# Quantitative risk assessment model of human salmonellosis linked to the consumption of ground beef

Abdunaser Dayhum

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# THÈSE

pour obtenir le grade de

**Docteur**

de

**l'Institut des Sciences et Industries du Vivant et de l'Environnement (Agro Paris Tech)**

Spécialité : Èpidémiologie

*Présentée et soutenue publiquement*
*par*

**Abdunaser DAYHUM**

**le 02 Juin 2008**

# Appréciation du risque de cotamination de l'homme par des *Salmonella* spp. a partir de produit d'origine bovine : viande hachée

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This thesis « *Quantitative risk assessment model of human salmonellosis linked to the consumption of ground beef*» was completed at the UEAR laboratory ("*Unité d'Epidémiologie et d'Analyse des Risques"*) at the Ecole Nationale Vétérinaire d'Alfort in collaboration with the MASQ laboratory (*"Unité Microbiologie des Aliments, Sécurité et Qualité"*). The work was supported financially in part by the Libyan Ministry of Higher Education, Tripoli, Libya, and in part by the "Unité d'Epidémiologie et d'Analyse des Risques (UEAR)"

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Quantitative risk assessment model of human salmonellosis linked to the consumption of ground beef.

**(ABSTRACT)**

Salmonellae are one of the most important causes of foodborne illness associated with raw dairy products. The assessment of the real risk associated with the consumption of these products is needed and the most appropriate method to achieve this goal is the risk analysis process which links pathogens in food to the public health problem. The main aim of this thesis is to quantitatively assess the risk of human salmonellosis linked to the consumption of ground beef. A data gap that is routinely identified in risk assessment is the lack of quantitative data on pathogens contaminated food. Therefore, as a first objective of this thesis, we developed a rapid, sensitive and reliable method for the quantification of *Salmonella* in artificially contaminated bovine fecal samples. The method combines the principles of most-probable-number (MPN) method with a real-time PCR assay. With this developed assay (MPN-real-time PCR) low levels of *Salmonella* (1-5 CFU/mL) in fecal could be enumerated after 8 h of non-selective enrichment in buffered peptone water. All estimated MPN counts corresponded well to the estimated contamination level of *Salmonella* inoculated into fecal samples. In order to evaluate the utility of this developed quantification assay, our second objective was to apply it to naturally contaminated fecal samples collected from slaughterhouse located in Meaux, France weekly in February and March 2006 (an average of 40 samples per visit). 9.12% (27/296), and 34.62% (9/26) fecal and environmental samples, respectively, were found *Salmonella*-positive, with estimated MPN values or counts of *Salmonella* ranging from <1.8 - 1609 MPN/g of fecal samples. The mean of the log10 concentration of *Salmonella* is 0.6189 MPN/g with standrdard deviations of 2.7112 by using the censored regression approach. Counts were generally low, with the exception of 6 animals (>1400 MPN/g), while all the other 21 *Salmonella* positive animals had faeces with less than

80 MPN/g (from them 13 animals with MPN values <1.8 MPN/g. The prevalence of Salmonella showed no significant difference (p=1) between French (8.63%, 17/197) and Belgian cattle (10%, 10/99). Furthermore, neither the animals' area of origin (p=0.75), age (p=0.18), race (p=0.94), breed (p=0.23), or movement of the animal (p=0.89) had any impact on the prevalence of *Salmonella*. The application of the MPN-real-time PCR assay for quantifying *Salmonella* in fecal proved to be rapid, easy-to-perform and highly sensitive. In the assessment of potential risks associated with *Salmonella* in ground beef it was necessary to examine the ability of *Salmonella* to grow in ground beef. Therefore, we presented in this thesis as a third objective, model of a growth / no growth interface of *Salmonella* in ground beef. A growth/no growth data were modeled by logistic polynomial regression on the available growth data for *Salmonella* in ground beef in published papers and all the data related to ground beef in ComBase in order to lead us to an accurate description of the conditions that *Salmonella* can growth / no growth. The overall results indicate clearly that the temperature is the most important and the only factor significant in the study. There was no growth observed at temperature less than 10°C. Where as the temperature 10°C and 12°C are the only temperature; we did observe growth / no growth (sometimes with the same conditions). Even though pH and water activity are important factors for microbial growth, in our study they have no effect due to meat structure. Finally, a quantitative risk assessment of human salmonellosis linked to the consumption of ground beef is presented. Different distributions were assumed for the parameters of the model and a Monte Carlo simulation was used to model the process and to quantify the risk associated with the consumption of 100 g serving of ground beef patty. The expected percentage of ground beef patties with contamination greater than 5, 10 and 100 *Salmonella* cells were 29%, 17.1% and 0.02%, respectively at the time of consumption. The risk of salmonellosis per 100 g serving ranged from 0 to 2.33E-06 dependent on the type of cooking and the fat content. For 10 million

servings of 100g, the expected number of cases of salmonellosis predicted by the model is in average of 11.04, 12.33 for fat content 7% and 24% respectively. The risk of salmonellosis was closed to zero when the 100 g serving ground beef patties consumed well done. The relative risk of getting salmonellosis from consumed the rare ground beef patties is 312, 61 times higher for fat content 7% and 24% respectively comparing to the consumption of well done patties. There are 35 batches with cases out of 2000 batches (1.8%). 15 of them have 2 cases or more (0.75%, 15/2000). Despite the limitations and the data gap, we demonstrated the benefit of risk assessment not only as a risk evaluation tool but also as a helping device in the decision-making and the risk management.

Appréciation du risque de contamination de l'homme par des *Salmonella* spp.
a partir de produit d'origine bovine : viande hachée

## (RESUME)

Les salmonelles sont l'une des causes les plus importantes de maladie transmise par les produits laitiers crus. L'appréciation du risque associé à la consommation de ces produits est nécessaire et la méthode la plus appropriée pour réaliser ce but est l'utilisation du processus d'analyse de risque qui associe les microbes pathogènes dans l'aliment au problème de santé publique. Le but principal de cette thèse est donc d'évaluer quantitativement le risque de salmonellose humaine lié à la consommation de viande hachée. Les lacunes qui sont en général identifiées pour l'appréciation des risques sont le manque de données quantitatives sur les microbes pathogènes contaminant les aliments. Donc, comme premier objectif de cette thèse, nous avons développée une méthode rapide, sensible et fiable pour la quantification des salmonelles dans les échantillons fécaux bovins artificiellement contaminés. La méthode a combiné les principes de la méthode du nombre-le plus-probable (NPP) avec une analyse PCR en temps réel. Avec cette analyse (NPP-PCR en temps réel) fiable niveau de la contamination (1-5 ufc/mL) du fécal peut être énuméré après 8 h d'enrichissement non-sélectif dans l'eau peptone tamponée. Toutes les valeurs de nombre le plus probable ont bien correspondu au niveau estimé de contamination des salmonelles inoculées dans les échantillons fécaux. Afin d'évaluer l'utilité de cette analyse de quantification, notre deuxième objectif était de l'appliquer aux échantillons fécaux naturellement contaminés recueillis de l'abattoir trouvé dans Meaux, la France chaque semaine en février et le mars de 2006 (une moyenne de 40 échantillons par visite). 9.12 % (27/296) et 34.62 % (9/26) les échantillons fécaux et de l'environnement, respectivement, ont été trouvés positif de *salmonella*, avec les valeurs de NPP estimées ou les comptes de Salmonella aux limites de <1.8 - 1609 MPN/g d'échantillons fécaux. The moyen de la concentration log10 de *Salmonella* est 0.6189 MPN/g avec les déviations standrdard de 2.7112 en utilisant l'approche de rétrogradation censurée.

