S. enterica* is divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salmae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica* (Popoff and Le Minor 1997; Reeves et al., 1989). Today, there are more than 2,500 known serotypes of *Salmonella* (WHO, 2005). Two of the most frequent serotypes in Danish herds are *S. Derby* and *S. Typhimurium*, representing 39 % and 21 % of the isolates, respectively (Anonymous, 2010). Therefore, these two serotypes were considered in this thesis represented by three different pig isolates originating from Denmark. The three isolates were from the genus *Salmonella*, species *enterica* and subspecies *enterica*, and two of those were serotype Typhimurium, Definitive Type 104 (DT104) and 12 (DT12), while the last isolate was serotype Derby.

2.1 Clinical aspects of salmonellosis

Salmonella enterica is a frequent agent of foodborne infections with the ability to cause a spectrum of diseases ranging from self-limiting gastroenteritis to the life-threatening systemic disease, typhoid fever (Fierer and Guiney, 2001). The disease outcome is mainly dependent on the serotype of *S. enterica* encountered. Gastrointestinal infections by *Salmonella* are a global problem and primarily caused by serotypes such as Enteritidis and Typhimurium (Jantsch et al., 2011). The incubation period ranges from 5 h to 5 days, but signs and symptoms usually begin 12-36 h after ingestion of a contaminated food. The shorter incubation periods are usually associated with higher doses of the pathogen or highly susceptible persons. Signs and symptoms include diarrhoea, nausea, abdominal pain, mild fever and chills. Sometimes vomiting, prostration, anorexia, headache and malaise occur. The syndrome usually lasts 2-5 days (ICMSF, 1996).

2.2 Source and transmission

Salmonella spp. are found worldwide and are universally recognized as zoonotic agents. Numerous animal reservoirs have been identified. The various serotypes of *S. enterica* show remarkable differences in their host range and specificity, with serotype Typhi being specific for humans and primates and serotypes Enteritidis or Typhimurium mainly infect humans, livestock animals, and various wild animals. *Salmonella* infections can also result in a carrier state with

patients shedding *Salmonella* with faeces for prolonged periods of time after asymptomatic encounter or acute disease (Jantsch et al., 2011).

Food, feed and water are the primary vehicles (ICMSF, 1996). Particularly foods of animal origin, and those subjected to sewage pollution, have been identified as vehicles for transmitting these pathogens to human beings and spreading them to processing and kitchen environments. *Salmonella* spp. reside in the intestinal tract of infected animals (including human beings). They are shed in the faeces and can be transmitted by contact to the hands of human beings following bowel movements and the feet, hair and skin of animals as they walk, sit or lie on faecally contaminated ground or litter.

Salmonella has been linked to many cases of foodborne illness across the world, and it is considered to be one of the main agents causing human gastroenteritis (Jiménez et al., 2009). In Denmark, the locally produced pork has been estimated as one of the most important sources of salmonellosis since the 1990s (Anonymous, 2004). In response to the rising number of human cases of salmonellosis, a Surveillance and Control Programme for Danish pork has been developed and implemented, generating knowledge both from laboratory and large-scale field studies (Mousing et al., 1997; Nielsen et al., 2001; Alban et al., 2002). Despite the reduction of the level of contamination of *Salmonella* in pork and the number of cases of salmonellosis nowadays (Baptista et al., 2010), *Salmonella* still remain to be controlled. In addition, as pointed out by Alban et al., (2012), the current Danish surveillance and control programme for *Salmonella* in pigs and pork does not include cutting plants and retailers. The most recent figures describing the prevalence of *Salmonella* in Danish pork measured at the retailers indicate that the prevalence at retail can be three to five times higher than at the abattoir. Several factors may have contributed to this, but the results underline the need for data as well as attention to hygiene throughout the entire food chain (Hansen et al., 2010). Therefore, the behaviour of *Salmonella* in pork processing has been the focus in the present thesis.

2.3 *Salmonella* in pork processing

Most countries in the world have a non-negligible prevalence of *Salmonella*, which makes it difficult for them to go for an eradication strategy (Alban et al., 2012). In the United States of America no pre-harvest surveillance exists, but decontamination at the abattoir has been in place for many years. Contrary, Sweden, Norway and Finland have implemented pre-harvest surveillance programmes in combination with an eradication strategy (Carlsson and Elvander, 2009; Jore et al., 2009, Huttunen et al., 2006), and therefore these countries have a very low prevalence of *Salmonella*. A prevalence of *Salmonella* in the main stages of pork production in Denmark is given in Figure 2.

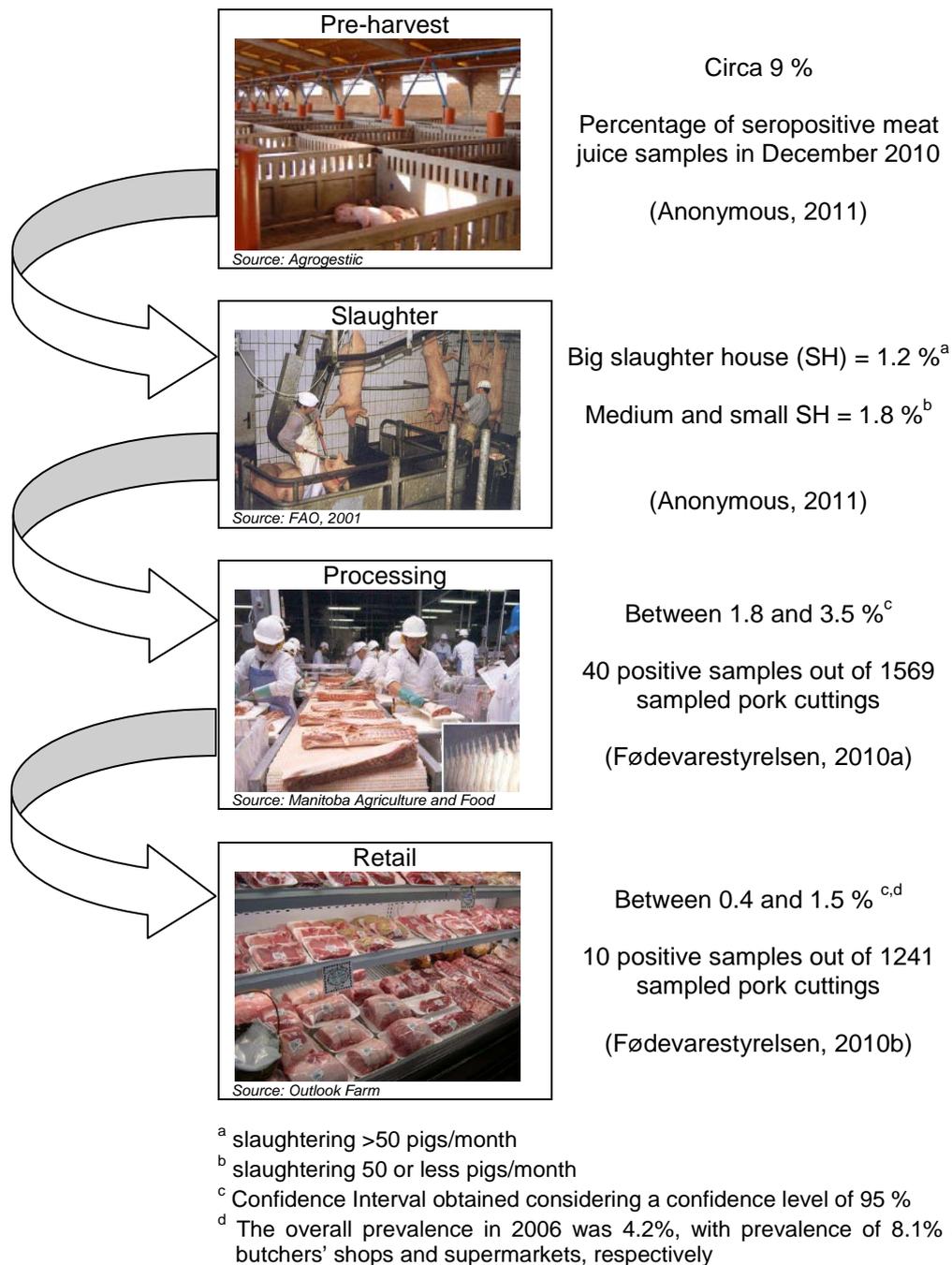


Figure 2. Prevalence of *Salmonella* in 2010 at different points of the production chain of pork in Denmark, according to Anonymous (2011) and Fødevarestyrelsen (2012a, 2012b).

In 2011, pork was estimated to attribute from 3.7 % to 11.2 % of salmonellosis cases in Denmark (Anonymous, 2012). Despite the fact that different sampling techniques were applied and unlike prevalence were obtained (serological or bacteriological), it is shown in Figure 2 that *Salmonella*

have been detected in all the main stages of pork production in Denmark, which indicates the importance of control measures in all stages of pork production.

Outbreaks of *Salmonella* infections in humans in the last twenty years have been the main trigger for intensified efforts to improve slaughter hygiene (post-harvest) and on-farm surveillance and control (Visscher et al., 2011). Despite the efforts, *Salmonella* continue to be one of the most important bacterial causes of acute foodborne diseases in humans (EFSA, 2010a).

Salmonella can enter the food chain at any point throughout its length, from livestock feed, via the on-farm production site, at the slaughterhouse or packing plant, in manufacturing, processing and retailing of food, through catering and food preparation in the home (Lo Fo Wong et al., 2002).

The slaughter process is a critical step of the pig meat chain with respect to pig and carcass contamination, and numerous aspects still remain unknown, e.g. possible airborne transmission of *Salmonella* in the abattoir. Therefore, studies need to be performed to properly assess the ways carcasses become contaminated. At the cutting plant stage, in regard to *Salmonella* contamination and concentration all over the carcass, there seems to be shortage of data in Europe. Also during preparation and consumption of pork there are many data gaps regarding time and temperature during transport from the factory to store, from factory to retail shops, and from retail to home. There is also a need for more knowledge about temperatures at retail level and household storage (EFSA, 2010b).

The prevalence of *Salmonella* in fresh pork cuttings in Denmark in the years 2002 and 2006 was investigated at retail and compared with the retail supply pattern. The overall prevalence of *Salmonella* in pork retail cuttings during the years of 2002 and 2006 was related to 1.2 % and 4.2 %, respectively. Hence, increases around 3- to 5-fold were found but *Salmonella* was rarely detected at levels higher than 400 CFU per gram. As the significantly increased prevalence and concentration of *Salmonella* could not be ascribed to a rise in *Salmonella* carcass prevalence at slaughter, a worsening in hygiene levels post-slaughter becomes the most probable explanation (Hansen et al., 2010).

Ground meat is one of the food items rather frequently associated with outbreaks of salmonellosis. This product is predisposed to contamination because many processing steps are involved in its manufacture, which all potentially contribute to an increase of *Salmonella* counts in the final product (Stock and Stolle, 2001).

Pork is one of the most important Danish food products to the national economy and is widely distributed across the world, indicating the need for attention and efforts in order to assure the food safety related to this product.

Salmonella remains a pervasive problem that survives well in the environment. The knowledge related to the effects of environmental factors on physiology of *Salmonella* is a key element that needs to be considered in any control strategy for *Salmonella*, and therefore these aspects will be addressed in this thesis.

2.4 Effects of environmental factors on physiology of *Salmonella* spp.

2.4.1 Growth of *Salmonella* spp.

The rate of growth of *Salmonella* is dependent on temperature, pH, salinity, water activity (a_w), and available nutrients, and reflects the synergistic and antagonistic interactions between these environmental factors (D'Aoust, 1989).

Salmonella spp. are usually grown at 35-37°C, but most *Salmonella* serotypes can grow at temperatures ranging between 5-47°C, although growth is reduced at temperatures below 10°C. Reports in literature suggest that some serotypes can grow at temperatures as low as 4°C (Kamat and Thomas, 1998; Uhart et al., 2006;), but this is not universally accepted. Lianou and Koutsoumanis (2009) have shown that the variability of growth kinetic parameters among *Salmonella* serotypes is not greater than that among strains. Growth of the serotypes Derby and Typhimurium used in the present thesis was investigated in both irradiated minced pork as well as minced pork with a natural microbiota. As shown in Figure 3, similar growth was observed from 8°C and above for both serotypes in irradiated minced pork and from 10.5°C and above in minced pork with a natural microbiota. However, the exact minimum temperature for growth, T_{min} , was not determined.

The T_{min} of *Salmonella* spp. is cardinal to the food industry because refrigeration constitutes one of the primary lines of defence against proliferation of this pathogen in the human food supply chain (D'Aoust, 1989). It has been reported that temperatures in domestic refrigerators can reach 15°C (James, 2003), however, in the present thesis, temperatures were not found to be higher than 6°C during observational studies performed in the food service sector. Although most *Salmonella* serotypes are unable to grow at refrigeration temperatures, the organism is able to survive for extended periods at chill temperatures, particularly under freezing conditions. Some foods (e.g.

raw meat) provide substantial protection from bacteria during freezing and frozen storage (Cosansu & Ayhan, 2012).

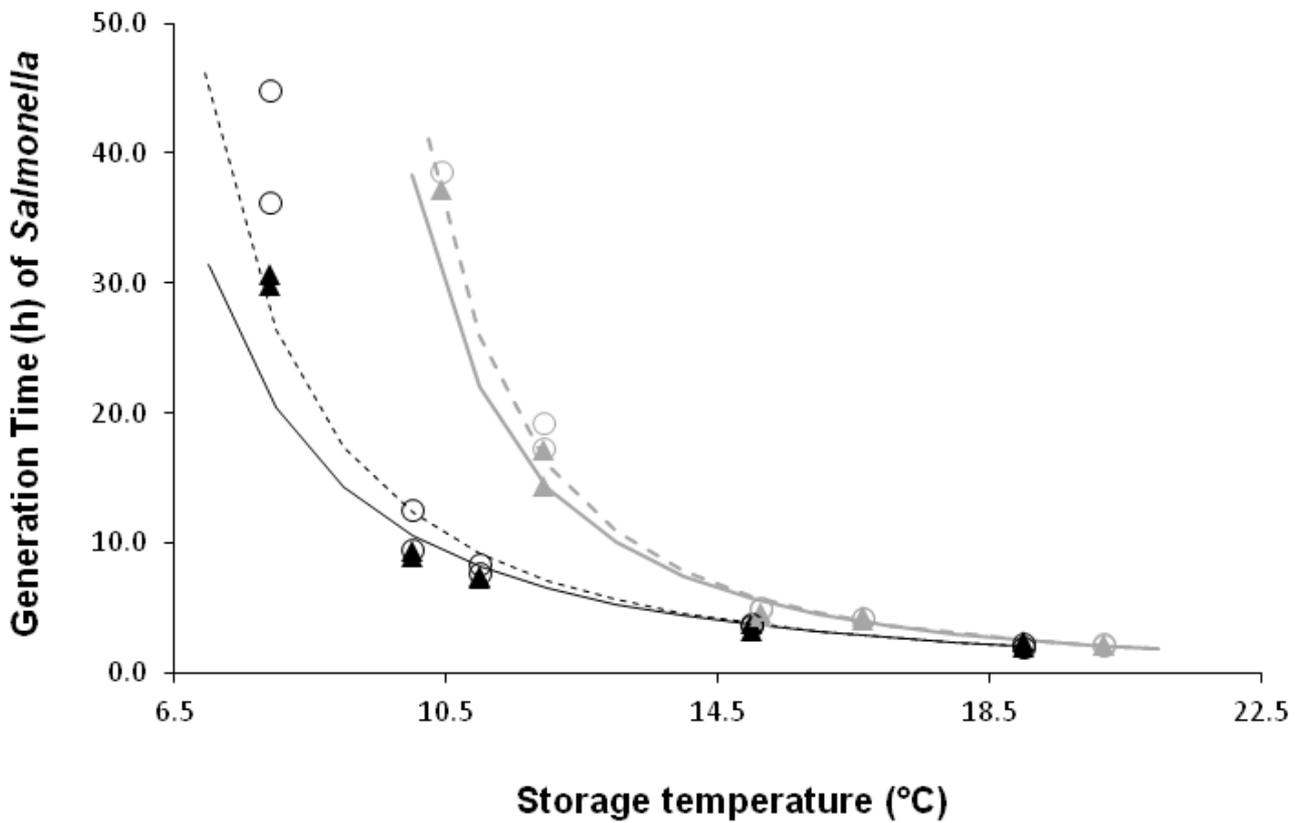


Figure 3. Influence of storage temperature (°C) on observed and fitted growth rates (*Generation Time*, h) of *Salmonella* Derby (▲, —) and *Salmonella* Typhimurium DT104 (○, - -) in irradiated minced pork (in black) and minced pork with a natural microbiota (in grey). Lines indicate models obtained from MANUSCRIPT I.

The optimal pH value for *Salmonella* growth is between 6.5 and 7.5, with possibilities for growth at pH values ranging from 4.5 to 9.0, while slow death occurs at more extreme conditions (Bryan et al., 1979). In the studies related to growth of *Salmonella* in pork in the present thesis, the pH of the ground pork during storage, ranged between 5.4 and 6.7, being around 5.8 at the beginning, follow until certain moment of the storage and increasing again later on, when the combination time/temperature of storage allowed (e.g. 96h at 10°C and 54h at 15°C) (NOTE II). Although *Salmonella* cannot grow under very acid conditions, the organism is able to survive for some time

in acid environments, e.g. $\text{pH} < 4.5$. Survival times are dependent on specific type of acid present and temperature, i.e. chilled temperatures favour survival (Xu et al., 2008).

Salmonella are able to grow at water activities down to 0.94, lower values are dependent on serotype, food sources, temperature and pH. *Salmonella* will die at water activities below those permitting growth, but inactivation can be extremely slow in some products (measured in years), particularly those with very low moisture and high fat content, such as chocolate (ICMSF, 1996).

The growth of *Salmonella* is generally inhibited in the presence of 3–4 % NaCl (w/w) in the water phase (D'Aoust, 1989). Inhibition was reported to increase in ground pork stored for 14 days at 10°C when the NaCl concentration was varied from 0 to 3.5 %, no growth was detectable for 5 % NaCl (Alford and Palumbo, 1969).

As discussed by Curtis (2007), *Salmonella* are facultative anaerobes (can grow with or without oxygen present) and growth is only slightly reduced under nitrogen. The organism is able to grow in atmospheres containing high levels of carbon dioxide (possibly up to 80 % in some conditions).

2.4.2 Survival of *Salmonella* spp.

Many factors can influence the survival of *Salmonella*, e.g. freezing, irradiation, gases, preservatives, disinfectants, drying, heat tolerance, etc. However, in this thesis, only heat inactivation of *Salmonella* is considered.

Salmonella spp. are sensitive to heat and heat-tolerant strains are rare. An example is the unique strain 775W of *S. Senftenberg*, which is considerably more tolerant than other salmonellae in moist foods. Heat tolerance is influenced by the water activity, nature of the solutes and pH of the suspending medium. Heat tolerance increases as the water activity of the substrate decreases. Reducing pH reduces heat tolerance. Higher heat tolerance is observed for cells in sucrose compared with NaCl at the same a_w values (ICMSF, 1996).

Susceptibility of *Salmonella* to thermal inactivation is frequently expressed in terms of the decimal reduction time (D-value), defined as the time required to affect a 90 % reduction in the number of viable cells at a given temperature. The z-value, the temperature increment necessary to obtain a 10-fold change in D-value in a given test system. (D'Aoust, 1989).

In a study performed by Velasquez et al. (2010) it was found that differences in *Salmonella* thermal inactivation between whole and ground pork were due only to differences in the physical structure of the meat. It seems that destruction of the original muscle structure during grinding results in a

change in water status and a more uniform distribution of the fat and protein within the product. Therefore, *Salmonella* was more heat tolerance in whole muscle than in ground pork.

Juneja et al. (2001) studied the heat tolerance of 35 *Salmonella* strains at 58 or 60°C using different substrates including commercially canned chicken broth (3 % fat), ground beef (12.45 % fat), ground pork (6.95 % fat), ground chicken (8.45 % fat), and ground turkey (8.85 % fat) and found that the D-values in meat (6.68 to 8.65 min at 58°C or 4.82 to 6.65 min at 60°C) were higher than those in chicken broth (2.98 to 1.85 min at 58°C or 1.3 to 0.75 min at 60°C). Similar conclusion was obtained by McCann et al. (2007) in a study performed in beef and broth systems at 55°C. They also found that chilled storage (4°C) of *S. Typhimurium* DT104 significantly reduced the thermal resistance of the pathogen in beef and broth systems. In the presence of high levels of gram-negative beef microbiota, the heat sensitivity of the pathogen was further increased on beef surfaces (McCann et al., 2007).

As pointed out by Velasquez et al. (2010), the literature contains limited information on thermal inactivation of *Salmonella* in ground pork, therefore, the heat inactivation models applied in the risk assessment model for pork meatballs conducted in the present thesis were based on a collection of data from heat inactivation studies applied to ground pork and also ground beef.

Chapter 3

Modelling the behaviour of *Salmonella*

3 Modelling the behaviour of *Salmonella* in food

As pointed out by McMeekin et al. (2008), a model can be defined as “the description of a system, theory, or phenomenon that accounts for its known or inferred properties and may be used for further study of its characteristics”. While, in common usage, a model is often a smaller replica of a real object, in science, engineering, finance etc., a model is an often simplified description of relationships between observations of the system (responses) and the factors that are believed to cause the observed responses. That description can be expressed in words or expressed quantitatively in one or more mathematical relationships or equations. Thus, a mathematical model can simply describe a collection of data or may represent a hypothesis or series of hypotheses about underlying relationships among the independent variables that lead to the observations or data. The first approach is often termed an ‘empirical’ model, while the latter is described as ‘mechanistic’. Both approaches have utility: the first simply to summarise data and the latter to summarise “understanding” or knowledge. Either can be used to predict the response of the system to changes in the variables (McMeekin et al., 2008).

Many different researchers have developed models for the behaviour of particular microorganisms in laboratory media and different food matrices, and made them available in the public domain through peer reviewed publications (Ross and Dalgaard, 2004). From an industry perspective, the utility of these public-domain models is often somewhat limited. In many cases, models have been developed under laboratory conditions, are based on specific combinations of parameters that might not be appropriate for the particular food products of an industry, and have not always been validated or even used in real food systems. Despite that, such models can be useful as long as their limitations are recognised and considered in their application (Membré and Lambert, 2008).

Changes in concentrations of *Salmonella* have been modelled at various points of the food chain. The behaviour of *Salmonella* during cross contamination has been studied by Mattick et al. (2003), where chicken and kitchen surfaces were investigated, respectively. Growth of *Salmonella* has been investigated by several authors in chicken and beef (Oscar, 1999a, 1999b, 2002, 2003, 2005, 2006, 2009; Juneja et al., 2007, 2009; Dominguez and Schaffner, 2008). Heat inactivation of *Salmonella* in ground beef and pork has also been examined (Juneja et al. 2001; Smith et al. 2001; Murphy et al. 2004; Juneja et al. 2010; Velasquez et al. 2010). However, these critical events, considering the behaviour of *Salmonella* in ground pork processed by the catering sector, still remain to be explored. As mentioned previously the transfer, growth and survival of *Salmonella* are the main subjects discussed in this work. The following three chapters will give a brief description of the factors involved in the selection of models applied to the performed risk assessment of *Salmonella* in pork processing at the catering sector.

3.1 Transfer of *Salmonella*

Salmonella contamination can occur at different points of the farm to fork continuum; at farm level through contaminated feed, premises, equipment, facilities or personnel (Forshell and Wierup, 2006; Mannion et al., 2007), during transport via *Salmonella* contaminated trucks (Mannion et al., 2008; Dorr et al., 2009) and in slaughterhouses as a result of poor dressing techniques or environmental contamination (Botteldoorn et al., 2003; Prendergast et al., 2008; Duggan et al., 2010). Once contamination with *Salmonella* takes place, there are many opportunities for cross contamination through the food chain to contaminate pork and pork products (Prendergast et al., 2009).

Most single-intervention and control measures are not sufficiently effective to reduce or remove *Salmonella* infection or contamination from the pig production chain. It is only by using a holistic approach that the number of cases of human salmonellosis can be reduced effectively. It is therefore, recommended that an intervention and control strategy be formulated, based on a combination of measures that are both practically and economically feasible (Delhalle et al., 2009).

Studies that simulate and model the distribution of pathogens during processing operations are of major relevance to risk analysts to ascertain the importance of equipment sanitation, sources of potential product contamination and improved equipment design (Flores et al., 2006). Table 1 shows a selection of cross contamination studies published from 1990 to 2012. At the beginning of the cross contamination modelling, the centre of attention was the consumers' behaviour and the transfer of pathogens for single events. Later on, the main purpose in modelling was related to providing distributions for single events of pathogen transfer to model consumers' behaviour in risk assessment investigations. Furthermore, the focus of modelling deflected from the consumer to the industrial processing, by starting risk assessment with inclusion of multiple cross contamination routes through the processing. At this point the main goal was to be able to model the prevalence changes in final products. Afterwards, this aim was extended to investigate the whole processing by applying empirical models. Recently, modellers have invested efforts in developing mechanistic models that are able to give better understanding of the dynamics involved in the events related to transfer of pathogens at the food processing.

A number of cross contamination models (Table 1) have been published describing transfer of *Listeria monocytogenes* (Vorst et al., 2006; Aarnisalo et al., 2007; Keskinen et al. 2008; Sheen, 2008), *Escherichia coli* O157:H7 (Pérez-Rodríguez et al., 2007; Sheen and Hwang 2010), and *Staphylococcus aureus* (Pérez-Rodríguez et al., 2007) during slicing of ready-to-eat products.

Table 1. Selected cross-contamination studies demonstrating the progress within this research area from 1990 to 2012.

Publication Year and authors	Process	Pathogen	Matrix	Model	Legends
de Boer and Hahné (1990)	Handling involving cutting boards and hands	<i>Salmonella</i> spp. <i>Campylobacter jejuni</i>	Raw chicken products	Transfer percentages of single events presented in text. Modelling not relevant.	Not relevant
Zhao et al. (1998)	Handling involving cutting boards and hands	<i>Enterobacter aerogenes</i> (indicator organism for <i>Salmonella</i>)	Chicken breast with skin Lettuce Cucumbers	Transfer (\log_{10} CFU/g), depending on disinfectant presented in table but no model was developed.	Not relevant
Chen et al. (2001)	Handling involving hand, cutting board and spigot	<i>Enterobacter aerogenes</i> (indicator organism for <i>Salmonella</i>)	Chicken Lettuce	Transfer percentages of single events presented in tables. Variability distributions were derived.	Not relevant
Flores and Tamplin (2002)	Grinding	<i>E. coli</i> O157:H7	Raw beef	Transfer (\log_{10} CFU/ml/g) presented in graphs but no model was developed	Not relevant
Rosenquist et al. (2003)	Handling involving cutting board	<i>Campylobacter</i>	Raw chicken Lettuce Prepared chicken	Distributions of single-event transfer percentages were used in risk modelling.	Not relevant
Ivanek et al. (2004)	Slicing	<i>Listeria monocytogenes</i>	Smoked fish	A compartmental mathematical model of cross-contamination among food products, food contact surfaces, employees' hands and the environment with which hands come in contact was developed to describe transmission over processing time with prevalence as output.	Not relevant

Publication Year and authors	Process	Pathogen	Matrix	Model	Legends
Nauta et al. (2005)	Slaughter	Campylobacter	Poultry carcass	$\begin{cases} N_{\text{ext},S}(i) = (1 - a_{\text{ext},S})(1 - c_{\text{ext},S}) N_{\text{ext},S-1}(i) \\ \quad + b_{\text{env},S} N_{\text{env},S} (1 - i) \\ \quad + (1 - a_{\text{fec},S}) N_{\text{fec},S}(i) \\ N_{\text{env},S}(i) = a_{\text{ext},S} N_{\text{ext},S-1}(i) \\ \quad + (1 - b_{\text{env},S}) (1 - c_{\text{env},S}) N_{\text{fec},S}(i) \\ \quad + a_{\text{fec},S} N_{\text{fec},S}(i) \end{cases}$	<p>$N_{\text{ext},S}(i)$ – number of campylobacters on carcass i at the end of stage S (and thus at the start of stage $S + 1$)</p> <p>$N_{\text{env},S}(i)$ – number of campylobacters in the environment after passage of carcass i (and thus prior to the entering carcass $i + 1$) in stage S</p> <p>$N_{\text{fec},S}(i)$ – number of campylobacters in the leaking feces of carcass i at stage S, that is the product of the concentration of campylobacter in the feces and the mass of leaking feces, $w_{\text{fec},S}(i) \times C_{\text{fec}}(i)$.</p> <p>Processing stages are linked as the output of stage $S - 1, N_{\text{ext},S-1}(i)$, is the input of stage S.</p>
Vorst et al. (2006)	Slicing	<i>Listeria monocytogenes</i>	Turkey breast Bologna Salami	Transfer (\log_{10} CFU/slice) presented in graphs but no model was developed	Not relevant
Aarnisalo et al. (2007)	Slicing	<i>Listeria monocytogenes</i>	'Gravad' salmon	$Y = a \cdot e^{(-X/b)}$	<p>Y – <i>L. monocytogenes</i> counts (\log_{10} CFU/g)</p> <p>X – Slice no.</p> <p>a, b – Constants</p>
Pérez-Rodríguez et al. (2007)	Slicing	<i>E. coli</i> O157:H7 <i>S. aureus</i>	Cooked meat product	<p>For low pathogen concentration: $\text{Log}(I_{\text{slice}}) = \text{log}(I_{\text{blade}}) - k \cdot N_{\text{slice}}/\ln(10)$</p> <p>For high pathogen concentration: $\text{Log}(I_{\text{slice}}) = \text{log}(I_{\text{blade}}) - (N_{\text{slice}}/a)^b$</p>	<p>N_{slice} – Slice no. ($N_{\text{slice}} = 1, 2, \dots, 20$)</p> <p>$I_{\text{slice}}$ – Pathogen concentration (\log_{10} CFU/cm²) on the slice</p> <p>I_{blade} – Regression parameter (the intercept)</p> <p>$k/\ln(10)$ – Regression parameter related to the slope through the calculation</p> <p>a – Parameter considered as a reaction rate constant</p> <p>b – Parameter behaviour index</p>

Publication Year and authors	Process	Pathogen	Matrix	Model	Legends
Keskinen et al. (2008)	Slicing	<i>Listeria monocytogenes</i>	Roast turkey breast	Transfer (\log_{10} CFU/slice), depending on biofilm formation, presented in graphs but no model was developed	Not relevant
Sheen (2008)	Slicing	<i>Listeria monocytogenes</i>	Salami	$Y = A \cdot e^{(-X/B)}$ with $A = a \cdot e^{(b \cdot n)}$ and $B = c \cdot n^{(d)}$ for inoculated blade to product or $A = a \cdot e^{(b \cdot n)}$ and $B = c \cdot e^{(d \cdot n)}$ for inoculated product to blade and back to product	Y – <i>L. monocytogenes</i> counts (\log_{10} CFU/slice) X – Slice no. n – Inoculation level (\log_{10} CFU/slice or \log_{10} CFU/blade) a, b, c, d – Constants to be estimated by fitting
Sheen & Hwang (2008)	Slicing	<i>Listeria monocytogenes</i>	Ham	$TR = dY / dt = dY / dt \cdot dX / dt$ with $dX / dt = k$ and $Y = Y_0 \cdot e^{(-X/B)}$ yields $TR = -dY / dt = Y_0 / B \cdot k \cdot e^{(-k \cdot t/B)}$	TR – transfer rate (\log_{10} CFU/min) Y – <i>L. monocytogenes</i> counts (\log_{10} CFU/slice) at slicing time, t (min) Y_0 – Initial <i>L. monocytogenes</i> counts (\log_{10} CFU/slice) k – Slicing rate (slices/min) B – Constant
Jiménez et al. (2009)	Handling involving cutting board	<i>S. Hadar</i>	Chicken skin	Transfer percentages, depending on carcass decontamination and storage temperature, presented in graphs but no model was developed	Not relevant
Sheen & Hwang (2010)	Slicing	<i>E. coli</i> O157:H7	Ham	From blade to product $Y = A \cdot X^B$ Form product to blade and back to product $Y = C \cdot e^{(-X/D)}$	Y – <i>E. coli</i> O157:H7 counts (\log_{10} CFU/slice) X – Slice no. A, B, C, D – Constants to be estimated by fitting for each inoculation level

Publication	Process	Pathogen	Matrix	Model	Legends
Møller et al. - PAPER I (2012)	Grinding	S. Typhimurium DT104	Raw pork	$\begin{cases} M_i = (1-a_1)(1-a_2)(1-c_2) S_i + (b_1 gr_{1,i-1}) + (b_2 gr_{2,i-1}) \\ gr_{1,i} = a_1 S_i + (1-b_1) (1-c_1) gr_{1,i-1} \\ gr_{2,i} = a_2 S_i + (1-b_2) (1-c_3) gr_{2,i-1} \end{cases}$	<p>M_i – number of <i>Salmonella</i> on ground meat portion i</p> <p>a_1 – transfer from meat slice to Environment1 (E1)</p> <p>a_2 – transfer from meat slice to Environment 2 (E2)</p> <p>b_1 – transfer from E1 to ground meat</p> <p>b_2 – transfer from E2 to ground meat</p> <p>c_1, c_2, c_3 – inactivation in E1, meat and E2, respectively</p> <p>gr_1, gr_2 – grinder environment 1 and 2, respectively</p>

Studies concerning transfer of *Salmonella* from chicken carcasses to cutting boards (Jiménez et al., 2009), from domestic washing-up sponge to kitchen surfaces and food (Mattick et al., 2003) and from raw chicken products during food preparation involving hands and cutting boards (de Boer and Hahné, 1990) have also been done. However, not much has been investigated or published regarding cross contamination events in fresh meat processing such as ground pork. Nevertheless, Flores and Tamplin (2002) have published a study related to transfer of *E. coli* O157:H7 during grinding of raw beef, but no model was developed.

As shown in Table 1, a remarkable approach was developed by Nauta et al. in 2005 to investigate and model the cross contamination of *Campylobacter* in poultry processing. Though not mechanistic on a detailed level, the model incorporates some mechanistic essential for the dynamics of the transmission of bacteria through the industrial-processing system. This provides insights into the effect of processing on numbers of bacteria per carcass that cannot be obtained from microbiological data and linear models alone. Therefore, a modified version of this modelling approach was suggested (Møller et al., 2012 - PAPER I) to be applied to the data obtained throughout the simulation of grinding of pork, which was performed according to observations of the practices in the catering sector.

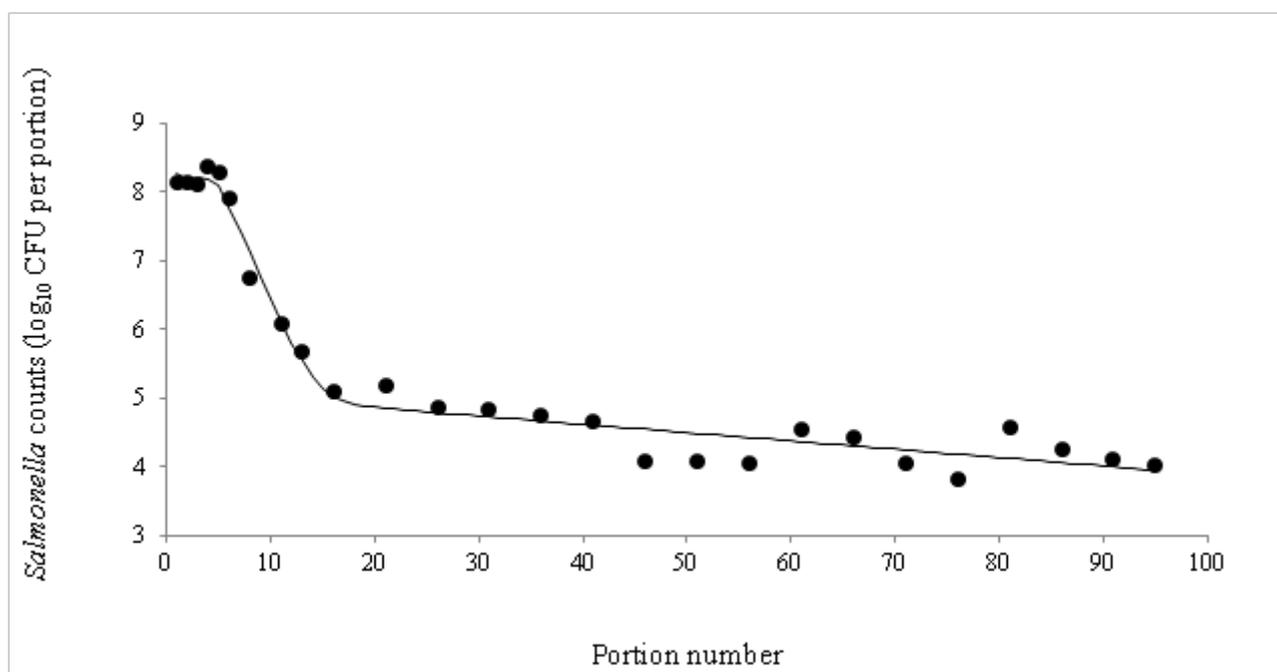


Figure 4. Transfer of *Salmonella* Typhimurium DT104 during grinding [five contaminated pork slices (10^9 log₁₀ CFU per slice) to grinder to uncontaminated pork slices] of 95 processed slices. The observed values (●) were fitted with the suggested modified version of Nauta et al. (2005) 5p-2ge model with 5 parameters considering two grinder environments (—) (adopted from Møller et al. 2012 - PAPER I).

Interestingly, a tailing phenomenon of the transfer of *Salmonella* was observed during the small-scale grinding process (Figure 4). A similar distinct tailing phenomenon during cross-contamination was observed in different studies (Flores and Tamplin, 2002; Vorst et al., 2006; Aarnisalo et al., 2007; Sheen, 2008; Sheen and Hwang, 2010). Considering the low RMSE and AICc-values supported by the F-tests results, the developed model (Møller et al., 2012 – PAPER I) satisfactorily predicted the observed behaviour of *Salmonella* during its cross contamination in the grinding of up to 110 pork slices corresponding to 21 kg meat. The tailing phenomenon occurred independently of the temperature applied to the processing, since no variation of the results was observed when performing the grinding of pork at 4°C or at 22°C (data not shown).

In the study of Sheen and Hwang (2010), the cross contamination of *E. coli* O157:H7 during slicing of ready-to-eat ham was modelled applying an empirical approach and the selected model characterized the transfer as decreasing following exponential law. Comparison of the model published by Sheen and Hwang (2010) to the model developed in the present study (Møller et al., 2012 – PAPER I) showed that the two models are mathematically similar. Despite this similarity, the proposed model should be preferred as it includes the pieces of meat that are contaminated before grinding and it gives clear explanations of all the parameters involved giving an overview of the dynamics of a grinding process. As it is easier to understand, it also holds the potential to be universal, *i.e.* transferrable to cross contamination dynamics for other food processing steps. The fitted model obtained in this study is of course specific to the studied grinding process including the particular grinder applied. However, the structure of the model, and particularly its ability to predict the tailing phenomenon, seems relevant for different cross contamination processes.

Testing the model structure on data published in other transfer studies, where different food products, microorganisms, concentration of pathogen and different routes of contamination (food product to slicer to food product or slicer to food product, using the same product or different products) were used, showed promising results (Figure 5) as those obtained by applying the suggested model (Møller et al., 2012 – PAPER I) to the data related to cross contamination of *E. coli* O157:7 during slicing of ham, published by Sheen and Hwang (2010). In addition, when applying the proposed 5p-2ge (Møller et al., 2012 – PAPER I) to literature data, improved R^2 values were obtained, which illustrates the promising results. For example, when applying the data published by Vorst et al. (2006), simulating cross contamination of *L. monocytogenes* during turkey slicing, $R^2 = 0.86$ was found, and when the data presented by Aarnisalo et al. (2007), regarding transfer of *L. monocytogenes* during slicing of gravad salmon, were used, $R^2 = 0.74$ was obtained.