Les comptes étaient généralement bas, à l'exception de 6 animaux (> 1400 MPN/g), pendant que tous les autres 21 *Salmonella* les animaux positifs avait des matières fécales avec moins de 80 MPN/g (d'eux 13 animaux avec les valeurs de MPN <1.8 MPN/g. La prédominance de *Salmonella* n'a montré aucune différence significative (p=1) entre le français (8.63 %, 17/197) et bétail belge (10 %, 10/99). En outre, aucun la région des animaux d'origine (p=0.75), l'âge (p=0.18), la course (p=0.94), la race (p=0.23), ou le mouvement de l'animal (p=0.89) n'avait aucun impact sur la prédominance de *Salmonella*. L'application de l'essai de PCR NPP-en-temps-réel pour quantifier la Salmonella dans fécal s'est avérée être rapide, facile à exécuter et extrêmement sensible. Dans l'appréciation de risques potentiels associés à la *Salmonella* dans la viande hachée il était nécessaire d'examiner la capacité des salmonelles à se développer dans la viande hachée._Donc, nous avons présenté dans cette thèse comme un troisième objectif, le modèle d'une croissance / aucune interface de croissance de *Salmonella* dans la viande hachée Des données de croissance / non croissance ont été modelées par la rétrogradation de polynôme logistique sur les données de croissance disponibles pour la *Salmonella* dans la viande hachée dans les papiers publiés et toutes les données rattachées a la viande hachée dans ComBase nous mènent afin d'à une description exacte des conditions que la *Salmonella* ne peut la croissance / aucune croissance. Les résultats généraux indiquent clairement que la température est la plus importante et le seul facteur significatif dans l'étude. Il n'y avait aucune croissance observée à la température moins que 10°C. Où comme la température 10°C et 12°C sont la seule température; nous n'avons vraiment observé la croissance / aucune croissance (quelquefois avec les mêmes conditions). Bien que pH et l'activité d'eau soient des facteurs importants pour la croissance microbienne, dans notre étude ils n'ont aucun effet en raison de la structure de viande. Finalement, un modèle d’appréciation du risque de salmonellose humaine liée à la consommation viande haché est présentée qui est basée sur les résultats des objectifs précédemment mentionnés dans cette thèse. Différentes

distributions ont été posées en hypothèse pour des paramètres du modèle et une simulation de Monte Carlo a été employée pour modeler le processus et pour mesurer le risque lié à la consommation de la portion de 100 g de bœuf haché. Le pourcentage attendu de portions de bœuf haché avec la contamination plus grande que 5, 10 et 100 cellules de *Salmonella* était 29 %, 17,1 % et 0,02 %, respectivement au moment de la consommation. Le risque de salmonellose par portion de 100g varie de 0 à 2,33 x $10^{-6}$ en fonction au type de cuisson et de la teneur en matière grasse. Pour 10 millions de portions de 100 g, le nombre attendu de cas de salmonellose prévu par le modèle est en moyenne 11, 12 pour les teneurs en matière grasse 7 % et 24. Le risque de salmonellose par portion de 100g varie de 0 à 2,33 x $10^{-6}$ en fonction au type de cuisson et de la teneur en matière grasse. Le risque de salmonellose est proche de zéro quand le bœuf haché est consommé bien cuit. Le risque relatif de salmonellose avec le bœuf haché saignant est 312 ou 61 fois plus élevé pour les teneurs 7 % et 24 % en matière grasse par comparaison avec la viande hachée bien cuit. Il y a 35 lots sur 2000 qui provoquent des cas (1,8 %). Quinze d'entre eux causent 2 cas ou plus (0,75 %, 15/2000).

# DEDICATION

This thesis is dedicated to my family without them this document, and the study and exploration it represents, would have been utterly impossible:

My parent who passed away before starting my thesis, may god bless them , for their instilling in me high values and a great work ethic.

My wonderful wife and best friend, Halima, for prayers, unending love, unwavering support, indulgence and encouagemen; my sweet angels, Lina and Lobna, for making my all days wonderful when I see their pretty face smiling so sweetly.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFFSA | Agence Française de Sécurité Sanitaire des Aliments |
| AOC | Appellation d'Origine Contrôlée |
| BEH | Bulletin Epidémiologique Hebdomadaire |
| BHI | Brain Heart Infusion |
| CAC | Codex Alimentation Commission |
| CDC | Center for Disease Control and Prevention |
| CFU | Colony Forming Units |
| $C_T$ | Crossing Threshold Point |
| dsDNA | Double-stranded DNA |
| ELISA | Enzyme-Linked ImmunoSorbent Assay |
| EU | European Union |
| FAO | Food and Agriculture Organization |
| GDS | Groupe de Defense Sanitaire |
| GHP | Good Hygiene Practice |
| GMP | Good Manufacturing Practice |
| GSS | Global Salm-Surv |
| HACCP | Hazard Analysis and Critical Control Points |
| IAC | Internal Amplification Control |
| InVs | Institut de Veille Sanitaire |
| LVD | Laboratoire Vétérinaires Départementaux |
| MR | Marginal Risk |
| MPN | Most-Probable-Number |
| NRC | National Reference Centre |
| PCR | Polymerase Chain Reaction |
| PFGE | Pulse-Field Gel Electrophoresis |
| PMP | Pathogen Modelling Program |
| RV | Rappaport-Vassiliadis |
| TIACs | Toxi-Infections Alimentaires Collectives |
| $T_m$ | Melting Temperature |
| QRA | Quantitative Risk Assessment |
| WHO | World Health Organization |
| WTO | World Trade Organization |

# Chapter 1: Introduction

**Background / Problem Statement**

Foodborne disease is an important and growing public health and economic problem in many countries. Factors that have been attributed to the increased risk for foodborne illness include new feeding practices, changes in animal husbandry, changes in agronomic process, increase in international trade, changes in food technology, increase in susceptible populations, increase in travel and changes in lifestyle and consumer demands. Foodborne diseases not only significantly affect people's health and well-being, but they also have economic consequences for individuals, families, communities, businesses and countries. These diseases impose a substantial burden on health-care systems and markedly reduce economic productivity. Estimating direct as well as indirect costs of foodborne disease is difficult. However, an estimate in the US places the medical costs and productivity losses in a population of approximately 250 millions inhabitants in the range of US$6.6-37.1 billion (Butzby and Roberts, 1997). In the European Union, , the annual costs incurred by the health care system as a consequence of *Salmonella* infections alone were estimated to be around 3 billion euros (Anonymous, 2004).