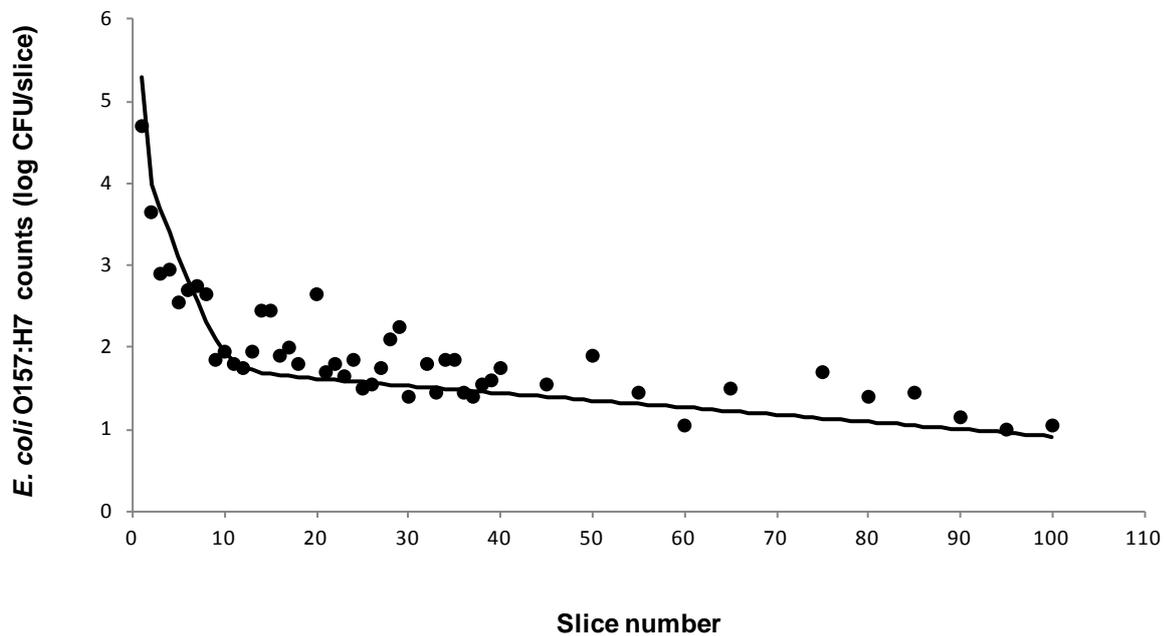


Figure 5. Transfer of *E. coli* O157:H7 (Sheen and Hwang, 2010) during slicing (contaminated blade to uncontaminated ham, 10^7 log₁₀ CFU per blade) of 100 processed slices. The observed values (●) were fitted with the suggested modified version of Nauta et al. (2005) 5p-2ge model (Møller et al., 2012 – PAPER I) with 5 parameters considering two grinder environments (—).

Despite the good performance of the model, there are some aspects related to the grinding that are not fully understood, i.e. the fact that only a fraction of the input of *Salmonella* have been found and recovered, as shown in Table 2. Since the method used to sample and detect the level of *Salmonella* in the inoculated meat has been shown to be fairly related to the level of *Salmonella* in the inoculums, it was assumed that the method of sampling and analysis performed was also effective to all the investigated meat. However, observing the tail of low contaminated portions related to the transfer of *Salmonella* during grinding, it seems that the way how *Salmonella* is organized inside the grinder could explain why most of the inoculated *Salmonella*, represented by an average of 77 % from a comparison of five experiments, could not be found. Therefore, investigations on the grinder are clearly necessary, and some efforts were applied in order to investigate the role of the grinder in the dynamics of transfer of *Salmonella*, and consequently to elucidate the mechanisms of cross contamination of pathogens during pork grinding.

Table 2. Input (CFU/slice) and Distribution (%) of *Salmonella* obtained after grinding of pork from five experiments performed in Møller et al., 2012 - PAPER I.

<i>Salmonella</i> on	Experiment ^a				
	1	2	3	4	5
Input from inoculated meat (CFU/slice)	8.68E+09	1.50E+09	4.35E+09	6.52E+09	9.54E+09
Processed meat (%)	34.24	25.44	19.50	15.42	20.39
Residual meat after grinding (%)	0.12	0.11	0.09	0.08	0.01
Grinder after processing (%) (by tape sampling)	0.00055	0.00173	0.00138	0.00001	0.00063
Not detected	65.64	74.45	80.41	84.50	79.60

^a Number of processed slices per experiment: 1 and 2) 45, 3) 95, 4) 100, and 5) 110

To increase our understanding of the dynamics of a grinding process, it would be useful to investigate the change in *Salmonella* level inside the grinder at different stages through the processing of the meat. In the present thesis, this was done in the end of grinding for each of the five experiments. Inspired from Schaffner et al. (2004), who performed a study related to cross contamination on cutting boards, a sampling technique involving “Con-Tact-It” adhesive tape, was applied. A distribution of *Salmonella* (%) inside the grinder is represented in Figure 6, indicating that the level of *Salmonella* is higher at the superior part of the machinery related to the location where the meat is inserted. However, the *Salmonella* concentration found when using this technique was not as high as the actual level of *Salmonella* inside the grinder (Table 2). Thus, the tape sampling technique was considered ineffective for this purpose and the surface material of the grinder was highlighted as a possible explanation. Since the surface inside of the grinder is rough it may support the built up of a matrix suited for capture and attachment of *Salmonella* cells for example through a fat overlay. Such an overlay would be maintained by the continuous contact with the meat throughout the period of processing, and could have increased the degree of difficulty to use tape sampling. Furthermore, the shape of some of the grinder pieces does not allow the tape sampling technique, which underlines the need for a sampling method that could physically remove the matrix with the attached *Salmonella* cells from the surface of the grinder such as washing, rinsing or swab sampling techniques. It is recognized that investigation of the

level of *Salmonella* inside the grinder need to be performed to get the whole picture of the dynamics of grinding, however, a sampling technique practical enough to be used throughout the grinding process which effectively represents the *Salmonella* counts in the grinder still need to be determined.

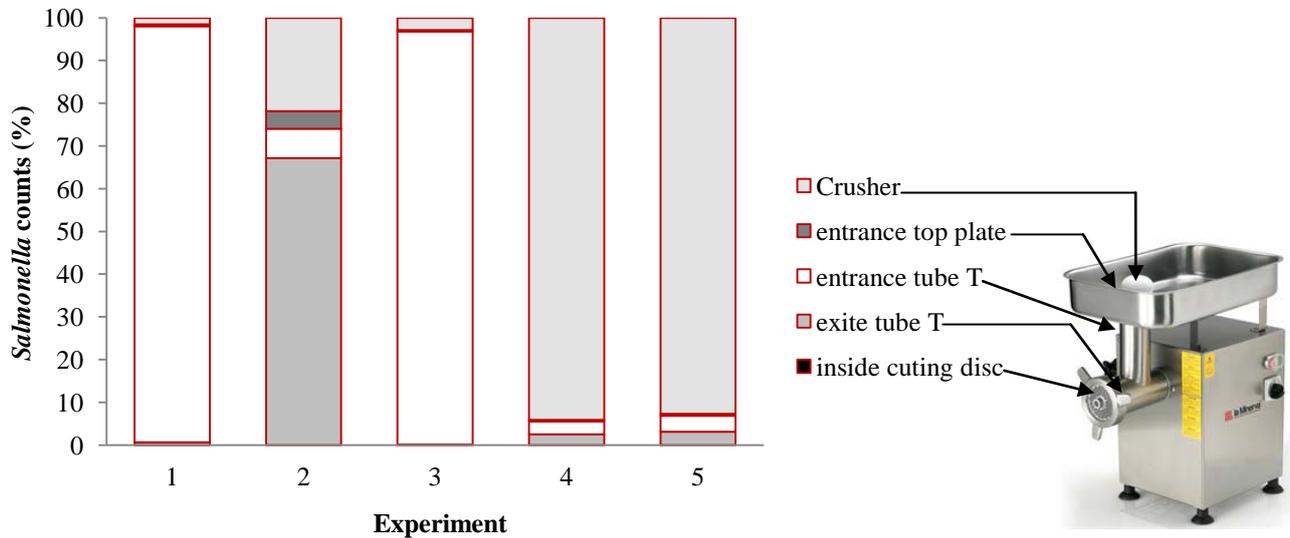


Figure 6. Distribution of *Salmonella* (%) inside the grinder after five episodes of processing, obtained by performing “Con-Tact-It” adhesive tape sampling

The suggested transfer of *Salmonella* model could well describe and explain the cross contamination event that occurs in low fat pork during grinding, however, the dynamics related to the grinder still remain to be explored. In addition, there are aspects that still need to be investigated because their effects on the mechanisms of cross contamination, on the structure of the model and consequently on the its parameter estimates, are so far unknown, i.e. types of meat (beef, lamb, etc.), fat content, variation on the size of the slices of meat to be processed, behaviour of other pathogens, influence of inoculums size, variety of process and processing with different pressure and friction, etc. Influence of inoculums size seems to be one of the most controversial aspects to be investigated, because despite the fact that Montville and Schaffner (2003) have not observed differences in cross contamination between surfaces related to varying inoculums size of *Salmonella*, Flores and Tamplin (2002) have changes in the transfer patterns of *E. coli* O157:H7 during beef grinding when applying different inoculation levels. In addition, Sheen and Hwang (2010) found the need for different model structures when applying different inoculation level of *E. coli* O157:H7 in slicing of ham.

3.2 Growth of *Salmonella*

Mathematical models of microbial growth are used increasingly for assessment and management of food safety and quality. Primary growth models describe or predict changes in concentrations of microorganisms over time, e.g. during processing, storage or distribution. Classical kinetic growth models are available to make predictions and to estimate the growth kinetic parameters lag time, growth rate and maximum population density from experimental data. The logistic model is particularly useful allowing the application of models to study the interaction between groups of bacteria to be conveniently predicted. Secondary growth models describe the relation between environmental conditions (processes and product criteria) and the growth response of microorganisms (Dalgaard, 2009).

While models are currently available to predict growth of *Salmonella* spp., some of these models were developed using laboratory media. Often, the predictions of *Salmonella* growth differ significantly from the observed growth in food products due to differences in the substrate and the intrinsic properties (pH and water activity) of the food or the presence of antimicrobials such as bacteriocins or anion effects from acidulants, phosphates, sorbates, and humectants other than sodium chloride. Further, these models do not consider the protective buffering effects of various food components when converting predictions from model systems to different and more solid food matrices. The effects of intrinsic properties are more apparent when a combination of these factors affects the microbial growth, such as pH and extremes of temperature during food processing (Velugoti et al., 2011).

Predictive models developed for specific food products, such as meat, are very useful for food processors. In 2009 (MANUSCRIPT I), a review of the literature was carried out in order to evaluate the performance of the available models for beef and chicken in predicting *Salmonella* spp in pork (Table 3). These models were compared with the herein developed growth model for pork (K), in relation to the observed growth of *Salmonella* during storage of sterile ground pork at different temperatures (Figure 7, Table 4). Despite the fact that the data used to develop the model for growth of *Salmonella* in ComBase Predictor was generated in broth, the predictive tool was included in this study because the model is validated and found to be fail safe, when challenged with data from literature developed in food, and it is wide accepted by the modelling community.

Table 3. Overview of models considered for prediction of growth rate of *Salmonella* in pork.

Reference for model *	<i>Salmonella</i> strain(s)	Growth medium	Secondary model	Growth rate response	Independent variables
(A) Oscar (1999b)	S. Typhimurium (ATCC 14028)	Cooked chicken	Polynomial	Specific growth rate ($\log_{10}\text{cfu h}^{-1}$)	Temperature (10-38°C) NaCl Previous growth NaCl
(B) Oscar (2002)	S. Typhimurium (ATCC 14028)	Cooked chicken	Ratkowsky 2	growth rate ($\log_{10}\text{cfu h}^{-1}$)	Temperature (10-38°C)
(C) Oscar (2003)	S. Typhimurium (TML)	Autoclaved chicken burgers	Cardinal temperature model	growth rate ($\log_{10}\text{cfu h}^{-1}$)	Temperature (10-38°C)
(D) Oscar (2005)	S. Typhimurium (ATCC 14028)	Cooked chicken	Modified version of square root	Maximum specific growth rate (h^{-1})	Temperature (10-38°C)
(E) Oscar (2006)	S. Typhimurium DT104 (ATCC 700408)	Raw ground chicken	Modified version of Logistic with delay	Maximum specific growth rate (h^{-1})	Temperature (10-38°C)
(F) Juneja et al. (2007)	Cocktail of S. Thompson S. Enteritidis S. Hadar S. Montevideo S. Heildelberg	Irradiated chicken	Modified Ratkowsky	Maximum specific growth rate (h^{-1})	Temperature (10-38°C)
(G) Dominguez and Schaffner (2008)	<i>Salmonella</i> spp. (collected from different literature studies)	Various kinds of meat and broth	Square-root or Ratkowsky	growth rate ($\log_{10}\text{cfu h}^{-1}$)	Temperature (8-37°C)
(H) Juneja et al. (2009)	Juneja et al. (2007)	Irradiated raw ground beef	Modified Ratkowsky	Maximum specific growth rate (h^{-1})	Temperature (10-38°C)
(I) Oscar (2009)	S. Typhimurium DT104 (ATCC 700408)	Chicken skin	Cardinal temperature model	growth rate ($\log_{10}\text{cfu h}^{-1}$)	Temperature (25-38°C)
(J) Anonymous - ComBase Predictor (accessed in 2009)	<i>Salmonella</i> spp.	Broth (BHI)	Standard quadratic multivariate polynomial	growth rate ($\log_{10}\text{cfu h}^{-1}$)	Temperature (8-38°C) pH NaCl
(K) MANUSCRIPT I (2012)	Cocktail of S. Typhimurium DT104 S. Typhimurium DT12 S. Derby	Irradiated pork	Simple square root model of Ratkowsky et al. (1982)	Maximum specific growth rate (h^{-1})	Temperature (8-38°C)

* the letters represent the references related to the data presented in Figure 7

In Figure 7, it is shown that between 8 and 38°C the growth rate of *Salmonella* increases with the rise in storage temperature of ground pork. However, at temperatures higher than 37°C this effect seems to stop and the growth rate decreases slightly. The growth model of *Salmonella* that was developed for ground pork (K) (MANUSCRIPT I) gives a good description of the observed data. As indicated in Figure 7, predictions made with the models developed by Dominguez and Schaffner in 2008 (G) and Oscar in 2002 (B), were close to the observed μ_{\max} -values.

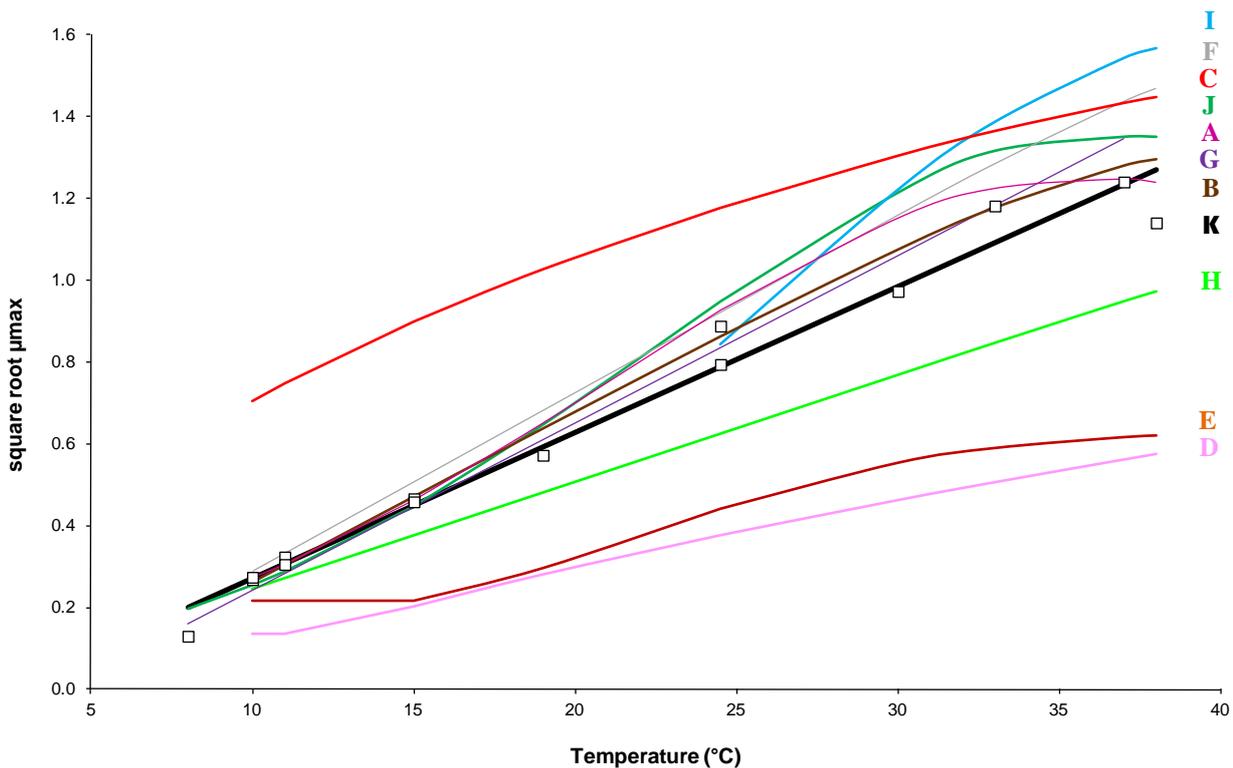


Figure 7. Visual evaluation of models predicting growth of *Salmonella*, where A) Oscar, 1999b; B) Oscar, 2002 (model 1); C) Oscar, 2003 (Typh); D) Oscar, 2005; E) Oscar, 2006; F) Juneja et al., 2007; G) Dominguez and Schaffner, 2008; H) Juneja et al., 2009, I) Oscar, 2009; J) Anonymous – ComBase Predictor (pH = 5.9 and NaCl in water = 0.15%), accessed in 2009; K) MANUSCRIPT I. The predictions of the models are compared to observed values (□) obtained from storage of sterile ground pork at different constant temperatures.

Bias (B_f) and accuracy (A_f) factors (Table 4) also confirm the conclusion obtained from the visual evaluation in Figure 7, indicating that the models with the best performances were the model suggested in the present thesis (MANUSCRIPT I) with $B_f = 1.04$ and $A_f = 1.14$, and those developed by Dominguez and Schaffner in 2008 ($B_f = 0.98$ and $A_f = 1.18$) and Oscar in 2002 ($B_f = 1.05$ and $A_f = 1.11$). The calculations of B_f and A_f for μ_{\max} were done as described by Ross (1996).

In both cases, a value of 1.0 is a perfect agreement between the model and the results of testing. For B_f a value below 1.0 indicates underestimation of the predicted values, while value higher than 1.0 indicates overestimation of the predicted values

Table 4. Performance of secondary models presented in Figure 7 for predicting μ_{\max} of *Salmonella* in irradiated minced pork: prediction bias and accuracy factors.

Reference for model	Bias factor	Accuracy factor
(A) Oscar (1999b)	1.09	1.12
(B) Oscar (2002)	1.05	1.11
(C) Oscar (2003)	2.96	2.96
(D) Oscar (2005)	0.21	4.67
(E) Oscar (2006)	0.33	3.02
(F) Juneja et al. (2007)	1.25	1.25
(G) Dominguez and Schaffner (2008)	0.98	1.18
(H) Juneja et al. (2009)	0.66	1.51
(I) Oscar (2009)	1.36	1.41
(J) Anonymous - ComBase Predictor (accessed in 2009)	1.16	1.28
(K) MANUSCRIPT I	1.04	1.14

Recently, Velugoti et al. (2011) have published a study where a dynamic model for predicting growth of *Salmonella* (a cocktail of *S. Tompson* FSIS 120, *S. Enteritidis* Phage Type 4 H3502, *S. Hadar* MF 60404, *S. Montevideo* FSIS 051, *S. Heidelberg* F5038BG1) in ground sterile pork was developed from growth experiments performed at static temperatures from 10 to 47°C. The

modified Ratkowsky equation (Zwietering et al., 1991) was used as the secondary model to describe the effect of temperature on μ_{\max} . As the parameter estimates for the fitted model were not made available by Velugoti et al. (2011), it was not possible to evaluate the performance of this particular model against the μ_{\max} -values observed in the present thesis. However, comparisons could be made to graphed μ_{\max} -values in the Velugoti et al. (2011) paper which showed good agreement between the two studies although b and T_{\min} obtained in the present study were slightly lower (MANUSCRIPT I). In addition, Oscar (2011) found that more than 30 % of residuals for survival and growth of *Salmonella* Kentucky on chicken skin at 11°C were outside the acceptable prediction zone and negative, indicating that the model developed with *Salmonella* Typhimurium DT104 overpredicted survival and growth of *Salmonella* Kentucky at this cold-storage temperature. Consequently, more research is needed to address these issues.

3.2.1 Interaction of *Salmonella* with the pork natural microbiota

The bacterial competition within the natural microbiota is a complex issue in modelling microbial evolution and it has been studied by several authors (Lotka, 1956; Jameson, 1962; Pin and Baranyi, 1998; Vereecken et al., 2000; Giménez and Dalgaard, 2004; Van Impe et al., 2005; Le Marc et al., 2009).

According to Jameson (1962), who studied the competitive enrichment of *Salmonella*, “when two intestinal organisms, which do not mutually interact by colicines or bacteriophage, are inoculated together into a liquid medium, each organism normally follows at first a growth pattern similar to that which would have followed from a similar inoculum in the same medium in the absence of a competitor. Neither organism normally exhibits its awareness, to any appreciable degree, of the other’s presence, until the bacterial density of one or other organism has risen to a level near to the molar concentration, when both organisms end their rapid multiplication”.

The lack of suitable predictive models for mixed microbial growth in spite of the need for such models for a number of industrial applications is primarily due to the complex nature of interspecies interactions. While single-species growth can mostly be characterized in terms of a lag phase, an exponential growth phase and a stationary phase, no such general outline exists for multiple-species growth (Vereecken et al., 2000).

In this work growth of *Salmonella*, growth of the dominating natural microbiota and their interaction in minced raw pork have been modelled and predicted. Growth of *Salmonella* in sterile raw pork between 4°C and 38°C was quantified and used for development of a predictive model. Data from the literature was used for development of a natural microbiota growth model at different

temperatures. Challenge tests at various temperatures and with *Salmonella* inoculated in minced raw pork were used for evaluation of growth and interaction models. As presented in MANUSCRIPT I, an expanded version of the Jameson-effect model by Gimenez and Dalgaard (2004) was suggested and evaluated (Equation 1). This new model includes a temperature dependent parameter (γ) that was estimated from mixed-culture studies and allowed the inhibiting effect of the natural microbiota on growth of *Salmonella* to differ depending on the storage temperature.

$$\left\{ \begin{array}{l} t < t_{lag-S}, \quad \frac{dS/dt}{S_t} = 0 \\ t \geq t_{lag-S}, \quad \frac{dS/dt}{S_t} = \mu_{max}^S \times \left(1 - \frac{S_t}{S_{max}}\right) \times \left(1 - \frac{\gamma \times NB_t}{NB_{max}}\right) \\ t < t_{lag-NB}, \quad \frac{dNB/dt}{NB} = 0 \\ t \geq t_{lag-NB}, \quad \frac{dNB/dt}{NB} = \mu_{max}^{NB} \times \left(1 - \frac{NB_t}{NB_{max}}\right) \times \left(1 - \frac{S_t}{S_{max}}\right) \end{array} \right. \quad \text{(Equation 1)}$$

Where γ is a coefficient of interaction. S and NB are the concentrations in CFU/g of *Salmonella* and the natural microbiota, respectively and S_{max} and NB_{max} are the maximum population densities in CFU/g. μ_{max}^S and μ_{max}^{NB} are the maximum specific growth rate in 1/h for *Salmonella* and for the natural microbiota, respectively.

It was found that high concentrations of the natural microbiota in ground pork reduced growth of *Salmonella*. This interaction effect was temperature dependent and mathematical models were developed to quantitatively predict the effect of both the storage temperature and of the natural microbiota on growth of *Salmonella* (Figure 8). The suggested interaction models for ground pork are new and important for future exposure and risk assessments where concentrations of *Salmonella* in this product at the time of consumption can be predicted to evaluate the risk of *Salmonella* infections.

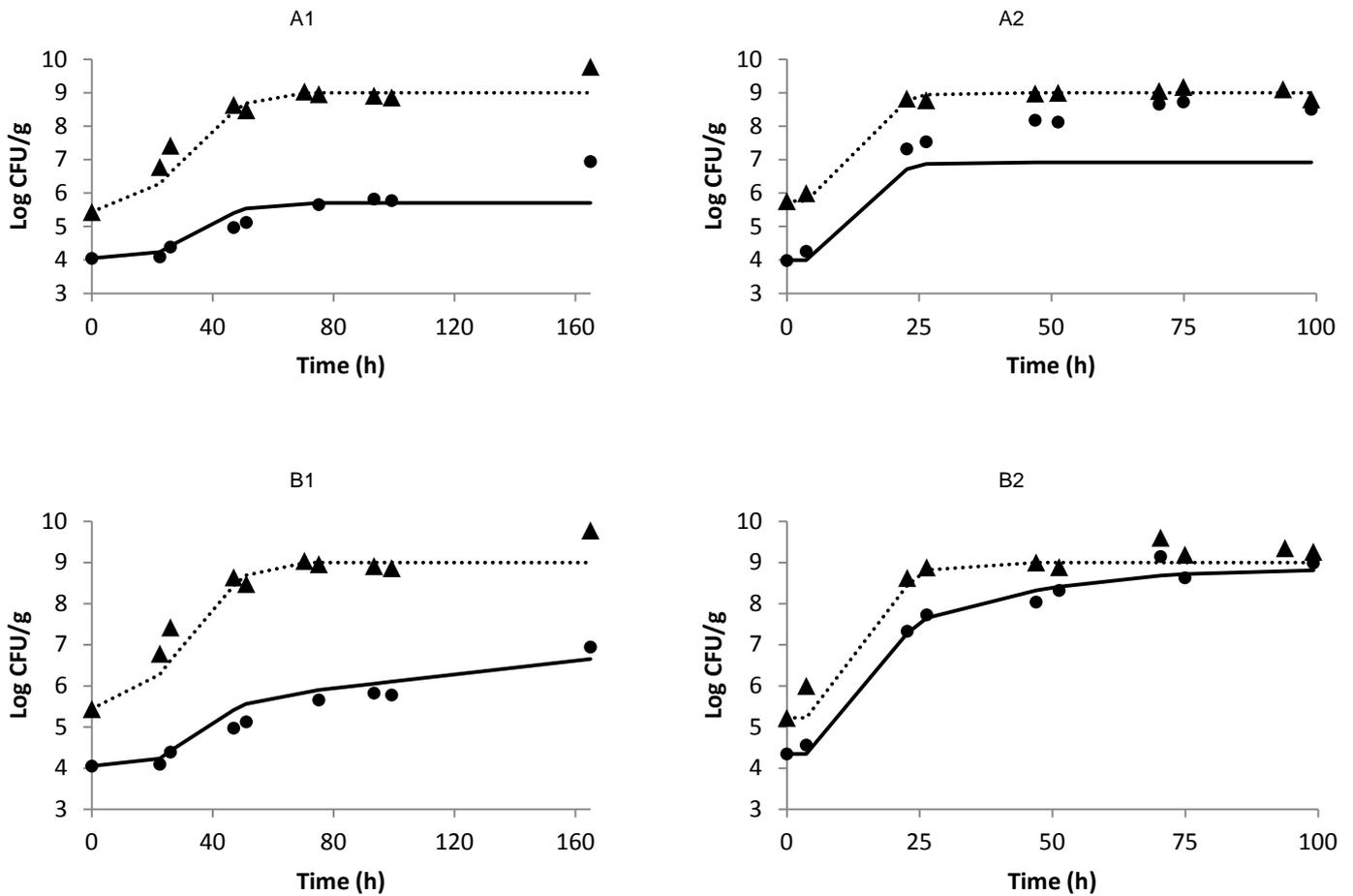


Figure 8. Effect of observed (\blacktriangle) and fitted (.....) pork natural microbiota counts (\log_{10} CFU g^{-1}) on growth of observed (\bullet) and fitted (—) *Salmonella* counts (\log_{10} CFU g^{-1}), during storage of ground pork at 11.9°C (1) and 20.2°C (2), using classical Jameson-effect model (A) and the new expanded Jameson-effect model (B).

The observed growth of *Salmonella* does not stop when the ground pork natural microbiota reaches its maximum growth, as the classical Jameson-effect model would predict (Figure 8A). When the natural microbiota stops to grow, the growth rate of *Salmonella* is reduced but the pathogen still continues to grow, what is well described by the suggested model (Figure 8B).

3.3 Heat inactivation of *Salmonella*

Isothermal microbial survival curves are usually described by either log-linear or non-log-linear time-dependent models (Chen and Campanella, 2012). Takhar et al. (2009) demonstrated that the survival curves of *Salmonella* spp. were non-log-linear exhibiting concavity. Therefore, the Weibull model (a flexible, yet simple model also applied to describe microbial heat inactivation by Peleg

and Cole, 1998, Fernandez et al., 1999, Peleg, 1999, Peleg and Penschina, 2000) was more successful than the log-linear model for predicting the inactivation of the pathogen when the three ground meats (turkey breast, turkey thigh and pork shoulder) were cooked at 50, 54, 58, 62 or 66 and held for various times. Juneja (2003) used a survival model for non-log-linear survival concave curves to describe the effect of heat inactivation on *Salmonella* spp. in beef with indigenous microflora. Different parameter estimates for linear models have been used to describe the behaviour of *Salmonella* during heat treatment, e.g. those models proposed by Juneja et al. (2001) applying a linear regression and a linear model to evaluate heat inactivation of *Salmonella* spp. in chicken broth, beef, pork, turkey and chicken. Murphy et al. (2004) applied other parameter estimates for linear model to compare heat inactivation of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in ground pork.

Several researchers have reported enhanced thermo-tolerance of *Salmonella* in whole muscle beef and turkey products compared with ground products of equivalent composition (Orta-Ramirez et al., 2005; Tuntivanich et al., 2008). However, these results also confirmed that thermo-tolerance is different between meat species (e.g., ground turkey versus ground beef). Therefore, there is a need for product-specific thermal resistance data and model parameters to ensure the highest degree of reliability in process validations (Velazquez et al., 2010).

In this thesis, log-linear inactivation kinetics was assumed for *Salmonella* and the concepts of decimal reduction times (D-values) and z-values were, therefore, applied. As already mentioned in chapter 2.4.2, D-value is defined as the time required to reduce microbial counts at a constant temperature by 90 %, whereas a z-value is the temperature change needed to achieve a 90 % change in D-value (Veeramuthu et al., 1998, Murphy et al., 2004). D-values for heating of *Salmonella* spp. in ground meat (pork and beef) were collected from different studies (Table 5). Highest D-values were found in ground pork (40.2 % fat) at 55°C (45.87 min) and 58.5°C (26.76 min) by Murphy et al. (2004) and in ground pork (2.5 % fat) at 55°C (23.40 min) by Velazquez et al. (2010). Lowest D-values were obtained in ground pork (40.2 % fat) at 70°C (0.08 min) by Murphy et al. (2004), in ground beef (4.8 % fat) at 64 °C (0.14 min) by Smith et al. (2001) and in lean ground beef at 71 °C (0.15 min) by Juneja et al. (2010). Variation on thermo-tolerance of *Salmonella* can be observed by investigating different serotypes, meat type, pH and fat content, however, it was not shown any correlation by looking at the highest and lowest D-values found in the listed studies (Table 5). D₆₀ was chosen for comparison of the effect of heating on D-values from the different evaluated survival curves.

Table 5. Selected heat inactivation studies demonstrating the efficiency of the treatment of *Salmonella* spp. in ground meat.

Ground matrix		Temperature (°C)	D (min.)	z value (°C)	Pathogen	Source
meat	Fat content (%)					
beef	4.8	55.0	9.05	5.1	<i>S. Typhimurium</i> DT104 – 10127 (human isolate)	Smith et al. (2001)
		58.0	2.26			
		60.0	0.91			
		61.0	0.57			
		64.0	0.15			
beef	4.8	55.0	10.55	4.1	<i>S. Typhimurium</i> DT104 – 10601 (human isolate)	Smith et al. (2001)
		58.0	2.15			
		60.0	0.68			
		61.0	0.41			
		64.0	0.07			
beef	4.8	55.0	10.27	4.8	<i>S. Typhimurium</i> DT104 – 01071 (human isolate)	Smith et al. (2001)
		58.0	2.06			
		60.0	0.84			
		61.0	0.43			
		64.0	0.14			
Lean beef		60.0	2.13	9.6	The same cocktail with 8 <i>Salmonella</i> serotypes used by Juneja et al. (2001)	Juneja et al. (2010)
		65.0	0.37			
		71.0	0.15			
beef	12.5	58.0	8.65	6.0	A cocktail with 8 <i>Salmonella</i> serotypes: <ul style="list-style-type: none"> • <i>S. Thompson</i> FSIS 120 (chicken isolate); • <i>S. Enteritidis</i> H 3527 phage 13A (human isolate); • <i>S. Enteritidis</i> H 3502 phage 4 (human isolate); • <i>S. Typhimurium</i> DT104 H 3380 (human isolate); • <i>S. Hadar</i> MF 60404 (turkey isolate); • <i>S. Copenhagen</i> 8457 (pork isolate); • <i>S. Montevideo</i> FSIS 051 (beef isolate); • <i>S. Heidelberg</i> F 5038 BG 1 (human isolate). 	Juneja et al. (2001)
		60.0	5.48			
		62.5	1.50			
		65.0	0.67			

Ground matrix		Temperature (°C)	D (min.)	z value (°C)	Pathogen	Source
meat	Fat content (%)					
pork	2.5	55.0	23.40	5.3	The same cocktail with 8 <i>Salmonella</i> serotypes used by Juneja et al. (2001)	Velasquez et al. (2010)
		58.0	3.43			
		60.0	1.74			
		62.0	0.77			
		63.0	0.74			
pork	8.5	58.0	6.68	7.1	The same cocktail with 8 <i>Salmonella</i> serotypes used by Juneja et al. (2001)	Juneja et al. (2001)
		60.0	6.65			
		62.5	1.62			
		65.0	0.87			
pork	40.2	55.0	45.87	5.7	A cocktail with 6 <i>Salmonella</i> serotypes: <ul style="list-style-type: none"> • S. Senftenberg; • S. Typhimurium; • S. Heidelberg; • S. Mission; • S. Montevideo; • S. California. 	Murphy et al. (2004)
		57.5	26.76			
		60.0	5.07			
		62.5	2.56			
		65.0	1.91			
		67.5	0.36			
70.0	0.08					

D₆₀ in grey were calculated with the other D-values available in the respective source.

Variation between the investigated strains of *Salmonella* could be the explanation for the very low D_{60} obtained from Smith et al. (2001). Higher D_{60} were observed in inactivation curves performed in meat with higher fat content, what indicates that the fat content could be the explanation for this phenomenon, since the protective effect of increasing fat content on *Salmonella* during heat inactivation is a phenomenon already recognized. The few data points observed in Juneja et al. (2010) could be the reason why the D_{60} obtained from this study was one of the lowest of all investigations using the same strains of *Salmonella*.

The classical method of D- and z-values, developed by Bigelow, Ball and Stumbo (Stumbo, 1973) was performed and a model describing the effect of heating temperature on the $\log_{10}D$ was derived and applied to the QMRA model developed in the thesis (MANUSCRIPT II). D-values for heat inactivation of *Salmonella* in minced meat (pork and beef) were collected from the literature (Juneja et al. 2001; Smith et al. 2001; Murphy et al. 2004; Juneja et al. 2010; Velasquez et al. 2010) and the classical model for the effect of temperature on D-values ($\log_{10}(D_T) = \log_{10}(D_{ref}) - (T - T_{ref})/z$) was used to estimate a z-value of 7.34 based on these data (Table 5).

There are not many published investigation of thermo-tolerance of a precise strain of *Salmonella* in ground pork with same fat content and pH. Therefore, in order to evaluate the results from different studies related to ground meat, the z-values shown in Table 5 were estimated by taking the absolute value of the inverse slope obtained by linear regression for a plot of temperature vs. $\log_{10}D$ for each individual study. Comparing the obtained z-values indicated variation between studies from 4.1 to 9.6°C, five of the eight listed curves being between 4.1 to 5.7°C, (Table 2). The highest observed z-value of 9.6°C was obtained in lean beef by Juneja et al. (2010), and the few data points observed in this study could be the explanation for the overestimation of the z-value.

Chapter 4

Application of predictive models

4 Application of predictive models

Current applications of predictive microbiology in an industrial context are wide and according to Membré and Lambert (2008) can be summarised into three groups of activities: 1) Product innovation, where new products and process are developed, existing products are reformulated, storage conditions and shelf-life are determined, by assessment of speed of microbial proliferation, growth limits, or inactivation rate associated with particular food formulations and/or process conditions; 2) Operational support, where predictive models are used as support decision tools to implement or run a food manufacturing operation, such as designing in-factory heating regimes, setting Critical Control Points (CCPs) in Hazard Analysis and Critical Control Points (HACCP), assessing impact of process deviations on microbiological safety and quality of food products; 3) Incident support, where the impact on consumer safety or product quality are estimated in case of problems with products on the market. An example of predictive modelling application in outbreak investigation is given by Hansen et al. (2009), where the ComBase Predictor and the Pathogen Modeling Program were used as support tools in a VTEC O25:H11 outbreak investigation, related to sausage in Denmark.

Beside this current use, predictive microbiology might also be utilised to transfer new risk

Table 6. Applications of predictive microbiology adopted from Fakruddin et al. (2011)

Area of Application	Example
<i>Hazard Analysis and Critical Control Points (HACCP)</i>	<ul style="list-style-type: none"> • Preliminary hazard analysis • Identification and establishment of critical control point(s) • Corrective actions • Assessment of importance of interaction between variables
<i>Risk assessment</i>	<ul style="list-style-type: none"> • Estimation of changes in microbial numbers in a production chain • Assessment of exposure to a particular pathogen
<i>Microbial shelf life studies</i>	<ul style="list-style-type: none"> • Prediction of the growth of specific food spoilers • Prediction of growth of specific food pathogens
<i>Product research and development</i>	<ul style="list-style-type: none"> • 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
<i>Temperature function integration and hygiene regulatory activity</i>	<ul style="list-style-type: none"> • Consequence of temperature in the cold chain for safety and spoilage
<i>Education</i>	<ul style="list-style-type: none"> • Education on safety, especially non-technical people
<i>Design of experiments</i>	<ul style="list-style-type: none"> • Defining the interval between sampling

management concepts into practical guidelines (Gorris, 2005; Membré et al., 2007). Fakruddin et al. (2011) have summarized some of the applications of predictive models (Table 6) and, back in 1994, Ross and McMeekin stressed the numerous potential advantages of predictive microbiology. These authors also underlined that the full realisation of predictive microbiology's potential, will depend upon a conscientious and rigorous approach to data gathering and modelling, ingenious solutions and strategies for the application of that data and models, and willingness on the part of the food industry to trial the approach and think in terms of the premises upon which predictive microbiology is based.

In this thesis, predictive models have been applied with different purposes related to the study of *Salmonella* behaviour in pork. Some of the aspects investigated and discussed through development of this work are going to be introduced as examples of application of predictive models.

4.1 Quantitative microbiological Risk Assessment (QMRA)

In this thesis predictive models have been used in the development of a QMRA where the risk of salmonellosis, from consumption of pork meatballs produced according to Danish catering practices, was estimated.

The CODEX Alimentarius Commission (1998) guidelines for conducting QMRA are one of the several approaches that have been proposed. It gives a list of principles and definitions, but does not present a modelling methodology. The modular process risk model (MPRM) proposed by Nauta (2001) describes the integration and application of QMRA methodology with scenario analysis and predictive microbiology. It states that the transmission of the hazard through the food pathway can be regarded as a series of basic processes. The MPRM structure is determined by this series of basic processes and may be used in any QMRA study, which range from industrial food processing to 'farm to table' risk assessment models. In this thesis, a QMRA following the Codex Alimentarius principles was conducted using the MPRM methodology and changes in prevalence, bacteriological concentration, and unit size within each module were modelled by means of five basic processes, three of which were food-handling process (*i.e.* cross contamination during grinding, mixing, and partitioning) and the remaining two were microbial processes (*i.e.* inactivation and growth).

As shown in Figure 9, a combination of observations and predictive models was applied. In order to reduce the uncertainty, models specifically developed studying *Salmonella* behaviour in pork (PAPER I: Møller et al., 2012; MANUSCRIPT I; Velasquez et al., 2010; Murphy et al., 2004; Juneja

et al., 2001), or other meat matrices (Juneja et al., 2010; Smith et al., 2001; Juneja et al., 2001) were used to build up the QMRA model. Through observational studies the production of meatballs made from pork was described as a serial of process modules for which time and temperature conditions was registered (MANUSCRIPT II).

The developed QMRA was used to evaluate the risk that *Salmonella* could pose to the consumers' health as a result of cross contamination, insufficient heat treatment and growth during cooling and storage of this product.