Millions of people suffer from foodborne illness yearly. It is difficult to obtain accurate estimates of the incidence of microbiological foodborne disease. In developed countries, the percentage of people suffering from microbiological foodborne disease each year has been reported to be up to 30%, while the problem is likely to be even more widespread in developing countries (WHO, 2002). However, it has been estimated that each year foodborne disease causes approximately 76 million illnesses, 325,000 hospitalizations, 5,000 deaths in the USA and 2,366,000 cases, 21,138 hospitalizations, 718 deaths in England and Wales (Mead et al., 1999; Adak et el., 2002). In France, The number of foodborne

hospitalizations has been reported to be between (10,200-17,800 cases) per year (Vaillant et al., 2005).

*Salmonella* is one of the leading causes of foodborne illness in the United States and the European Union (EU) (Anonymous, 2007; Mead et al., 1999), with estimated incidences of 15.1 cases per 100,000 persons in the United States (Anonymous, 2002) and 38.2 cases per 100,000 persons in the EU (Anonymous, 2007). Most *Salmonella* infections do not require treatment and result in temporary gastroenteritis. In some cases, severe diarrhoea requires medical interventions such as intravenous fluid rehydration. In cases where the pathogen enters the bloodstream, i.e. septicaemia or bacteraemia, symptoms include high fever, malaise, pain in the thorax and abdomen, chill and anorexia. In some patients, long-term effects or sequelae may occur, such as arthritis, osteoarthritis, appendicitis, endocarditis or meningitis (Bell, 2002).

Raw poultry and meat products remain the principal source of *Salmonella* in many countries (Bansel et al., 2006). Meat is a highly perishable food product which, unless correctly stored, packaged and distributed, spoils quickly and becomes hazardous due to microbial growth / especially if the pathogens are present. Potential for microbial contamination is influenced by the condition of animals prior to slaughter, abattoir practices, extent of handling and subsequent storage conditions (Jackson et al., 1997). The increasing number and severity of food-poisoning outbreaks world-wide has increased public awareness about the safety of meat. The epidemiological literature and outbreak investigations still implicate ground beef in salmonellosis outbreaks in France (Haeghebaert et al., 2000a, Gilles 2000, Haeghebaert et al., 2000b). Therefore, the threat to human health linked to the ingestion of *Salmonella* spp. in ground beef should not be underestimated because all raw meat can

have some level of microbial contamination present and cannot be expected to be otherwise without further processing. Depending on the species and whether they are present, pathogens such as *Salmonella* spp. can grow and cause illness by the ingestion of bacterial cells. The presence of pathogens in food in low number is undesirable and is considered a major cause of gastrointestinal diseases world-wide (Buchanan and Whiting 1986).

The importance of meat products in human diet requires systems that ensure meat products safety. Although food safety plans such as good hygiene practice (GHP), good manufacturing practice (GMP), and implementing hazard analysis critical control points (HACCP) along the whole food chain have been established by both regulatory authorities and industry all over the world, the success of these approaches in decreasing the incidence of human salmonellosis has been minor because of improper use and/or incomplete implementation. It is important to focus our efforts towards the real risks in the population. The challenge is therefore to use a multidisciplinary approach to identify the best mitigation strategies along the food-chain to prevent foodborne disease, especially at the primary production level, and then implement appropriate prevention programs. The most appropriate method to achieve this goal is through the use of the risk assessment process which links pathogens in food to the public health problem.

The overall objective of risk assessment is to provide estimates on the probability of disease occurrence using a well structured approach based on four steps: hazard identification, exposure assessment, hazard characterization (dose-response), and risk characterization (Codex Alimentatrius, 1995). There is therefore a strong need to provide data on the frequency and level of *Salmonella* contamination in meat and meat products. A data gap that is routinely identified in risk assessment is the lack of quantitative information on the

contamination of food with pathogens (Coleman and Marks, 1999). Most data are qualitative rather than quantitative because enumeration of pathogens in food requires more labor and time than does determining pathogen incidence, especially since the advent of rapid detection methods (Tietjen and Fung, 1995). Although low numbers of *Salmonella* cells in fecal samples can be enumerated using the traditional most-probable-number (MPN) method, this method is labor and time-consuming. However, with the advent of molecular methods such as real-time polymerase chain reaction (PCR) it is now possible to develop enumeration methods that require only preenrichment, thus saving labor and time. One of the objectives of this thesis is therefore to develop a rapid and a sensitive method to enumerate *Salmonella* in fecal samples.

Predictive food microbiology is a promising and rapidly developing area of food microbiology, which has drawn significant scientific attention in recent years. The prediction of *Salmonella* growth in ground beef under environmental conditions such as temperature, pH, and water activity are needed to adequately describe the changing conditions associated with processing and storage of these products. One of the most important environmental factors that affect *Salmonella* growth in meat is temperature. Scientific data related to the effect of temperature on the growth of *Salmonella* in meat and ground beef are present in literature. Thus, to assess the risks associated with *Salmonella* in ground beef it is necessary to predict the growth of this bacterium in meat and meat products under different temperature profiles from literature review. Therefore, one of our objectives is to develop model of a growth / no growth interface of *Salmonella* in ground beef. The available growth data for *Salmonella* in ground beef in published papers and all the related data to ground beef in ComBase are used in order to lead to an accurate description of the conditions where *Salmonella* can grow or not.