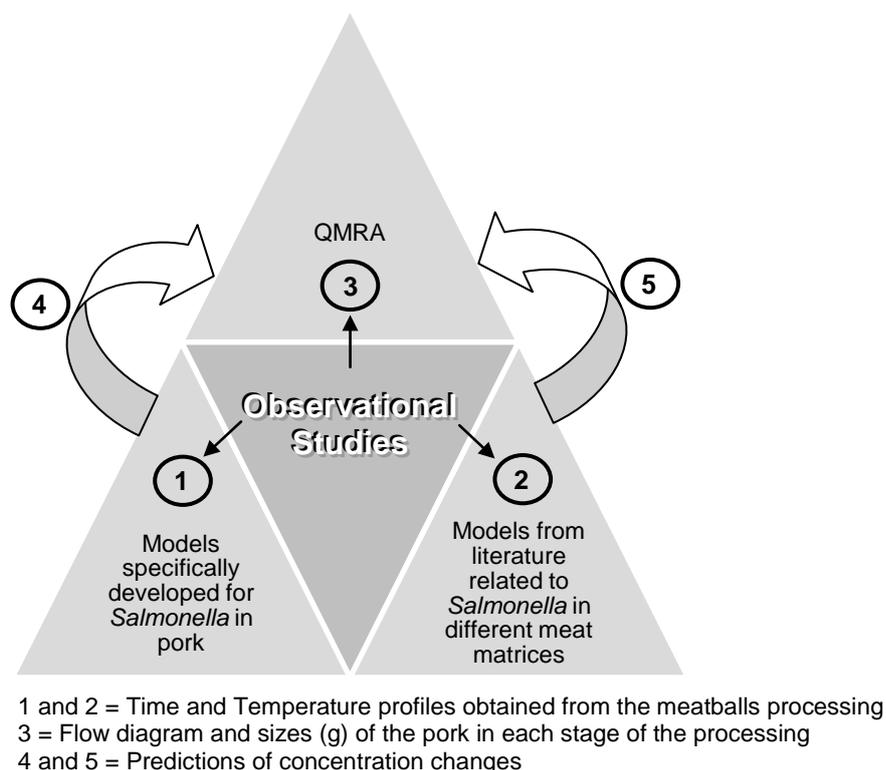


Figure 9. Schematic representation of the process to build up the QMRA model, developed in this thesis, to describe the risk estimates related to the meatballs processing according the Danish catering sector.

According to the observational studies, pork meatballs are served about 24 times a year in the establishments we surveyed. When the proposed baseline model is considered (MANUSCRIPT II) and inferring that about 20 % of the Danish population (5.6 million people in 2011) is eating catered meals and that the conditions of production of the meatballs are the same as observed in this study, it would be possible to conclude that: 1) following the Danish heating recommendations, there would be no cases of salmonellosis by consumption of meatballs subjected to heat treatment in oven until it had reached 75°C or even 72°C; 2) However, if the heat treatment in oven would reach only 65°C, about 27 people could become ill from salmonellosis annually; 3) About 69 cases of salmonellosis could happen if the step of heat inactivation was not applied; 4) In case of cooling

of meatballs in room temperature in the kitchen (RTK) equal to 30°C, followed by refrigerated storage of those meatballs not heat treated in oven, about 16,300 cases are predicted.

This practical example showed reasonable estimates when comparing with the incidence of salmonellosis caused by consumption of pork in Denmark in 2011, and considering that the risk estimates obtained here correspond to a fraction of the 44-131 reported cases (Anonymous, 2012). The very high predicted number of cases (16,300) was obtained simulating a scenario that does not correspond to the practices recommended by the authorities and it was only a hypothetical situation that could happen only if errors in the processing were made.

4.2 Quantitative detection of counts below detection

The complexity and difficulty of low pathogen level transfer, faced in this thesis during the studies related to transfer of *Salmonella* through the pork grinding, was also described by Sheen and Hwang (2010), who stressed how relevant it is to obtain more information and knowledge to facilitate realistic risk predictions. It also suggests that new methods of analysis should be developed in order to improve sensitivity, and still continue to be simple enough for that large scale studies to conduct. Enrichment in the meat at relatively low temperatures from 11 to 16°C combined with accurate predictive models appears as an obvious solution.

In this thesis, it is proposed to use the reversed version of the developed growth model of *Salmonella* considering the interaction with the pork natural microbiota (MANUSCRIPT I) to trace the level of the *Salmonella* present prior to enrichment at about 12°C. As input to predict the log₁₀-count of *Salmonella* present before enrichment, the observed log₁₀-count of pork natural microbiota (NB) prior to enrichment, the enrichment time (days), and temperature (°C), and the observed log₁₀-counts of *Salmonella* and NB after the enrichment time were used. More details are provided in NOTE I.

According to Figure 10, the predictions obtained with the proposed recovery model (□) indicated a slight overestimation of some data points (0.13 - 0.67 log CFU/g) when compared to the observed *S. Typhimurium* DT104 counts obtained immediately after grinding and prior to enrichment. Since a deviation of 0.5 log correspond to an expected uncertainty inherent to the plate count method, only one of the nine observed data points were considered slightly overestimated. However, two of the data points from the tail of low contaminated portions were underestimated at approximately 1 log CFU/g. Otherwise, the proposed recovery model seemed to give a good description of the shape of contaminated portions. In addition, the results indicate that the recovery model can be a promising alternative when it is further developed by performing more studies and applying levels of inoculation even lower than those already tested, since it has been reported that levels of

Salmonella spp. are lower than 3 log CFU/g in pork cuttings at the Danish retail market (Pires, 2009; Anonymous, 2010; Hansen et al., 2010).

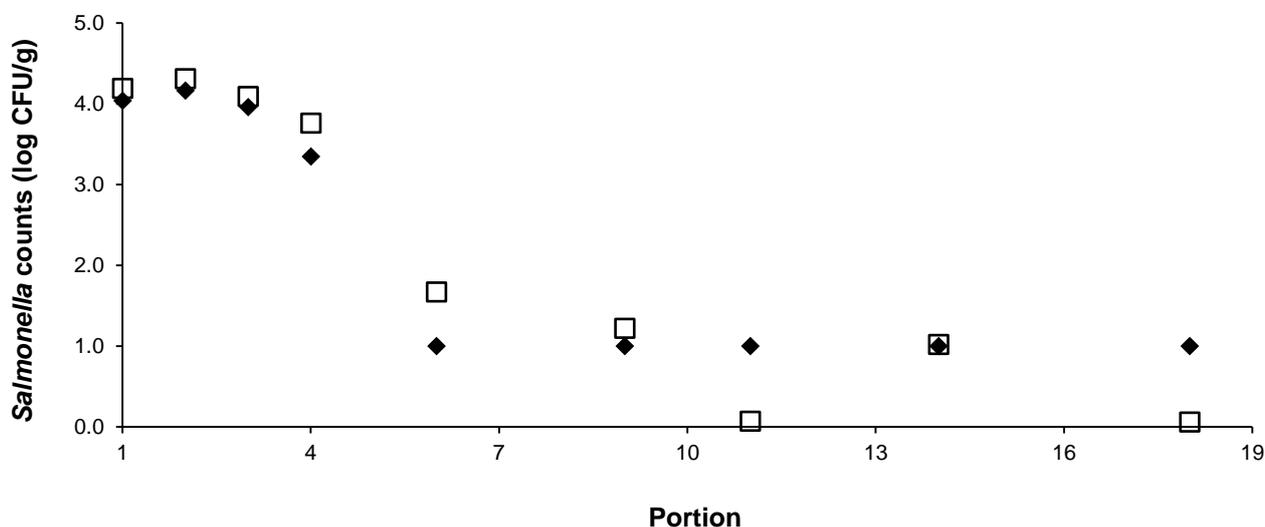


Figure 10. Transfer of *Salmonella* Typhimurium DT104 during grinding (three contaminated pork slices to grinder to uncontaminated pork, 10^4 log₁₀ CFU/g) of 18 processed slices. The observed values (◆) were predicted with the proposed recovery model (□) (NOTE I).

4.3 Shelf life estimation considering product safety

Food quality is obviously an important issue. Quality in a very broad sense means satisfying the expectation of the consumer; in other words, quality experience delivered by a food should match quality expectation of a consumer. Incorporating quality into product and process design is the big challenge for a food manufacturer. Product and process designs need to be flexible these days for several reasons, and reaching quality by trial and error does not seem the best way anymore. A cheaper and more systematic way is by use of modelling and several types of models have been described and are currently used in food science to model quality indicators such as the predictive shelf-life model used as a tool for the improvement of quality management in pork and poultry chains, developed by Bruckner et al. (2011), or the modelling of in-mouth flavour release during eating of dairy gels, presented by Souchon et al. (2011). The kinetic modelling of food quality attributes can be reached by modelling chemical reactions, temperature dependence of chemical reactions, enzymatic reactions, physical reactions, changes in sensory parameters and microbiological changes (van Boekel, 2008).

Traditionally, the microbiological safety of food has always relied on microbial examination of raw materials and final products, coupled with monitoring process parameters and hygiene standards.

Challenge tests, or inoculated pack experiments, can be established to simulate the effects of environmental conditions on food, in terms of growth and proliferation of spoilage and pathogenic microorganisms. They can provide useful data for determining the safety and shelf-life of food under a set of conditions. The concept of predictive microbiology has been used to evaluate the effect of processing, distribution and storage operations on food safety (Lebert and Lebert, 2006).

The challenge is to model food quality taking into consideration the food safety issues as well. In this thesis, an example of modelling combining food quality and food safety, where performed. For the same samples of fresh minced pork, assessment of colour and odour was carried out by a 4-member panel in parallel with microbiological analyses during storage at seven different temperatures (9.4 – 24.1°C). The shelf-life was determined as the last sampling time where the meat was assessed by the panel to be acceptable for use. In all samples, acidic odour arose before putrid odour and also before significant colour changes. Acidic odour was listed as the

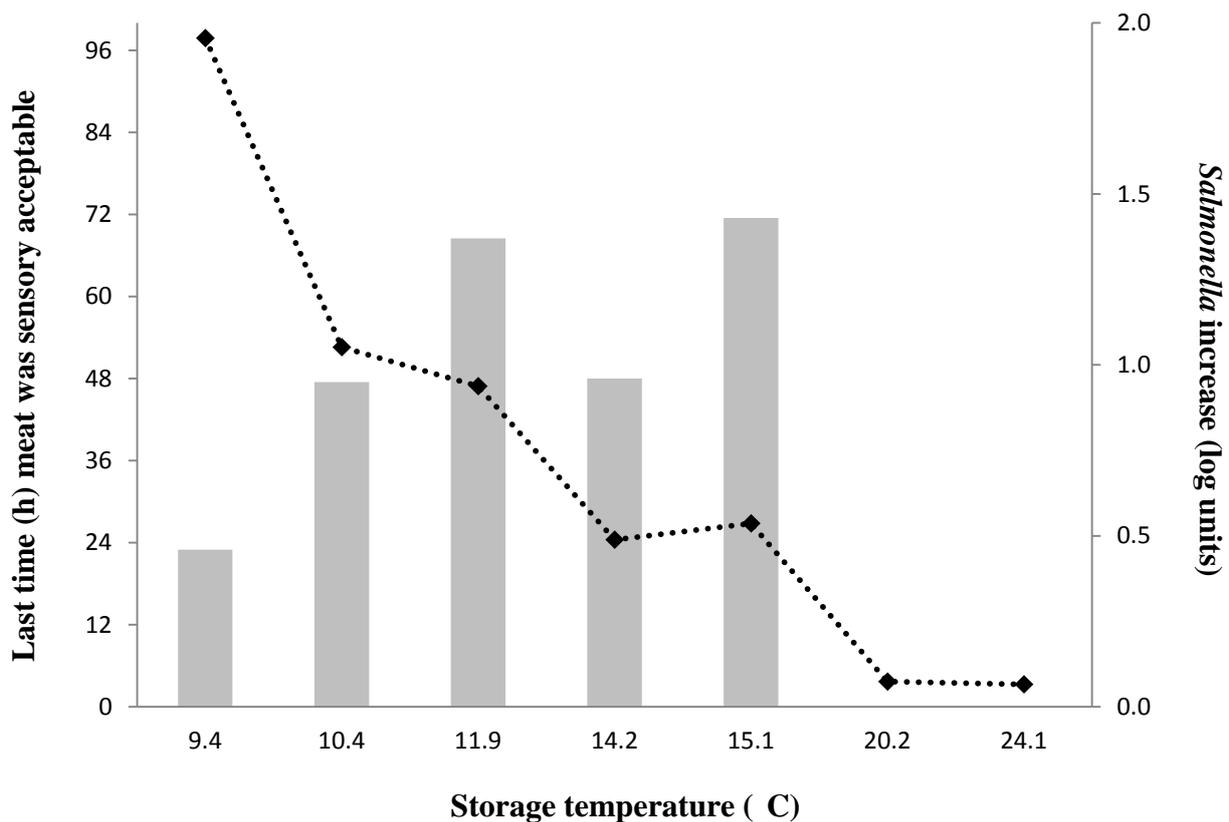


Figure 11. Predicted growth of *Salmonella* with MANUSCRIPT I (grey bars) and shelf-life (...◆...) of fresh minced pork during storage (h) at different temperatures (°C) (NOTE II).

determining factor for rejection of the meat by the panel. At the time of rejection, the level of natural microbiota in minced pork averaged $8.7 \log_{10}\text{CFU/g}$ (S.D.: $0.44 \log_{10}\text{CFU/g}$) and no systematic effect of temperature, on this level, was observed (NOTE II). Combining these observations with the developed growth model of *Salmonella* considering the interaction with the pork natural microbiota (MANUSCRIPT I) makes it possible to evaluate the growth potential of *Salmonella* with the determined shelf-life. The result of doing so is shown in Figure 11.

As shown, shelf-life was *approx.* 3 – 4 hours at 20.2 and 24.1°C and at those temperatures no increase in *Salmonella* count would be expected during this period. However, in the temperature interval from 9.4 to 15.1°C, increases in *Salmonella* counts of *approx.* $0.5 \log_{10}\text{CFU/g}$ (9.4, 14.2 and 15.1°C) and $1 \log_{10}\text{CFU/g}$ (10.4 and 11.9°C) could take place before the meat was assessed unacceptable for use. This indicated that safety, rather than spoilage, could be the shelf-life limiting factor of fresh pork meat at these abusive storage conditions.

Chapter 5

PAPER I

Modelling transfer of *Salmonella* Typhimurium DT104 during simulation of grinding of pork

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ORIGINAL ARTICLE

Modelling transfer of *Salmonella* Typhimurium DT104 during simulation of grinding of porkC.O.A. Møller¹, M.J. Nauta¹, B.B. Christensen², P. Dalgaard³ and T.B. Hansen¹¹ National Food Institute, Technical University of Denmark, Søborg, Denmark² Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Copenhagen, Denmark³ National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark**Keywords**cross-contamination, grinding, modelling, pork, retail, *Salmonella*.**Correspondence**

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Abstract**Aims:** The aim of this study was to develop a model to predict cross-contamination of *Salmonella* during grinding of pork.**Methods and Results:** Transfer rates of *Salmonella* were measured in three experiments, where between 10 and 20 kg meat was ground into 200-g portions. In each experiment, five pork slices of about 200 g per slice were inoculated with 8–9 log-units of *Salmonella* Typhimurium DT104 and used for building up the contamination in the grinder. Subsequently, *Salmonella*-free slices were ground and collected as samples of *c.* 200 g minced pork. Throughout the process, representative samples were quantitatively analysed for *Salmonella*. A model suggested by Nauta *et al.* (2005) predicting cross-contamination of *Campylobacter* in poultry processing and two modified versions of this model were tested.**Conclusions:** The present study observed a tailing phenomenon of transfer of *Salmonella* during a small-scale grinding process. It was, therefore, hypothesized that transfer occurred from two environmental matrices inside the grinder and a model was developed. The developed model satisfactorily predicted the observed concentrations of *Salmonella* during its cross-contamination in the grinding of up to 110 pork slices.**Significance and Impact of the Study:** The proposed model provides an important tool to examine the effect of cross-contamination in quantitative microbial risk assessments and might also be applied to various other food processes where cross-contamination is involved.**Introduction**

Salmonella has been linked to many foodborne illness cases across the world, and it is considered to be one of the main agents causing human gastroenteritis (Jiménez *et al.* 2009). In Denmark, the locally produced pork was estimated as one of the most important sources of salmonellosis in 2009 (Anonymous, 2010). Contamination and cross-contamination with *Salmonella* start inside the pig slaughterhouse (De Busser *et al.* 2011) and originates from animals carrying *Salmonella* in their intestine. Along the slaughter line, several steps can be critical for *Salmonella* contamination: dehairing, polishing, removal of the

intestines, removal of the pluck set and meat inspection procedures (Borch *et al.* 1996). During these steps, the carcass can be contaminated with faeces and bacteria can be spread all over the carcass and to subsequent carcasses. In the following handling and processing of pork through the cutting and retail steps, recontamination and cross-contamination with *Salmonella* may continue because infected and noninfected meat share the same cutting surfaces and processing equipment without cleaning and disinfection in between (EFSA, 2010). According to Hansen *et al.* (2010), this behaviour was most likely responsible for an increased prevalence of *Salmonella* in pork cuttings at retail in Denmark between 2002 and 2006, as the rise

observed in 2006 at retail could not be ascribed to a rise in *Salmonella* carcass prevalence at slaughter.

The food-processing environment is an important but still poorly recognized and understood source of recontamination. Detailed proof and facts concerning this issue have only been published occasionally. Fortunately, the number of publications on investigations of processing environments is slowly increasing, demonstrating an increased awareness, but there are still only few data available that allow us to quantify the rate of transfer of pathogens from food to contact surfaces and *vice versa* during processing (De Boer and Hahné 1990; Reij and Den Aantrekker 2004). Studies that simulate and model the distribution of pathogens during processing operations are of major relevance to risk analysts to ascertain the importance of equipment sanitation, sources of potential product contamination and improved equipment design (Flores *et al.* 2006). Recently, a number of cross-contamination models have been published describing transfer of *Listeria monocytogenes* (Vorst *et al.* 2006; Aarnisalo *et al.* 2007; Keskinen *et al.* 2008; Sheen 2008), *Escherichia coli* O157:H7 (Pérez-Rodríguez *et al.* 2007; Sheen and Hwang 2010), and *Staphylococcus aureus* (Pérez-Rodríguez *et al.* 2007) during slicing of ready-to-eat products. Studies concerning transfer of *Salmonella* from chicken carcasses to cutting board (Jiménez *et al.* 2009), from domestic washing-up sponge to kitchen surfaces and food (Mattick *et al.* 2003) and from raw chicken products during food preparation (De Boer and Hahné 1990), have also been performed. A few studies using *Enterobacter aerogenes* with attachment characteristics similar to *Salmonella* have also been conducted on chicken to investigate cross-contamination (Zhao *et al.* 1998; Chen *et al.* 2001). However, not much has been investigated regarding cross-contamination events in fresh-meat processing and according to Pérez-Rodríguez *et al.* (2008), pathogens may transfer to foods through many different types of events, such as recontamination and cross-contamination, which might be decisive in many of outbreaks. A work published by Berends *et al.* (1998), describing the ecology and epidemiology of *Salmonella* spp. in pork cutting lines of Dutch cutting plants and in butchers' shops, has already mentioned the impossibility of accurate identification and quantification of all risks involved in contamination of pork with *Salmonella*, because of a lack of data. To our knowledge, models describing the transfer of *Salmonella* occurring during grinding of fresh meat have not yet been published. Therefore, this study investigated the transfer of *Salmonella* during grinding of pork. Different models, derived from the model developed by Nauta *et al.* (2005) to describe cross-contamination of *Campylobacter* in poultry processing, were applied to the obtained data, and a

mathematical model was selected describing the grinding and pointing out how long the contamination was maintained during processing and in what concentration *Salmonella* was transferred. Finally, the model was challenged with different *Salmonella* concentrations inserted at several points during the grinding process.

Materials and methods

Meat

Pieces of vacuum-packaged fresh, deboned, skinned pork leg, frequently used to produce cooked ham, containing 6–8% fat, measuring about $8 \times 10 \times 15$ cm and weighing 1289 ± 109 g were obtained from a central distributor. With the exception of one pack, which was reserved for inoculation, all the others were divided into five slices. From each of the slices that was selected for analysis, representative samples of 25 g were aseptically collected to be sure that the meat to be processed was *Salmonella* free. After removing of these 25 g, the investigated slices averaged 212 ± 50 g per slice. Appropriately diluted samples in Maximum Recovery Diluent (Oxoid Ltd – Thermo Fisher Scientific Inc., Greve, Denmark) were enumerated by spread plate ($100 \mu\text{l}$) on XLD – xylose lysine deoxycholate agar (Oxoid) and incubation at 37°C for 16–24 h.

Salmonella culture

As prior investigations had shown that *Salmonella* Typhimurium was the predominant serotype in retail pork cuttings in Denmark (Hansen *et al.* 2010), a strain of *Salm.* Typhimurium DT104 (77-20547-1 provided by Anders M. Hay Sørensen, National Food Institute, DTU, Denmark) isolated from pigs and carrying resistance to ampicillin, chloramphenicol, florfenicol, streptomycin and sulfa was chosen for the experiments and grown in 20-ml lysogeny broth (Oxoid) with shaking (200 rev min^{-1}) overnight at 37°C . Subsequently, and prior to inoculation of pork, the culture was kept at 5°C for 24 h and then used directly ($c. 10^9 \text{ CFU ml}^{-1}$) in experiments conducted for building up and validation of the model, or diluted to $c. 10^7 \text{ CFU ml}^{-1}$ for validation challenge tests.

Inoculation of meat

To mimic the muscle structure that normally is infected in case of contamination with *Salmonella*, the whole piece of the obtained pork was surface inoculated with 10 drops of $100 \mu\text{l}$ of the *Salmonella* culture corresponding to $2 \times 10^9 - 4 \times 10^9 \text{ CFU}$ per piece of meat, instead of inoculating the meat already sliced. The culture was

spread on the whole surface of the side facing up using a Drigalski spatula (VWR International Ltd – Bie & Berntsen, Herlev, Denmark) and, subsequently, the meat was kept for 40 min to allow attachment of cells. The inoculated piece of pork was then divided into five slices, resulting in 10^8 – 10^9 CFU of *Salmonella* per slice. In total, the piece of pork was left uncovered on a table in the laboratory for 1 h at 21–22°C and 40% relative humidity before grinding took place.

Transfer experiment

A semi-industrial grinder (la Minerva® food service equipment, Italy – obtained from H.W. Larsen A/S, Copenhagen, Denmark) was used in a refrigerated room with temperature of about 5°C. Five slices of noninoculated meat were processed to create a matrix inside the grinder. Five inoculated samples were then ground and 40–90 slices of noninoculated pork meat were processed. Individual portions (213 ± 56 g) corresponding to each processed slice were collected in separate sterile Stomacher® bags (Seward, Worthing, UK). To ensure a homogenous distribution of *Salmonella* in the whole portion of meat, it was mixed two times for 1 min in a Stomacher® 400 circulator (Seward), intercalated with a step of manual blending, before sampling. Subsequently, 25 ± 0.2 g samples were diluted in 225 ml of brain heart infusion broth (Oxoid) and mixed in a Stomacher® 400 circulator for 2 min. Further appropriate dilutions were made in maximum recovery diluent (Oxoid) and drop-plated ($3 \times 10 \mu\text{l}$) or spread-plated ($1 \times 100 \mu\text{l}$ or $3 \times 333 \mu\text{l}$) onto XLD agar with and without 100 mg l^{-1} ampicillin (Bristol-Myers Squibb, New York, NY, USA) at 37°C for 16–24 h. This procedure was repeated three times resulting in dataset 1, 2 and 3.

Model development and validation

Initially, results from the three *Salmonella* transfer studies were fitted to a model developed for predicting cross-contamination of *Campylobacter* in poultry processing (Nauta *et al.* 2005). This model is characterized by a four-parameter equation that assumes that the grinder can be described as one single environment, with constant transfer rates between the grinder and the meat. As a result of lack of fit, an extended model was studied: two new models were derived from the hypothesis, that the input of *Salmonella* is organized in two different matrices inside the grinder; one matrix where *Salmonella* reveals high transfer ability and a second where *Salmonella* demonstrates low transfer ability from the grinder to the meat. Mathematical details of the different models are described in the Results section. For estimation of model parame-

ters, the residual sum of squares (RSS) was minimized using the solver function in MS EXCEL (Microsoft® Office Excel® 2007). The three models fitted to own experimental transfer results were compared by *F*-tests (Zwietering *et al.* 1990). Considering the RSS of the model, the number of observations and the number of model parameters, the root mean sum of squared errors (RMSE) (Ratkowsky 2004) and the bias-corrected version of Akaike information criterion (AICc) were calculated as measures for goodness of fit (Hurvich and Tsai 1989).

To validate the best-fit model, two challenge tests were performed. The first challenge test A (100 processed slices) was conducted by adding slices contaminated with 10^8 – 10^9 CFU of *Salmonella* per slice processed as 1st, 2nd and 3rd, 29th and 55th slices. A second challenge test B processed 110 portions where 1st, 2nd and 3rd slices were contaminated with 10^7 CFU of *Salmonella* per slice and the 19th and 35th slices had 10^8 – 10^9 CFU and 10^6 – 10^7 CFU of *Salmonella*, respectively. The inoculation procedure used in the validation trials was the same as adopted for model development. It is, however, important to mention that individual pieces of meat were used for each different concentration. As usual, the inoculated piece of pork was divided in five slices, then the selected slices were processed in the validation challenge test, and the remaining slices were discarded. In addition to the visual inspection of the data, bias and accuracy factors (Ross 1996) with \log_{10} CFU per slice as the response variable were used to evaluate the performance of selected models.

Model predictions with low inoculation levels

Transfer of *Salmonella* in pork at the Danish retail level usually occurs at low concentrations (Hansen *et al.* 2010); therefore, the suggested model was used to predict transfer of *Salmonella* for low initial concentrations (10^3 , 10^4 and $10^5 \log_{10}$ CFU per slice), as at such low levels, the transfer of pathogens during processing is recognized as very challenging (Aarnisalo *et al.* 2007; Sheen 2008; Sheen and Hwang 2010).

Results

Salmonella transfer model

Despite different number of processed slices in each of the three transfer trials, the profile of transfer of *Salmonella* during grinding of pork followed the same pattern. The cross-contamination of *Salmonella* occurred at two distinct rates through the process as shown in Fig. 1. First, the contamination builds up with ground meat from five contaminated slices, showing a (slightly increasing) plateau

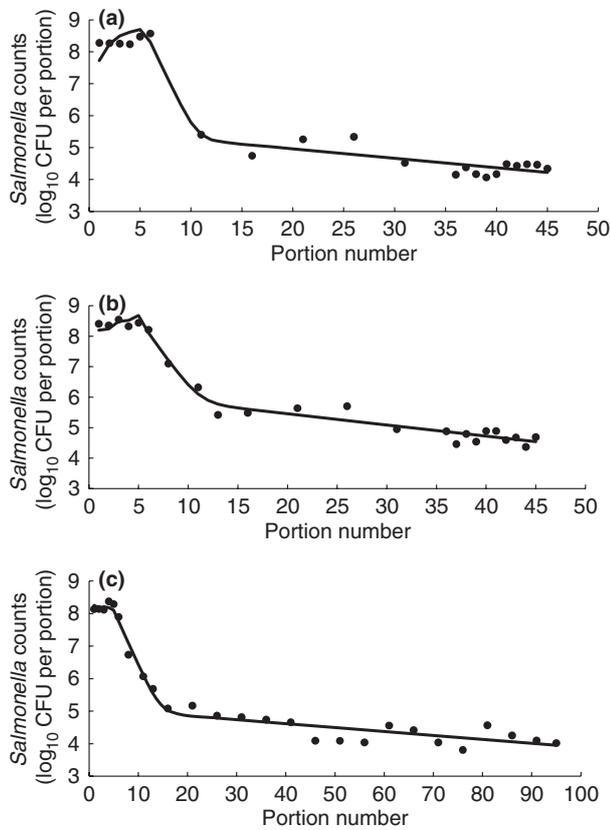


Figure 1 Transfer of *Salmonella* Typhimurium DT104 during grinding (contaminated pork to grinder to uncontaminated pork, 10^9 log₁₀ CFU per slice) of (a) (dataset 1, 45 processed slices), (b) (dataset 2, 45 processed slices) and (c) (dataset 3, 95 processed slices). The observed values (●) were fitted with the suggested 5p-2ge model with five parameters considering two grinder environments (—)

that balances the number of bacteria from each contaminated slide with the transfer from and to the grinder and inactivation in the grinder. At the first descending slope, fast transfer of *Salmonella* from the grinder to the minced meat occurred, whereas at the second slope, a ‘tail’ of low contaminated portions of ground pork represented a much slower transfer of *Salmonella*.

The model suggested by Nauta *et al.* (2005), predicting cross-contamination of *Campylobacter* in poultry processing, could efficiently describe the observed transfer of *Salmonella* during grinding of the first 20 slices but could not explain the ‘tail’ of low contaminated portions (results not shown). Therefore, it was hypothesized that the input of *Salmonella* is not organized in one environment as in the original model, but in two different matrices inside the grinder, as represented in Fig. 2. One matrix, where *Salmonella* is relatively loosely attached, is responsible for the fast transfer to the minced meat and from a second matrix, *Salmonella*’s transfer occurs at a slower rate. Based on this hypothesis, a modified version of Nauta *et al.* (2005) model was implemented. Modifications of the parameters and addition of an extra parameter to the model to describe the whole transfer were tested as shown in the following model equations:

$$\begin{cases} M_i = (1-a_1)(1-a_2)(1-c_2)S_i + (b_1gr_{1,i-1}) + (b_2gr_{2,i-1}) \\ gr_{1,i} = a_1S_i + (1-b_1)(1-c_1)gr_{1,i-1} \\ gr_{2,i} = a_2S_i + (1-b_2)(1-c_3)gr_{2,i-1} \end{cases} \quad (1)$$

This new model has seven parameters, a_1 , a_2 , b_1 , b_2 , c_1 , c_2 and c_3 , which represent probabilities of transfer (a , b) or inactivation (c) per bacterial cell, as explained in Fig. 2. However, as it was possible to recover the inoculated number of *Salmonella* from pork slices (results not shown) and pork in general is an excellent substrate for survival and growth of *Salmonella* (Escartín *et al.* 2000), inactivation in the meat was assumed not to take place and c_2 , therefore, set to zero. Likewise, inactivation of *Salmonella* in environment 1 in the grinder was assumed unlikely to occur and c_1 set to zero. In this case, the assumption was based on the fact that transfer from this environment would happen too fast for *Salmonella* to be inactivated and recovery of *Salmonella* from the ground meat portions was designed to minimize stress factors. As c_1 and c_2 both was set to zero, the model can be considered a five-parameter model (all with values between 0

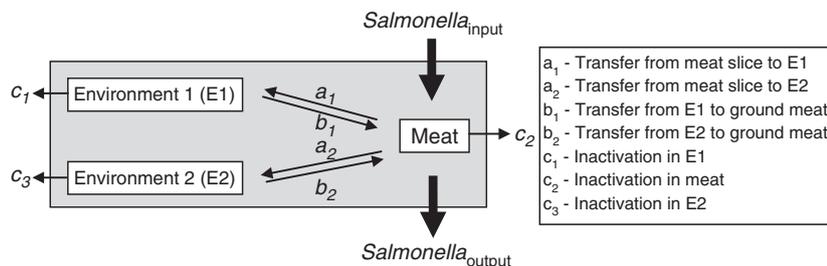


Figure 2 Diagram of the transfer model for *Salmonella* during grinding of pork. Three versions are compared: 4p-1ge ($a_2 = 0$; $b_2 = 0$; $c_2 = 0$); 4p-2ge ($c_1 = 0$; $c_2 = 0$; $c_3 = 0$), 5p-2ge ($c_1 = 0$; $c_2 = 0$). Grinder (□).

and 1), which considers k slices of meat that are processed in a grinder to k portions of minced meat ($i = 1, 2, \dots, k$). The i th slice carries S_i *Salmonella* (CFU per slice), and the resulting minced meat portion from slice i carries M_i *Salmonella* (CFU per portion). The 'contamination status' of the grinder is gr_i . The probability of transfer per *Salmonella* cell from meat to grinder, environment 1 and to grinder, environment 2 is represented by a_1 and a_2 , respectively. The backward transfer probabilities from the grinder (environments 1 and 2) to ground meat are given by b_1 and b_2 . The survival in environment 2 of the grinder is represented by $1 - c_3$.

Fitting of models and goodness of fit

As shown in Table 1, three different models were fitted to the datasets obtained from the three transfer experiments. The first model, named 4p-1ge, was a four-parameter model that considered only one grinder environment and is identical to the Nauta *et al.* model from 2005. The second model, 4p-2ge, was a modified version of this model also with four parameters but taking into account two grinder environments (Eqn 1 with $c_1 = c_2 = c_3 = 0$), and the third, 5p-2ge, was the new suggestion for a transfer model with five parameters as described previously (Eqn 1 with $c_1 = c_2 = 0$). The suggested 5p-2ge model was evaluated as the best choice as it resulted in the lowest RMSE value and AICc score for all three datasets (Table 1). Low RMSE values, as those obtained for the 5p-2ge model, show that the observed and predicted transfer of *Salmonella* were very close (Valero *et al.* 2007) and the model with the lower AICc score, like the proposed 5p-2ge model, is more likely to be correct (Motulsky and Christopoulos 2003), and therefore, it is considered to have substantial support. This conclusion was also supported statistically by significant F -tests ($P \leq 0.033$) when used to compare the suggested model (eqn 1) to the two other models for dataset 1, 2 and 3, respectively (Table 1). The difference between the three tested models was most pronounced for dataset 3, where the F -tests were highly sig-

nificant ($P < 0.001$). Figure 3 is a visual example of fitting the three models to the dataset 2. It illustrates why the 5p-2ge was the superior model. The model 4p-2ge could not describe appropriately the observed build-up of *Salmonella* in the grinder while model 4p-1ge could not describe the observed data as it was not able to fit the 'tailing' phenomenon. Parameter estimates obtained from fitting the 5p-2ge model to each of the three datasets are shown in Table 2.

Validation of transfer model

As opposed to the experiments performed when building the suggested model 5p-2ge, the five input slices carrying *Salmonella* were not only added in the beginning of the grinding process, but also at two later processing points in the two validation trials. As shown in Fig. 4, *Salmonella*-contaminated slices were added as 1st, 2nd, 3rd, 29th and 55th slices in trial A (Fig. 4a) and as 1st, 2nd, 3rd, 19th and 35th slices in trial B (Fig. 4b). In validation trial A, all five contaminated slices contained 10^8 – 10^9 CFU *Salmonella*, whereas in validation trial B, the

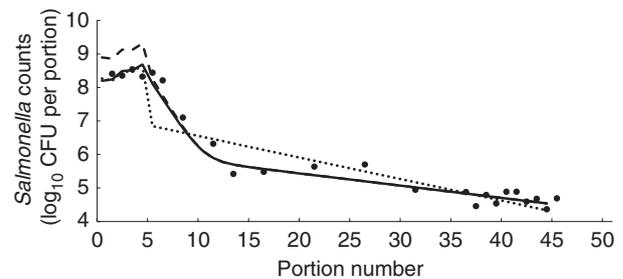


Figure 3 Transfer of *Salmonella* Typhimurium DT104 during grinding of 45 slices of 200-g boneless skinless pork leg (dataset 2) where the observed values (●) were fitted using the suggested model (5p-2ge) with five parameters considering two grinder environments (—), the modified version of the Nauta *et al.* (2005) model (4p-2ge) considering two grinder environments (---), and the Nauta *et al.* (2005) model (4p-1ge) considering one grinder environment (.....).

Table 1 Performance of the developed models when fitted to three different datasets

Model*	Dataset 1			Dataset 2			Dataset 3		
	RMSE	AICc	F -test†	RMSE	AICc	F -test	RMSE	AICc	F -test
5p-2ge	1.2029	29.78	a	1.1345	-7.04	a	1.1378	11.23	a
4p-2ge	1.2612	88.55	b	1.2364	100.47	b	1.2291	157.69	b
4p-1ge	1.4534	315.11	b	1.2862	166.49	b	1.3596	440.80	b

RMSE, root mean sum of squared error; AICc, Akaike information criterion.

*5p-2ge = suggested model with five parameters considering two grinder environments; 4p-2ge = modified version of Nauta *et al.* (2005) model considering two grinder environments; 4p-1ge = Nauta *et al.* (2005) model considering one grinder environment.

†For each dataset, different letters denote statistical difference according to the F -test.

Table 2 Parameter estimates from three datasets obtained from the transfer of *Salmonella* during grinding of pork, with initial level of 10^8 – 10^9 \log_{10} CFU per each of the five contaminated slices

Source of parameters	Number of processed slices	Parameter				
		a_1	b_1	a_2	b_2	$1 - c_3$
Dataset 1	45	0.0008	0.0655	0.7924	0.1331	0.2475
Dataset 2	45	0.0020	0.0809	0.8166	0.0555	0.3692
Dataset 3	95	0.0010	0.0275	0.8909	0.0558	0.4887

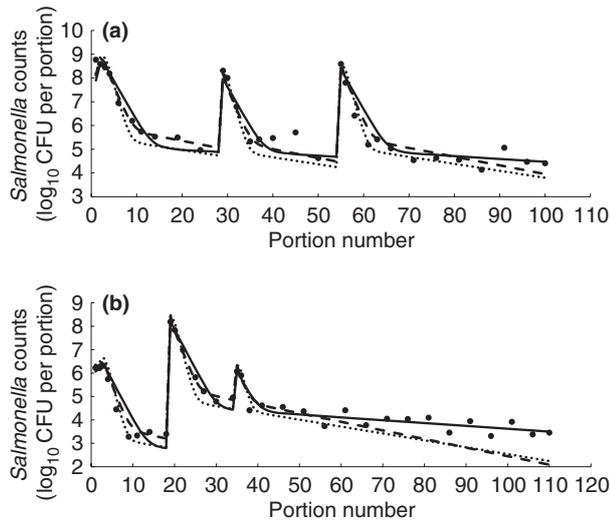


Figure 4 Observed (•) and predicted transfer of *Salmonella* Typhimurium DT104 during challenge test (a) (grinding of 100 slices of 200-g boneless skinless fresh pork leg with insertion of contaminated slices with 10^9 of *Salmonella* at three different points through the process) and during challenge test (b) (grinding of 110 slices of 200-g boneless skinless fresh pork leg with insertion of contaminated slices with 10^7 of *Salmonella* at the beginning of the process, the processed slice number 19 was a contaminated slice with 10^9 of *Salmonella* and the slice 35 was a contaminated slice with 10^7 of *Salmonella*). The transfer was predicted using parameter estimates obtained when applying the suggested model (5p-2ge) with five parameters considering two environments to dataset 1 (.....), dataset 2 (- - -) and dataset 3 (—).

Salmonella concentration was changed so that the first three slices contained 10^6 – 10^7 CFU, the fourth 10^8 – 10^9 CFU and the fifth 10^6 – 10^7 CFU. For validation trial A, comparisons of observed and predicted values resulted in bias factors of 0.95, 0.99 and 1.01 and accuracy factors of 1.07, 1.05 and 1.06, when using the parameter estimates from dataset 1, 2 and 3, respectively. Likewise for validation trial B, bias factors of 0.91, 0.93 and 1.01 and accuracy factors of 1.14, 1.12 and 1.07 were obtained applying parameters estimates from dataset 1, 2 and 3, respectively. These values of bias and accuracy factors indicate how good the model performed as the perfect agreement between predictions and observations will lead to a bias or accuracy factor of 1 (Ross 1996). Figure 4 shows that using parameter estimates obtained from fit-

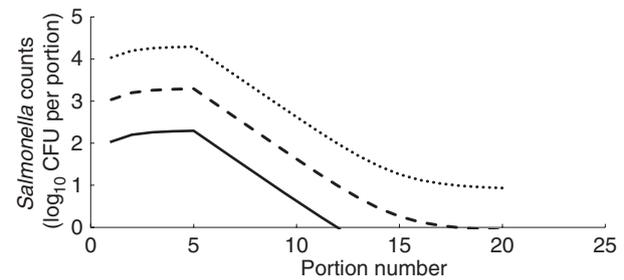


Figure 5 Simulated transfer of *Salmonella* Typhimurium DT104 during grinding of pork (contaminated pork to grinder to uncontaminated pork) was predicted with the parameter estimates from dataset 3 obtained by applying the suggested model (5p-2ge) with five parameters considering two grinder environment. The predictions were related to 10^3 \log_{10} CFU (—), 10^4 \log_{10} CFU (- - -), and 10^5 \log_{10} CFU (.....) hypothetical levels of contamination of the first five slices before the processing of 15 *Salmonella* free slices.

ting of dataset 3 to the 5p-2ge model predicted the observed ‘tailing’ phenomenon most accurately both in validation trial A and B. In large-scale studies as those related to cross-contamination, it is difficult to obtain a similarity in the size of the samples, what can be an explanation for the variation of the parameter estimates from different datasets. However, it was observed that the number of processed slices seems to have influence on the shape of the tailing phenomena and with larger experiment better parameter estimates were obtained, like those presented by dataset 3.

How the model can be used

The suggested model, 5p-2ge with parameter estimates obtained from dataset 3, was applied to simulate transfer of *Salmonella* at hypothetical lower levels of contamination. The cross-contamination was simulated with input concentrations of 10^5 , 10^4 and 10^3 *Salmonella* per slice as illustrated in Fig. 5. Looking at the profile of cross-contamination, it is possible to observe that in all examples, the tailing started after the 15th–16th processed slice but the contamination level of the tail corresponded to different levels depending on initial input of *Salmonella*, that is input of five slices with 10^4 *Salmonella* per slice resulted in a tail of minced portions containing *c.* 1 CFU per

200 g, and an increase of ten times this value was noticed using input of 10^5 that results in tailing around 10 CFU per 200 g (Fig. 5). However, it is important to be aware that when using input of five slices with 10^5 *Salmonella* per slice, at the 95th processed slice, levels of about 1 CFU of *Salmonella* could be present in a 2-kg processed piece of pork and then successively. The same elucidation can be applied to explain other hypothetical levels of *Salmonella*. Preliminary experiments, using input concentrations around 10^4 – 10^5 *Salmonella* per slice, resulted in *Salmonella* counts under the quantification limit ($<2 \log_{10}$ CFU g^{-1}) from portion 7 to portion 25. As a supplement to the direct counting methods, all these portions were incubated at 11–12°C for 2 days and then analysed again using the direct counting method. At this point, *Salmonella* not only could be detected in all portions but also appeared to be present in almost the same concentration (result not shown) confirming a tailing phenomenon.

Discussion

During the grinding of pork, *Salmonella* present on a single piece of meat may be transferred to many portions of minced meat as a result of cross-contamination in the grinder. It is, therefore, important to be able to describe the cross-contamination by grinding, mathematically, to predict how many portions of ground meat may be contaminated with the pathogen from one single piece of meat. In this study, such mathematical model was developed and it was shown that it can be used for this purpose. During the development of the model, it was also taken into consideration that a tailing phenomenon of the transfer of *Salmonella* was observed during a small-scale grinding process. It was suggested that transfer occurred from two environmental matrices inside the grinder and the model was developed to match this hypothesis. Considering the low RMSE and AICc values supported by the *F*-tests results, the developed model satisfactorily fitted the observed behaviour of *Salmonella* during its cross-contamination in the grinding of up to 110 pork slices corresponding to 21 kg meat. During the past decade, a number of studies investigating the transfer of pathogens during slicing of ready-to-eat products (Vorst *et al.* 2006; Aarnisalo *et al.* 2007; Sheen 2008; Sheen and Hwang 2010) have been published. However, no other studies have yet modelled the transfer of *Salmonella* during a grinding process and only in few other studies, a similar distinct tailing phenomenon during cross-contamination was observed (Vorst *et al.* 2006; Aarnisalo *et al.* 2007; Sheen 2008; Sheen and Hwang 2010). In the study of Sheen and Hwang (2010), the cross-contamination of *E. coli* O157:H7 during slicing of ready-to-

eat ham was modelled applying an empirical approach, and the selected model characterized the transfer as decreasing following exponential law. Comparison of the Sheen and Hwang (2010) model to the model developed in the present study revealed that the two models are mathematically similar, and it can be shown that the Sheen and Hwang (2010) model is basically the same as the model termed 4p-1ge (Nauta *et al.* 2005) considering one environment only. Despite this similarity, the proposed model should, however, be preferred as it includes the pieces of meat that are contaminated before grinding and it gives clear explanations of all the parameters involved providing an overview of the dynamics of a grinding process. As it is easier to understand, it also holds the potential to be universal, that is transferrable to cross-contamination dynamics for other food-processing steps. The fitted model obtained in this study is of course specific to the studied grinding process including the particular grinder applied. However, the structure of the model, and particularly its ability to predict the tailing phenomenon, seems relevant for different cross-contamination processes. Testing the model structure on data published in other transfer studies, where different food products, micro-organisms, concentration of pathogen and different routes of contamination (food product to slicer to food product or slicer to food product, using the same product or different products) were used, showed promising results. Applying the proposed 5p-2ge to literature data and obtaining R^2 values close to one illustrates this claim. For example, when applying the data published by Vorst *et al.* (2006), simulating cross-contamination of *L. monocytogenes* during turkey slicing, $R^2 = 0.86$, was found. When the data presented by Aarnisalo *et al.* (2007), regarding transfer of *L. monocytogenes* during slicing of gravad salmon, were used, $R^2 = 0.74$ was obtained, and for the data published by Sheen and Hwang (2010) related to cross-contamination of *E. coli* O157:H7 during ham slicing, R^2 was 0.78.

The good bias and accuracy factors, with values close to one, obtained when validating the suggested model, are an indication that the hypothesis of two matrices being responsible for the transfer could in fact be the explanation for the observed tailing. Nevertheless, it has yet to be elucidated what the two suggested environmental matrices consist of and how the transfer takes place at the physical level. The tailing phenomenon could also be interpreted as two subpopulations behaving differently in one environment, that is having different susceptibility to the environmental stress experienced during grinding and, thereby, different transfer abilities. More investigations are needed to determine the exact cause of the observed two-phase transfer of *Salmonella* during grinding. Furthermore, as *Salmonella* levels as high as 7–9 \log_{10} CFU

per slice were used for developing the model, it is not known whether tailing could be an artefact of high concentrations. Although levels of *Salmonella* lower than $3 \log_{10}$ CFU per slice (Hansen *et al.* 2010) would have been more relevant to study, this was not performed because transfer modelling becomes very challenging at such low concentrations because of the random transfer pattern resulting in concentrations below the detection limit when using direct plate counting methods (Aarnisalo *et al.* 2007; Sheen and Hwang 2010). However, when applying *Salmonella* concentrations as low as $4\text{--}5 \log_{10}$ CFU per slice and combining it with enrichment for 2 days at $11\text{--}12^\circ\text{C}$ in the minced meat, the tailing phenomenon was confirmed. The complexity and difficulty of low-pathogen-level transfer faced in the present study were also described by Sheen and Hwang (2010), who stressed how relevant it is to obtain more information and knowledge to facilitate realistic risk predictions. It also suggests that new methods of enumeration should be developed to improve sensitivity and still continue to be simple enough for low concentration, large-scale studies to be realistic. Enrichment in the meat at relatively low temperatures from 11 to 16°C combined with accurate predictive models appears as an obvious solution.

Other factors such as varying fat contents of the meat, varying sizes of the meat pieces to be minced and growth of *Salmonella* inside the grinder could also influence the cross-contamination dynamics and perhaps be responsible for the observed tailing. Biological variation between the pieces of meat and *Salmonella* populations, as well as randomness, may also explain a large part of the variation between the parameter values as obtained from the three experimental datasets compared in this study (Table 2). In the present study, the effect of these factors was minimized by (i) use of lean meat with the same low fat content for all experiments, (ii) use of meat slices with approximately the same dimensions for all experiments and (iii) use of processing temperature and time conditions not supporting growth of *Salmonella*. Therefore, these factors could be excluded as main decisive factors of the observed tailing phenomenon.

Different models describing transfer behaviour of pathogens in food processing have already been published in the scientific literature with relatively good performance. However, most of them are empirical models and cannot explain the meaning behind the model parameters. Therefore, it is still important to develop models not only capable of describing the observed occurrence of cross-contamination taking into consideration the precise level of contaminant in the whole process, but also of giving a reasonable explanation to the phenomenon implicated in transfer of pathogens during food processing. This was achieved by the model proposed in the present

study, and it is believed that the model structure is transferable to other cross-contamination scenarios. Furthermore, the proposed model presents an important tool to examine the effect of cross-contamination in case of low concentrations, for example in relation to quantitative microbial risk assessment investigations.

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Chapter 6

MANUSCRIPT I

Effect of natural microbiota on growth of *Salmonella* spp. in fresh pork – a predictive microbiology approach

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Abstract

This study was undertaken to model and predict growth of *Salmonella* and the dominating natural microbiota, and their interaction in ground pork. Growth of *Salmonella* in sterile ground pork at constant temperatures between 4°C and 38°C was quantified and used for developing predictive models for lag time, max. specific growth rate and max. population density. Data from literature was used to develop growth models for the natural pork microbiota. Challenge tests at temperatures from 9.4 to 24.1°C and with *Salmonella* inoculated in ground pork were used for evaluation of interaction models. The existing Jameson-effect and Lotka-Volterra species interaction models and a new expanded Jameson-effect model were evaluated. F-test indicated lack-of-fit for the classical Jameson-effect model at most of the tested temperatures and at 14.1 to 20.2°C this was caused by continued growth of *Salmonella* after the natural microbiota had reached their max. population density. The new expanded Jameson-effect model and the Lotka-Volterra model performed better and appropriately described the continued growth of *Salmonella* after the natural microbiota had reached their max. population density. The expanded Jameson-effect model is a new and simple species interaction model, which has performed as well as the more complex Lotka-Volterra model.

1. Introduction

Pork meat is a substantial source of human *Salmonella* infections in many countries and in Denmark 9 % of the domestic and sporadic cases of *Salmonella* infections has been attributed to pork meat (Anonymous, 2009; Hald et al., 2004). Substantial efforts have been allocated to control of *Salmonella* in primary pig production (Hurd et al., 2008; Rajic et al., 2007). Nevertheless, Danish prevalence of *Salmonella* in raw pork cuts in retail ranged between 0.4 % and 9.6 % depending on cuts, year and type of retailer (Hansen et al., 2010). Both the risk of infection and the risk of illness given infection increase with the amount of *Salmonella* being consumed (FAO/WHO, 2002; Teunis et al., 2010). Therefore, to reduce risk of *Salmonella* infections it is important to limit growth in pork products (e.g. Hansen et al., 2010; Prendergast et al., 2009), reduce cross contamination (e.g. Jiménez et al., 2009; Møller et al., 2012) and to use efficient decontamination procedures when possible (e.g. Hugas and Tsigarida, 2008; Morild et al., 2011).

Predictive microbiology models can be used to evaluate the potential growth of microorganisms in food. For growth of *Salmonella* in pork several models and software are available to predict the effect of constant and fluctuating storage temperatures, pH, water activity and organic acids (Ingham et al., 2009; Min and Yoon, 2010; Pin et al., 2011; Velugoti et al., 2011). *Salmonella* is

relatively resistant to organic acids and bacteriocins, which, together with the translocation from the intestinal rumen to the intracellular environment, allow them to avoid detrimental competition from microorganisms in the mammalian gastrointestinal tract (Ahmer and Gunn, 2011). However, in environments where *Salmonella* cannot escape interaction, growth can be reduced. This has been observed by the competing microbiota in raw meat (Zaher and Fujikawa, 2011) and in enrichment cultures (Jameson, 1962). Hence, to predict concentrations of *Salmonella* in pork, interaction with the food microbiota can be important, but no predictive microbiology models have yet been developed for the inhibiting effect of the pork meat microbiota on the growth of *Salmonella* at different storage temperatures.

High concentrations of microorganisms can have a substantial inhibiting effect on growth of pathogens in pork meat and in other muscle foods (Cornu et al., 2011; Vermeiren et al., 2006). In relation to exposure and risk assessments it is important to take into account the quantitative effect of these microbial interactions at the same time as the quantitative effect of relevant product characteristics and storage conditions (Pouillot et al., 2007; Ross et al., 2009). Several mathematical models have been developed to quantitatively describe the effect of microbial interaction in foods e.g. the inhibiting effect of lactic acid bacteria (LAB) on growth of *Listeria monocytogenes* (Cornu et al., 2011; Giménez and Dalgaard, 2004). A simple model relies on the assumption of the Jameson effect *i.e.* that the dominating microbiota quantitatively inhibits growth of the pathogen in the same way that they inhibits their own growth and that the pathogen therefore stops growing at the time when the dominating microbiota reach their maximum population density (MPD). This simple growth pattern can be predicted without interaction parameters that have to be estimated from mixed-culture experiments and this facilitates predictions at different storage temperatures (Giménez and Dalgaard, 2004). A modified version of this interaction model allows a microbiota concentration lower than their MPD to dampen and stop growth of the pathogen (Le Marc et al., 2009). The more general Lotka-Volterra model of predator-prey interaction can also be used for groups of microorganisms in food when values of interaction parameters have been estimated (Cornu et al., 2011; Lotka, 1956; Volterra, 1931). However, interaction models appropriate for the inhibiting effect of the natural microbiota on growth of *Salmonella* in raw pork meat remain to be suggested and evaluated.

The objectives of the present study were to model and predict growth of *Salmonella*, growth of the dominating natural microbiota and their interaction in ground raw pork. Growth of *Salmonella* in sterile ground raw pork between 4°C and 38°C was quantified and used for development of a predictive model. Data from the literature was used for development of a natural microbiota growth model at different temperatures. Challenge tests at various temperatures and with *Salmonella* inoculated in ground raw pork were used for evaluation of growth and interaction models.

2. Materials and methods

2.1 Challenge tests and *Salmonella* growth models

Primary and secondary *Salmonella* growth models were developed based on experimental data generated from inoculation of sterile ground pork meat.

Packages of approx. 500 g of modified atmosphere packaged (MAP) ground lean pork were obtained from a local retailer. The packages used for preparing sterile meat were mixed manually in a sterile bag for 10 min. Portions of 100 ± 3 g were vacuum packaged and frozen at -18°C . Sterilization of volumes of 5 times 100 g of frozen samples was done by irradiation at a dose of 5 kGy for 523 min, followed by freezing at -18°C . Prior to challenge tests 100 g portions of vacuum packed sterilized ground meat were defrosted in water at a temperature of approx. 40°C for 30 min.

Three *Salmonella* strains, previously isolated from pigs and characterized by the National Food Institute at the Technical University of Denmark, were studied. *Salmonella* Typhimurium DT104 (77-20547) carried resistance to ampicillin, chloramphenicol, florfenicol, streptomycin and sulfa. *Salmonella* Typhimurium DT12 (R77) was resistant to rifampicin and *Salmonella* Derby (77-20390) carried resistance to gentamycin, streptomycin, sulfa and spectinomycin. The strains were maintained as frozen (-80°C) stock cultures.

For each *Salmonella* isolate, one loop of the frozen stock culture was transferred to a test tube containing 10 ml of Lysogeny Broth (OXOID A/S – Thermo Fisher Scientific Inc., Greve, DK) and incubated at 37°C overnight with shaking (200rpm). Subsequently, these stationary phase cultures were kept at 5°C for 3 days. Prior to inoculation of the sterile meat samples, the three cultures were diluted in maximum recovery diluent (OXOID) to obtain a concentration of approx. 10^6 CFU ml^{-1} . A *Salmonella* cocktail was prepared by mixing equal volumes of the three cultures in a sterile tube.

Each of the 100-g-meat samples was aseptically transferred to separate sterile stomacher bags (Seward, Worthing, UK) and inoculated with 1 ml of the *Salmonella* cocktail. To distribute the added cells, each inoculated minced sample (with ca. 10^4 CFU g^{-1}) was mixed two-times during one minutes in a stomacher (Seward).

Inoculated meat samples in stomacher bags were stored at 4, 8, 10, 11, 15, 19, 24.5, 30, 33, 37 and 38°C . At appropriate time intervals, the whole meat sample was mixed in a stomacher for two times one minute. Subsequently, 5.0 ± 0.2 g of meat was aseptically transferred to a sterile filter bag (Seward) and the stomacher bag with the remaining amount of meat was placed back in the incubator within 5 min. The 5-g samples were diluted in 45 ml of maximum recovery diluent and

mixed in a stomacher for two minutes. Further 10-fold dilutions were performed using maximum recovery diluent and appropriate dilutions were drop-plated (three drops of 10 µl each) onto XLD agar (OXOID). The plates were incubated at 37°C for 16 to 24 h. Two growth curves were generated at four of the 11 studied temperatures, resulting in a total of 15 growth curves.

The primary log-transformed Logistic model with delay was fitted to *Salmonella* growth curves at each storage temperature to estimate lag phase duration (λ , h), maximum specific growth rate (μ_{\max} , h⁻¹) and maximum population density (N_{\max} , log₁₀CFU g⁻¹) (Dalgaard, 2009). The obtained μ_{\max} -values were modeled as a function of temperature using the simple square root model of Ratkowsky et al. (1982) (Equation 1):

$$\sqrt{\mu_{\max}} = b \cdot (T - T_{\min}) \quad (1)$$

where T is the temperature in °C, T_{\min} the theoretical minimum temperature of growth in °C and b a constant.

In order to model the influence of temperature on λ (h), a one parameter model (Equation 2), derived from the μ_{\max} model, was applied (Ross and Dalgaard, 2004; Zwietering et al., 1994):

$$\lambda = RLT \cdot \ln(2) / \mu_{\max} \quad (2)$$

where RLT is the relative lag time which is assumed to be independent of storage temperature. When fitted to experimental lag time data μ_{\max} in Equation 2 was replaced by Equation 1 i.e. $(b \cdot (T - T_{\min}))^2$ with fitted values of the parameters b and T_{\min} .

The performance of the developed *Salmonella* growth models for the effect of storage temperature on μ_{\max} and λ were evaluated by comparison with data collected from the literature (Table 1). Bias and accuracy factors were calculated as suggested by Ross (1996). The bias factor measures the average relative deviation between predictions and observations, whereas the accuracy factor measures how close predicted values on average are to the observed. Bias factors for μ_{\max} and λ were calculated so that a value of 1.10 indicated the predicted values to be on average 10 % higher than the observed values.

2.2 Models for growth of natural microbiota

Secondary models for growth of natural microbiota in raw pork meat were developed based on data from the literature. A literature search was conducted and 27 studies were found to contain data suitable for estimation of growth parameters of the natural aerobic mesophilic or psychrotolerant microbiota of pork meat stored under aerobic conditions at different temperatures (Table 2). Values of λ (h), μ_{\max} (h^{-1}) and N_{\max} ($\log_{10}\text{CFU}\cdot\text{g}^{-1}$), were obtained directly as reported, determined from tables and graphs or calculated from reported generation time or growth rates (GR, $\log_{10}\text{CFU}$ per time unit) values as $\mu_{\max} = \text{GR} \cdot \ln(10) = \ln(2) / \text{generation time}$. Equations 1 and 2 were used, respectively, to model the effect of storage temperature on μ_{\max} and on λ for growth of the natural microbiota. As this model was developed directly based on data from various studies with naturally contaminated raw pork meat no product validation of the developed model was performed.

2.3 Evaluation of interaction models for inhibition of *Salmonella* by the natural microbiota

To select and evaluate interaction models for the inhibiting effect of the natural microbiota on growth of *Salmonella* in ground raw pork 17 challenge tests were performed at eight different storage temperatures between by 9.4 and 24.1°C.

2.3.1 Challenge tests with *Salmonella* and natural microbiota in ground raw pork

Packages with 500 g of MAP ground pork were obtained from local retailers and divided into 100 g portions. The 100-g-samples were transferred to sterile bags and inoculated with the three-strain *Salmonella* cocktail prepared and pre-cultured as described above (See 2.1). At pre-determined time intervals, 5-g samples were 10-fold diluted using maximum recovery diluent, drop-plated (three 10- μl -drops) onto Plate Count Agar (OXOID) and incubated at $21 \pm 1^\circ\text{C}$ during 3 d for enumeration of aerobic viable bacteria and on XLD agar (37°C , 16 – 24 h) to enumerate *Salmonella*. Challenge tests were performed at 9.4, 10.5, 11.9, 14.2, 15.1, 16.6, 20.2 and 24.1°C.

2.3.2 Selection of primary interaction models

To predict the inhibition of *Salmonella* by the natural microbiota of pork meat at various storage temperatures, three microbial interaction models were tested.

The model proposed by Giménez and Dalgaard (2004) for interaction between LAB and *L. monocytogenes* was evaluated (Equation 3). This model corresponds to the Jameson effect and

predicts that one microorganism stops growing when the other has reached its maximum population density (MPD). An important characteristic of this equation is that it does not include parameters that need to be fitted from mixed-culture studies. Equation 3 includes the lag phase duration for both *Salmonella* (t_{lag-S}) and the natural microbiota (t_{lag-NB}):

$$\left\{ \begin{array}{l} t < t_{lag-S}, \quad \frac{dS/dt}{S_t} = 0 \\ t \geq t_{lag-S}, \quad \frac{dS/dt}{S_t} = \mu_{max}^S \times \left(1 - \frac{S_t}{S_{max}}\right) \times \left(1 - \frac{NB_t}{NB_{max}}\right) \\ t < t_{lag-NB}, \quad \frac{dNB/dt}{NB} = 0 \\ t \geq t_{lag-NB}, \quad \frac{dNB/dt}{NB} = \mu_{max}^{NB} \times \left(1 - \frac{NB_t}{NB_{max}}\right) \times \left(1 - \frac{S_t}{S_{max}}\right) \end{array} \right. \quad (3)$$

where S and NB are the concentrations in CFU g^{-1} of *Salmonella* and the natural microbiota, respectively and S_{max} and NB_{max} are the maximum population densities in CFU g^{-1} . μ_{max}^S and μ_{max}^{NB} are the maximum specific growth rate in h^{-1} for *Salmonella* and for the natural microbiota, respectively.

An expanded version of the Jameson model by Gimenez and Dalgaard (2004) was suggested and evaluated (Equation 4). This new model includes a temperature dependent parameter (γ) that was estimated from mixed-culture studies (See 2.3.1) and allowed the inhibiting effect of the natural microbiota on growth of *Salmonella* to differ depending on the storage temperature. This expanded Jameson-effect-model is more flexible than equation 3 and depending on γ -values equation 4 allows the predicted concentration of *Salmonella* to (i) increase after the natural microbiota has reached its MPD ($\gamma < 1$) or (ii) to decrease after the natural microbiota has reached its MPD ($\gamma > 1$). With an γ -value of 1.0 equation 3 and 4 are identical.

$$\left\{ \begin{array}{l}
t < t_{lag-S}, \quad \frac{dS/dt}{S_t} = 0 \\
t \geq t_{lag-S}, \quad \frac{dS/dt}{S_t} = \mu_{max}^S \times \left(1 - \frac{S_t}{S_{max}}\right) \times \left(1 - \frac{\gamma \times NB_t}{NB_{max}}\right) \\
t < t_{lag-NB}, \quad \frac{dNB/dt}{NB} = 0 \\
t \geq t_{lag-NB}, \quad \frac{dNB/dt}{NB} = \mu_{max}^{NB} \times \left(1 - \frac{NB_t}{NB_{max}}\right) \times \left(1 - \frac{S_t}{S_{max}}\right)
\end{array} \right. \quad (4)$$

where γ is a coefficients of interaction and other parameters are as indicated for equation 3.

The classical Lotka-Volterra model for predator-prey interaction (Cornu et al., 2011; Lotka, 1956; Vereecken et al., 2000; Volterra, 1931) was evaluated and coefficients of interaction ($\alpha_{S,NB}$ and $\alpha_{NB,S}$) were estimated from mixed-culture studies (Equation 5). We assumed, however, that *Salmonella* did not influence growth of the natural microbiota and used a fixed value of zero for the $\alpha_{NB,S}$ coefficients of interaction in Equation 5 whereas values of $\alpha_{S,NB}$ was fitted at the different storage temperatures.

$$\left\{ \begin{array}{l}
t < t_{lag-S}, \quad \frac{dS/dt}{S_t} = 0 \\
t \geq t_{lag-S}, \quad \frac{dS/dt}{S_t} = \mu_{max}^S \times \left(1 - \frac{S_t + \alpha_{S,NB} \cdot NB_t}{S_{max}}\right) \\
t < t_{lag-NB}, \quad \frac{dNB/dt}{NB} = 0 \\
t \geq t_{lag-NB}, \quad \frac{dNB/dt}{NB} = \mu_{max}^{NB} \times \left(1 - \frac{NB_t + \alpha_{NB,S} \cdot S_t}{NB_{max}}\right)
\end{array} \right. \quad (5)$$

where $\alpha_{S,NB}$ and $\alpha_{NB,S}$ are coefficients of interaction and other parameters are as indicated for equation 3 and 4 above.

2.3.3 Secondary interaction models

The effect of temperature on coefficients of interaction i.e. γ in Equation 4 and $\alpha_{NB,S}$ in Equation 5 was described by a simple polynomial model (Equation 6).

$$\text{coefficients of interaction} = a_0 + a_1 \cdot T + a_2 \cdot T^2 \quad (6)$$

where T is the temperature in °C and a_0 , a_1 and a_2 are constants.

2.3.4 Evaluation of interaction models

Performance of the interaction models was evaluated by lack-of-fit F-tests as suggested by Zwietering et al. (1990). The residual sum of squares (RSS) of growth curves simulated by the interaction models was compared to RSS of the growth curves fitted individually to the log-transformed Logistic model with delay for temperatures at 11.9°C and above and to the log-linear model for 9.4 and 10.4°C, representing the pure error.

2.4 Curve fittings and simulations

Curve fittings and simulations were done in five steps. In the first step, individual bacterial growth curves of *Salmonella* spp. in sterile pork were fitted to the log-transformed Logistic with delay model by minimizing the RSS using the solver function in Microsoft Excel. In the second step, the PROC NLIN in SAS Enterprise was applied for fitting the secondary models, Equations 1 and 2, describing the effect of temperature on growth parameters for *Salmonella* spp. in sterile ground pork and for the natural pork microbiota, respectively. In the third step, the parameter estimates from Equations 1 and 2 were used in the Equations 4, and 5 for the estimation of the coefficients of interaction γ and $\alpha_{S,NB}$, respectively. The individual growth curves of *Salmonella* spp., obtained from the challenge tests in ground pork with a natural microbiota, were fitted to Equations 4, and 5 using the SAS computation procedure as described above. In the fourth step, the SAS computation procedure was applied for fitting the secondary model, Equation 6, describing the effect of temperature on the coefficients, γ and $\alpha_{S,NB}$, for the microbial interaction between *Salmonella* spp. and the natural pork microbiota. In the fifth step, simulations of the microbial interaction between *Salmonella* spp. and the natural microbiota in ground pork were performed in Microsoft Excel, using the Equations 3, 4, and 5 with parameter estimates from the fitted Equations 1, 2, and 6. The simulations were compared with observed growth of both *Salmonella* spp. and the natural

microbiota in ground raw pork stored under aerobic conditions at various temperatures.

3. Results

3.1 Challenge tests and *Salmonella* growth models

The starting levels of *Salmonella* in the samples were $4.5 \pm 0.34 \log_{10}\text{CFU g}^{-1}$. For one sample stored at 4°C, no growth was observed within the 7.1-day time frame of the experiment. For samples stored at 8°C to 38°C the estimated kinetic parameters for growth of *Salmonella* (λ , μ_{\max} and N_{\max}) are shown in Figure 1 (closed symbols). Eqn. 1 and 2, respectively, provided a good description of the observed μ_{\max} - and λ -values as shown in Figure 1 and by the RMSE-values of 0.059 for μ_{\max} and 6.8 for λ . Fitted parameter values are shown in Table 3. No systematic effect of temperatures on N_{\max} ($\log_{10}\text{CFU g}^{-1}$) was observed (Figure 1c) and this parameter was determined as average \pm standard deviation of the N_{\max} -values obtained by fitting of the primary growth model (Table 3).

The fitted growth rate model (Figure 1b) predicted μ_{\max} -values that on average was in good agreement with the 20 μ_{\max} -values collected from literature and used to evaluate the developed model for the effect of temperature on μ_{\max} -values of *Salmonella* spp. in ground raw pork. The bias- and accuracy-factor values were 0.90 and 1.41, respectively. As shown in Figure 1b, the under-prediction of μ_{\max} -values appeared to be occurring at temperatures above 18°C. A bias of 0.76 could be calculated for the data-points above 18°C whereas below 18°C, bias and accuracy factors were 0.94 and 1.29, respectively. The fitted lag time model (Figure 1a) also predicted values that on average were in good agreement with literature data and this resulted in bias- and accuracy-factor values of 0.98 and 1.34, respectively. The average N_{\max} -value ($\log_{10}\text{CFU g}^{-1}$) determined in the present study for growth of *Salmonella* spp. in inoculated ground raw pork was 16 % higher than the average value obtained from literature data (Table 1, Figure 1c).

3.2 Models for growth of the natural microbiota

Sixty seven μ_{\max} -, 54 λ - and 32 N_{\max} -estimates were obtained and used to model the effect of temperature between -1.1°C and 25°C on growth of the natural microbiota in pork meat stored under aerobic conditions (Table 2). While experimental aims, methods, substrates and presentations of the results varied among these studies, an overall consistency was observed as shown in Figure 2 and by the RMSE-values of 0.068 for μ_{\max} and 40.3 for λ . Fitted parameter

values are shown in Table 3. No systematic effect of temperatures on N_{\max} ($\log_{10}\text{CFU g}^{-1}$) was observed but the values obtained from the literature showed considerable variability (Figure 2c, Table 3).

3.3 Interaction models

High concentrations of the natural microbiota in ground raw pork reduced the growth of *Salmonella* spp. and this effect was temperature dependent. At 9.4°C, 10.4°C, 11.9°C, and 14.2°C growth of *Salmonella* spp. was either not observed or dampened by the natural microbiota corresponding approximately to the Jameson effect. However, at 15.1°C, 16.6°C and 20.2°C growth of *Salmonella* spp. continued after the natural microbiota had reached its MPD (Figure 3). As expected, this temperature dependent growth pattern was not appropriately described by the simple Jameson-effect-model (Figure 3, Table 4). In contrast, both the expanded Jameson-effect-model (Equation 4) and the Lotka-Volterra model (Equation 5) provided reasonable fits for all the co-culture kinetics observed with natural microbiota and *Salmonella* spp. between 9.4°C and 24.1°C (Figure 3, Table 4). Fitted values of the coefficients of interaction, *i.e.* γ in equation 4 and $\alpha_{NB,S}$, in equation 5, reflected the temperature dependent effect of the natural microbiota on growth of *Salmonella* spp. in ground raw pork (Figure 4, Table 5).

4. Discussion

We found high concentrations of the natural microbiota in ground pork to reduce growth of *Salmonella*. This interaction effect was temperature dependent and mathematical models were developed to quantitatively predict the effect of both the storage temperature and the natural microbiota on growth of *Salmonella* (Figure 3). The suggested interaction models for ground pork are new and important for future exposure and risk assessments where concentrations of *Salmonella* in this product at the time of consumption can be predicted for estimation of the risk of *Salmonella* infections.

The models developed in the present study for *Salmonella* spp. predicted growth that quantitatively corresponded to independent product studies with pork meat and model parameters were similar to previously developed models (Table 1, Table 3). The fitted RLT -value of 3.10 (2.50 – 3.70) corresponded to the average value of 3.1 reported by Velugoti et al. (2011) for growth of *Salmonella* spp. in ground pork and it did not differ from the average value of 2.5 reported by Juneja et al. (2007) for growth in chicken. The fitted T_{\min} -value of 2.33 (0.40 – 4.26) was not significantly lower than the value of 4.27 ± 0.35 reported by Pin et al. (2011) for growth of

Salmonella spp. in broth at different temperatures.

High concentrations of the natural microbiota in meat can reduce growth *Salmonella* spp. as observed for raw ground chicken (Oscar, 2006; Zaher and Fujikawa, 2011) and in the present study for ground pork at storage temperatures below ca. 20°C (Figure 3). In this situation, to estimate the inhibiting effect of the natural microbiota, the time required for their growth to high concentrations must be predicted. We have found no models for the effect of storage temperature on growth of the natural microbiota in ground pork. Growth models have previously been published on pseudomonades, *Brochothrix thermosphacta*, lactic acid bacteria and *Enterobacteriaceae* in pork (Koutsoumanis et al., 2006; Koutsoumanis et al., 2008) but not for the total psychrotrophic microbiota. Therefore, a model based on available data from the literature was developed (Table 2, Table 3). The fitted T_{min} -value of -5.48 (-6.91 to -4.05) corresponds to values previously determined for growth of pseudomonades at different temperatures (Dominguez and Schaffner, 2007).

Interestingly, the concentrations of *Salmonella* spp. attained in ground pork with a natural microbiota increased markedly with storage temperatures from 9.4°C to 24.1°C (Figure 3). A similar growth pattern has been observed for *Salmonella* spp. in ground chicken with a natural microbiota (Zaher and Fujikawa, 2011). However, mathematical models, to describe and predict this growth pattern, have not previously been developed.

At storage temperatures of 14.2°C and below little or no growth of *Salmonella* spp. was observed after the time when the natural microbiota reached their MPD. In this temperature range the three evaluated interaction models provided a reasonable description of the growth kinetics for *Salmonella* spp. (Figure 3). In contrast, kinetics at 15.1°C, 16.6°C and 20.2°C showed growth of *Salmonella* spp. after the natural microbiota had reached its MPD. This pattern clearly was not appropriately descended by the simple Jameson effect model (Equation 3) but much better, and about equally well, described by the expanded Jameson-effect model (Equation 4) and by the Lotka-Volterra model (Equation 5) (Figure 3, Table 4). Importantly, these models that included one coefficients of interaction each provided reasonable predictions for growth of *Salmonella* spp. throughout the studied range of storage temperatures from 9.4°C to 24.1°C.

Previously the simple Jameson effect model or its modification suggested by Le Marc et al. have been used to predict growth of microorganisms in food at different storage temperatures (Le Marc et al., 2009; Mejlholm and Dalgaard, 2007; Vermeulen et al., 2011). In those studies, however, the effect microbial interaction on growth patterns was independent of the studied storage temperatures. The Lotka-Volterra model has been used to predict the effect of interaction on growth of microorganisms in food (Cornu et al., 2011; Giuffrida et al., 2009) but we have not found previous studies describing the effect of temperature on its coefficients of interaction.

The efficiency of the tested models (Equation 4 and 5) was temperature dependent. Both models showed generally good fit at temperatures from 15.1 to 20.2°C but otherwise lack-of-fit was observed (Table 4). One reason for the lack-of-fit of these interaction models was speculated to be a result of the strongly biased lag phase model for the natural pork microbiota (Figure 2). In 10 out of 17 challenge tests in the present study, the natural microbiota was observed to have no lag phase. Therefore, fittings and simulations were repeated for the Lotka-Volterra and expanded Jameson-effect models assuming a lag time of zero for the natural pork microbiota. As indicated by lower F-values, improvements were observed at 6 and 5 storage temperatures for Lotka-Volterra and expanded Jameson-effect model, respectively (results not shown). These results underlined the inherent difficulty in predicting microbial interactions as in the case of *Salmonella* spp. in fresh pork, where two models might be needed; one including lag time for the natural microbiota suited for the situation where the contamination with *Salmonella* spp. occurs during the slaughter process, and another excluding the lag time for the natural microbiota suited for the situation where the contamination with *Salmonella* spp. occurs when the natural microbiota already has started to grow, e.g. at the retail.

Another possible source for the erroneous predictions of *Salmonella* spp. could be the *Salmonella* growth model itself. As indicated by the accuracy factors, discrepancies of predictions of *Salmonella* lag times and growth rates were observed when compared to literature (Alford and Palumbo, 1969; Ingham et al., 2007; Mann et al., 2004,). Alternatively a model based on our *Salmonella* spp. in pork results and literature pork growth data could be used. In this case, the parameter estimates of 0.0384, 2.582°C, 2.93 and 8.45 log₁₀CFU g⁻¹ (S.D.: 0.74) for b , T_{\min} , RLT and N_{\max} , respectively, could be used. If a model representing a broader variety of meats, e.g. including cooked or lightly cured products, is wanted, the *Salmonella* μ_{\max} model published by Dominguez and Schaffner (2008) would be the choice.

Since Lotka-Volterra and the proposed version of Jameson with gamma models show similar good fits of the observed effect of natural microbiota on growth of *Salmonella* during storage of ground pork, it is important to stress the simplicity of the proposed model since it works with just one interaction coefficient, gamma. The Lotka-Volterra model includes two interaction coefficients: one alpha to model changes in growth rate of *Salmonella* as affected by the natural pork microbiota concentration and another alpha to model changes in growth rate of the natural pork microbiota as affected by the concentration of *Salmonella*. However, as *Salmonella* concentrations in fresh pork are low and only rarely exceed 400 CFU/g (Pires, 2009; Anonymous, 2012; Hansen et al., 2010) *Salmonella* will always be present in a lower concentration than the natural microbiota in pork making it unlikely that the growth rate of the natural microbiota will be affected by *Salmonella*. Therefore, the alpha is not applicable in the case of *Salmonella* in fresh pork and can be ignored

during the interaction modelling with the Lotka-Volterra model. In that way, the Lotka-Volterra model and the expanded Jameson-effect model become equally simple.

In conclusion, the inhibiting effect of high levels of the natural pork microbiota on growth of *Salmonella* was temperature dependent, *i.e.* below 15°C and above 20°C, growth of *Salmonella* stopped when the natural microbiota reached its MPD, whereas between 15 to 20°C, growth of *Salmonella* continued after the natural microbiota had reached its MPD. This effect was described well by the new expanded Jameson-effect model and the classical Lotka-Volterra model. We believe the developed interaction models for ground pork will be valuable for future exposure and risk assessments to predict concentrations of *Salmonella* and thereby contribute to evaluation of the risk of *Salmonella* infections.

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Table 1. Published growth parameter estimates used for validation of the *Salmonella* models. All data were calculated from tabulated values.

Pork type	pH	<i>Salmonella</i> serotypes	Temperature (°C)	Growth parameters			Reference
				λ (h)	μ_{max} (h ⁻¹)	N_{max} (log ₁₀ CFU g ⁻¹)	
Fresh ground	N.R.	<i>S. Typhimurium</i> , <i>S. Enteritidis</i>	4.4	No growth in 72 h			Mann et al. 2004
			7.2	48.0	0.050	N.E.	
			10.0	12.0	0.054	N.E.	
			22.2 – 23.3	4.0	0.572	N.E.	
Thawed loin, ground	5.7	<i>S. Heidelberg</i> , <i>S. Hadar</i> , <i>S. Enteritidis</i>	10.0	54.7	0.066	N.R.	Ingham et al. 2007
			12.8	17.2	0.166	N.R.	
			15.6	8.8	0.276	N.R.	
			18.4	8.2	0.815	N.R.	
			21.1	5.7	0.801	N.R.	
			23.9	4.3	0.995	N.R.	
			26.7	4.4	1.520	N.R.	
			29.5	1.8	1.050	N.R.	
			32.2	2.6	1.630	N.R.	
			35.0	1.7	1.230	N.R.	
37.8	1.6	2.017	N.R.				
Thawed fresh ham, ground	5.7 – 6.4	<i>S. Chester</i> , <i>S. Derby</i> , <i>S. Typhimurium</i> , <i>S. Thompson</i> , <i>S. Enteritidis</i>	4.0	No growth in 16 d			Alford & Palumbo 1969
	5.75		10.0	29.3	0.083	7.7	
Boneless chops	N.R.	<i>S. Typhimurium</i> , <i>S. Enteritidis</i>	4.4	No growth in 72 h			Mann et al. 2004
			7.2	No growth in 72 h			
			10.0	No growth in 72 h			
			22.2 – 23.3	4.0	0.250	N.E.	
Thawed fresh ham, ground, added 2 % NaCl	5.7 – 6.4	<i>S. Derby</i> , <i>S. Thompson</i> , <i>S. Enteritidis</i> , <i>S. Chester</i> , <i>S. Derby</i> , <i>S. Typhimurium</i> , <i>S. Thompson</i> , <i>S. Enteritidis</i>	10.0	10.8	0.049	8.2	Alford & Palumbo 1969
	5.7 – 6.4		10.0	29.9	0.053	8.3	
	5.7 – 6.4		10.0	0	0.058	6.3	
	5.75		10.0	53.4	0.069	7.9	

N.E.: Could not be estimated within the duration of experiment. N.R.: Not reported by authors.

Table 2. Published studies used as sources for growth parameters in the natural microbiota models.

Pork type	Temperatures (°C)	No. of data points			pH	Source	Reference
		λ	μ_{\max}	N_{\max}			
Ground	6, 25	2	2	2	-	C	Fukushima and Gomyoda 1986
Ground	2	0	1	0	6.02	C	Chung-Wang et al. 1997
Ground	4.4, 7.2, 10, 22.7	8	8	0	-	C	Mann et al. 2004
Ground	0, 10	2	2	2	6.13	C	Koutsoumanis et al. 2006
Minced	4	1	1	1	5.4	C	Ogden et al. 1996
Minced	4	1	1	0	5.5 – 5.7	B	Carramiñana et al. 2008
Minced	5	1	1	1	-	C	Koutsoumanis et al. 2008
Minced	2, 4, 7, 10, 15, 20	6	6	6	-	C	Kreyenschmidt et al. 2008
Boneless chops	4	2	2	2	5.8	C	Manu-Tawiah et al. 1993
Boneless chops	4.4, 7.2, 10, 22.7	8	8	0	-	B	Mann et al. 2004
Slices	1	1	1	1	5.3 – 5.6	C	Asensio et al. 1988
Slices	9.9	1	1	1	5.5	B	Muermans and Stekelenburg (ComBase) ID: BA_25
Slices	1, 7	4	4	4	5.3	B	Departamento de Nutrición y Bromatología (ComBase) ID: SY1, SY31
Slices	1, 7	2	2	2	5.3	B	Mano et al. 1995 (ComBase) ID: SL1, SL27
Slices	7	1	1	1	6.0	B	Mano et al. 1995 (ComBase) ID: SL31
Slices	1, 7	2	2	2	5.3	B	Mano et al. 2000 (ComBase) ID: SA17, SA51
Slices	7	1	1	1	6.0	B	Mano et al. 2000 (ComBase) ID: SA31
Slices	1, 7	2	2	2	5.4	B	Mano et al. 2002 (ComBase)
Loin slices	5	2	1	2	-	C	Lambert et al. 1992
Loin slices	3	0	1	0	-	C	Zhao et al. 1996
Loin chops	-1.1	1	1	1	-	C	Huffman 1974
Loin chops	4	0	1	0	5.0 – 5.9	B	Enfors et al. 1979
Loin chops	2	0	2	0	-	C	Igbinedion et al. 1983
Loin chops	10	3	3	0	5.39 – 6.43	A	Greer and Murray 1991
Loin chops	4	0	1	0	5.46 – 5.82	C	Jensen et al. 2003
Loin pieces	1	1	1	1	-	C	Silliker et al. 1977
Loin pieces	0	0	1	0	5.7	B	Lee et al. 2003
Loins	4, 14	0	2	0	5.6	C	Blickstad et al. 1981
Backfat pieces	4	0	1	0	5.5	C	Zhang et al. 2010
Not stated	4	2	6	0	5.4	B	Dipartimento di Sicurezza Alimentare (ComBase) ID: Carntt_1_1
In total	-1.1 to 25	54	67	32	5.3 to 6.43		27 different studies

A) data reported directly, B) calculated from tabulated values, C) calculated from graphs.