In general, only relatively few papers dealing with quantitative risk assessment models (QRAM) for foodborne *Salmonella* have been published since the first suggested Codex definition was published in 1995 (FAO/WHO, 1995). For example, pasteurized liquid eggs (Whiting and Buchanan, 1997), cooked poultry (Whiting, 1997), whole chicken (Oscar, 1998, 2004), shell eggs and eggs products (FSIS, 1998; Whiting et al., 2000), eggs and broiler chickens (WHO/FAO, 2002), turkey cordon bleu (Bermrah et al., 2002) and pork products (Giovannini et al., 2004). However, risk assessment models linked to the consumption of ground beef have previously been developed for other foodborne pathogens such as *Escherichia coli* O157:H7 (Cassin et al., 1998; FSIS, 2001) but not for *Salmonella*. Therefore, the present work attempts to estimate the risk for public health from the consumption of ground beef contaminated by *Salmonella.*

# Chapter 1: Introduction (en français)

## Contexte

Les maladies infectieuses transmises par les aliments sont demeurées un problème de santé publique et un problème économique important dans beaucoup de pays au cours des deux dernières décennies. Les causes qui ont été attribuées au risque accru de maladies infectieuses transmises par les aliments incluent de nouvelles pratiques d'alimentation, des changements en production animale, des changements des procédés agronomiques, l'augmentation du commerce international, les changements en technologie alimentaire, l'augmentation des populations fragiles, l'augmentation des voyages et les changements de style de vie et des demandes du consommateur. Les maladies infectieuses transmises par les aliments affectent non seulement de manière significative la santé et le bien-être des personnes, mais elles ont également des conséquences économiques pour des individus, des familles, des communautés, des entreprises et des pays. Ces maladies imposent un fardeau substantiel aux systèmes de santé et réduisent nettement la productivité économique. L'estimation des coûts directs et indirects des maladies infectieuses transmises par les aliments est difficile. Cependant, une évaluation aux USA situe les coûts médicaux et les pertes de productivité dans une population d'environ 250 millions d'habitants dans une fourchette de 6,6 à 37,1 milliards de dollars US (Butzby et Roberts, 1997). Dans l'Union européenne, les coûts annuels encourus par le système de santé par suite des seules infections causées par les salmonelles sont estimés à 3 milliards d'euros (Anonyme, 2004).

Des millions de personnes souffrent de maladies infectieuses transmises par les aliments annuellement. Il est difficile d'obtenir des évaluations précises de l'incidence des maladies infectieuses transmises par les aliments. Dans les pays développés, on a rapporté que le pourcentage annuel des personnes souffrant de maladies infectieuses transmises par les aliments atteint 30%, alors que le problème est susceptible d'être bien plus répandu dans les

pays en voie de développement (WHO, 2002). Cependant, on a estimé que tous les ans les maladies infectieuses transmises par les aliments causent approximativement 76 millions de malades, 325.000 hospitalisations, et 5.000 décès aux Etats-Unis et 2.366.000 cas, 21.138 hospitalisations, 718 décès en Angleterre et au Pays de Galles (Mead et al., 1999; Adak et al., 2002). En France, on a rapporté que le nombre d'hospitalisations du fait des maladies infectieuses transmises par les aliments est compris entre 10.200 et 17.800 par an (Vaillant et al., 2005).

La salmonelle est l'une des causes principales des maladies infectieuses transmises par les aliments aux Etats-Unis et dans l'Union européenne (Anonyme, 2005; Mead et al., 1999), avec des incidences estimées à 15,1 cas par 100,000 personnes aux Etats-Unis (Anonyme, 2002) et à 38,2 cas par 100,000 personnes l'Union européenne (Anonyme, 2007). La plupart des infections causées par les salmonelles n'exigent pas de traitement et se limitent à une gastroentérite passagère. Dans certains cas, une diarrhée grave exige des interventions médicales telles que la réhydratation par voie intraveineuse. Dans les cas où le pathogène entre dans la circulation sanguine, c.-à-d. septicémie ou bactériémie, les symptômes incluent une fièvre élevée, un malaise, une douleur dans le thorax et l'abdomen, des frissons et une anorexie. Chez quelques patients, des effets à long terme ou des séquelles peuvent être observés tels que une arthrite, une ostéoarthrite, une appendicite, une endocardite ou une méningite (Bell, 2002).

Les produits crus et à base de volaille et de viande restent la source principale des salmonelles dans beaucoup de pays (Bansel et al., 2006). La viande est un produit alimentaire extrêmement périssable qui, à moins que correctement ne conservé, emballé et distribué, s’altère vite et devient le dangereus du fait de la croissance microbienne, surtout si des

pathogènes sont présents. Le potentiel de contamination microbienne dépend de la condition des animaux avant l'abattage, des pratiques d'abattage, des mesures prises pour la manipulation et l'entreposage ultérieurs (Jackson et al., 1997). Le nombre croissant et la sévérité des épidémies d'origine alimentaire dans le monde entier a accru la conscience que le public a des problèmes liés à l'innocuité de la viande. La littérature épidémiologique et les enquêtes sur les épidémies impliquent toujours la viande haché dans les épidémies de salmonellose en France (Haeghebaert et el., 2000a, Gilles, 2000, Haeghebaert et el., 2000b). Donc, la menace pour la santé humaine due à l'ingestion de *Salmonella* spp. dans la viande hachée ne devrait pas être sous-estimée parce que toute la viande crue peut avoir un niveau de contamination microbienne notable et il ne peut pas en être autrement sans traitement. Selon les espèces et s'ils sont présents, les pathogènes comme *Salmonella* spp. peuvent cultiver et provoquer la maladie par l'ingestion de cellules bactériennes. La présence de pathogènes dans les aliments en faible nombre est indésirable et est considérée comme une cause majeure de maladies gastrointestinales dans le monde entier (Buchanan et Whiting, 1986).

L'importance des produits carnés dans les régimes alimentaires de l'homme exige des systèmes qui garantissent la sécurité de ces produits. Bien que les plans de maîtrise sanitaire, les bonnes pratique d'hygiène (BPH), les bonnes pratiques de fabrication (BPF), et l'application des principes HACCP (analyse des dangers – points critiques pour leur maîtrise) le long de la chaîne alimentaire soient imposées par les autorités compétentes et l'industrie partout dans le monde, le succès de ces outils sur la réduction de l'incidence de la salmonellose humaine a été mineure en raison de leur utilisation imparfaite ou incomplète. Il est important de focaliser nos efforts sur les vrais risques dans la population. Le défi est donc d'employer une approche multidisciplinaire pour identifier les meilleures stratégies de réduction le long de la chaîne alimentaire pour réduire l'incidence des maladies infectieuses

transmises par les aliments, particulièrement au niveau de la production primaire, et de mettre en application des programmes appropriés de prévention. La méthode la plus appropriée pour réaliser ce but est l'utilisation du processus d'analyse du risque qui associe des microbes pathogènes des aliments au problème de santé publique.

L'objectif global de l'appréciation des risques est de fournir des estimations de la probabilité de survenue de la maladie en utilisant une approche structurée fondée sur quatre étapes: l'identification du danger, l'appréciation de l'exposition, l'appréciation des effets (dose-réponse), et l'estimation du risque (FAO/WHO, 1995). Il y a donc un besoin fort de rassembler plus de données sur la fréquence et le niveau de contamination de *Salmonella* dans la viande et les produits carnés. Les lacunes habituellement identifiées dans l'appréciation des risques sont le manque d'information quantitative sur la contamination de l'aliment par des pathogènes (Coleman et Marks, 1999). La plupart des données sont qualitatives plutôt que quantitatives parce que le dénombrement des pathogènes dans l'aliment demande plus de travail et de temps que la détermination de la prévalence des pathogènes, particulièrement depuis l'arrivée des méthodes de détection rapides (Tietjen et Fung, 1995). De faibles nombres de salmonelles dans échantillons fécaaux peuvent être estimés en utilisant la méthode classique du nombre le plus probable (NPP), mais cette méthode demande du travail et du temps. Cependant, avec l'arrivée des méthodes moléculaires telles que la réaction d'amplification en chaîne par polymérase en temps réel (PCR en temps réel) il est maintenant possible de développer les méthodes de dénombrement qui nécessitent seulement un pré-enrichissement, ce qui entraîne une économie de temps. Un des objectifs de cette thèse est donc de développer une méthode rapide et sensible pour dénombrer des salmonelles dans les échantillons fécaux.

La microbiologie prévisionnelle est un domaine prometteur qui se développe rapidement en microbiologie des aliments, qui a retenu une attention scientifique significative ces dernières années. La prédiction de croissance des salmonelles dans la viande hachée en fonction des conditions environnement comme la température, le pH et l'activité d'eau est nécessaire pour décrire correctement les conditions environnementales changeantes liées au traitement et au stockage de ces produits. Un des facteurs environnementaux les plus importants qui affecte la croissance des salmonelles dans la viande est la température. Des données scientifiques rattachées à l'effet de température sur la croissance des salmonelles dans la viande et la viande hachée sont présentes dans la littérature. Ainsi, pour apprécier les risques dus aux salmonelles dans la viande hachée il est nécessaire de prévoir la croissance de cette bactérie dans la viande et les produits carnés qui sont soumis à différents profils de température, en utilisant les données publiées., Un de nos objectifs est donc de développer des modèles qui décrivent mathématiquement la croissance des salmonelles dans la viande hachée.

En général, relativement peu d'articles traitant des modèles quantitatifs d'appréciation des risques (QRAM) pour des salmonelles transmises par les aliments ont été publiés depuis les recommandations du Codex alimentarius de 1995 (FAO/WHO, 1995). On peut citer des travaux sur les aliments suivants : les œufs liquides pasteurisés (Whiting et Buchanan, 1997), la volaille cuite (Whiting, 1997), le poulet entier (Oscar, 1998, 2004), les œufs en coquille et les ovoproduits (FSIS, 1998 ; Whiting et al., 2000), les œufs et les poulets de chair (WHO/FAO, 2002), le « cordon bleu » de dinde (Bemrah et al., 2002) et les charcuteries (Giovannini et al., 2004). Cependant, des modèles d'appréciation des risques associés à la consommation de la viande hachée ont été développés précédemment pour d'autres pathogènes transmis par les aliments tels que *Escherichia coli* O157:H7 (Cassin et el., 1998;

FSIS, 2001) mais pas mais pas pour les salmonelles. Par conséquent, le travail actuel essaye d'estimer le risque pour la santé publique de la consommation de la viande hachée contaminée par salmonelle.

# Thesis objectives

## **Personal objectives**

By undertaking the research objectives I hope to enhance my ability to independently plan and conduct investigations of population issues. Upon completion of the PhD I will be working for the Department of Preventive Medicine at the Faculty of Veterinary Medicine, Tripoli, Libya and will likely face a wide variety of issues and be involved in training veterinarians. To handle these tasks I will require competence in a wide range of epidemiological techniques. For example, I will need to develop and assess new methodology, make adaptations of existing methodology, analyse sophisticated data sets and model complex systems.

The safety of products of animal origin is an issue of increasing importance to governments, producers of raw products, manufacturers and consumers. Hence, it is very appropriate that I develop epidemiological expertise in this area. This will strengthen my general knowledge of food safety, and help me to develop expertise in the public health importance of enteric pathogens derived from livestock. These are skills that are becoming increasingly important to the market driven outlook now adopted by all governments.

Quantitative risk assessment (QRA) is a relatively new approach. Through my research I hope to make a contribution in this area by applying QRA to specific issue (S*almonella* in ground beef). Thus, I am aiming to help clarify the role of QRA as a method of scientific investigation and to generate an understanding of its role relative to observational and experimental studies.

**Research objectives**

The goal of this work is to quantitatively assess the risk of human salmonellosis from the consumption of ground beef

More specifically, the four main objectives of this work are:

1. To develop a microbiological method based on the principles of the Most-Probable-Number (MPN) statistics and LightCycler real-time PCR (MPN-real-time PCR) to enumerate *Salmonella* in artificially contaminated fecal samples.

2. To detect *Salmonella* in fecal samples collected from slaughterhouse located in Meaux, France using SYBR Green real-time PCR and to evaluate the utility of the developed (MPN-real-time PCR) method to enumerate *Salmonella* in positive samples.

3. To develop model of a growth / no growth interface of *Salmonella* in ground beef from the available growth data in published papers and the data related to ground beef in ComBase to lead to an accurate description of the conditions where *Salmonella* can grow or not.

4. To develop a quantitative risk assessment model of human salmonellosis linked to the consumption of ground beef.

## Thesis outline

**Chapter 2** is a literature review regarding data of detection, quantification, and growth of *Salmonella* in meat and meat products as well as *Salmonella* outbreaks implicating ground beef. These aspects are related to most issues studied and discussed in this thesis. **Chapter 3** tests the sensitivity and specificity of SYBER Green real-time PCR for the detection of *Salmonella* in broth culture as well as in artificially contaminated fecal samples. This chapter also presents the development of MPN-real time PCR assay for the enumeration of *Salmonella* in artificially contaminated fecal samples and also presents the on-slaughter study for the detection of *Salmonella* in fecal samples collected from slaughterhouse located in Meaux, France. This chapter also validates the developed MPN-real-time PCR for enumerating *Salmonella*-positive fecal samples. **Chapter 4** describes the growth limits of a mixture of *Salmonella* strains, *Salmonella* Typhimurium, *Salmonella* Dublin, *Salmonella* Enteritidis or *Salmonella* Senftenberg at different environmental conditions. **Chapter 5** reports a quantitative risk assessment model of human salmonellosis linked to the consumption of ground beef in France. The complete process of ground beef making is modeled, from slaughter to consumption. Finally, **Chapter 6** presents the general conclusions, implications, limitations and recommendations for further studies.

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# Chapter 2: Literature Review

### Salmonella general characteristics

*Salmonella* is a rod-shaped motile non-spore forming Gram-negative bacterium (the exceptions for non-motile serotypes are *S*. Pullorum and *S*. Gallinarum) (FDA, 1998). Currently there are 2541 *Salmonella* serotypes (Popoff et al; 2004). These are classified in two species, *S. enterica* and *S. bongori*. *S. enterica* is divided into six subspecies, *Salmonella enterica* subsp. *arizonae*, *Salmonella enterica* subsp. *diarizonae*, *Salmonella enterica* subsp. *enterica*, *Salmonella enterica* subsp. *houtenae*, *Salmonella enterica* subsp. *indica*, and *Salmonella enterica* subsp. s*alamae* (Popoff et al; 2004). Although this new nomenclature has not been yet validated by the Bacteriological Code, it is widely used by the scientific community, including the World Health Organization (WHO) and the Institut Pasteur in Paris, France (Popoff et al., 2004).