Table 3. Estimated parameters for secondary growth models of *Salmonella* and natural microbiota in ground raw pork.

Parameters	Fitted values and 95 % confidence limits	
	<i>Salmonella</i>	Natural microbiota
<i>b</i>	0.0356 (0.0323 – 0.0389)	0.0267 (0.0239 – 0.0295)
T_{\min} (°C)	2.33 (0.40 – 4.26)	-5.48 (-6.91 to -4.05)
<i>RLT</i>	3.10 (2.50 – 3.70)	4.07 (3.31 – 4.84)
N_{\max} (\log_{10} CFU g ⁻¹)	8.829 ± 0.325 ^a	8.997 ± 0.804 ^a

^aValues indicate average and standard deviation

Table 4. Lack-of-fit tests for three microbial interaction models used to describe simultaneous growth of the natural microbiota and *Salmonella* in ground raw pork at different storage temperatures.

Temp. (°C)	Pure error			Lack-of-fit F-test ^a								
	Model	RSS	DF	Jameson model			Lotka-Voltera model			Expanded Jameson		
				RSS	DF	F-value	RSS	DF	F-value	RSS	DF	F-value
9.4	Log-linear	1.556	14	15.603	16	63.20	7.493	16	26.71	7.621	16	27.29
10.4	Log-linear	4.447	16	11.747	18	13.13	10.553	18	10.98	10.603	18	11.07
11.9	Log-logistic with lag	0.957	15	3.248	19	8.98	2.771	19	7.11	2.774	19	7.12
14.2	Log-logistic with lag	1.403	18	2.245	22	2.70	4.877	22	11.14	5.474	22	13.06
15.1 ^b	Log-logistic with lag	2.541	18	39.572	22	65.58	6.084	22	6.27	7.117	22	8.10
15.1	Log-logistic with lag	0.728	8	16.607	12	43.60	0.445	12	0.78	0.558	12	0.47
16.6	Log-logistic with lag	2.804	16	60.550	20	82.36	3.236	20	0.61	3.631	20	1.18
20.2	Log-logistic with lag	1.383	14	16.607	18	38.52	1.534	18	0.38	1.014	18	0.93
24.1	Log-logistic with lag	0.275	16	3.919	20	53.04	0.783	20	7.39	2.101	20	26.58

^a Boldface F- values indicate lack-of-fit of given model compared to pure error model

^b Initial levels of *Salmonella* spp. in these trials were 1 - 2 log₁₀CFU g⁻¹ lower than the other trials

Table 5. Estimated parameter values describing the effect of storage temperatures on coefficients of interaction.

Parameters	Fitted values for equation 6 describing coefficients of interaction ^a	
	γ in equation 4	$\alpha_{NB,S}$ in equation 5
a_0	3.88 (3.04 – 4.70)	2.41 (1.89 – 2.95)
a_1	-0.37 (-0.48 to -0.27)	-0.211 (-0.28 to -0.15)
a_2	0.011 (0.008 – 0.014)	0.005 (0.003 – 0.007)

^a Numbers in parentheses are 95 % confidence limits

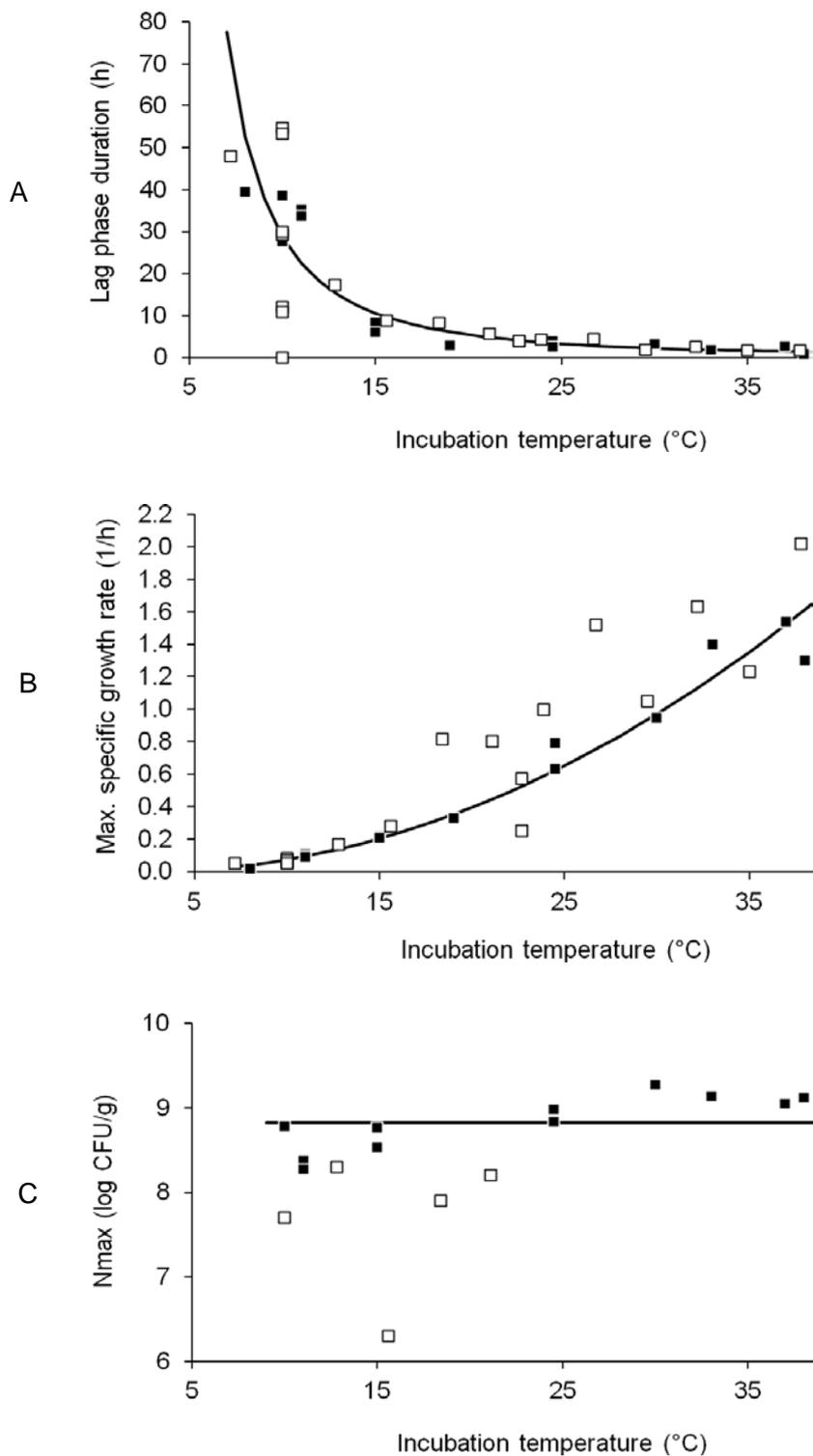


Figure 1. Effect of storage temperature on lag time (A), maximum specific growth rate, μ_{max} (B) and maximum population density, N_{max} (C) for growth of *Salmonella* in pork. Data were determined in challenge tests as part of the present study (■) or obtained from the literature (□). Solid lines represent secondary growth models fitted to data determined in the present study.

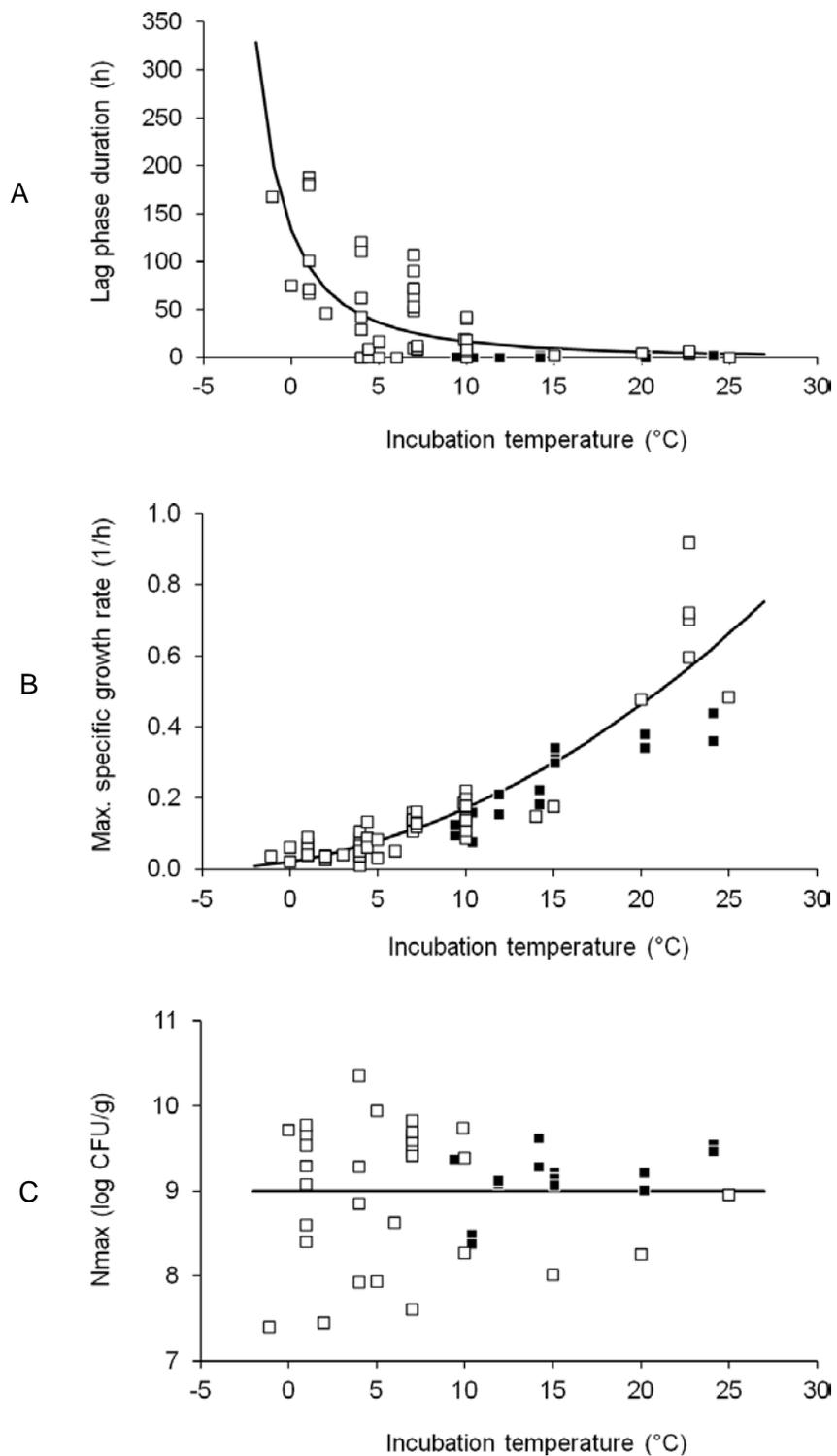


Figure 2. Effect of storage temperature on lag time (A), maximum specific growth rate, μ_{max} (B) and maximum population density, N_{max} (C) for growth of natural microbiota in pork. Data were obtained from the literature (\square) or determined in challenge tests as part of the present study (\blacksquare). Solid lines represent secondary growth models fitted to data obtained from the literature.

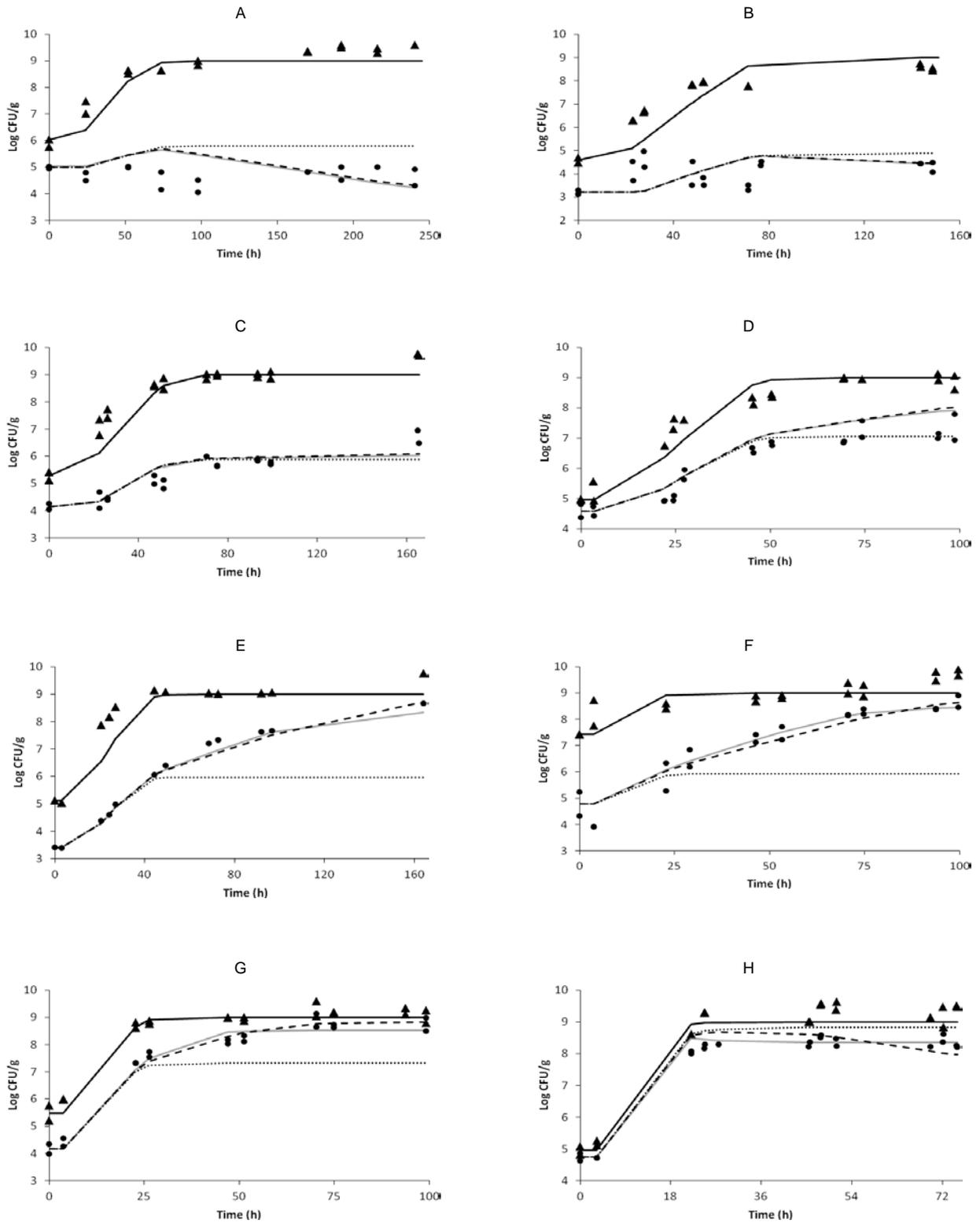


Figure 3. Observed and fitted growth of the natural microbiota (▲, —) and observed growth of *Salmonella* (●) in ground raw pork at different storage temperatures. Growth kinetics for *Salmonella* was fitted using the Jameson-effect-model (Equation 3:), the expanded Jameson-effect-model (Equation 4: - - -) and the Lotka-Volterra model (Equation 5: —).

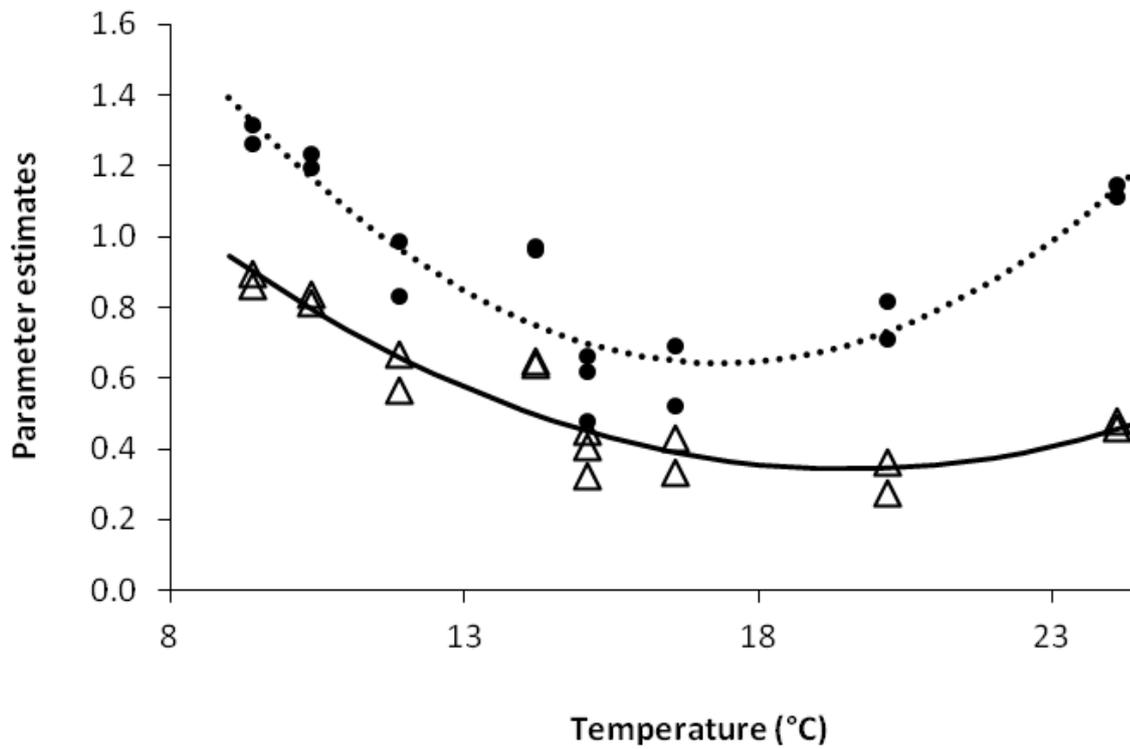


Figure 4. Estimates of $\alpha_{S,NB}$ (Δ) and γ (\bullet) parameters obtained applying the Lotka-Volterra and the expanded Jameson-effect model, respectively to describe the effect of the natural microbiota on growth of *Salmonella*, during storage of ground pork at different temperatures (9.4, 10.4, 11.9, 14.2, 15.1, 16.6, 20.2 and 24.1°C). The fit of the $\alpha_{S,NB}$ (—) and γ (.....) parameters were obtained by using equation 6.

Chapter 7

MANUSCRIPT II

Risk assessment of *Salmonella* spp. in Danish meatballs produced at the catering sector

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In preparation

Abstract

A risk assessment was conducted to assess health risks associated with *Salmonella* spp. by consumption of the Danish meatballs, made with fresh pork, produced at a catering unit. Using the modular process risk model (MPRM) approach, meatball production and consumption were described as a series of processes (modules), from 1.3 kg meat pieces to 70 g meatballs, followed by a dose response model to assess the risk of illness from consumption of these meatballs. Changes in bacterial prevalence, concentration, and unit size were modelled within each module. The risk assessment was built by using observational data and models that were specific for *Salmonella* spp. in meatballs produced at the catering sector. According to the guidelines of the Food Authorities, Danish meatballs are supposed to be heat treated. After frying of the meatballs, normally heating in the oven is applied before the consumption. However, in practice this last heat treatment may be omitted. Therefore, eleven production scenarios were evaluated with the model, to test the impacts of heat treatment and cool storage. Meatballs temperatures higher than 72°C were found sufficient to inactivate *Salmonella*, indicating that the recommendation from the Danish Food Safety Authorities of 75°C are sufficient. Survival and growth of *Salmonella* during cooling had relevant impact on the risk estimates, and therefore should be considered as critical during meatballs processing.

1 INTRODUCTION

The incidence of foodborne diseases is increasing globally and international food trade is disrupted by frequent disputes over food safety and quality requirements. Many food control systems need to be revised and strengthened if improvements are to be realized (FAO/WHO, 2003). Food prepared outside of the home can be a significant source of foodborne illness (Jones and Angulo, 2006), and food safety is crucial in catering, considering the vast number of meal served away from home every year (Poumeyrol et al., 2012). In many countries established food safety standards for food service are enforced through routine inspections in order to prevent restaurant-associated foodborne disease outbreaks (Filion and Powell, 2011). In Denmark food safety has been improved by a control system based on HACCP self-inspection and the Smiley system (Nielsen, 2006). Nevertheless, outbreaks do occur and, for example in Denmark, 55 of 77 reported outbreaks in 2010 were associated with outside-the-home settings including restaurants, canteens, hotels, schools, shops, institutions and sporting events (Anonymous, 2010).

Salmonellosis is an important cause of foodborne human gastroenteritis in most European countries (EFSA, 2010), and pork contributes significantly to the public health disease burden

caused by *Salmonella* infections (van Hoek et al., 2012). A traditional and popular pork product often consumed in Denmark is fried pork meatballs or 'frikadeller'. They are consumed as a component in hot meals but are also widely used as filling in cold sandwiches. During cooling and storage there may be an opportunity for growth of *Salmonella*. The meatballs are occasionally consumed cold, without prior heating, which may offer higher risk of illness to the consumers.

According to Filion and Powell (2011), Hensen et al. (2006), Jones et al. (2004), Lee and Middleton (2003), catering is an important segment, since 70 % of the foodborne illness worldwide distributed are estimated to be linked to food prepared at food service establishments. Because of their popularity, pork meatballs are often part of the product range served in Danish catering settings and as recently as in 2009, meatballs served on a buffet at a sport event were the most probable source for an outbreak of *Yersinia enterocolitica* involving 30 cases of illness (personal communication: Lone J. Porsbo, 2012). One of the few examples regarding outbreaks of *Salmonella* by consumption of a meatballs-like product in the food service sector in Denmark took place in 1995. Fish cakes served at a local hospital resulted in 40 cases of salmonellosis among patients of whom 6 died (Statens Seruminstitut, 1995).

Despite the large consumption and potential risk, there is a lack of knowledge on the human health risk related to Danish meatballs and *Salmonella* produced at the Danish catering sector. Quantitative Microbiological Risk Assessment (QMRA) can be used to evaluate this risk, and explore the potentials for control. The application of QMRA modelling as a tool to investigate the fate of microbiological hazards during food processing chains is a relatively new approach. Several process chain models have been published (e.g. Afchain et al., 2008; Aziza et al., 2006, Billoir et al., 2011, Bollaerts, et al., 2009; Clough et al., 2009; Delhalle et al., 2009; Ebel et al., 2004; Lindqvist et al., 2002; Nauta, 2001; Nauta et al., 2005; Nauta et al., 2007). However the behaviour of *Salmonella* through pork processing by food services remained unexplored until now. Some risk assessment related activities are however undertaken in the *Salmonella* pig/pork area. In 2009 Boone et al. published a work where NUSAP method was used for evaluating the data quality in a QMRA model for *Salmonella* in the pork production chain. Moreover, Bollaerts et al. (2009) developed a QMRA for human salmonellosis through household consumption of fresh minced pork meat in Belgium. In addition, Delhalle et al. have published a study in 2009, where they assessed interventions by applying QMRA tools to reduce the risk of human salmonellosis from fresh minced pork meat, also in Belgium. Furthermore, Bollaerts et al (2010) evaluated scenarios for reducing human salmonellosis through household consumption of fresh minced pork meat. Recently, van Hoek et al. (2012) published a work pointing out that a quantitative approach towards a better understanding of the dynamics of *Salmonella* spp. in pork slaughter-line.

In this study, a QMRA following the Codex Alimentarius Principles (Codex, 1998) was conducted in

order to assess health risks associated with *Salmonella* spp. by consumption of Danish meatballs, made with fresh pork, produced by a catering unit. The aim of the study was to develop a model able to provide more accurate estimates for the pork processing by combining observational studies with models developed specifically to pork processing.

In order to establish a representative process flow as well as to identify where errors such as temperature abuse, inappropriate inactivation and cross contamination could occur, two observation studies were performed in Danish catering units, one school and one worksite canteen. As part of the studies, time/temperature profiles were collected for the various steps during the production of pork meatballs. Next, using the modular process risk model (MPRM) methodology proposed by Nauta (2008), changes in bacterial prevalence and concentration, and unit size were modelled. This was done by identifying one of five basic processes to each process step. Three of which were food-handling process (i.e. cross contamination during grinding, mixing, and partitioning) and the remaining two were microbial processes (i.e. inactivation and growth). The observational studies on the processing of Danish meatballs at catering sector, were combined with experimental results and models developed for *Salmonella* in pork (Møller et al. 2012a; Møller et al. 2012b). This allowed us to develop a QMRA model that was specifically targeted for our purpose. Different scenarios were tested in order to evaluate the efficiency of the Danish food safety guidelines for prevention of illness caused by *Salmonella* during preparation of meatballs in a catering unit.

2 MATERIAL AND METHODS

2.1 Establishment of process flow and modules for the QMRA model

To build up the modules in the QMRA model, observations of the production of meatballs were carried out in one school canteen, serving 220 meals per day, and in one worksite canteen, serving 250 meals per day. Based on the observation, a main flow diagram was selected to represent the process (Figure 1). No growth of *Salmonella* was anticipated during cold storage of the fresh pieces of meat and the ground meat, due to the effectiveness of storage practices under monitored temperatures lower than 6°C (Velugoti, 2011). Therefore only the cold storage of the read-to-eat meatballs was included in the model.

Inputs to the QMRA model (Table 1), such as temperatures, duration and weight of the different units at the distinct steps of production of meatballs were obtained from the observational study. The obtained data were applied to models specifically developed to describe *Salmonella* behaviour

in pork. In the cases where observational data or models related to *Salmonella* in pork were not available, supplementary data from the literature were obtained.

The model was developed to describe the probability of illness from consumption of the Danish meatballs, produced in a unit from the catering sector, considering the prevalence (%) and concentration (CFU/g) of *Salmonella* spp. in pork of Danish origin (Hansen et al., 2010). Eight different steps were followed in order to build up the structure of the model:

- (1) Reception of the ten pork pieces weighting 1.289 ± 109 g (Møller et al., 2012a);
- (2) Cutting each of the ten pieces of pork in five slices, considering the partitioning approach developed by Nauta (2005) to insert a random distribution of *Salmonella* spp. on the slices of meat;
- (3) Grinding of slices of pork using the model developed by Møller et al. (2012a) to describe the transfer of *Salmonella* during pork grinding;
- (4) Mixing of five of those ten processed pieces of pork into a batter consisting of 2/3 meat and 1/3 of other (non contaminated) ingredients (rye flakes, pasteurized eggs and milk, tap water, chopped onions, flour, salt and pepper);
- (5) Partitioning into meatballs of each of the two batches of batter in meatballs weighting 73 ± 6 g (Appendix A), using the same approach applied to the slicing (2);
- (6) Heat inactivation of meatballs in a pan, where 2/3 of the meatballs was assumed to have no surviving *Salmonella*, and the remainder (in the middle) was subjected to lower temperatures, potentially allowing survival;
- (7) Growth during holding, serving and storage, applied only to the scenarios where the meatballs were not subjected to heat inactivation in oven, using the growth model proposed by Møller et al. (2012);
- (8) Heat inactivation in oven, where the meatballs were subjected to heat inactivation according to the \log_{10} -reduction model in Table 2, which also was derived from observations collected in the present study (Appendix B) and literature data;
- (9) Estimation of the risk, where the dose response model suggested by FAO/WHO 2002 was applied to estimate the number of salmonellosis cases per lot of 300 meatballs.

2.2 Collection of observational data and use of supplementary models

Observational studies were carried out at two units from the Danish catering sector. Information on process flows were collected and used for constructing a common pathway (Figure 1) for the QMRA model. Furthermore, temperatures, durations, and weight of the different units at the distinct steps of production of meatballs were measured and used as basis for modelling. The supplementary data were obtained by applying predictive models to the obtained observed data.

2.2.1 Heat inactivation models

Heat inactivation models were applied at step (6) and (8) in the QMRA model. In both cases, log-linear inactivation kinetics was assumed for *Salmonella* and the concepts of decimal reduction times (D-values) and z-values were applied. D-values for heat inactivation of *Salmonella* in minced meat (pork and beef) were collected from the literature (Juneja et al. 2001; Smith et al. 2001; Murphy et al. 2004; Juneja et al. 2010; Velasquez et al. 2010) and the classical model for the effect of temperature on D-values ($\log_{10}(D_T) = \log_{10}(D_{ref}) - (T - T_{ref})/z$) was used to estimate a z-value of 7.34°C based on these data (Figure 2).

2.2.1.1 Log₁₀-reduction by heating in pan

For each of 60 meatballs, the time/temperature data obtained from the observational study, presented in Appendix B, were converted to effective time at 60°C using the concept of lethal effect described by Flambert et al. (1977).

The temperature increase during frying of meatballs was considered to be linear, since the heat was applied for a short period of time (5 – 11 min) and there was a large difference between the temperature of the meatball batter (5.1 – 10.2°C) and the surface of the hot pan. For estimation of the effective time, the linear curve for each meatball was divided in five equally long time intervals of constant temperatures.

For each of the 60 temperature profiles from the observed meatballs, the Effective Time (EF) of the heat treatment was calculated based on the concept of lethal effect applying Equation 1.

$$EF_{T_{ref}} = \sum_{start}^{end} 10^{\frac{T(t)-T_{ref}}{z}} \Delta t \quad , \quad \text{with } T_{ref} = 60^{\circ}\text{C} \quad \text{Equation 1}$$

where $EF_{T_{ref}}$ is the effective time in minutes from the *start* to the *end* at a reference temperature, T_{ref} , z is the z-value for heat inactivation of *Salmonella* in minced meat, and T is the temperature (°C) in the middle of each time interval.

From the obtained EF during frying in pan, the Log₁₀-Reduction (LR) of *Salmonella* was determined for each of the 60 meatballs by applying Equation 2.

$$LR_{heating} = \frac{EF_{T_{ref}}}{D_{T_{ref}}} \quad \text{Equation 2}$$

where $EF_{T_{ref}}$ is the effective time (min) at the chosen reference temperature, T_{ref} , and $D_{T_{ref}}$ is the D-value of *Salmonella* in minced meat at 60°C. The obtained $LR_{heating}$ for the 60 individual meatballs are presented in Figure 3.

2.2.1.2 Log₁₀-reduction by heating in oven

During heating of the meatballs in the oven, the time/temperature profile in the core of one meatball was recorded (Figure 4). In order to determine effective time of this profile, it was fitted to Equation 3, which describes the temperature change in the meatball as an exponential relation, depending on the initial temperature of the meatball and the temperature of the surrounding air, according to Newton's Law (Burmeister, 1993).

$$T(t) = T_a + (T_o - T_a)e^{-kt} \quad \text{Equation 3}$$

where $T(t)$ is the temperature (°C) of the meatball at time t (min), T_o is the initial temperature (°C) of the meatball, T_a is the temperature (°C) of the surrounding air and k (1/min) is a constant describing the rate of change.

Fitting by the method of least squares resulted in the following estimates: $T_a = 121^\circ\text{C}$, $T_o = 41^\circ\text{C}$, $k = 6.1 \text{ min}^{-1}$ and a Root mean sum of squared errors (RMSE) of 1.22. Using these fitted estimates for simulation of the temperature curve for the meatball, the time to reach 95, 75, 70 and 65°C was determined to be 11, 5.5, 4.5 and 3.5 min, respectively. As it was observed that T_o of the meatballs could vary considerably, we could not assume that all meatballs followed the same profile. Therefore, we simulated a temperature curve for each of eight observed T_o (25.4, 33.2, 33.6, 35.7, 38.5, 40.8, 41.0 and 45.1°C) assuming T_a and k to be the same for all meatballs and using $t = 11, 5.5, 4.5$ and 3.5 min as heating time. For each of these temperature curves, the effective time of heating in oven was calculated using Equation 1. The curves were split into eight evenly distributed time intervals of constant temperatures. For the curves with heating time, $t = 11$ min $T_{ref} = 95^\circ\text{C}$ was used and for $t = 5.5, 4.5$ and 3.5 min $T_{ref} = 75, 70$ and 65°C , respectively, were used (Table 2). From the obtained $EF_{T_{ref}}$ during heating in oven, the LR_{oven} of *Salmonella* was determined for each of the eight meatballs by applying Equation 2. The log transformed results are shown in Table 2.

2.2.2 Growth model

The combination of time and temperature (Figure 5) during holding time (0 – 90 min) and serving time (120 min) may support growth of *Salmonella*, therefore the dynamic time/temperature profile was simulated applying Equation 3.

The effect of start temperature on growth of *Salmonella* was tested considering T_o between 18.2 and 66.8 (Appendix B) with constant $k = 1.8$ (obtained applying Equation 3 to the cooling profile measured in a meatballs from each of the two investigated catering units, (see Figure 6), $T_a =$ Room temperature in the kitchen (20, 24, 25, 26, 27, 28, 29 and 30°C), and combinations of different holding time (Figure 5) and constant serving time (120 min) plus constant storage time at 6°C (210 min).

In addition, the models proposed by Møller et al. (2012b) for prediction of μ_{max} (Equation 4) and Lag Time (Equation 5) of growth of *Salmonella* in sterile ground pork during storage at different temperatures (3.6 - 38°C), were applied to each five minutes interval (of the sum of holding, serving and storage time) in order to determine the moment when *Salmonella* had conditions of time and temperature to start growing.

$$\mu_{max} = (b (T - T_{min}))^2 \quad \text{Equation 4}$$

where b is a constant = 0.04, T is the investigated temperature and $T_{min} = 2.33^\circ\text{C}$.

$$\text{Lag time} = \text{RLT} * \ln(2)/\mu_{max} \quad \text{Equation 5}$$

where RLT is the Relative Lag Time = 3.10 and μ_{max} is determined from Equation 4.

The growth of *Salmonella*, was calculated considering a scenario where remaining meatballs after serving, not heat treated in the oven, were placed at cold storage (6°C), before being served in cold sandwiches. The growth of *Salmonella* was obtained applying the growth model (Equation 6) developed by Møller et al. (2012b) to the meatballs that ranged the exact moment where *Salmonella* had conditions of time and temperature to grow, until the moment that the meatball cooled down to a temperature of 6°C under cold storage. This part of the study was carried out to investigate the effect of dynamic temperature on growth of *Salmonella* at the mentioned interval for meatballs with starting temperature of 45°C. The typical starting temperature was picked from those occurring in observed meatballs (Appendix B) since no differences were observed when testing start temperatures between 18 to 66.8 °C.

$$\log(S_t) = \frac{1}{\ln(10)} \sum_{i=k}^{\tau} \mu_{max}(T_i) \Delta t + \log(S_0) \quad \text{Equation 6}$$

where $\log(S_t)$ is the concentration of increase of *Salmonella* (S) ($\log_{10}\text{CFU/g}$) after t (hours), that is at the end of the cooling process. The time for cooling of meatballs, t , was divided in i intervals of $\Delta t = 5$ minutes, where $i=k$ is the first interval where $t >$ Lag Time and τ is the last time interval, ending at time t . $\mu_{max}(T_i)$ represents the growth rate at each specific temperature in the beginning

of each 5 minutes interval. The sum of log increase at all intervals during the cooling process is added to the level of *Salmonella* at the moment before the cooling start $\log(S_0)$.

The total \log_{10} increase of *Salmonella* was estimated from the sum of the calculated growth for each time interval from each of the combinations of holding, serving and storage time at a determined room temperature, as presented in Figure 5. The Lag Time was only applied once, during holding time and cold storage, until the meatballs reached 6°C.

2.3 QMRA Model

2.3.1 Overview of the model

Several steps, as described in Figure 7, were followed at the catering unit during the production of the meatballs and taken into consideration to build up the model, as shown in Table 1 and Appendix C:

2.3.1.1 Reception

Prevalence ($R_{\text{prevalence}}$) and concentration ($R_{\text{concentration}}$) of *Salmonella* spp. in fresh pork pieces were obtained from a Danish retail study published by Hansen et al. (2010), where *Salmonella* was detected in 37 samples out of a total of 887 in 2006 (4.2 %). Information on concentration of *Salmonella* in fresh pork pieces was adopted from another Danish retail study conducted in 2002 (Hansen et al., 2010). Out of 52 *Salmonella* positive samples, 39 were found to contain 0.04 – 0.4 *Salmonella* per gram, seven contained 0.4 – 4 *Salmonella* per gram, five contained 4 – 40 *Salmonella* per gram and a single sample contained more than 40 *Salmonella* per gram (in the QMRA model it was assumed to be 400).

The quantity of meat used for production of one lot of meatballs in the observed catering units corresponded to 10 to 12 kg in the form of ten fresh pork pieces. The size of the pieces (1.289 ± 109 g) was adopted from the study published by Møller et al. (2012a).

To simulate the number of *Salmonella* per piece of contaminated meat (in cfu) $R_{\text{conc piece}}$, the concentration of *Salmonella* (cfu/g) for each piece was sampled from the data set, assuming uniform distributions of each log concentration, and multiplied by a sample from the normal distribution of the weight of the pieces of pork (g). Assuming a prevalence of 4.2 %, contaminated pieces of meat were obtained by a Bernoulli trial (Papoulis, 1984). The obtained result was rounded to obtain an integer value for the number of cfu per piece of meat.

2.3.1.2 Slicing

In order to use the semi-industrial grinder normally employed in most catering units, size of the meat pieces must be reduced to 200 – 300 g each. Therefore, the simulation assumed that each of the pork pieces was cut into $S_{n \text{ slices}} = 5$ slices. The modeling approach for homogeneous partitioning with equal sized units as developed by Nauta (2005) was chosen to describe the random distribution of *Salmonella* spp. on the slices of meat, and the Equation 7 was applied:

$$S_{\text{conc slice}} \sim \text{Multinomial} (R_{\text{conc piece}}, \{1/ S_{n \text{ slices}}\}) \quad \text{Equation 7}$$

2.3.1.3 Grinding of slices into portions

A tool for examining the transfer of *Salmonella* during fresh pork grinding was developed by Møller et al. (2012a). This tool was used to add the effect of *Salmonella* cross contamination during the grinding process, and obtain the distribution of *Salmonella* in a realistic scenario. The *Salmonella* randomly distributed in the slices of pork were the input to Equation 8 and the parameter estimates $G_{p a_1}$ (0.0010), $G_{p b_1}$ (0.0275), $G_{p a_2}$ (0.8909), $G_{p b_2}$ (0.0558) and $1 - G_{p c_3}$ (0.4887), published by Møller et al. (2012a) were applied.

$$\begin{cases} M_i = (1 - G_{p a_1})(1 - G_{p a_2}) C_{\text{conc slice}} + (G_{p b_1} gr_{1,i-1}) + (G_{p b_2} gr_{2,i-1}) \\ gr_{1,i} = G_{p a_1} \times C_{\text{conc slice}} + (1 - G_{p b_1}) gr_{1,i-1} \\ gr_{2,i} = G_{p a_2} \times C_{\text{conc slice}} + (1 - G_{p b_2}) (1 - G_{p c_3}) gr_{2,i-1} \end{cases} \quad \text{Equation 8}$$

Refer to Møller et al. (2012a) for explanation of parameters.

The minced meat processed from the first 25 slices was collected as one batch in the same container and the final 25 slices as a second batch in another container. These two batches were processed into two batters in two separate mixing processes.

2.3.1.4 Mixing with ingredients

Each batch of minced meat was mixed with other ingredients (chopped onions, pasteurized eggs and milk, tap water, rye flakes, flour, salt and pepper) in a semi-industrial blender to obtain the batter. In a homogeneous batter, where all ingredients are well mixed, usually the meat corresponds to 2/3 of the total weight, so the total weight is multiplied by 3/2.