Serotyping and phage typing, together with ever-improving molecular subtyping techniques, become increasingly important in *Salmonella* epidemiology, surveillance, better infection control measures, and support of public health policy. Despite its utility, *Salmonella* serotyping is not without drawbacks. Technologically, serotyping is reasonably unchanged since it was introduced decades ago with most *Salmonella* antisera still produced in animals and requiring a good deal of effort to obtain the required specificity. Determination of serotype is performed in an antigen-antibody agglutination reaction. This process, while simple to perform, requires the maintenance of thousands of antisera, which can be expensive and time-consuming. Traditional serotyping techniques also require the growth of the isolate and are dependent upon antigen expression. In order to simplify the identification of *Salmonella* serotypes, modern molecular technology is now being used.

Molecular typing tools such as Pulsed Field Gel Electrophoresis (PFGE) (Ridley et al., 1998) or amplified fragment length polymorphism (AFLP) (Aarts et al., 1998), random

amplified polymorphic DNA PCR (RAPD-PCR), arbitrary primed PCR (AP-PCR), and repetitive element PCR (Rep-PCR) are very useful techniques to study the epidemiology of outbreaks and to track isolates of *Salmonella*. Recent typing advances utilize DNA sequence polymorphisms within specific sequence targets to differentiate strains. One of these techniques includes multiclocus sequence typing method (MLST) (Kotetishvili et al., 2002; Lindstedt, et al., 2004). Nowdays, PFGE remains the "gold standard" method and is used worldwide for surveillance and for outbreak investigation (Bender et al., 2001; Torpdahl et al., 2005). Notably because of the high frequency of isolation of a limited number of serotypes, serotyping is considered to be a poor discriminating marker for outbreak investigations. Therefore, different phenotypic methods, including antimicrobial resistance profiling and phage typing have been used to study the diversity among *Salmonella* serotypes, and remain an important part of epidemiological investigation.

**Detection, isolation, and quantification of *Salmonella* in food**

The majority of cases of human salmonellosis are due to the consumption of contaminated foods. *Salmonella* control is therefore necessary at all the key steps of food production to ensure safe products for consumers. This control requires rapid and reliable methods in the detection, isolation, characterization and quantification of *Salmonella*. It is essential that methods for detection of *Salmonella* in foods have the ability to detect low levels of pathogens that are healthy, as well as those that are stressed / injured due to conditions in the food and/or during food processing. The detection of low numbers of cells is particularly important for *Salmonella* spp., since epidemiological evidence suggests that low doses of certain *Salmonella* strains can cause disease in a significant proportion of the consumers (Hedberg et al., 1992).

Culture and colony counting methods, Polymerase Chain Reaction (PCR) as well as immunology-based methods are the most common tools used for pathogens detection including *Salmonella* isolation (Lazcka et al., 2006), they involve counting of bacteria, DNA analysis and antigen-antibody interactions, respectively. These methods are often combined together to yield more robust results.

Detection of *Salmonella* in foods by conventional culture methods include pre-enrichment culturing, selective enrichment in different media, plating on selective and indicative media and subsequent biochemical and serological identification of suspected colonies (ISO 6579, 1993). The culture method is time-consuming and labour intensive when handling many samples requiring a minimum of 4-6 days (Andrew et al., 2003; Uyttendaele et al., 2003). Pre-enrichment of *Salmonella* in buffered peptone water is commonly used for most foods, though some foods require a more specific medium (European Committee for Standardization, 1997). Selective enrichment aims at increasing the number of salmonellae, while at the same time reducing the non-*Salmonella* population. In ISO Standards 6579 Rappaport–Vassiliadis (RV) broth and selenite cystine (SC) broth are used in parallel for the enrichment of salmonellae. The additional use of SC broth often does not result in more positive results (O'Donoghue and Winn, 1993) and in practice when only one enrichment medium is used, RV broth or a modification of this medium is usually used. The superiority of RV medium to other selective enrichment media for salmonellae has been shown in several studies (Maijala et al., 1992; June et al., 1996). The selectivity of enrichment media at elevated temperature (42-43°C for RV) is greater than at lower temperatures (35-37°C); prolonged (48h) enrichment usually does not result in a substantial increase in the recovery of salmonellae (D'Aoust et al., 1992a). Waltman et al. (1993) found that the optimum time for incubating the enrichment cultures was 24 h followed by delayed secondary enrichment after 5 days. D'Aoust et al. (1995) found that interruption of the *Salmonella* analysis by

refrigeration of pre-enrichment and enrichment cultures during the weekend did not result in lower recoveries. In several comparative studies, motility enrichment on Modified Semisolid Rappaport–Vassiliadis (MSRV) medium has been further confirmed as a very effective procedure for the isolation of salmonellae from foods (Bolderdijk and Milas, 1996; Afflu and Gyles, 1997). The results with MSRV medium are usually better than or equal to those obtained with RV broth.

Besides being time consuming, the conventional culture method has also been reported to show poor sensitivity for low-level contamination in samples (D'Aoust, 1992b). Because only a minority of bacterial cells present in any given environmental samples can be cultured with these techniques, and since the correlation between culturability and infectivity has not been properly determined, this technique remains questionable, especially in the light of increasing numbers of *Salmonella* cases worldwide. In addition, works have been published indicating that viable but non-culturable bacterial cells might still be infectious (McKay, 1992). Rapid isolation and identification of *Salmonella* in food will increase the chances of preventing diseases caused by this pathogen. For this purpose, a variety of so called rapid methods have emerged, the majority of these methods however including immunological and molecular methods.

The field of immunology-based methods for *Salmonella* detection provides very powerful analytical tools for a wide range of targets. For example, immunomagnetic separation (IMS), a pre-treatment and/or pre-concentration step, can be used to capture and extract the targeted pathogen from the bacterial suspension by introducing antibody coated magnetic beads in it. IMS can then be combined with almost any detection method. For example, Rijpens et al. (1999) described a method based on PCR and IMS for the detection of

*Salmonella* in different dairy and egg products. Other detection methods are only based on immunological techniques; in this case the enzyme-linked immunosorbent assay (ELISA) test is the most established technique nowdays. ELISAs combine the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme (Lazcka et al., 2006).

Nucleic acid-amplification technology predominantly including standard or real-time polymerase chain reaction (PCR) has been suggested to shorten conventional methods. It is based on the isolation, amplification and quantification of a short DNA sequence including the targeted bacteria's genetic material. Methods based on the PCR offer the advantages of high specificity and sensitivity. A number of PCR-based kits are commercially available for testing of food or other samples for the presence of *Salmonella* nucleic acids: Probelia™ *Salmonella* spp. of Sanofi Diagnostics Pasteur (Marnes La Coquette, France), the *Taq*man™ *Salmonella* PCR amplification detection kit of Perkin Elmer (Normalk, CT, USA) and BAX™ for screening/*Salmonella* of Qualicon (Wilmington, DE, USA) (Rijpens et al., 1999), etc.

For PCR, several primers specific for *Salmonella* spp. have been described (Gooding and Choudary, 1999). However, to detect low numbers of S*almonella* in food, PCR with specific primers has to be preceded by appropriate procedures of enrichment and DNA template preparation. Enrichment in a single non-selective medium (Chen et al., 1997; Wang et al., 1997), enrichment in a non-selective medium followed by two parallel selective media (Cohen et al., 1996) and a successive enrichment in three media (Aabo et al., 1995) have been used prior to DNA extraction. Template DNA was obtained from the cells by boiling in water (Iida et al., 1993; Kwang et al., 1996), by the treatment with proteinase K (Soumet et al.,

1997), detergents (Aabo et al., 1995; Wang et al., 1997) or using various commercially-available DNA extraction kits (Jones et al., 1993; Chen et al., 1997).

The application of the PCR assay after cultural enrichment offers distinct advantages in testing of food samples:

1. It ensures that target DNA is obtained from viable cells.
2. It increases the amount of target DNA, which is beneficial because the concentration of target organisms may be initially low and they may not be uniformly distributed.
3. It allows proliferation of *Salmonella* while reducing or diluting out non-*Salmonella* organisms and other components (inhibitors) that might interfere in the PCR assay.
4. Preenrichment allows recovery of stressed organisms.

Several conventional PCR methods for detecting *Salmonella* cells have been published which use specific gene sequences as targets (Fratamico and Strobaugh, 1998; Gou et al., 2000; Ferretti et al., 2001). Although most research has focused on diagnostic and clinical microbiology, only recently have commercial PCR test for food-safety been introduced (Bailey, 1998; Hines, 2000). Unfortunately, unlike the specificity of S*almonella* detection by PCR, which was satisfactory, the detection limit of $10^0$ cfu 25 $g^{-1}$ remained problematic for most of the conventional PCR methods described, in particular with naturally-contaminated food sample (Shearer et al., 2001).

The recent development and availability of rapid real-time PCR assays have allowed for advancement of conventional PCR techniques. Real-time PCR permits to obtain quicker

results without too much manipulation. This technique bases its detection on fluorescent emission by a specific dye as it attaches itself to the targeted amplicon. Given that fluorescence intensity is proportional to the amount of amplified product, it is possible to follow the amplification in real time, thus eliminating laborious post-amplification processing steps such as gel electrophoresis.

Real-time PCR assays for the sensitive and specific detection of *Salmonella* have targeted genes such as *invA* (Eyigor and Carli, 2003; Hong et al., 2003), *himA* (Chen et al., 2000), *iagA* (Liming and Bhagwat, 2004), *fimA* (Jothikumar et al. 2003), *agfA* (Doran et al., 1993), *sefA* (Medici et al. 2003), and the 16S rRNA (Trkov and Avgustin, 2003; Lin et al., 2004). Several papers have currently appeared where real-time PCR application for pathogen detection has been reported (Jothikumar and Griffiths, 2002; Medici et al., 2003; Liming and Bhagwat, 2004). The main advantages of real-time PCR are high sensitivity, high specificity, excellent efficiency, reduced amplicon size and less risks of cross-contamination (Lazaro et al. 2003).

The real-time PCR has many PCR-based detection methods such as fluorogenic detection methods which utilizes the 5'nuclease activity of *Taq* DNA polymerase to hydrolyse an internal fluorogenic probe for monitoring amplification of DNA targets (referred to as *Taq*Man assay) (Chen et al., 1997; Hoorfar et al., 2000; Rodriguez-Lazaro et al., 2003) while the other like iQ-check system utilizes a fluorogenic probe which has flanking GC-rich sequences complementary to one another (Liming and Bhagwat, 2004) (referred as molecular beacon, MB). These fluorescent-probe-based assays require the availability of primers and probes that must be selected according to very rigid conditions, which can not always be easily applied.

Use of the double-stranded DNA (dsDNA) binding dye SYBR Green I for detection of PCR products has overcome this limitation by allowing real-time PCR to be applied without the need for probes linked to fluorescent molecules (Aarts, 2001). Therefore, recently, more researchers rely upon real-time PCR studies using simple and less expensive SYBR Green dye. A number of SYBR Green real-time PCR assays for detection of microbial pathogens such as *Escherichia coli* (Jothikumar and Griffiths, 2002), *Campylobacter* (Inglis and Kalischuk, 2004) have been reported. In addition, several SYBR Green real-time PCR assays for detection of *Salmonella* from different types of samples have been described. Medici et al. (2003), Bhagwat (2004) and Hyang-Mi et al., (2005) reported SYBR Green based PCR assay with poultry samples, vegetable rinse water and dairy farm environmental samples, respectively. Additionally, multiplex real-time PCR using SYBR Green for simultaneous detection of *Salmonella* and *Listeria monocytogenes* in raw sausage meat (Wang et al. 2004), and spiked tap water and pasteurized milk samples (Jothikumar et al., 2003) have been reported.

One limitation to the adoption of PCR for *Salmonella* detection is that despite the number of validation studies reported in the literature there are few studies that report the sensitivity and specificiy of PCR for the detection of *Salmonella* in naturally contaminated samples (Oliveira et al., 2003). The viability of bacteria from samples artificially contaminated with stock cultures may differ from that of naturally contaminated samples, which have been exposed to a variety of unfavorable conditions or suffered some degree of injury while in transport, storage and/or in processing (Gouws et al., 1998). In addition, the sensitivity of a test when applied to an artificially dosed sample may differ significantly from its ability to detect the much smaller bacterial loads that can be anticipated in a sample with naturally occurring bacterial contamination (Gouws et al., 1998). Comparing results between

studies for the evaluation of PCR is also made difficult by the lack of standard protocols for sample handling and equipment. Previous studies have resulted in recommended standard protocols for conducting the PCR assay and use of an internal amplification control to indicate false-negative results (Malorny et al., 2003; Hoorfar et al., 2004). However, standard protocols for collection, handling, enrichment, and selective enrichment of *Salmonella* isolates being detected by PCR due to naturally occurring contamination of food products have yet to be established (Myint et al., 2006).