2.3.1.5 Dividing into meatballs

The same modeling approach used in the slicing step (Chapter 2.3.1.2) (Nauta, 2005) was used to ensure a random distribution of *Salmonella* spp. from the batter to the meatballs (73 ± 6 g) during the partitioning step.

$$P_{\text{conc Meatball}} \sim \text{Multinomial} (M_{\text{conc batter batch}}, \{1/ P_{\text{weight Meatball of } P_n \text{ MB samples}}\}) \quad \text{Equation 9}$$

Where: $P_{\text{conc Meatball}}$ is the concentration of *Salmonella* spp. in meatballs. $M_{\text{conc batter batch}}$ is the number of *Salmonella* spp. per processed batter, $P_{\text{weight Meatball}}$ is the weight of meatballs and $M_{\text{weight batter batch}}$ is the weight of the batter processed per batch.

Although each batch generated about 150 units of meatballs, just a sample of 25 units from each of the two produced batches was investigated, since this number was considered representative and enough to include the variation between the 300 units of meatballs in the model.

2.3.1.6 Heat inactivation in pan

Danish meatballs are typically roasted in a pan following a procedure comparable to that of hamburgers, where the main difference is the height of the meatballs which is about 3 cm. Therefore, the critical zone, where *Salmonella* could survive (since the temperatures measured in this zone varied from 18.2 to 66.8°C), is the central part of the meatball, which was assumed to correspond to 1/3 of the total weight. The concentration of *Salmonella* present in this part of the meatball was assumed to be reduced following a log normal distribution of the log₁₀-reduction calculated partly from observed time/temperature data for this particular step, partly from literature data on heat tolerance of *Salmonella* in minced meats (Figure 3).

The log₁₀ reduction of each measured sample was log₁₀-transformed (Appendix B), and the average (-3.06) and standard deviation (1.57) were applied to the level of *Salmonella* present in each of the considered meatballs in the QMRA model, using a normal distribution.

2.3.1.7 Heat inactivation in oven

The recommendation from the Danish food authorities suggests that the meatballs reach the

temperature of 75°C in order to assure the safety of this product. Therefore, the meatballs are subjected to heat inactivation in oven. The heat inactivation approach was the same as applied for heat inactivation in pan, considering D_{65} , D_{70} , D_{75} , and D_{95} (Table 2).

The mean and standard deviation of \log_{10} -transformed \log_{10} -reduction in oven from all end temperatures at the same heating time (Table 2), were applied to the level of *Salmonella* present in each of the considered meatballs in the QMRA model, using a normal distribution for each of the tested scenarios, where each of the tested temperatures, 65°C, 70°C, 75°C and 95°C, was investigated separately.

2.3.1.8 Growth during cooling considering holding and serving time plus cold storage

In Denmark it is common to consume meatballs in hot meals but also to use cold meatballs as filling in sandwiches, after refrigerated storage. Therefore, scenarios taking into account a period of the cooling plus cold storage and the probability of growth of the remaining *Salmonella* cells were simulated.

It was shown in Figure 6 that the k parameter (1.8 min^{-1}), was obtained by applying Newton's Law (Papoulis, 1984) to two observed cooling profiles. This k parameter was then used to predict growth of the different scenarios.

The \log_{10} -increase of each measured sample (Figure 5), was \log_{10} -transformed and the average and standard deviation of all values for each room temperature in the kitchen, between 25 and 30°C, were applied to the level of *Salmonella* present in each of the considered meatballs in the QMRA model, using a normal distribution. Each specific room temperature (20°C, 24°C, 25-30°C) was tested as an individual scenario.

2.3.1.9 Estimation of the risk

The dose response model in Equation 9 (FAO/WHO, 2002) was applied to each studied meatball. The mean of the obtained probabilities of illness, i.e. the mean probability of illness, was used as risk estimate.

$$P_{\text{ill}_{\text{dose}}} \sim 1 - (1 + (GC_{\text{conc after growth}} / \beta)^{\alpha})^{-1} \quad \text{Equation 9}$$

Where $GC_{\text{conc after growth}}$ is the concentration of *Salmonella* spp. in a meatball after Growth at Storage, $\beta = -0.3126$ and $\alpha = 2836$ (FAO/WHO, 2002).

2.4 Model implementation

The entire model was simulated with Monte Carlo techniques (10,000 iterations) using @Risk software (version 5.7, Palisade Corporation, Newfield, NY, U.S.). For each scenario, simulation was repeated five times and the results expressed as the average risk per serving. For scenario and sensitivity analyses, baseline scenarios were defined and for each alternative scenario the risk was expressed relative to the risk obtained for these baseline scenarios.

2.5 Alternative scenarios to test control measures

The QMRA model was used to test different scenarios (Table 3), describing the catering sector practices and challenging the Danish food safety guidelines for heat treatment.

The process flow of meatballs produced at the Danish catering sector is shown in Figure 1. Meatballs are usually served hot after heating in the oven, but can also be served cold, after chilled storage. Sometimes, however, heating in the oven is forgotten (e.g. because the oven capacity is too small), which is likely to provide scenarios with higher risk. The three scenarios shown in Figure 8 are compared in this study.

Scenario 1A, where the core temperature in meatballs in the oven reached 75°C in the coldest spot, the Danish recommendation for heat treatment, was used as baseline to test the effect of heating in oven on *Salmonella* survival at three different core temperatures, generating scenarios 1B (73°C), Scenario 1C (70°C), and Scenario 1D (65°C).

Scenario 2 differs from scenario 1A by the fact that the step of heating in the oven was omitted for all meatballs.

Scenario 3A, where the storage with a Room Temperature in the kitchen (RTk) of 20°C was tested, was used as baseline to test the effect of holding, serving and storage time until the meatballs (not heat treated in oven) reached 6°C, and scenarios testing different room temperatures in the kitchen as 3B (25°C), 3C (26°C), 3D (27°C), 3E (28°C), 3F (29°C) and 3G (30°C) were generated.

2.6 Sensitivity analysis

A sensitivity analysis was performed on some of the model parameters, by evaluating alternative scenarios regarding:

- Prevalence and concentration

Instead of using a prevalence of 4.2 % (Hansen et al., 2010), an alternative prevalence of 20 % was analyzed, as well as a modified distribution of concentrations where the maximum value is two \log_{10} -units higher than in the baseline model, was tested.

- Parameters of heat inactivation

There is little information regarding the inactivation on *Salmonella* during heating of pork ground meat, therefore, the literature data collected was applied in two different ways : 1) Using a $\log_{10}D(T)$ model derived from all the data presented in Figure 2 in all scenarios that included effect of heat inactivation in oven (1A, 1B, 1C, 1D – related to end temperatures of 75, 73, 70 and 65°C, respectively); 2) Using a $\log_{10}D(T)$ model derived from the data related to studies of heat inactivation of *Salmonella* Typhimurium in fresh pork from Juneja et al. (2001) and Murphy et al. (2004) for testing scenarios investigating the effect of heat inactivation in oven until core temperatures of 75, 70°C and 65°C (Table 4);

- Concentration of *Salmonella* in meatball centres

We assumed that *Salmonella* will only survive in the centre of the meatballs where the temperature is likely to be lowest. The size of the centre (1/3 of the meatball) is an own assumption. The sensitivity of that assumption has been tested by using 1/2 and 1/4 of the meatballs as alternative sizes of the centre.

- Relative Lag Time (RLT)

The RLT 3.1 used in the baseline model was obtained as an average estimate between RLT 1.1 and 4.8, from Møller et al. (2012b) who measured the growth of *Salmonella* in sterile ground pork during storage at different temperatures (3.6 - 38°C). No growth was observed when RLT was higher than 4.8, even in the worst-case scenario (Room Temperature at kitchen = 30°C). RLT equal to one was applied to all scenarios tested in the baseline model plus scenarios investigating temperatures of storage between 21 and 24°C (Figure 5) were tested.

3 RESULTS

3.1 Baseline and alternative processing

Using scenario 2 (no heating in oven and no cold storage) as a baseline ($P_{\text{ill}} = 7.3 \times 10^{-5}$), the risk estimates of all scenarios tested were compared as shown in Figure 9. It shows that heating in the oven (scenarios 1) and storage (scenarios 3) had an important effect on the risk estimates. This effect was more evident at higher temperatures, during both heating in the oven as well as during cooling. No differences were observed between heating to core temperatures of 73°C and heating in oven at 75°C, since no cases of salmonellosis would be obtained in any of these scenarios (Figure 9).

3.2 Sensitivity analysis

The probability of illness (P_{ill}) presented in Figure 9 was relatively low in scenarios 2 ($P_{\text{ill}} = 7.3 \times 10^{-5}$) and 1D ($P_{\text{ill}} = 2.52 \times 10^{-5}$). In scenario 3F ($P_{\text{ill}} = 1.49 \times 10^{-2}$), where growth of *Salmonella* was allowed, the probability of illness is almost 1000 times larger. The results for the sensitivity analyses are shown in (Figure 10).

In all scenarios, the effect of concentration had the highest impact on the risk of illness, whereas the critical zone had the lowest impact. The RLT was the second most important aspect when looking at scenario 3F and 2. It is important to mention that when using RLT = 1 there is growth of *Salmonella* when the room temperature is as low as 20°C. The use of the alternative heat tolerance data (Table 4) had a low impact on estimation of the risk at scenario 1D.

3.3 Incidence estimations

According to our observational studies pork meatballs made with pork are served about 24 times a year in the establishments we surveyed. When the proposed baseline model is considered (Figure 9) and inferring that about 20 % of the Danish population (5.6 million people in 2011) consume catered meals and that the conditions of production of the meatballs are the same as observed in this study, it would be possible to conclude that: 1) following the Danish heating recommendations, there would be no cases of salmonellosis from consumption of meatballs subjected to heat treatment in oven until they had reached 75°C or even 73°C in the core; 2) However, if the core temperature would reach only 65°C, about 27 people could become ill from salmonellosis annually; 3) About 69 cases of salmonellosis could happen if the step of heat inactivation in oven was not applied; 4) In case of cooling (RTK = 30°C) and storage of those meatballs not heat treated in

oven, about 16 300 cases are predicted.

4 DISCUSSION

4.1 The QMRA model

In the present study, the concentration and prevalence of *Salmonella* in pork from Danish retail (Hansen et al., 2010) was used as the input in a QMRA to assess the risk of illness caused by consumption of pork meatballs prepared in the catering sector. The QMRA model which was based on data from observational studies of the Danish meatballs processing by catering units, and on models specifically developed to study the behaviour of *Salmonella* during pork grinding (Møller et al., 2012a) and storage of ground pork (Møller et al., 2012b). Supplementary data and models were obtained from literature to cover the basic processes related to slicing (Møller et al., 2012a), partitioning (Nauta, 2005) and heat inactivation (Figure 2). A practical example estimating the risk of Danish population getting ill with salmonellosis per year by consumption of pork meatballs prepared in catering was performed applying the proposed QMRA baseline model. It confirmed the results obtained when testing the different scenarios with the QMRA baseline model, and showed reasonable estimates when comparing with the incidence of salmonellosis caused by consumption of pork in Denmark in 2011, and considering that the risk estimates obtained here correspond to a fraction of the 44-131 reported cases (Anonymous, 2012). The very high predicted number of cases (16 300) was obtained simulating a scenario that does not correspond to the practices recommended by the authorities and it was only a hypothetical situation that could happen only if errors on the processing were made.

The combination of observational studies and models specifically developed to *Salmonella* in pork is a novel approach in QMRA that will improve the accuracy of risk estimates in pork processing at the catering sector. The quality of the estimates obtained from the model may still be further improved by collection of data through additional observational studies, and the insertion of a model of heat inactivation specifically developed according to those data. Nevertheless, the results showed the reasonable outputs applying the proposed model, when simulating and testing different scenarios.

4.2 Scenarios

To explore the impact of different practices during the processing and preparation of Danish meatballs in the catering sector, 11 hypothetical scenarios (Figure 9) were tested and compared,

At domestic kitchens, the Danes typically use the frying of pork meatballs in a pan as the only heat treatment applied to this product, therefore, this scenario was used as a baseline to compare the other scenarios. In order to fulfil the recommendation of the Danish Food authorities suggesting 75°C to be achieved in the coldest point of the meatball, the catering sector included an extra step of heat treatment performed in an oven. Since the Danish recommendation (Fødevarestyrelse, 2008) is stricter than in other countries as e.g. in Canada (Health Canada, 2012) and USA (FDA, 2007; USDA, 2012) where the official guidance is 71°C for pork products, four different temperatures were tested in order to challenge those recommendations. The risk estimates revealed that a process comprising heat treatment of meatballs to core temperatures higher than 71°C, and subsequent holding at room temperatures lower than 25°C, for not longer than 3.5 hours prior to refrigeration at max. 6°C, was very effective against *Salmonella*. However, there are other more thermo-tolerant strains of *Salmonella*, like *Salmonella* Senftenberg in ground turkey (Veeramuthu et al., 1998), with D-values two-four times higher than applied in this study (temperature dependent: e.g. $D_{55} = 211.35$ min, and $D_{65} = 3.43$ min). In addition, a factor recognized to increase the thermo-tolerance of *Salmonella* in ground meat has been prior heat shock (Mackey and Derrick, 1987). Heat shock could be introduced during frying of meatballs in pan, before heating in oven. This aspect was not considered in the present model as the cells of *Salmonella* were not exposed to sublethal temperatures for more than 10 - 15 min, which was assumed to be too short time for inducing heat shock proteins. This could, however, be an interesting aspect for future investigations. Furthermore, safety related to other pathogens associated with pork, e.g. *Listeria monocytogenes*, also should be covered by the official food safety guidelines. As *L. monocytogenes* has been known to be more thermo-tolerant than *Salmonella* spp. in ground pork (Doyle et al., 2001), higher core temperatures might be needed to ensure safety considering this particular pathogen.

Since pork meatballs are consumed not only as component in hot meals but also as filling in cold sandwiches, scenarios testing different room temperatures in the kitchen, during the cooling of the meatballs and until reaching 6°C at refrigerated storage, were investigated. No growth of *Salmonella* was observed when the room temperature in the kitchen was lower than 24°C during the 3.5 hours necessary for cooling of the pork meatballs before being subjected to refrigeration at temperatures lower than 6°C. Microorganisms like *L. monocytogenes* (Chan and Wiedmann, 2009) and *Yersinia enterocolitica* (Strotmann et al., 2008), which are psychrotolerant, may have a different growth potential than *Salmonella* when surviving the heat treatment, and facilitate the occurrence of outbreaks like the one happened in 2009, when meatballs served on a buffet at a Danish sport event were the most probable source for an outbreak of *Yersinia enterocolitica* involving 30 cases of illness (personal communication: Lone J. Porsbo, 2012). Therefore, these

pathogens with high relevance for pork should be included in future studies of safety aspects of Danish meatballs.

4.3 Sensitivity analysis

It has been recognized that salmonellosis can arise due to cross contamination (de Boer and Hahné, 1990; Jiménez et al., 2009; Mattick et al., 2003; Møller et al., 2012a), insufficient heat treatment (Juneja et al., 2001; Murphy et al., 2004) and growth during cooling and storage (Alford & Palumbo, 1969; Ingham et al., 2007; Mann et al., 2004; Møller et al., 2012b). However, in order to evaluate the impact of the individual stages of Danish meatballs production in the catering sector on the risk estimates of *Salmonella*, sensitivity analyses were performed for steps of the processing considered more critical. Comparison of the different scenarios tested using the proposed model, can also be an alternative to generate hypotheses during investigations of outbreaks, since different aspects as concentration, prevalence, conditions of growth and heat treatment can be explored as demonstrated by the sensitivity analysis performed in this study.

According to the sensitivity analysis (Figure 10), high concentration and prevalence of *Salmonella* in fresh pork are the aspects that have the greatest influence on the risk estimates. Therefore, special attention to the credibility of that information should be given. Reduction of the Relative Lag Time (RLT = 1), which implies a better opportunity for growth, also has a high impact on the obtained risk estimates in scenarios where the meatballs are not heated in the oven. This impact of RLT = 1 was strongest in the scenario where the temperature in the kitchen was 30°C. These findings pointed out the importance of temperature control in the kitchen during the meatballs production. There is also a need of better understanding of the lag time of *Salmonella*, since no growth was predicted at temperatures lower than 25°C for the same period of cooling, when the average of RLT = 3.1 obtained by Møller et al. (2012b) and (Velugoti et al., 2011) was tested. The large standard deviation of lag time mentioned in different studies (Møller et al., 2012b; Velugoti et al., 2011), indicates that determination of lag time continues to be challenging during investigation of growth of *Salmonella* in ground pork.

4.4 Conclusion

A QMRA model has been built for *Salmonella* in Danish pork meatballs processed and prepared in the catering sector. It is based on i) data from an observational study of the Danish meatballs processing by catering units, ii) models specifically developed to study the behaviour of *Salmonella*

in pork grinding and in storage of ground pork, and iii) supplementary data from the available literature. Whereas other QMRA food chain models frequently have to use surrogate data and expert estimates, the availability of specific data on the pathogen, food matrix and food processing allowed us to derive most of the models and parameter estimates from suitable data. This increases the confidence in the model results, even though the uncertainty about the dose response relation remains important when the credibility of the risk estimates (i.e. the human incidence estimates following the consumption of Danish meatballs) are considered. Also, the high impact of lag time and heat inactivation on the risk estimates of salmonellosis suggest that more efforts should be considered in order to clarify the behaviour of *Salmonella* during storage and inactivation.

The model shows that, in terms of risk for salmonellosis, production of meatballs with heating in the oven with a core temperature of 72°C or more is safe from *Salmonella*. Also, if heating in the oven is skipped, the risk is small as long as the meatballs are not exposed to a conditions that allow growth. This confirms that the Danish guidelines (Fødevarestyrelse, 2008) are accurate, and that the risk of salmonellosis from Danish meatballs can be controlled quite easily.

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Table 1. Summary of the QMRA model applied to assess the risk of salmonellosis from consumption of pork meatballs processed in Danish catering units

module	basic process	Source/model reference	unit	output
Reception	1. initial contamination	Hansen et al. (2010)	CFU/1.289±109 g piece of pork	A_{conc} piece
Slicing	2. partitioning	Nauta (2005)	CFU/212±50 g pork slices	C_{conc} slice
Grinding slices into portions	3. cross contamination	Møller et al. (2012)	CFU/213 g±56 g portions of ground meat	G_{conc} portion
Mixing of ingredients	4. mixing	Nauta (2005)	CFU/about 9.6 kg batch of ground meat and other ingredients	M_{conc} batter batch
Dividing into meatballs	5. partitioning	Nauta (2005)	CFU/72.87±5.9 g meatballs	P_{conc} Meatball
Heating inactivation in pan	6. inactivation	this study	CFU/72.8 ±5.9 g meatballs	HP_{conc} after inac
Holding time	7. growth	Møller et al. (2012b), this study	CFU/72.87±5.9 g meatballs	
plus Heating inactivation in oven	8. inactivation	this study	CFU/72.87±5.9 g meatballs	HO_{conc} after inac
Serving time plus cold storage until 6°C is reached	7. growth	Møller et al. (2012b), this study	CFU/72.87±5.9 g meatballs	GS_{conc} after growth
Estimation of the risk	9. dose response	FAO/WHO (2002)	cases / lot of 300 meatballs	P_{ill} mean

Table 2. Data used to determine degree of heat inactivation in oven applying $\text{Log}_{10}D = -0.136 \times T + 8.433$

Observed meatball	Start temperature (°C)	End temperature (°C)	Heating time (min)	T_{ref} (°C)	Log (log (EF _{Tref})) (min)
1	25.4	89.8	11	95	3.76
		66.3	5.5	75	0.33
		63.5	5.0	73	0.19
		60.5	4.5	70	-0.51
		54.0	3.5	65	-1.44
2	33.2	92.3	11	95	4.14
		70.8	5.5	75	0.97
		68.2	5.0	73	0.87
		65.4	4.5	70	0.20
		59.5	3.5	65	-0.66
3	33.6	92.4	11	95	4.16
		71.0	5.5	75	1.01
		68.4	5.0	73	0.90
		65.7	4.5	70	0.23
		59.8	3.5	65	-0.62
4	35.7	93.1	11	95	4.26
		72.2	5.5	75	1.18
		69.7	5.0	73	1.08
		67.0	4.5	70	0.42
		61.2	3.5	65	-0.41
5	38.5	94.0	11	95	4.40
		73.8	5.5	75	1.41
		71.4	5.0	73	1.33
		68.8	4.5	70	0.68
		63.2	3.5	65	-0.13
6	40.8	94.8	11	95	4.51
		75.1	5.5	75	1.60
		72.8	5.0	73	1.53
		70.2	4.5	70	0.89
		64.8	3.5	65	0.11
7	41.0	94.9	11	95	4.52
		75.3	5.5	75	1.62
		72.9	5.0	73	1.55
		70.4	4.5	70	0.91
		65.0	3.5	65	0.13
8	45.1	96.2	11	95	4.73
		77.6	5.5	75	1.96
		75.3	5.0	73	1.91
		73.0	4.5	70	1.29
		67.8	3.5	65	0.54

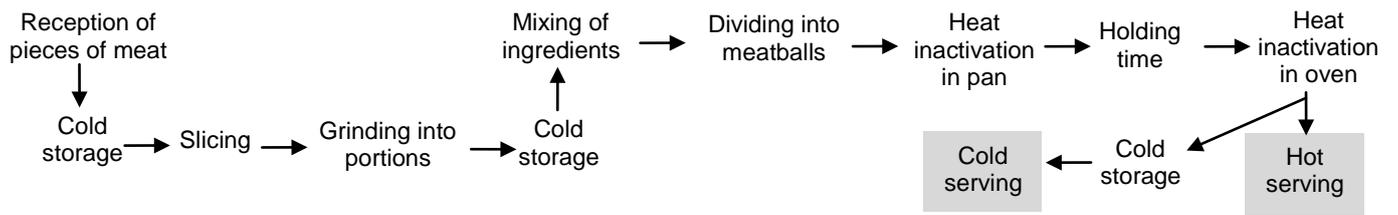
Table 3. Scenarios testing different temperatures during heating in oven and cooling of meatballs, with the proposed QMRA baseline model

Scenario	Heat treatment in oven to core temperature (°C)	Room temperature in the kitchen during cooling (°C)
1A	75	20
1B	73	20
1C	70	20
1D	65	20
2	—	20
3A	—	25
3B	—	26
3C	—	27
3D	—	28
3E	—	29
3F	—	30

— treatment not applied

Table 4. Data used to determine degree of heat inactivation in oven testing $\log_{10}D = -0.170 \times T + 10.974$

Observed Meatball	Start temperature (°C)	End temperature (°C)	Heating time (min)	T_{ref} (°C)	$\log(\log(EF_{Tref}))$ (min)
1	25.4	66.3	5.5	75	0.094
		60.5	4.5	70	0.014
		54.0	3.5	65	0.009
2	33.2	70.8	5.5	75	0.277
		65.4	4.5	70	0.088
		59.5	3.5	65	0.068
3	33.6	71.0	5.5	75	0.293
		65.7	4.5	70	0.097
		59.8	3.5	65	0.076
4	35.7	72.2	5.5	75	0.392
		67.0	4.5	70	0.159
		61.2	3.5	65	0.131
5	38.5	73.8	5.5	75	0.579
		68.8	4.5	70	0.308
		63.2	3.5	65	0.272
6	40.8	75.1	5.5	75	0.797
		70.2	4.5	70	0.529
		64.8	3.5	65	0.494
7	41.0	75.3	5.5	75	0.820
		70.4	4.5	70	0.555
		65.0	3.5	65	0.521
8	45.1	77.6	5.5	75	1.455
		73.0	4.5	70	1.465
		67.8	3.5	65	1.517



• Grey box indicates the end of the processing

Figure 1. Main flow diagram representing the processing of meatballs in the Danish catering sector

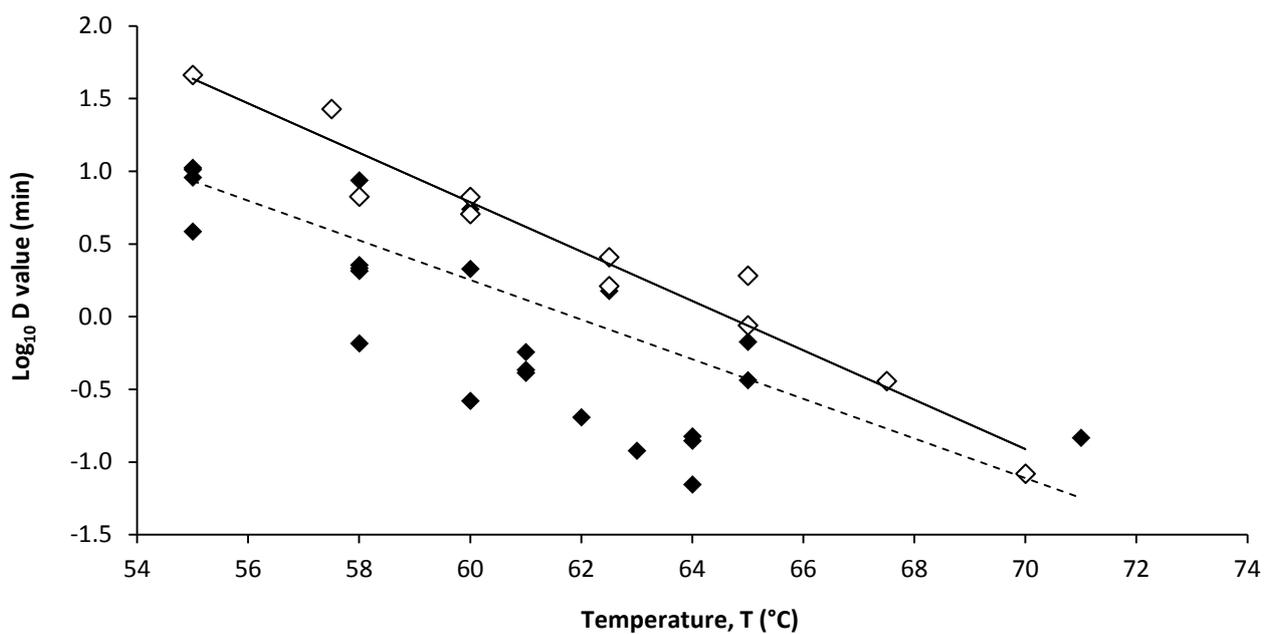


Figure 2. Effect of temperature on the decimal reduction times of *Salmonella* in minced meat collected from the literature, pork (◇) and beef (◆). All data (◇, ◆) fitted by the linear trendline (- - -) $\text{Log}_{10}D = -0.136 \times T + 8.433$, and pork data only (◇) fitted by the linear trendline (—) $\text{Log}_{10}D = -0.170 \times T + 10.974$

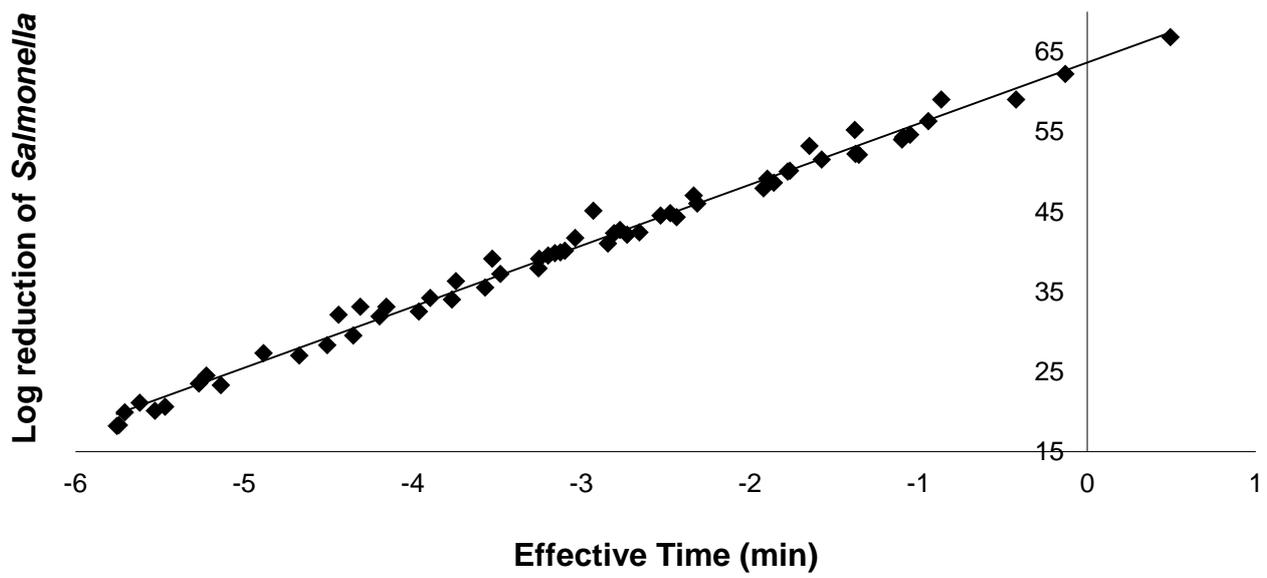


Figure 3. Log₁₀-reductions (LR_{heating}) obtained from the observed data for 60 meatballs (♦) and fitted by a linear trendline (—) using $T_{\text{ref}} = 60^{\circ}\text{C}$ and $z = 7.34^{\circ}\text{C}$ in $EF_{60} = 7.63 \times LR_{\text{heating}} + 63.62$

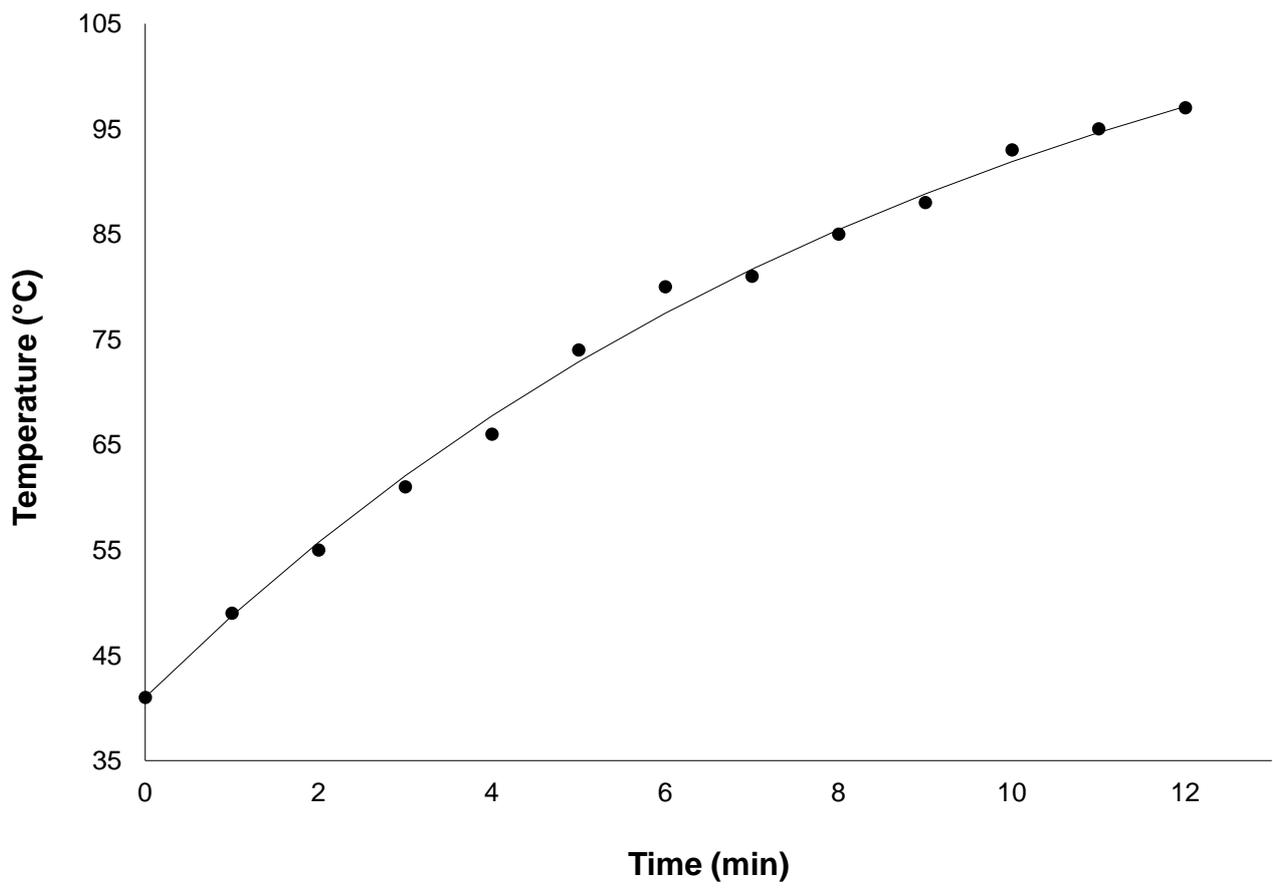


Figure 4. Observed (•) and fitted with Newton's Law equation (—) temperature profiles of heating of meatball in oven until an end temperature of 95°C

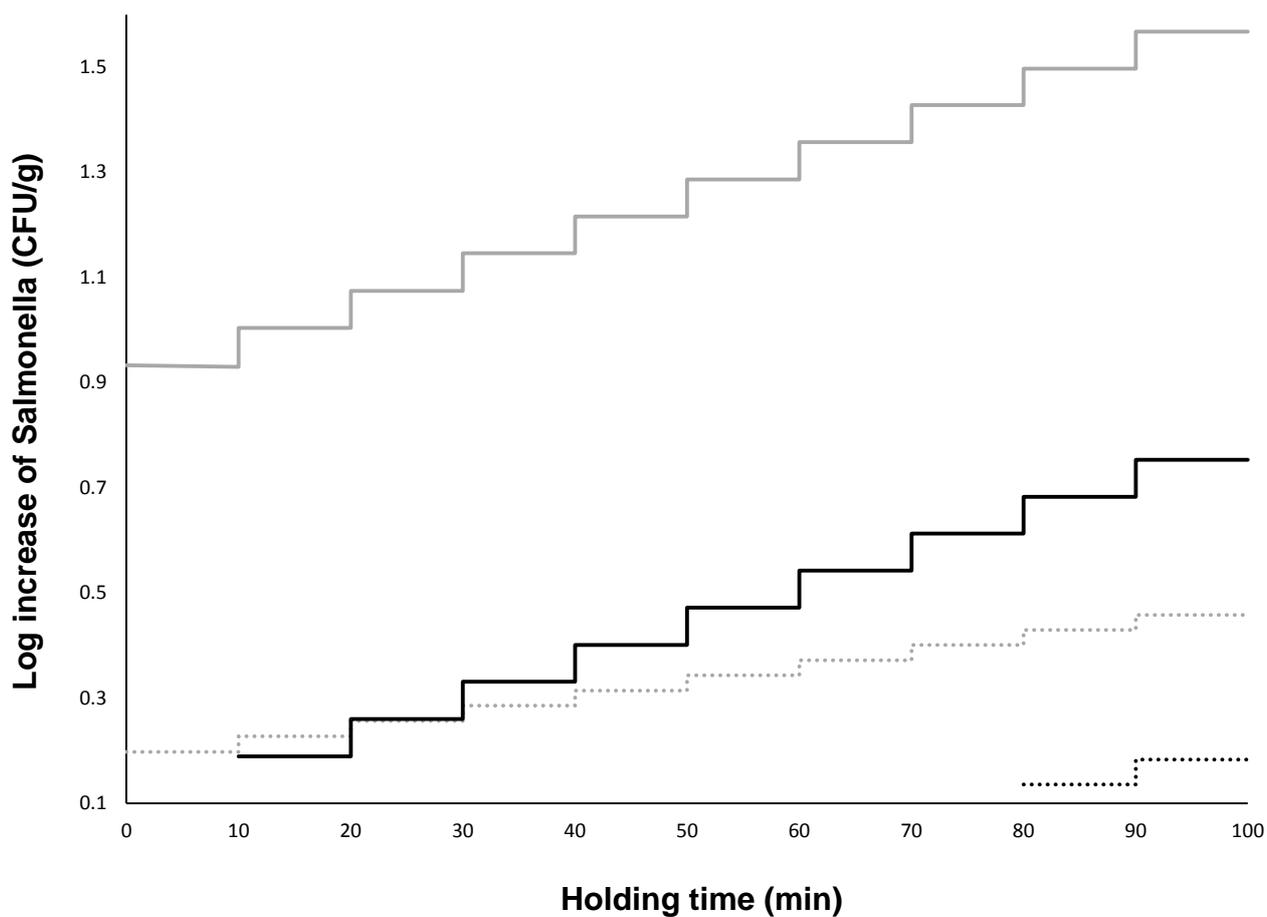


Figure 5. Distribution obtained from modelling the effect of different combinations of holding time (0, 10, 20...90 min), serving time (120 min) and storage time (210 min) on growth of *Salmonella* spp. in a meatballs with start temperature at 45°C and end temperature 6°C, considering Relative Lag Time (RLT) = 1 at room temperature in the kitchen (RTk) = 20°C (.....) and 30°C (—), and RLT = 3.1 at RTk = 25°C (.....) and 30°C(—).

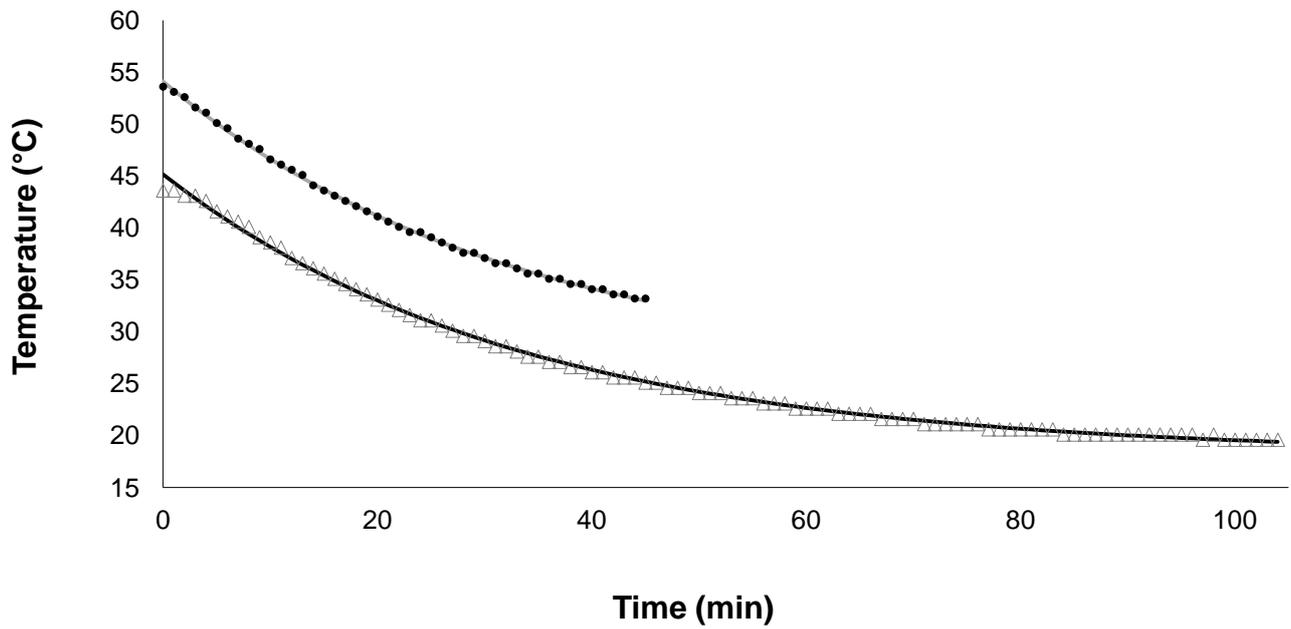


Figure 6. Observed (●, △) and fitted (—, —) temperature profiles of the cooling of meatballs in kitchens one and two, respectively. Fitting done with Newton's Law resulting in $k = 1.8 \text{ min}^{-1}$ in both catering units.

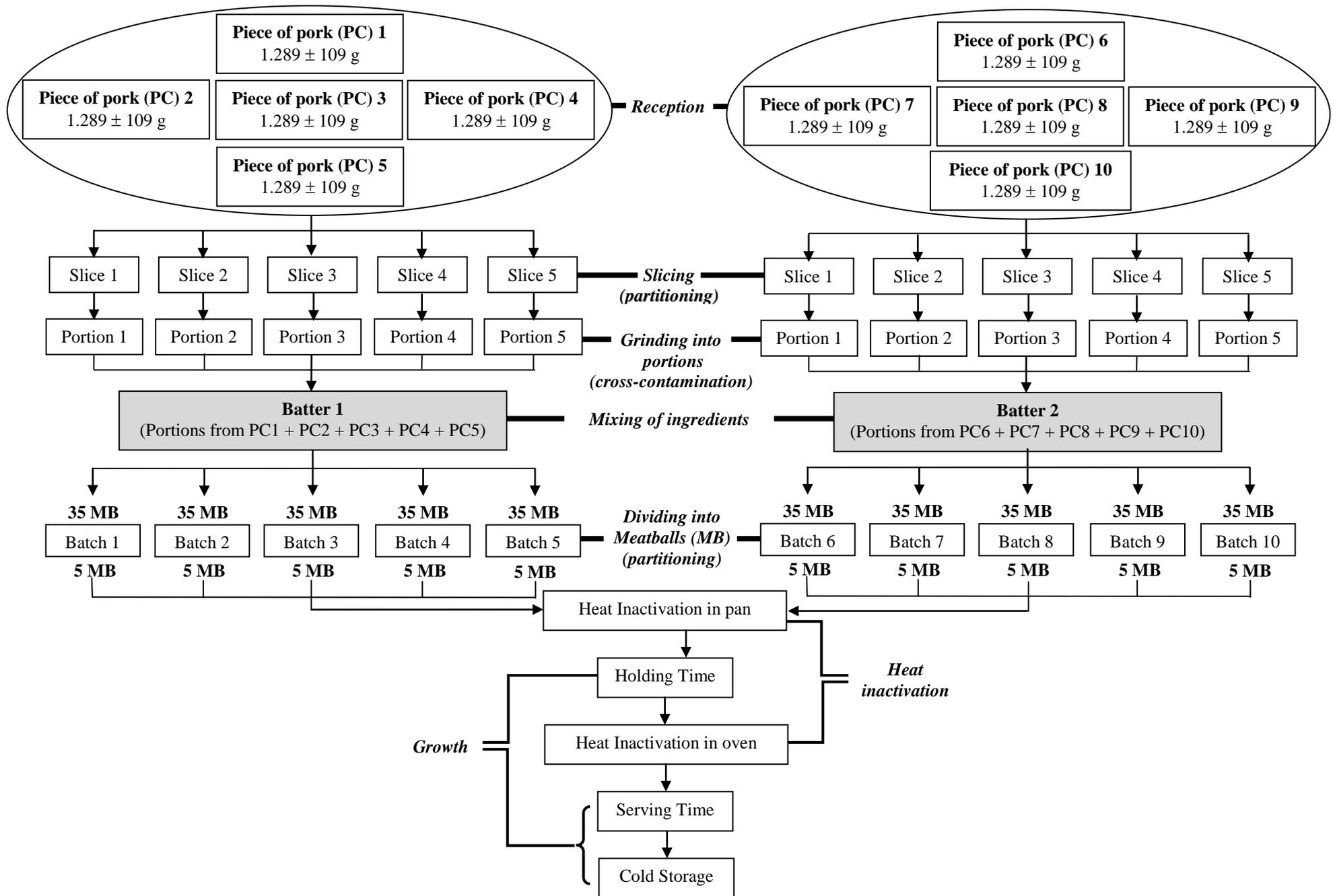


Figure 7. Schematic presentation of the pathway describing the processing of one lot of Danish Meatballs (MB) at a catering unit.

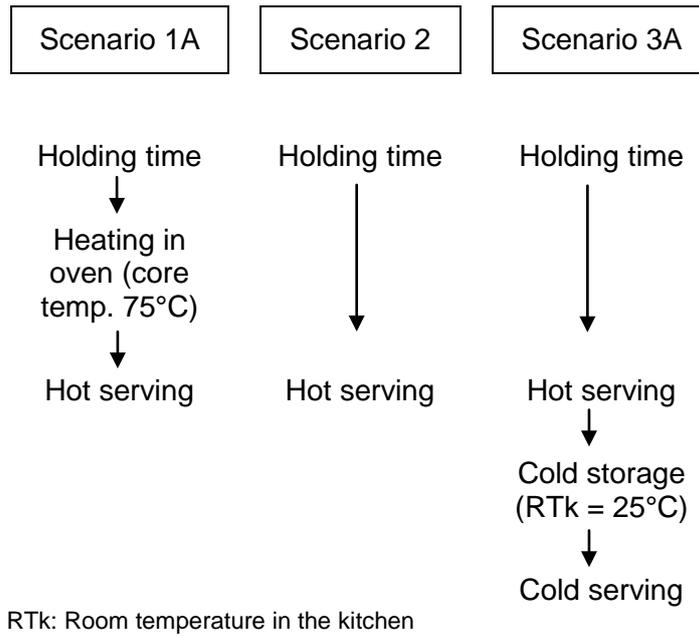


Figure 8. Variation from the main flow according to the studied scenarios simulating different flow processing of meatballs according to observation in two Danish catering units

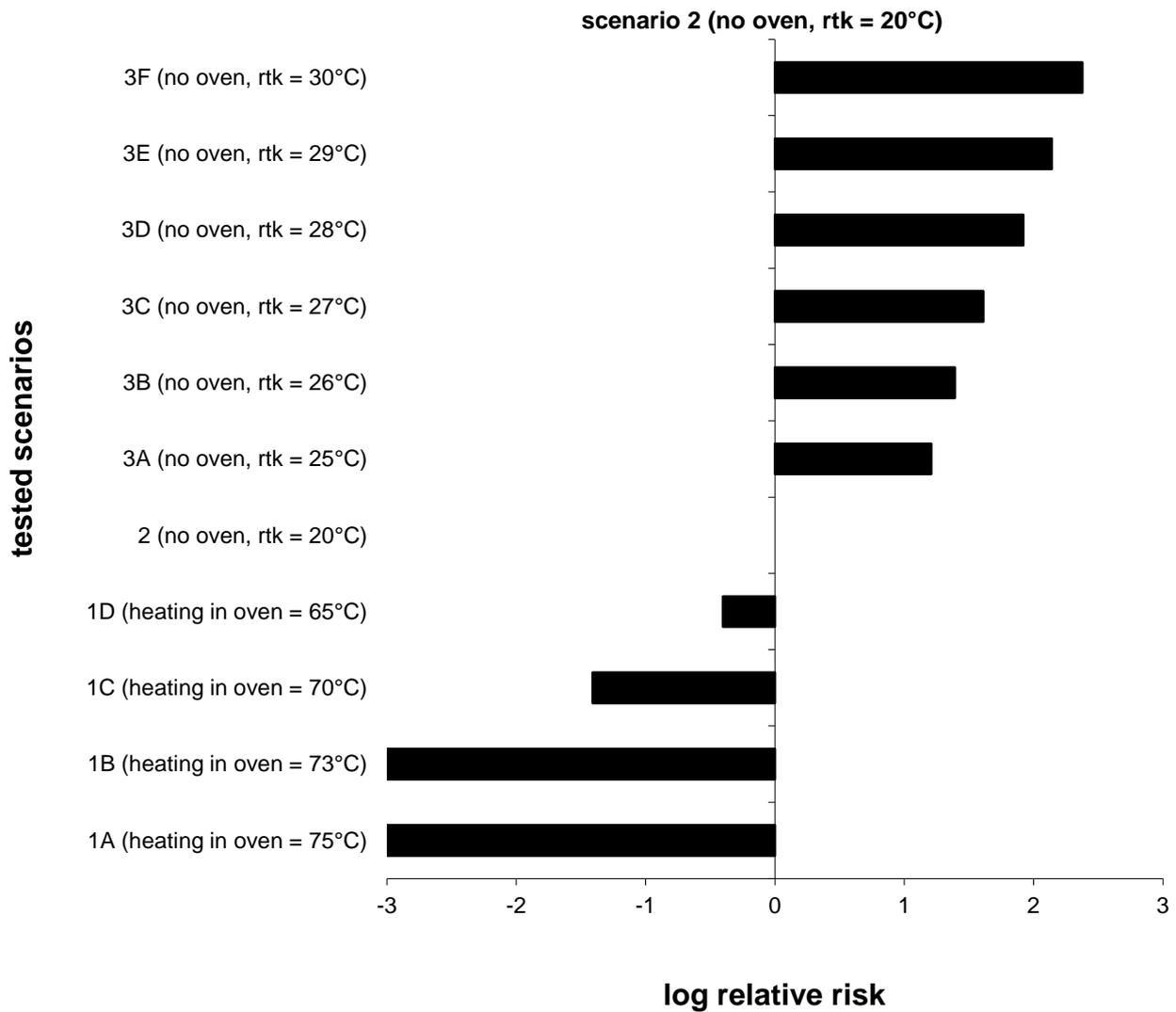


Figure 9. Log₁₀ relative risks from all scenarios tested using the proposed QMRA baseline model in relation to scenario 2, where meatballs were not heat treated in oven ($P_{III} = 7.3 \times 10^{-5}$), in order to evaluate the effect of temperature in meatballs heat treated in oven, and cooling of meatballs not heat treated in oven but maintained at room temperature in the kitchen (rtk) during holding, serving and refrigerated storing times.

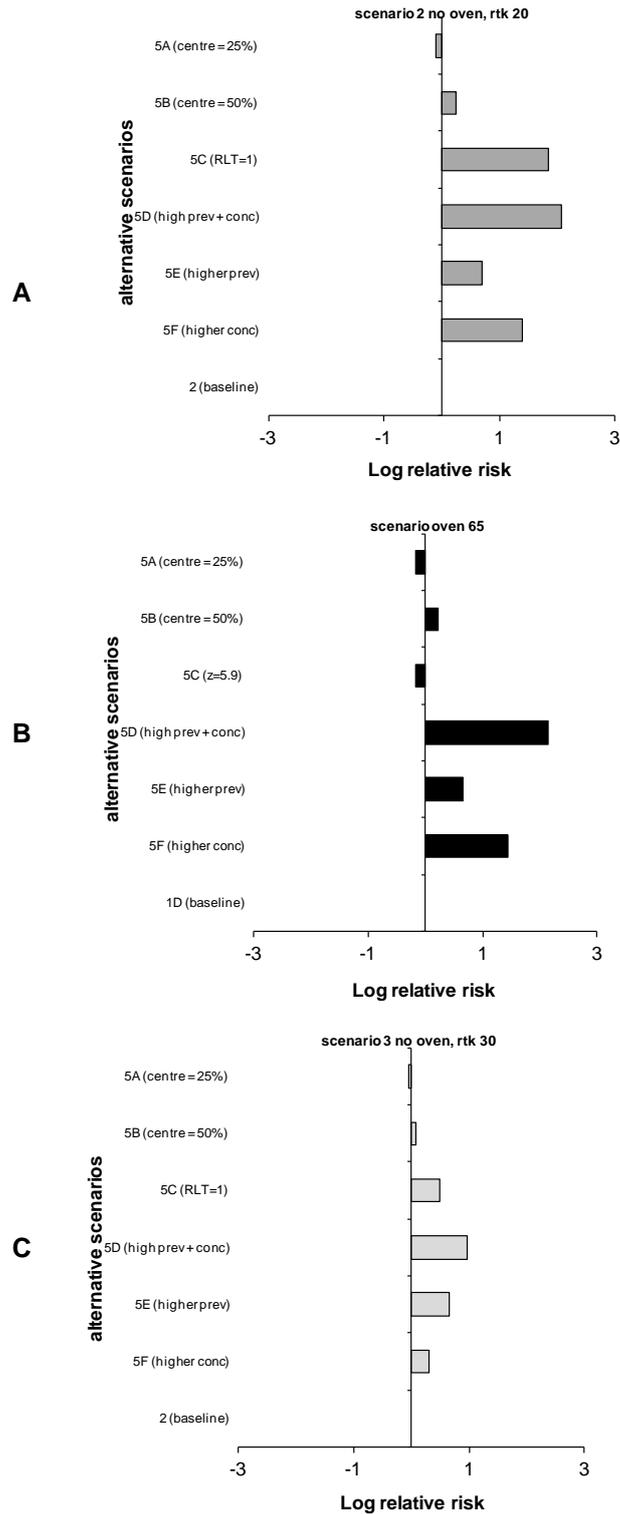


Figure 10. Log₁₀ relative risks from sensitivity analysis testing different scenarios considered as baseline: A) scenario 2, without heat treatment of the meatballs in the oven and considering room temperature in the kitchen (rtk) = 30°C; B) scenario 1D, where the temperature of the meatballs in the oven reached 65°C; and C) scenario 3F, where the meatballs not heat treated in oven were subjected to cooling at rtk = 30°C.

Appendix A. Number and average weight of meatballs per observed batch

Batch	Number of meatballs per batch (unit)	Average weight of meatballs per batch (g)
1	34	71
2	34	72
3	34	74
4	41	66
5	33	71
6	32	84
Average	35	73
Standard deviation	3	6

Appendix B. Parameters used to model heat inactivation of *Salmonella* during pan frying

Observed Batch	Start temperature ^a (°C)	Heating time (min)	End temperature (°C) per measured Meatball (MB)									
			MB 1	MB 2	MB 3	MB 4	MB 5	MB 6	MB 7	MB 8	MB 9	MB 10
2	5.1	7	19.9	23.5	31.9	34.2	39.1	39.5	39.8	42.7	44.5	56.3
3	5.1	7	18.2	18.3	20.1	20.6	27.0	28.3	29.5	41.0		
4	5.1	7	23.3	32.5	34.0	35.5	37.9	42.4	47.9	52.1	54.0	59.0
5	5.6	10	42.1	44.3	48.6	52.2	54.3	54.6				
6	7.2	10	37.2	46.0	49.1	50.1	62.2	66.8				
7	7.2	10	39.9	40.1	42.3	44.8	50	51.5				
8	8.4	6	32.1	33.1	39.1	53.2	55.2	59				
9	10.2	11	21.1	24.5	27.3	33.1	36.3	41.7	47			
10	10.2	5	45.1									

^a = measured in the batter

Appendix C. Overview of simulation variables and parameters applied to achieve the QMRA of meatball

Abbreviation	Variable	Value	Unit	Source
	Salmonella present in pork at Reception (R)			
R _{prev total}	Total investigated samples for <i>Salmonella</i> spp. in pork at retail (DK, 2006)	887	50g sample	Hansen et al., 2010
R _{prev positive}	Positive samples for <i>Salmonella</i> spp. in pork at retail (DK, 2006)	37	50g sample	Hansen et al., 2010
R _{prevalence}	Prevalence of <i>Salmonella</i> spp. in fresh pork cuttings at retail in Denmark (2006)	$= (R_{\text{prev positive}} / R_{\text{prev total}}) \times 100$	%	Calculated
R _{prev piece}	Presence of <i>Salmonella</i> spp. in a fresh pork piece	$\sim \text{Binomial}(1, R_{\text{prevalence}})$	Presence/Absence	Calculated
R _{conc cfu}	Concentration of <i>Salmonella</i> spp in positive samples of pork at retail (DK, 2002)	0.04, 400	CFU/g	Hansen et al., 2010
R _{conc positive}	Distribution of positive samples for <i>Salmonella</i> spp. per concentration in pork at retail (DK, 2002)	39, 7, 5, 1	50g sample	Hansen et al., 2010
R _{concentration}	Concentration of <i>Salmonella</i> spp. in fresh pork cuttings at retail in Denmark (2002)	$\sim \text{Histogram}(R_{\text{conc cfu}}\{R_{\text{conc positive}}\})$	CFU/g	Calculated
R _{avg weight grin}	Average of weight of fresh pork pieces used in simulation of grinding at a catering unit	1289	g	Møller et al., 2012a
R _{st dev weight grin}	Standard deviation of weight of fresh pork pieces used in simulation of grinding at a catering unit	109	g	Møller et al., 2012a

$R_{\text{weight grinding}}$	Weight of fresh pork pieces used in simulation of grinding at a catering unit	\sim Normal ($R_{\text{avg weight grin}}$, $R_{\text{st dev weight grin}}$)	g	Calculated
$R_{\text{conc piece}}$	Number of <i>Salmonella</i> spp. on pork piece	\sim Round ($R_{\text{concentration}} \times R_{\text{weight grinding}}$, 0)	CFU/piece	Calculated
<i>Salmonella</i> present in pork at Slicing (S)				
$S_{\text{n slices}}$	Number of slices normally obtained by piece during cutting and before grinding	5	slices	Møller et al., 2012a
$S_{\text{conc slice}}$	Number of <i>Salmonella</i> spp. in fresh pork slices normally cut at the observed catering unit	\sim Multinomial($R_{\text{conc piece}} \{1/ S_{\text{n slices}}\}$)	CFU/slice	Calculated
<i>Salmonella</i> present in pork at Grinding (G)				
$G_{\text{n portions}}$	Number of portions normally obtained by 5 slices after grinding	5	portions	Møller et al., 2012a
$G_{\text{p a1}}$	transfer per <i>Salmonella</i> cell from meat to grinder, environment 1	0.0013		Møller et al., 2012a
$G_{\text{p b1}}$	backward transfer probabilities of <i>Salmonella</i> from the grinder, environments 1	0.0498		Møller et al., 2012a
$G_{\text{p c1}}$	inactivation of <i>Salmonella</i> in environment 1	0		Møller et al., 2012a
$G_{\text{p a2}}$	transfer per <i>Salmonella</i> cell from meat to grinder, environment 2	0.8989		Møller et al., 2012a
$G_{\text{p b2}}$	backward transfer probabilities of <i>Salmonella</i> from the grinder, environments 2	0.0652		Møller et al., 2012a
$G_{\text{p c2}}$	inactivation of <i>Salmonella</i> in meat	0		Møller et al., 2012a
$G_{\text{p c3}}$	inactivation of <i>Salmonella</i> in environment 2	0.5462		Møller et al., 2012a

$G_{\text{conc portion}}$	Number of <i>Salmonella</i> spp. in fresh pork portions normally ground at the observed catering unit	$= (1 - G_{p a1}) (1 - G_{p a2}) (1 - G_{p c2}) C_{\text{conc slice}} + (G_{p b1} \times gr_{1,i-1}) + (G_{p b2} \times gr_{2,i-1})$ $gr_{1,i} = G_{p a1} \times S_{\text{conc slice}} + (1 - G_{p b1}) (1 - G_{p c1}) gr_{1,i-1}$ $gr_{2,i} = G_{p a2} \times S_{\text{conc slice}} + (1 - G_{p b2}) (1 - G_{p c3}) gr_{2,i-1}$	CFU/portion	Calculated
	Salmonella present in pork at Mixing (M)			
$M_{\text{n pieces batter}}$	Number of pork pieces normally processed by batter at the observed catering unit	5	pieces	Estimated
$M_{\text{weight pork batter}}$	Weight of pork pieces normally processed by batter at the observed catering unit	$= A_{\text{weight grinding}} \times M_{\text{n pieces batter}}$	g	Calculated
$M_{\text{weight ingr batter}}$	Weight of other ingredients normally processed by batter at the observed catering unit	$= M_{\text{weight pork batter}} / 2$	g	Calculated
$M_{\text{weight batter batch}}$	Weight of the batter processed per batch at the observed catering unit	$= M_{\text{weight pork batter}} + M_{\text{weight ingr batter}}$	g	Calculated
$M_{\text{conc batter batch}}$	Number of <i>Salmonella</i> spp. per processed batter according the conditions observed in a catering unit	$= \text{sum of } G_{\text{conc portion}} \text{ of } G_{\text{n portions}}$	cfu	Calculated
	Salmonella present in Meatball at Partition (P)			
$P_{\text{n MB batter}}$	Number of meat balls produced from a batter	150	unit	Estimated
$P_{\text{n batches}}$	Number of batches of meatballs produced from a batter	5	unit	Estimated
$P_{\text{n MB batch}}$	Number of meatballs produced from in a batch	$= P_{\text{n MB batter}} / P_{\text{n batches}}$	unit	Calculated
$P_{\% \text{ MB samples}}$	Percentual of meatballs sampled from a batch	15	%	Calculated
$P_{\text{n MB samples}}$	Number of meatballs sampled from a batch	$= P_{\text{n MB batch}} \times P_{\% \text{ MB samples}}$	unit	Calculated

$P_{\text{avg weight MB}}$	Average of weight of meatball produced at a catering unit	72.87	g	Estimated
$P_{\text{st dev weight MB}}$	Standard deviation of weight of meatball produced at a catering unit	5.90	g	Estimated
$P_{\text{weight Meatball}}$	Weight of meatball produced at a catering unit	$\sim \text{Normal}(P_{\text{avg weight MB}}, P_{\text{st dev weight MB}})$	g	Calculated
$P_{\text{conc Meatball}}$	Concentration of <i>Salmonella</i> spp. in meatball processed at the observed catering unit	$\sim \text{Multinomial}(M_{\text{conc batter batch}}, \{1/P_{\text{weight Meatball of } P_n \text{ MB samples}}\})$	CFU/meatball	Calculated, see Nauta 2005
	<i>Salmonella</i> present in Meatball after Heating Inactivation in Pan (HP)			
HP_{layers}	Number of layers in a meatball, considering the contact with the heating surface	3		Estimated
$HP_{\text{conc crit area}}$	Concentration of <i>Salmonella</i> in the central layer of the meatball, considered as a critical area.	$= P_{\text{conc Meatball}} / HP_{\text{layers}}$	CFU/central layer	Calculated
$HP_{\text{avg log log red}}$	Average of log log reduction of <i>Salmonella</i> spp. in meatball predicted at 60°C with observed start temperatures, final temperatures and heating times	-3.55	log log CFU/g	Estimated

HP _{st dev log log red}	Standard deviation of log log reduction of <i>Salmonella</i> spp. in meatball predicted at 60°C with observed start temperatures, final temperatures and heating times	1.76	log log CFU/g	Observed
HP _{log log reduction}	log log reduction of <i>Salmonella</i> spp. in meatball predicted at 60°C with observed start temperatures, final temperatures and heating times	~ Normal (HP _{avg log log red} , HP _{st dev log log red})	log log CFU/g	Calculated
HP _{conc after inac}	Concentration of <i>Salmonella</i> spp. in meatball after heating inactivation in pan	~ Poisson (10 ^{log10(HP_{conc crit area}) - 10^(HP_{log log reduction}))}	CFU/g	Calculated
	Salmonella present in Meatball after Heating in Oven (HO)			
HO _{avg log log red}	Average of log log reduction of <i>Salmonella</i> spp. in meatball predicted at specific a D _{value} with observed start temperatures, final temperatures and heating times	1.56 if D ₉₅ -2.24 if D ₇₅ -3.74 if D ₇₀ -5.36 if D ₆₅	log log CFU/g	linear trend of log D from Juneja et al. (2001), Smith et al. (2001) and Murphy et al. (2004) applied to observed data
HO _{st dev log log red}	Standard deviation of log log reduction of <i>Salmonella</i> spp. in meatball predicted at a specific D _{value} with observed start temperatures, final temperatures and heating times	2.05 if D ₉₅ 2.88 if D ₇₅ 3.96 if D ₇₀ 4.38 if D ₆₅	log log CFU/g	linear trend of log D from Juneja et al. (2001), Smith et al. (2001) and Murphy et al.

				(2004) applied to observed data
HO _{log log reduction}	log log reduction of <i>Salmonella</i> spp. in meatball predicted at specific a D _{value} with observed start temperatures, final temperatures and heating times	~ Normal (HO _{avg log log red} , HO _{st dev log log red})	log log CFU/g	Calculated
HO _{conc after inac}	Concentration of <i>Salmonella</i> spp. in meatball after heating inactivation in oven	~ Poisson (10 ^{log10(HO_{conc crit area}) - 10^(HO_{log log reduction}))}	CFU/g	Calculated
	Salmonella present in Meatball after Growth at cooling (GC)			
GC _{min log inc}	Minimum log increase by growth of <i>Salmonella</i> in meatball at storage since after heating inactivation at a specific Room Temperature (RT) until it reach 6°C at refrigerator	0.17 if RT = 30°C 0.03 if RT = 29°C 0.04 if RT = 28°C 0.05 if RT = 27°C 0.06 if RT = 26°C 0.11 if RT = 25°C	log CFU/g	Calculated from the observed data
GC _{max log inc}	Maximum log increase by growth of <i>Salmonella</i> in meatball at storage since after heating inactivation at a specific room temperature until it reach 6°C at refrigerator	0.75 if RT = 30°C 0.21 if RT = 29°C 0.20 if RT = 28°C 0.18 if RT = 27°C 0.17 if RT = 26°C 0.16 if RT = 25°C	log CFU/g	Calculated from the observed data
GC _{log increase}	log increase by growth of <i>Salmonella</i> in meatball at storage since after heating inactivation at a specific room temperature until it reach 6°C at refrigerator	~ Uniform (GC _{min log inc} , GC _{max log inc})	log CFU/g	Calculated

GC _{conc after growth}	Concentration of <i>Salmonella</i> spp. in meatball after Growth at Storage	$= (10^{(\log_{10}(HO_{\text{conc after inac}})} + 10^{(GC_{\text{log increase}})})$		Calculated
	Probability of consumers getting ill by ingestion of Meatball contaminated with <i>Salmonella</i> at an investigated Danish catering unit (Pill)			
α		-0.3126		(FAO and WHO, 2002)
β		2885		(FAO and WHO, 2002)
Pill _{dose}		$\sim \text{Poisson } (1 - (1 + (GC_{\text{conc after growth}} / \beta)^\alpha)$		Calculated
Pill _{mean}		$\sim \text{Mean (—$		Calculated

Chapter 8

NOTE I

Using a two species interaction model for quantifying levels of *Salmonella* Typhimurium DT104 under detection limit in minced pork by cold-enrichment

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Introduction

The complexity and difficulty of low pathogen level transfer, faced in the studies performed by Møller et al. 2012 (PAPER I) was also described by Sheen and Hwang (2010), who suggests that new methods of analysis should be developed in order to improve sensitivity and still continue to be simple enough for low concentration, large-scale studies to be realistic to conduct.

According to different studies, the concentration of *Salmonella* in pork rarely exceeds 3 log₁₀CFU/g (Pires, 2009, Hansen, 2010, Anonymous, 2012). According to the study of Møller et al. (2012 - PAPER I), using this concentration as start level for simulation of a grinding process will result in a 'tail' of portions having a contamination level lower than the detection limit of the available techniques of quantitative analysis, and therefore, *Salmonella* positive portions of meat could erroneously be considered as *Salmonella* free portions.

The colony count technique on selective agar is only appropriate for higher levels of *Salmonella* (10² to 10³ CFU/g or more) and it is not feasible when high levels of background microbiota are present. For low numbers of *Salmonella*, and/or when the amount of background microbiota is relatively high, the labor-intensive MPN method is recommended, which provides confirmed results within 3 to 6 days. Recently, Krämer et al. (2011) have developed and validated a novel real-time PCR-based tool suitable for generation of quantitative data on minimum levels of 1.4 CFU/20cm (approximately 10 g) of *Salmonella* for risk assessment on naturally and artificially contaminated cork borer samples of pig carcasses, as an alternative to the laborious MPN method. However, the method still needs to be tested in different food matrices, and as pointed out by Lam et al. (2011) because it involves the polymerase chain reaction, there is a requirement for skilled technicians and specific laboratory facilities. Therefore, enrichment in the meat at relatively low temperatures from 11 to 16°C combined with accurate predictive models appears as an obvious alternative.

The objective of the present study was to evaluate the possibility to carry out a *Salmonella* enrichment step in the meat itself and use the two species interaction model, presented in MANUSCRIPT I, for quantifying levels of *Salmonella* Typhimurium DT104 in minced pork.

Materials and methods

Experimental set up. Preparation of *S. Typhimurium* DT104 inoculum culture, preparation of minced pork portions as well as the inoculation procedure were performed as described in Møller et al. (2012 - PAPER I). In short, *S. Typhimurium* DT104 was inoculated in averaged concentrations of 2.58, 2.93, 3.57, 4.70, 4.96, 6.51, 6.53, 6.84, and 7.04 log₁₀CFU/g on the surface of whole pork pieces from nine studies, which after attachment time were cut into slices and minced in a semi-industrial grinder. Subsequent to these infected pork pieces a number of non-

infected pieces were processed in the same grinder giving rise to minced pork portions with varying concentrations of *S. Typhimurium* DT104. Concentrations of *S. Typhimurium* DT104 as well as levels of natural microbiota in these portions were determined immediately after grinding and again after incubation of the minced meat samples for *approx.* 48 h at 11 and 16°C. This incubation was perceived as a kind of enrichment procedure performed in the meat itself.

Enumeration of *S. Typhimurium* DT104 and the natural microbiota was carried out on XLD and XLD+ampicillin incubated at 37°C for 18 – 24 h as well as PCA incubated at 22°C for 2 d, respectively, using the methods described by Møller et al. (2012 - PAPER I).

Data analysis. A rearranged version of the expanded Jameson-effect species interaction model, suggested by Møller et al. in MANUSCRIPT I, was applied for prediction of the *Salmonella* concentration in the minced pork portions.

The model was rearranged to have the *Salmonella* count (\log_{10} CFU/g) prior to enrichment and the natural microbiota count (\log_{10} CFU/g) after enrichment as outputs. Inputs to the model were enrichment temperature (°C) and time (d), count of the natural microbiota (\log_{10} CFU/g) prior to enrichment and count of *Salmonella* and natural microbiota (\log_{10} CFU/g) after the enrichment period. The *Salmonella* count (\log_{10} CFU/g) prior to enrichment was then predicted by finding the value that resulted in a difference of zero between observed and simulated count of *Salmonella* (\log_{10} CFU/g) after the enrichment. The solver function in Microsoft Excel was used for this purpose.

Observed and predicted counts of *S. Typhimurium* DT104 (\log_{10} CFU/g) prior to enrichment were compared visually and by paired t-test. In addition, the bias factor (Ross 1996) with \log_{10} CFU/g as the response variable was used to evaluate the performance of the model for this particular purpose.

All statistical analyses were performed in Microsoft Excel.

Results and discussion

Observed and predicted counts of *S. Typhimurium* DT104 (\log_{10} CFU/g) prior to enrichment are compared visually in Figure 1. As shown, a relatively good agreement between predicted and observed values was seen, which was confirmed by a non-significant paired t-test ($P = 0.20$). However, a tendency of the model to overestimate was observed for counts from 3 log-units and above, whereas underestimation to some extent was seen for counts below 3 log-units (Figure 1). These findings resulted in a bias factor of 0.75 indicating an underestimation of 25 % of the log-count on average.

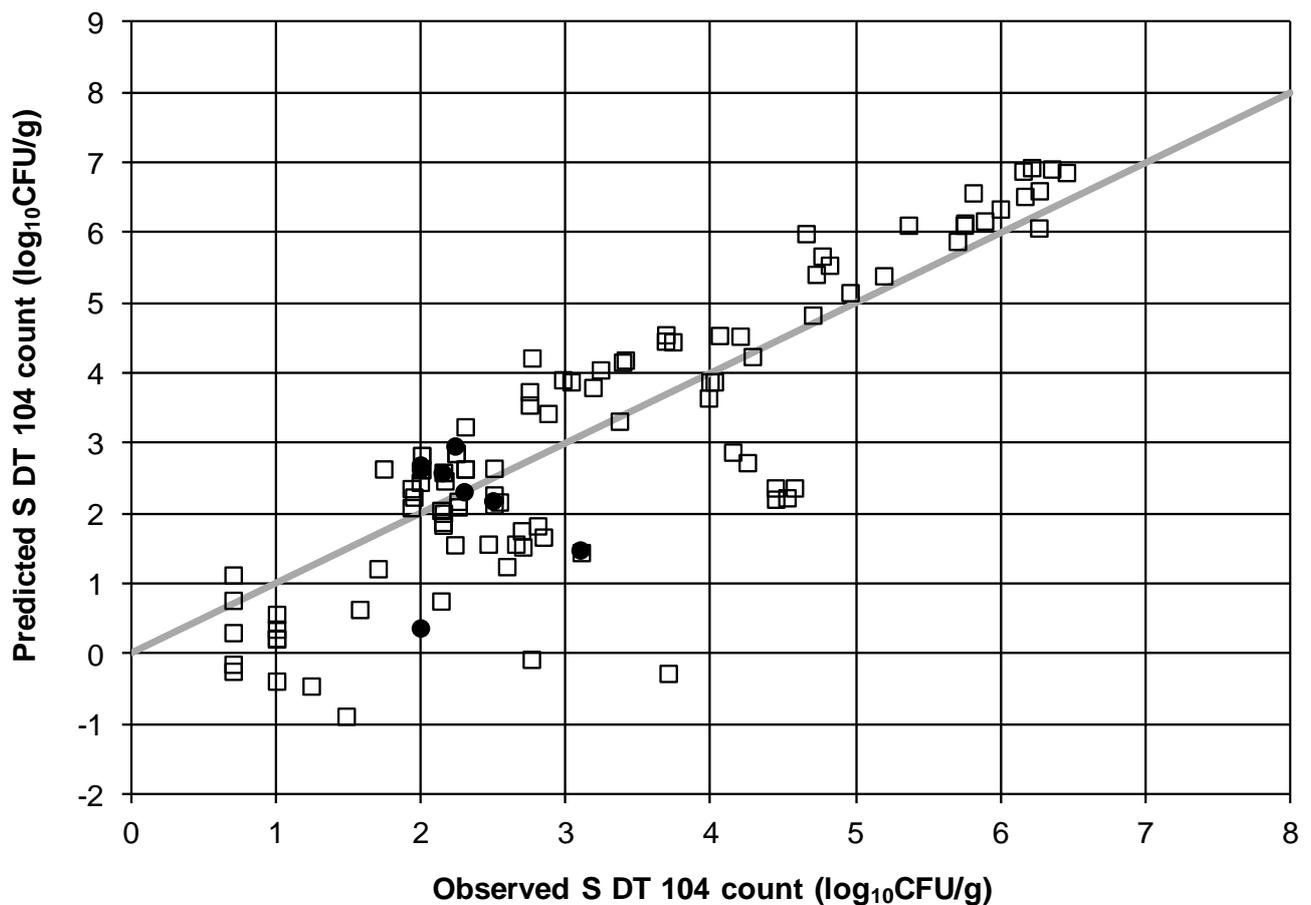


Figure 1. Visual comparison of observed *S. Typhimurium* DT104 (log₁₀CFU/g) and predicted *Salmonella* count (log₁₀CFU/g) prior to enrichment in the minced pork sample itself for 40 – 48 h at 11 (□) and 16°C (●).

The overestimation could most likely be explained by uncertainty of the lag-time model for *Salmonella*, *i.e.* a short lag time would result in a lower initial count to get to the same count after enrichment as compared to a long lag time. In contrast, the underestimation appeared to coincide with competitive growth of *Citrobacter braakii* and *Hafnia alvei* on the XLD and XLD+ampicillin agars and, therefore, more likely was a result of underestimation of the *Salmonella* count after enrichment. Whether competition between these species also took place in the meat during enrichment is not known. However, as the observed levels of the competitive species were below 5.5 log₁₀ CFU/g it is questionable whether interaction with *S. Typhimurium* DT104 in the pork could have occurred. More work is needed to clarify these observations.

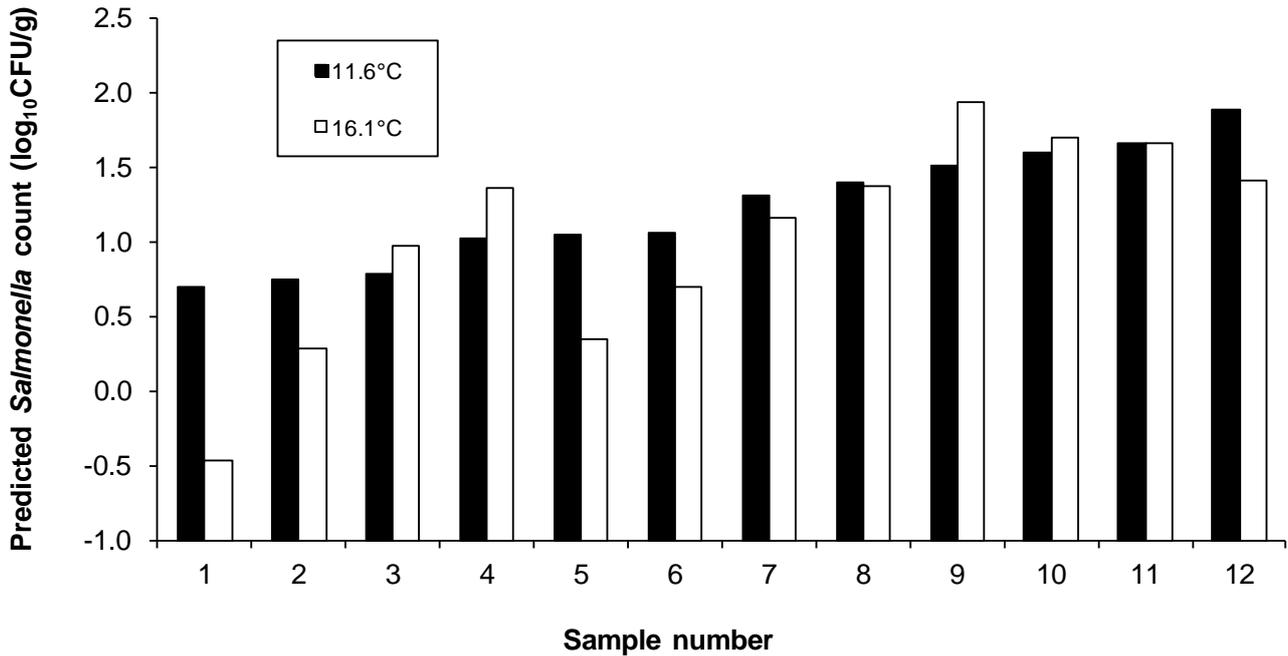


Figure 2. Predicted *Salmonella* counts (log₁₀CFU/g) in minced pork prior to enrichment in the pork sample itself for 42 – 47 h at 11.6°C or 40 – 42 h at 16.1°C.

Eight of the data points shown in Figure 1 were predicted from enrichment at 16.1°C, the rest from enrichment at 11.1 to 12.1°C. No systematic effect of enrichment temperature was observed for these data for which *S. Typhimurium* DT104 counts prior to enrichment was quantifiable on XLD or XLD+amp agar. Neither for the 12 data points where *S. Typhimurium* DT104 counts before enrichment were below the detection limit, significant differences were found when comparing predicted values obtained after enrichment at 16.1°C to enrichment at 11.6°C (paired t-test, $P = 0.18$) (Figure 2).

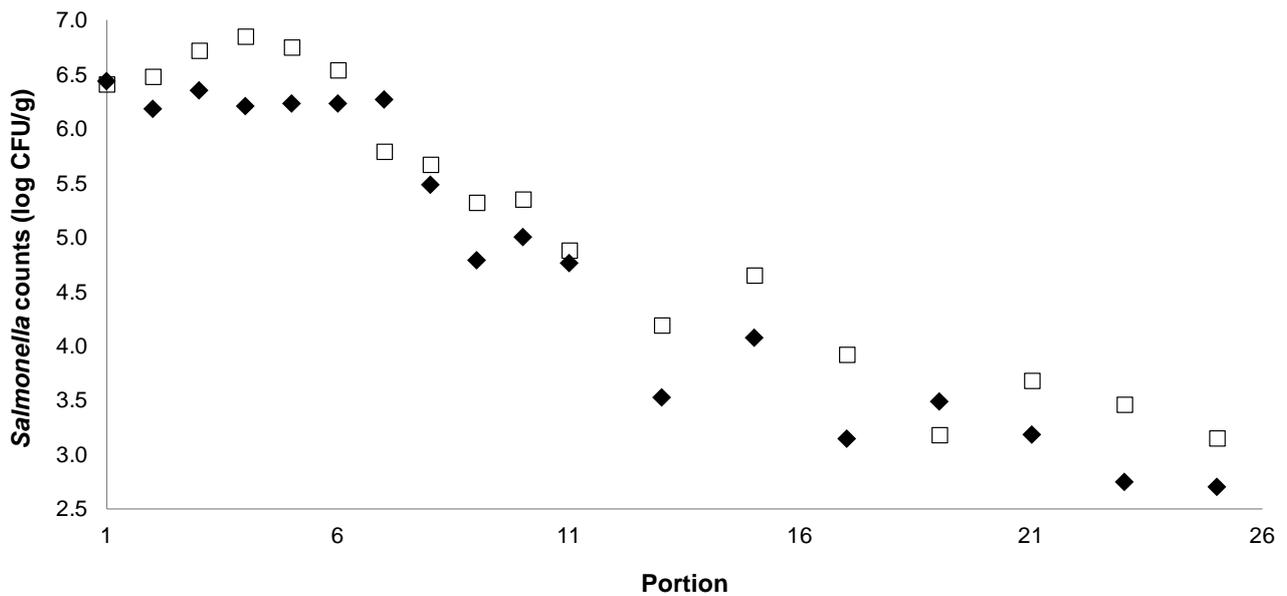


Figure 3. Levels of *Salmonella* Typhimurium DT104 (\log_{10} CFU/g) observed immediately after grinding at T0 (◆) and levels at T0 predicted with the proposed recovery model using observed counts of *S.* Typhimurium DT104 after 48-h-incubation at 11.6°C (□).

According to Figure 3, the predictions obtained with the proposed recovery model (□) indicated a slight overestimation of some data points (0.03 - 0.78 log CFU/g) when compared to the observed *S.* Typhimurium DT104 counts obtained immediately after grinding and prior to enrichment. Since a deviation of 0.5 log correspond to an expected uncertainty inherent to the plate count method, only four of the 18 observed data points were considered slight overestimated (0.64 – 0.78 log CFU/g). Otherwise the proposed recovery model seemed to give a good description of the shape of contaminated portions.

Conclusion

A novel approach for determining *Salmonella* counts at low concentrations was proposed. Applying a simple plate count method, after cold enrichment (11-12°C for 2 d) in the ground pork sample itself in combination with predictive growth models, showed promising results. It indicates the potential of this approach as an alternative to meet the need for more sensitive methods, there are simple enough to be used in large-scale series of analysis. However, supplementary studies, testing levels of contamination of *Salmonella* even lower, still need to be performed in order to confirm the effectiveness of the approach and improve the accuracy of the predictions.

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Chapter 9

NOTE II

Facing safety against quality in fresh minced pork by quantifying the potential for *Salmonella* growth within the shelf-life period

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Introduction

Globally, the incidence of foodborne diseases is increasing and international food trade is disrupted by frequent disputes over food safety and quality requirements. The terms food safety and food quality can sometimes be confusing. Food safety refers to all those hazards, whether chronic or acute, that may make food injurious to the health of the consumer. It is not negotiable. Quality includes all other attributes that influence a product's value to the consumer. This includes negative attributes such as spoilage, contamination with filth, discoloration, off-odours and positive attributes such as the origin, colour, flavour, texture and processing method of the food. (FAO/WHO, 2003)

As pointed out by Grunert (2005), food quality and safety have been highly topical for the past 10 years - in the public debate, in food policy, in industry, and, last but not least, in research. Several factors have driven this debate. First, a variety of food scares has directed public attention to food safety issues. As a result, safety issues have for some years figured prominently on the political agenda, with the EU white PAPER on food safety (Commission of the European Communities, 2000) and the foundation of the European Food Safety Agency as visible outcomes. Second, segments of the general public have become interested and often critical with regard to certain ways of producing food - both at the farm level and at the processing level. As a result, we have had discussions on organic production, animal welfare, and the use of genetically modified organisms (GMOs) in food production, to mention only the most prominent debates. Third, and partly related to the previous factor, consumers in developed countries have become more demanding, more critical, and more fragmented in their food choices, leading to situations where quality differentiation of food products, both vertical and horizontal, has become necessary in order to satisfy consumers.

For delivery of a safe food product with the desirable quality requirements, a balance between food safety and quality is needed and can be achieved if both aspects are considered when evaluating the shelf-life. Keeping this concept in mind, this study was performed in order to evaluate the potential for growth of *Salmonella* Typhimurium DT104 and *Salmonella* Derby within the shelf-life of fresh minced pork at abusive temperatures.

Materials and methods

Preparation of Salmonella cultures. A cocktail of *Salmonella* Typhimurium DT104, carrying

resistance to ampicillin, chloramphenicol, florfenicol, streptomycin and sulfa, and *Salmonella* Derby, carrying resistance to gentamycin, streptomycin, sulfa and spectinomycin, were used. Both *Salmonella* serovars had been isolated from pigs. One loop of a stock culture (-80°C) of each isolate was cultured separately in 10 ml LB-broth by overnight shaking at 37°C. Subsequently, the tubes were stored at 5°C for 3 days. Prior to inoculation, the cultures were diluted in phosphate buffered saline to obtain a concentration of *approx.* 10⁶ CFU ml⁻¹ and equal volumes of each was mixed and used as the inoculation cocktail.

Storage experiments. Packages of modified atmosphere packaged minced pork were obtained from local retailers one day before each test round and divided into 100 g portions at the following day. Each 100-g-meat sample was aseptically transferred to separate sterile stomacher bags and inoculated with 1 ml of the *Salmonella* cocktail to a final concentration 10⁴ CFU g⁻¹. To ensure even distribution of the cocktail in the whole meat sample, it was mixed by stomaching for 2 times 1 min. The inoculated samples were stored in normal atmosphere at selected temperatures between 4°C and 20°C. At appropriate time intervals, the whole meat sample was removed from the incubator and mixed in a stomacher for 2 times 1 min. Subsequently, 5 g meat was sampled aseptically and transferred to a sterile filter bag and the remaining meat sample was returned to the incubator within 5 min. The samples were diluted in 45 ml of buffered peptone water and mixed in a stomacher for 2 min. For bacterial enumeration, further 10-fold dilutions were performed using isotonic saline solution and appropriate dilutions were drop-plated (10 µl) onto XLD+ampicillin and XLD+gentamycin agars to enumerate *S. Typhimurium* DT104 and *S. Derby*, respectively. The plates were incubated at 37°C for 16 to 24 h.

Sensory evaluation. Throughout the incubation periods, appearance and odour of the meat samples was evaluated by a four-member panel. Besides describing the appearance and odour, the panel was also asked to evaluate whether they found the meat acceptable for consumption.

Results and discussion

Table 1 summarizes the appearance and odour of minced pork samples as assessed by the sensory panel throughout incubation at 10.5, 15 and 20°C. These observations determined for how long the meat was acceptable for consumption at different storage temperatures. In all samples, acidic odour arose before putrid odour and also before significant colour changes. Acidic odour

Table 1. Sensory changes during storage of minced pork as assessed by a four-member panel

Storage		pH	Acceptable odour?		Colour intensity ^a		
Temp. (°C)	Time (h)		Yes	No	Red	Brown	Other
9.5	0 – 24	5.9	X		High	Low	-
	48 – 96	5.5 – 5.9	X		Medium	Low	-
	168 – 192	5.7		X	Medium	Medium	Yellow & green
	210 – 240	5.5 – 6.3		X	Low	High	Green
10.5	0 – 24	5.8	X		High	Low	-
	30 – 54	5.5 – 5.7	X		Medium	Low	-
	72 – 80	5.4		X	Medium	Medium	-
	96 – 102	5.8		X	Low	Medium	-
12	0 – 30	5.7 – 5.8	X		High	Low	-
	48 – 54	5.6		X	Medium	Low	-
	72 – 96	5.5 – 5.6		X	Medium	Low	Orange
	102 – 168	5.6 – 6.3		X	Low	Medium	Orange
15	0 – 24	5.5 – 5.9	X		High	Low	-
	27 – 48	5.5 – 5.8		X	Medium	Low	Yellow
	54 – 165	5.8 – 6.7		X	Low	Medium	Yellow & green
20	0 – 4	5.8	X		High	Low	-
	6 – 48	5.5 – 5.8		X	Medium	Low	Orange
	54 – 72	5.5 – 5.6		X	Medium	Low	Orange & green

^aThe colour attributed for fresh meat in this study (high red and low brown colours) was supported by colour 1A displayed in the colour reference standards (AUS-MEAT, 2005). The other colours were registered according observations at the latest stages of the studied storage periods.

was listed as the determining factor for rejection of the meat by the panel. At the time of rejection, the level of natural microbiota in minced pork averaged 8.7 log₁₀CFU/g (S.D.: 0.44 log₁₀CFU/g) and no systematic effect of temperature, on this level, was observed (results not shown).

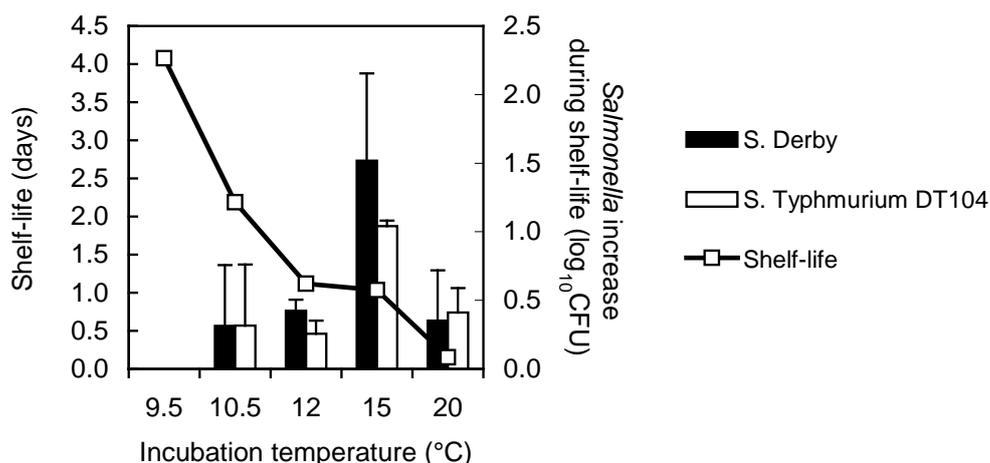


Figure 1. Average increase (\log_{10} CFU) of *Salmonella* Derby and *Salmonella* Typhimurium DT104 in minced pork with a natural microflora measured on the final day of shelf-life. (Log-increase was defined as the difference between the count found at the end of shelf-life and the initial count).

As shown in Figure 1, the shelf-life of minced pork was found to be 4 days at 9.5°C and below 4 hours at 20°C. At storage temperatures above 10°C, both *S. Typhimurium* DT104 as well as *S. Derby* started to grow before the meat was rejected for consumption (Figure 1). Growth was most pronounced around 15°C, where an average increase of more than 1 log-unit was found, but also at 12°C and 20°C, *Salmonella* was observed to initiate growth before the meat was spoiled (Figure 1). This indicated that safety, rather than spoilage, could be the shelf-life limiting factor of fresh pork at temperatures from 12 to 20°C.

Conclusion

It was observed that temperature abuse, even in the chilled temperature area, may induce critical *Salmonella* growth before spoilage of fresh minced pork occurs. This is important in relation to the setting of critical limits in the cold chain, *i.e.* for temperature shifts during handling. In addition, the results indicate that safety, rather than spoilage, could be the shelf-life limiting factor of fresh pork meat at the studied abusive storage conditions. This supports that food quality and safety should not be evaluated independently, but there is a need for a balance between all the requirements involved in a safe food product with high quality.

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Chapter 10

Discussion

Discussion

Despite efforts applied to control *Salmonella* and consequently to improve food safety, *Salmonella* still continue to be one of the pathogens of concern in Europe and pork is one of the most important sources of salmonellosis (EFSA, 2010b; van Hoek et al., 2012). The spread, growth and survival of *Salmonella* in pork processing, especially regarding the practices at the catering sector, still remain to be identified. Understanding of the dynamics related to these aspects is crucial to improve the safety of pork products produced in the catering sector.

One way to increase our understanding in this field is the development and application of models. Several process chain QMRA models have already been published (Afchain et al., 2008; Aziza et al., 2006, Billoir et al., 2011, Bollaerts, et al., 2009; Clough et al., 2009; Delhalle et al., 2009; Ebel et al., 2004; Lindqvist et al., 2002; Nauta, 2001; Nauta et al., 2005; Nauta et al., 2007;). However, the behavior of *Salmonella* through pork processing by catering units remained unexplored until now, which increased the level of difficulty to estimate the risk of salmonellosis regarding pork production by this sector, since most of the necessary data to build up the QMRA model was not available, e.g. pathway, time and temperature profiles at each step of pork meatballs processing, volume of production, etc. According to Filion and Powell (2011), Hensen et al. (2006), Jones et al. (2004) and Lee and Middleton (2003), catering is an important segment, since 70 % of the foodborne illness distributed worldwide are estimated to be linked to food prepared at food service establishments, underlining the relevance of the proposed QMRA model to improve the safety of pork products in the catering sector.

In a recent outbreak where Danish meatballs, produced in the catering sector, were involved, many production errors were pointed out to have caused the problem, like cross contamination, inefficient heating, cooling, cold storage, etc., but no specific point was determined due to uncertainty of the investigation results (personal communication: Lone J. Porsbo, 2012). However, it is important to mention that the use of more accurate modelling could help risk assessors to improve the quality of risk estimates, by reducing the level of uncertainty, which would result in more effective outbreak investigations. In addition, more accurate models could also support the food processors if such models were used to establish critical limits in the process steps and impact of changes in processing as well, especially regarding time and temperature adjustments. Furthermore, to implement modelling and perform scenario analysis could also assist the food industry to failure diagnostic in a specific step of processing or even find the most hazardous combination of failures through the process, what could contribute to development of products and also improve the safety of processing.

This aspect has been taken into consideration through the studies performed in the present thesis where modelling tools have been developed and applied, allowing the quantification and assessment of changes in concentration of *Salmonella* from a selected raw pork product through a catering process to a final meal, using scenario analysis.

A practical example of the value of the developed models is shown in MANUSCRIPT II where a quantitative risk assessment model was built to investigate *Salmonella* in pork processing, exemplified by the production

of meatballs by catering units. Applying the proposed QMRA model, risk estimates of illness from meatballs processed by the catering sector per year in Denmark were obtained and considered reasonable, when comparing with the epidemiological data from 2011 for *Salmonella* cases in Denmark related to pork (Anonymous, 2012). Scenario analysis showed that complying with the Danish official guidelines stating a minimum temperature of 75°C in the coldest spot during heating, would result in no cases of salmonellosis upon consumption of those meatballs. However, when the temperature in the coldest spot only reached 65°C, about 27 people could become ill per year. Since the Danish recommendation (Fødevarestyrelse, 2008) is stricter than in other countries, e.g. Canada (Health Canada, 2012) and USA (FDA, 2007; USDA, 2012), where the official guidance is 71°C for pork products, scenarios testing different temperatures in the coldest spot of meatballs were performed in order to challenge those recommendations. As shown in MANUSCRIPT II, no statistical differences were observed when testing temperatures between 72 and 75°C, showing that this range of temperature was very effective against *Salmonella*. This information could be used by the food processors for maintaining the quality of their products by applying lower heating temperatures. However, it needs to be considered that there are more thermo-tolerant strains of *Salmonella*, than those applied in this study like *Salmonella* Senftenberg (Veeramuthu et al., 1998). In addition, the safety regarding other more thermo-tolerant pathogens associated with pork, e.g. *L. monocytogenes* (Doyle et al., 2001), also should be covered by the official food safety guidelines. Nevertheless, such scenario analysis could also support the setting of critical limits for heat processing considered as Critical Control Point (CCP).

In relation to the heat treatment of meatballs in oven, it showed to be difficult to extrapolate the observed heating profile from one facility to another and, therefore, the module used to simulate the effect of heating in oven should be used with care. Preferably the module should be adjusted to the specific heating profile obtained in the new situation that is investigated. Juneja et al. (2001), Murphy et al. (2004) and Velasquez et al. (2010) have demonstrated that *Salmonella* in ground pork seems to be a little more heat tolerant when compared with the behaviour of *Salmonella* in different matrices. However, this aspect had very little impact on the risk estimates when sensitivity analyses were applied.

According to the observational study which was performed as part of MANUSCRIPT II in the present thesis, it may occur that some of the meatballs do not receive the final heat treatment in the oven. When the number of meatballs was higher than the capacity of the oven, it was observed that the heat treatment of the excessive meatballs in the oven in practice was not performed. Therefore, a scenario where the step of heat inactivation in oven was not applied to any of the served meatballs was tested. In such a situation, about 69 cases of salmonellosis could occur which primarily was a result of an ineffective heat inactivation by applying temperatures lower than the necessary, which allowed survival of *Salmonella* at dangerous levels. Effect of heat injured *Salmonella* was not considered in this QMRA model due to need of data and models specifically developed to address this aspect, what could be a detail to be explored and added to the QMRA, and consequently reduce the level of uncertainty of the effect of heat inactivation.

Since pork meatballs is consumed not only as component in hot meals, but also as filling in sandwiches, scenarios looking at growth potential of *Salmonella* during cooling of the meatballs (not subjected to heat treatment in oven) and until reaching 6°C at refrigerated storage, were investigated (MANUSCRIPT II). The

effect of kitchen temperatures (20 – 30°C) on growth of *Salmonella* were tested applying a model based on Newton's law of cooling (Burmeister, 1993) for simulation of the temperature profile of meatballs. The temperature reached in the meatballs during pan frying was between 18.2 and 66.8°C but had no impact on growth of *Salmonella*. However, the kitchen temperature (RTK) combined with the sum of holding time (time necessary to fry all the batches of meatballs = 0 – 90 min), serving time (90 min), time necessary for packing and placing in refrigerator to storage (30 min), and time during cold storage necessary for the meatballs to reach 6°C (3.3 – 3.5 h), was demonstrated to have an important effect on the growth of *Salmonella* and consequently on the risk estimates. In case of cooling (RTK = 30°C) and refrigerated storage (6°C) of those meatballs not heat treated in oven, about 16.299 people could become ill with salmonellosis by consumption of pork meatballs by the catering sector per year in Denmark. When the kitchen temperature was below 24°C, no growth of *Salmonella* could be predicted, independent on holding times up to 90 min. However, at temperatures higher than 29°C, growth of *Salmonella* was predicted in all possible combinations of time during cooling of fried meatballs not heated in oven (MANUSCRIPT II).

By applying the developed QMRA model (MANUSCRIPT II), it was found that the temperatures during heating in oven and in the kitchen (RTK) during cooling of meatballs are critical parameters that need to be controlled in order to assure the safety of the product. Sensitivity analyses, testing different alternative scenarios regarding different aspects related to *Salmonella* in meatballs processing: prevalence, concentration, lag time, D-values, and concentration after frying, show the high impact of concentration and prevalence of *Salmonella* in the raw pork before the process, what justify the global efforts to keep the levels of *Salmonella* as low as possible (Wegener et al., 2003).

In order to build up the discussed QMRA model, there was a need for data and models specifically focused on *Salmonella* in pork processing following the catering sector conditions. Therefore, two of those models were developed in this thesis. The first model that is going to be discussed below is the tool developed to describe the transfer of *Salmonella* during grinding of pork.

As pointed out by Flores and Tamplin (2002), epidemiological studies of foodborne illnesses caused by *Escherichia coli* O157:H7 and *Salmonella* spp. show that many of these illnesses are associated with contaminated ground beef (Banatvala et al., 1996; Roels et al., 1997). In two of these studies, the meat grinder was implicated in the transfer of these pathogens to ground beef. Banatvala et al. (1996) traced an *E. coli* O157:H7 infection outbreak in the Bethel area (Connecticut, USA, 1994) to supermarket grinders and other cutting utensils. Roels et al. (1997) indicated that inadequate cleaning and sanitation of the meat grinder in a butcher's shop was associated with an outbreak illness caused by *Salmonella* Typhimurium in which 158 persons were infected in Wisconsin (USA) during the 1994 Christmas holiday period.

Considering that grinding is a very important step for the spread of *Salmonella* from contaminated pieces to non-contaminated portions of pork, a study has been developed in this thesis in order to model the transfer of *Salmonella* during grinding of pork, according to the conditions mimicking those observed at the catering sector.

Looking at the results obtained with the modelling of cross contamination (PAPER I), and considering the low

RMSE and AICc-values supported by the F-tests results, the developed model satisfactorily predicted the observed behaviour of *Salmonella* during its cross contamination in the grinding of up to 110 pork slices corresponding to 21 kg meat. During the past decade a number of studies investigating the transfer of pathogens during slicing of ready-to-eat products (Vorst et al., 2006; Aarnisalo et al., 2007; Sheen, 2008; Sheen and Hwang, 2010) have been published. However, no other studies have yet modelled the transfer of *Salmonella* during a grinding process and only in few other studies, a similar distinct tailing phenomenon during cross contamination was observed (Flores and Tamplin, 2002; Vorst et al., 2006; Aarnisalo et al., 2007; Sheen, 2008; Sheen and Hwang, 2010). In the study of Sheen and Hwang (2010), the cross contamination of *E. coli* O157:H7 during slicing of ready-to-eat ham was modelled applying an empirical approach and the selected model characterized the transfer as decreasing following exponential law. Comparison of the Sheen and Hwang (2010) model to the model developed in the present study showed that the two models are mathematically similar. Despite this similarity, the model proposed in the present thesis (PAPER I) should be preferred as it includes the pieces of meat that are contaminated before grinding and it gives clear explanations of all the parameters involved giving an overview of the dynamics of a grinding process, that are important aspects as pointed out by other research groups (Flores and Tamplin, 2002; Carrasco et al., 2012). As it is easier to understand, it also holds the potential to be universal, *i.e.* transferrable to cross contamination dynamics for other food processing steps. The fitted model obtained in this study is of course specific to the studied grinding process including the particular grinder applied. However, the structure of the model, and particularly its ability to predict the tailing phenomenon, seems relevant for different cross contamination processes. Testing the model structure on data published in other transfer studies, where different food products, microorganisms, concentration of pathogen and different routes of contamination (food product to slicer to food product or slicer to food product, using the same product or different products) were used, showed promising results (Figure 5, Chapter 3.1).

As indicated by the trial of low contaminated portions obtained when grinding low as well as high contaminated beef (Flores and Tamplin, 2002) or pork (PAPER I), the grinder is an important source to spreading of pathogens. This means that when contamination gets started it will last for a long period of processing and affect a high amount of previous pathogen free meat. In case of *Salmonella* in Danish pork, where the concentration is typically below 1.000 CFU/g (Anonymous, 2012, Pires, 2009, Hansen, 2010), this tail phenomena of low contaminated portions would result in a number of portions with level lower than the detection limit of the available techniques of analysis. Therefore, meat portions positive for *Salmonella* could be considered as *Salmonella* free portions. Non-compliance in subsequent stages of meat processing, like temperature abuse, could stimulate growth of *Salmonella* until levels that could compromise the safety of the meat.

As demonstrated in Table 2 (Chapter 3.1) a high amount of the *Salmonella* used as input to contaminate the grinder was not transferred to the meat but continued to be inside the grinder making the grinder itself a critical point. However, the *Salmonella* concentration found when using "Con-Tact-It" adhesive tape technique (Schaffner et al, 2004) was not as high as the actual level of *Salmonella* inside the grinder (Table 2, Chapter 3.1). Thus, the tape sampling technique was considered ineffective for this purpose and the

surface material of the grinder was highlighted as a possible explanation. It is recognized that investigation of the level of *Salmonella* inside the grinder need to be performed to get the whole picture of the dynamics of grinding, however, a sampling technique practical enough to be used throughout the grinding process which effectively represents the *Salmonella* counts in the grinder still need to be determined.

According to the observational study, cleaning and disinfection of the grinder are performed only once a day, after the end of processing. To small scale producers (20 kg meat/day), like the investigated food service units, the already applied cleaning and disinfection routines may be enough, however, large scale production sites might need to be controlled by more frequent cleaning and disinfection. According to Borch and Arinder (2002) slaughter is a difficult process from a hygienic point of view. HACCP and GMP must be focused on controlling the sources of contamination during slaughter, including meat inspection (Borch et al., 1996; Nesbakken, 2001). For most operations, it is only possible to reduce the extent of contamination during processing, i.e. improving the hygiene level. Improved cleaning/disinfection routines would limit the level of contamination in meat processing (Borch and Arinder, 2002).

In this thesis only cross contamination in the grinder was considered, however, other stages of the food processing at the catering sector could pose a risk of cross contamination. As pointed out by Carrasco et al. (2012), studies related to cross contamination and recontamination by *Salmonella* in foods have been performed on cutting boards (Schaffner, 2004; Jiménez et al., 2009), stainless steel surfaces (Kusumaningrum et al., 2002) and kitchen surfaces (Mattick et al., 2003), and they have shown its relevance on the impact of the risks generated by transfer of pathogens, usually when Good Kitchen Practices were not followed as intended. Therefore, to include the effect of cross contamination in other relevant stages of the food preparation could be important for providing tools to more accurate risk assessment investigations. The proposed QMRA model (MANUSCRIPT II) could certainly be improved by covering cross contamination in other relevant stages of processing i.e. cutting of meat. The approach developed by Gomes-Neves et al. (2002) could be an alternative for this purpose, since *Salmonella* cross-contamination in swine abattoirs was investigated and it was demonstrated that besides a high level of *Salmonella* swine contamination at pre-harvest level, slaughtering, dressing, cutting and deboning operations are contributing to the occurrence of *Salmonella* in pork products.

The good bias and accuracy factors, with values close to one, obtained when validating the suggested transfer of *Salmonella* (Møller et al., 2012 - PAPER I), indicate that the hypothesis of two matrices being responsible for the transfer could in fact be the explanation for the observed tailing. Nevertheless, it has yet to be elucidated what the two suggested environmental matrices consist of and how the transfer takes place at the physical level. Furthermore, as *Salmonella* levels as high as 7 to 9 log₁₀CFU per slice were used for developing the model, it is not known whether tailing could be an artefact of high concentrations. In fact, influence of inoculum size seems to be one of the most controversial aspects to be investigated, because despite the fact that Montville and Schaffner (2003) have not observed differences in cross contamination between surfaces related to varying inoculum sizes of *Salmonella*, Flores and Tamplin (2002) observed changes in the transfer patterns of *E. coli* O157:H7 during beef grinding when applying different inoculation levels. In addition, Sheen and Hwang (2010) found the need for different model structures when applying

different inoculation level of *E. coli* O157:H7 in slicing of ham.

After the pork has been contaminated in the grinder, the conditions applied to the subsequent steps of processing need to be controlled, otherwise survival and growth of *Salmonella* may occur and the safety of the meat product can be questionable (MANUSCRIPT II). The second model specifically developed to investigate *Salmonella* in pork processing according the catering sector conditions, and used to build up the already discussed QMRA model (MANUSCRIPT II), was the model describing the effect of natural microbiota on growth of *Salmonella* during storage of ground pork at different temperatures (MANUSCRIPT I) and it is going to be discussed in the following section.

Until today the effect of pork microbiota on growth of *Salmonella* still remained to be investigated, therefore, studies were performed in the present thesis (MANUSCRIPT I) in order to model and predict growth of *Salmonella*, growth of the dominating natural microbiota and their interaction in ground raw pork stored at temperatures between 4°C and 38°C.

Primary and secondary *Salmonella* growth models were developed based on experimental data generated from inoculation of sterile ground pork. The models developed for *Salmonella* spp. predicted growth that quantitatively corresponded to independent product studies with pork and model parameters were similar to previously developed models (Table 1 and Table 3 in MANUSCRIPT I). No models were found for the effect of storage temperature on growth of the natural microbiota in ground pork. Growth models have previously been published on pseudomonades, *Brochothrix thermosphacta*, lactic acid bacteria and *Enterobacteriaceae* in pork (Koutsoumanis et al., 2006; Koutsoumanis et al., 2008) but not for the total psychrotrophic microbiota. Therefore, a model based on available data from the literature was developed (Table 2 and Table 3 in MANUSCRIPT I).

In order to investigate the effect of pork natural microbiota on growth of *Salmonella*, the existing Jameson-effect and Lotka-Volterra species interaction models and a new expanded Jameson-effect model were evaluated. F-tests indicated lack-of-fit for the classical Jameson-effect model at most of the tested temperatures and at 14.1 to 20.2°C this was caused by continued growth of *Salmonella* after the natural microbiota had reached their max. population density (MPD). The inhibiting effect of high levels of the natural pork microbiota on growth of *Salmonella* was temperature dependent, *i.e.* below 15°C and above 20°C, growth of *Salmonella* stopped when the natural microbiota reached its MPD, whereas between 15 and 20°C, growth of *Salmonella* continued after the natural microbiota had reached its MPD. This effect was described well by the new expanded Jameson-effect model and the classical Lotka-Volterra model. Both models showed generally good fit at temperatures from 15.1 to 20.2°C but otherwise lack-of-fit was observed (Table 4 in MANUSCRIPT I). One reason for the lack-of-fit of these interaction models was speculated to be a result of the strongly biased lag time model for the natural pork microbiota (Figure 2 in MANUSCRIPT I). In 10 out of 17 challenge tests in the present study, the natural microbiota was observed to have no lag phase. Therefore, fittings and simulations were repeated for the Lotka-Volterra and expanded Jameson-effect models assuming a lag time of zero for the natural pork microbiota. As indicated by lower F-values, improvements were observed at 6 and 5 storage temperatures for Lotka-Volterra and expanded Jameson-

effect model, respectively (results not shown). These results underlined the inherent difficulty in predicting microbial interactions as in the case of *Salmonella* spp. in fresh pork, where two models might be needed; one including lag time for the natural microbiota suited for the situation where the contamination with *Salmonella* spp. occurs during the slaughter process, and another excluding the lag time for the natural microbiota suited for the situation where the contamination with *Salmonella* spp. occurs when the natural microbiota already has started to grow, e.g. at the retail.

Another possible source for the erroneous predictions of *Salmonella* spp. could be the *Salmonella* growth model itself. As indicated by the accuracy factors, discrepancies of predictions of *Salmonella* lag times and growth rates were observed when compared to literature (Alford and Palumbo, 1969; Ingham et al., 2007; Mann et al., 2004.). Alternatively, a model based on the growth of *Salmonella* spp. in pork results presented in this thesis and literature pork growth data could be used. In this case, the parameter estimates of 0.0384, 2.582°C, 2.93 and 8.45 log₁₀CFU/g (S.D.: 0.74) for b , T_{\min} , RLT and N_{\max} , respectively, could be used. If a model representing a broader variety of meats, e.g. including cooked or lightly cured products, is wanted, the *Salmonella* μ_{\max} model published by Dominguez and Schaffner (2008) would be the best choice.

Previously, the simple Jameson-effect model or its modification suggested by Le Marc et al. (2009) have been used to predict growth of microorganisms in food at different storage temperatures (Le Marc et al., 2009; Mejlholm and Dalgaard, 2007; Vermeulen et al., 2011). In those studies, however, the effect of microbial interaction on growth patterns was independent of the studied storage temperatures. It is believed that the developed interaction models for ground pork will be valuable for future exposure and risk assessments to predict concentrations of *Salmonella* and, thereby, contribute to evaluation of the risk of *Salmonella* infections. Despite the good performance demonstrated, the suggested interaction model could be further improved by investigation of some issues, e.g. other inoculation levels rather than that applied in this study (ca. 10⁴ CFU/g), and lag time.

The obtained relative lag time (RLT)-value of 3.10 (2.50 – 3.70) found in this study (MANUSCRIPT I) corresponded to the average value of 3.1 reported by Velugoti et al. (2011) for growth of *Salmonella* spp. in ground pork and it did not differ from the average value of 2.5 reported by Juneja et al. (2007) for growth in chicken. However, as indicated by the sensitivity analysis, this variation adds uncertainty to the QMRA developed in this thesis using the growth model of *Salmonella* here investigated (MANUSCRIPT II). Lag phase has been considered challenging because based on the observed variability of single-cell lag, and considering that the limit of growth for a microbial cell can be identified to a certain level of an environmental factor where lag becomes infinite, variability of the growth limits of individual cells would also be expected (Aguirre and Koutsoumanis, 2011). Baranyi et al. (1995) have applied an approach incorporating the lag-phase duration (LPD) and growth rate (GR) in a single mathematical function to predict growth of *Brochothrix thermosphacta* in a laboratory medium during nonisothermal conditions. Zwietering et al. (1994) developed separate predictions for LPD and GR of *Lactobacillus plantarum* in a laboratory medium and concluded that exposing lag-phase cells to a shift in temperature resulted in a 25 % increase in LPD at the new temperature beyond the expected remaining proportion of lag phase. These authors did propose that ignoring these adaptation-related delays was a convenient approach to prediction. However, as pointed out by Ingham et al.

(2009), ignoring the adaptation times would be expected to increase the likelihood of a fail-safe prediction, i.e., it would more likely result in over-prediction of growth when the temperature increased over time – such as when temperature control is lost in raw meat processing. In the model proposed in this thesis (MANUSCRIPT I), *RLT* averaged 3.10 was adopted, however, in cases when the *RLT* is shorter than that value, the proposed model may underestimate the growth of *Salmonella* and the reciprocal might also happen. Better understanding of factors affecting the lag time duration of growth of *Salmonella* in pork would generate more accurate predictions and improve risk estimates of salmonellosis in pork processing.

Strain variation could be another issue, since the growth kinetic parameters among *Salmonella* could differ according the strain, as pointed out by Lianou and Koutsoumanis (2009), and by Pin et al. (2011). However, when comparing the growth curves obtained from two of the three investigated *Salmonella* strains in this study (*S. Typhimurium* DT104 and *S. Derby*) by visual evaluation it was not possible to observe differences between the behavior of the strains neither in sterile ground pork nor in ground pork with natural microbiota (Figure 3, Chapter 2.4.1)

Some applications of the proposed species interaction model, modelling the effect of pork natural microbiota on growth of *Salmonella*, have been considered. The main objective would be to find alternatives to solve some aspects that still continue to be challenging within food safety and food quality. The first suggested application of the proposed species interaction model is to apply the model as an alternative for quantifying levels of *Salmonella* under detection limit in minced pork after cold-enrichment (NOTE I). A model able to recover *Salmonella*, at levels lower than the available detection methods, is widely demanded (Krämer et al., 2011; Lam et al., 2011). A good example for the application of the suggested recovery model would be the studies related to transfer of *Salmonella* during grinding or slicing, which so far only were possible to perform with inoculation levels of 3 log₁₀CFU/portion or slice, and above. However, as already mentioned the concentration of *Salmonella* in contaminated pork is usually lower than 3 log₁₀CFU/g, and therefore, the proposed recovery model seems to be a promising alternative as pointed out by the presented results (Figure 10 in Chapter 4.2 and Figure 3 in Note I). When the proposed approach was applied to recover the level of *Salmonella* transferred from five inoculated pieces (6.18 – 6.44 log₁₀CFU/g) to 20 uninoculated pieces during grinding of pork (Figure 3 in Note I), the predictions obtained with the proposed recovery model (□) indicated a slight overestimation of some data points (0.03 - 0.78 log CFU/g) when compared to the observed *S. Typhimurium* DT 104 counts obtained immediately after grinding and prior to enrichment. Since a deviation of 0.5 log₁₀-units correspond to an expected uncertainty inherent to the plate count method, only four of the 18 observed data points were considered slight overestimated (0.64 – 0.78 log CFU/g). Otherwise the proposed recovery model seemed to give a good description of the pattern of contaminated portions. This good description was also obtained when applying and evaluating the proposed approach to describe the level of *Salmonella* transferred during grinding of pork in another data set, where three inoculated pieces (3.96 – 4.16 log₁₀CFU/g) were used to contaminate the 15 uninoculated pieces subsequently processed (Figure 10 in Chapter 4.2). However, supplementary studies, testing levels of contamination of *Salmonella* even lower, still need to be performed in order to confirm the effectiveness of the approach and improve the accuracy of the predictions.

Another suggested application of the proposed interaction model, that describes the effect of pork natural microbiota on growth of *Salmonella*, is to use the model as an alternative to face safety against quality in fresh minced pork by quantifying the potential for *Salmonella* growth within the shelf-life period (NOTE II). Food quality is obviously an important issue for food producing companies, including catering units. Food quality can be quantified by modelling the temperature dependence of chemical reactions, enzymatic reactions, physical reactions as well as microbiological changes that may affect the product's sensory characteristics (van Boekel, 2008). To model food quality taking into consideration safety issues as well is a challenging task as described by Chen et al. (2011), when investigating the application of linear/non-linear classification algorithms in discrimination of pork storage time. According to their study, on one side, the meat industry needs rapid analytical methods or tools to determine and select suitable processing procedures for their raw material and predict the remaining shelf life of their products. On the other side, inspection authorities need reliable methods for control purposes, while the wholesale and retail sectors need these valid methods to ensure the freshness and safety of their products and to resolve potential disputes between buyers and sellers. Therefore, rapid identification of pork storage time associated with pork safety and spoilage, and development and application of practical means such as analytical methods or devices and robust model systems are of great importance in assuring consumers that food safety and quality are ensured. Balasubramanian et al. (2012) have investigated different gas sensor-based artificial olfactory systems for screening *Salmonella* Typhimurium contamination in beef, and they pointed out that with complex food systems like fresh beef and in food safety applications where miscalculation rates could result in serious health risks to the consumer, sensor integration approach could result in increased efficiency of food quality and safety attributes prediction.

In this thesis, an example of combining food quality and food safety through the application of predictive models, where performed for fresh minced pork (NOTE II). Combining the developed growth model of *Salmonella* considering the interaction with the pork natural microbiota (MANUSCRIPT I) with sensory observations indicating the end of ground pork shelf-life during storage (odour, colour intensity and pH), makes it possible to evaluate the growth potential of *Salmonella* within the determined shelf-life. The result of doing this indicated that safety, rather than quality, could be the shelf-life limiting factor of fresh pork meat at abusive storage conditions such as 10 to 15°C (Figure 11 in Chapter 4.3 and Figure 1 in NOTE II). The growth potential of *Salmonella* was most pronounced in the lower temperature range from 10 to 12°C where an increase of *approx.* 1 log₁₀CFU/g could take place before the meat was assessed unacceptable for use. It is relevant to consider that aspects like strain variation and lag time, already discussed on the proposed species interaction model (MANUSCRIPT I), could also represent issues in the accuracy of this suggested approach related to modelling the combined effect of food quality and food safety. If lag time of *Salmonella* would be lower than predicted by the obtained *RLT*-value of 3.10 (2.50 – 3.70), then the safety of the pork would be compromised in a previous stage than the one being evaluated (NOTE II). On the other hand, if the lag time of *Salmonella* during storage of the investigated ground pork would be higher than that used to built up the proposed model, then the safety of the meat would be compromised at a later stage, what would extend the predicted shelf life (considering safety and quality attributes) obtained by applying the proposed

approach (NOTE II).

Chapter 11

Conclusions and future perspectives

Conclusions and future perspectives

In addition to observational studies, models specifically developed studying transfer and growth of *Salmonella* in pork (PAPER I and MANUSCRIPT I), and data related to *Salmonella* spp. in different meat matrices were used to build up a process chain QMRA model (MANUSCRIPT II). The combination of data from these three different sources resulted in a new approach that may improve the quality of estimates in risk assessments related to *Salmonella* in pork processed at the catering sector. However, it is necessary to be aware of some limitations related to the developed models, and therefore, that further investigations are needed in order to elucidate some aspects that will improve the modelling of *Salmonella* behaviour at during pork processing like grinding and storage. Consequently, the accuracy of the risk estimates related to salmonellosis by consumption of pork meatballs produced according to the Danish catering sector will increase.

In this thesis, considerations and suggestions for further improvement, related to the three main developed models, are made:

- The transfer of *Salmonella* during grinding of pork was successfully modelled in processing of up to 110 pork slices corresponding to 21 kg meat (PAPER I). This model includes the pieces of meat that are contaminated before grinding and it gives clear explanations of all the parameters involved giving an overview of the dynamics of a grinding process. The structure of the model, and particularly its ability to predict the tailing phenomenon, seems relevant for different cross contamination processes. However, different matrices, pathogens, inoculum levels and types of processing still remain to be investigated. Despite the good description of transfer of *Salmonella* during pork grinding related to the meat, the dynamics inside the grinder is not fully understood. A high level of the input of *Salmonella* (approx. 70 %) was not detected due to an inefficient enumeration technique and, as a consequence, there is a need for determining an applicable sampling method.
- Effect of pork natural microbiota on growth of *Salmonella* was modelled and predicted during storage of ground pork at temperatures between 4°C and 38°C (MANUSCRIPT I). Continued growth of *Salmonella* after the natural microbiota had reached their max. population density was observed. This effect was described well by the complex Lotka-Volterra species interaction model as well as a new expanded Jameson-effect model but not by the classical Jameson-effect model. As the new expanded Jameson-effect model is more simple and practical to use, it may be preferred to the Lotka-Volterra model. However, the performance of these two models was temperature dependent, presenting good results at temperatures from 15.1 to 20.2 °C. Part of the reason for lack-of-fit at lower temperatures was speculated to be poor accuracy of the lag time models. Therefore, investigations related to the lag time of the pork microbiota and *Salmonella* should be performed in order to improve the model. Also the influence of different inoculum sizes needs to be looked further into.
- Applying the proposed QMRA model (MANUSCRIPT II), risk estimates of salmonellosis from meatballs processed by the catering sector per year in Denmark were obtained and considered reasonable, when compared to the epidemiological data from 2011 for *Salmonella* cases in Denmark

related to pork. Together with the CODEX guidelines, the modular process risk model approach was the base of the suggested QMRA. This flexible structure allows scenario analysis, and consequently the application of the QMRA model from outbreak investigations to product development and other food safety evaluation activities, e.g. efficiency of guidelines, etc. However, the module related to heat inactivation should be adjusted to the specific heating profile obtained in the new situation that is investigated. A better solution would be to develop a model specifically to the conditions applied to pork meatballs at the catering sector, since D-values found in different kinds of meat were used to build up the current version of the model, and heat injured *Salmonella* cells were not considered. Improvement of the two previously developed models would generate better risk estimates with a new version of the suggested QMRA model.

In addition to the investigation of the behaviour of *Salmonella* during pork meatballs processing according to conditions found at the Danish catering sector, and the development of models able to describe this behaviour, some applications of the proposed species interaction model are suggested:

- Application of the model as an alternative for quantifying levels of *Salmonella* under detection limit in minced pork after cold-enrichment (NOTE I). According to preliminary experiments using this model to recover and predict low levels of *Salmonella*, it seemed to give a good description of the observed values, when testing different inoculation levels (3.96 – 6.44 log₁₀ CFU/g). However, supplementary studies, testing levels of contamination of *Salmonella* even lower, still need to be performed in order to confirm the effectiveness of the approach and improve the accuracy of the predictions.
- The use of the model as an alternative to face safety against quality in fresh minced pork, by quantifying the potential for *Salmonella* growth within the shelf-life period (NOTE II), also seems to be promising. Doing this indicated that safety, rather than quality, could be the shelf-life limiting factor of fresh pork at abusive storage conditions such as 10 to 15°C. It is important to mention, however, that aspects like strain variation and lag time, already mentioned in MANUSCRIPT I, could also represent issues in the accuracy of this suggested approach, and therefore, it need to be better investigated.

Chapter 12

Reference List

Reference list

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