The real-time PCR assays is substantially faster than conventional PCR, and can be employed as a routine procedure for the definitive identification of *Salmonella* in a diverse range of food matrices, and when combined with subculture of enrichment broths from PCR-positive samples, provide in most cases an isolate of the pathogen. Therefore, these methods should be of great benefit to the food industry and to regulatory or public health authorities engaged in establishing the safety of food products and the management of salmonellosis. In a routine basis for diagnosis, it should be considered that a large number of samples may be processed in a relative short period of time using the PCR assays. However, rapid tests for *Salmonella* identification might contribute to, but not replace, bacteriological culture techniques. Indeed, organism isolation is still needed for serotyping and determination of resistance profiles, and also for epidemiological studies.

Quantification of *Salmonella* in foods can be done by the standard plate count (SPC), or by traditional Most-Probable-Number (MPN) method. MPN method provides statistical estimates of viable cell concentration. The method is most frequently used to estimate low populations of foodborne pathogens in foods (Gooch et al., 2001). The traditional MPN uses conventional culture and biochemical techniques to identify isolates. Generally, the MPN can

be used to estimate numbers of pathogens cells present at less than 10 or 100 CFU/g of food, which otherwise cannot be enumerated by direct plating. The MPN results are more likely than the standard plate count method to be consistent from one laboratory to another. However, the traditional MPN technique is limited by some drawbacks. These include the large volume of glassware that is required, which is costly in terms of both time and labor, the selectivity of culture media, the lack of opportunity to observe the colonial morphology of the organisms (Seo et al., 2006).

Quantitative real-time PCR assay are often used for quantification of initial target DNA. Unfortunately, amplification efficiencies can be different from sample to sample due to the effects caused by inhibition of amplification, human failures or preparation errors. This implies that quantification, even with external controls, does not always represent a correct calculation of initial amount of target in each sample (Klerks et al., 2004). To eliminate part of these drawbacks, different approaches of using an internal amplification control (IAC) in each real-time PCR have been described (Hoorfar et al., 2000; Vandesompele et al., 2002).

Recently, an alternative approach is presented based on the combination of most-probable-number method (MPN) and conventional-PCR assay. Martin et al., (2004) reported that MPN-PCR assay proved to be a rapid and reliable method for enumerating *Listeria monocyogenens* in fermented sausages, including low contaminated samples. Mäntynen et al., (1997) developed a simple and sensitive MPN-PCR assay for the detection and enumeration of enterotoxin C producing *Staphylococcus aureus* from fresh cheese. The analysis with this MPN-PCR took one day to perform compared with three days analysis time with plate counting. The MPN-PCR assay was also used for the detection and quantification of specific flagellate species in soil (Fredslund et al., 2001). The MPN-PCR method yielded significant

labor and time savings, as opposed to the traditional methods. Since the real-time PCR is substantially faster and more sensitive than conventional PCR. This real-time PCR assay, therefore, can be combined with MPN statistics for the enumeration of pathogens in a diverse range of food matrices which could lead to more shortening of the time for analysis compared to MPN-conventional PCR assays. One of the objectives of this thesis was to develop a MPN-real-time PCR for the detection and enumeration of *Salmonella* in artificially and naturally contaminated fecal samples. This rapid method should be of great benefit to food industry and regulatory or public health authorities engaged in establishing the safety of food products and the management of salmonellosis.

**Salmonella surveillance and monitoring programs**

Bacterial enteropathogens account for up to 20% of acute diarrhea observed worldwide, with *Salmonella*, *Shigella*, and *Campylobacter* being the principle species. These same pathogens were the primary (89%) causes of bacterial gastroenteritis infections within the European and Latin American medical centers (Streit et al., 2006). To minimize risk to consumers particularly with respect to the global food supply, surveillance of foodborne disease is becoming an increasingly high priority in the public health and food safety agenda in many countries. Currently, only a few countries in the world have fully adequate surveillance programs. All other countries, including all the developing countries, are in the process of establishing and improving their national systems.

A variety of surveillance systems exist but the most common form encountered throughout the world is epidemiologic surveillance wherein the incidence of specific illnesses is monitored. Depending on the public health system, compulsory notification of illnesses may be involved or the occurrences of illnesses may be passively collected through

physicians. In general terms, foodborne disease surveillance is essential for: (1) estimating the burden of foodborne disease, and monitoring trends; (2) identifying priorities and setting policy in the control and prevention of foodborne diseases; (3) detecting, controlling and preventing foodborne disease outbreaks; (4) identifying emerging food safety issues; and (5) evaluating foodborne disease prevention and control strategies (WHO, 2002). In addition to the programs addressing human illnesses, epidemiologic surveillance of diseases in animal populations is also important as such data are used for both implementation and evolution of disease control programs as well as for international trade. Some examples of French and international epidemiologic surveillance system are described below.

**French Surveillance systems**

The National Institute for Public Health Surveillance (InVs), in collaboration with other structures, plays a central role in the organization and collection of data concerning the impact of foodborne diseases due to pathogens on human health (Leclerc et al., 2002). InVS is a public technical agency that coordinates the public health surveillance system in France (http://www.invs.sante.fr). The general aim of InVs is to monitor continuously the health of the population and its evolution.

Foodborne disease outbreaks surveillance programs in France are being established to track foodborne disease outbreaks and their geographical distributions. These foodborne disease outbreaks including *Salmonella* outbreaks are reported as *Toxi-Infections Alimentaires Collectives* (*TIAC*s) (De Buyser et al., 2001). *TIAC*s are investigated by local agencies (district veterinary services, or DSV, Direction des services vétérinaires) of the Ministry of Agriculture and Fisheries, *Direction général de l'alimentation*, and/or by local agencies (district health directorates) of the Ministry of Public Health, the *Directions departementales*

*des affaires sanitaires et sociales (*DDASS). All reports are centralized by the coordinator of the national institute for public health surveillance (http://www.invs.sante.fr) which records the outbreaks and publishes them in the weekly *Bulletin Epidemiologique Hebdomadaire* (BEH). *TIAC* reports in BEH constitute the main source of information for *Salmonella* outbreaks.

The National Reference Centres (Pasteur Institute, Paris), network of laboratories or hospitals and sentinel networks are involved in human health surveillance. This surveillance is based on characterization of the strains isolated from human specimen. These data coupled to those obtained from notification of the diseases are used to inform InVs and DDASS of a public health problem (Leclerc et al., 2002). This centre is another source of information for *Salmonella* outbreaks, which serotypes *Salmonella* strains isolated from patients and submitted by field laboratories.

Surveillance of antimicrobial resistance in bacteria from animal origin in France is organized by the French Agency for Food Safety (Agence française de sécurité sanitaire des aliments) (AFSSA) through two types of networks (Martel et al., 2000). The first collects non-human zoonotic *Salmonella* strains in one centre (AFSSA-Paris) where they are tested for their antimicrobial susceptibility. The others, managed by AFSSA-Lyon, deal with bovine pathogenic strains and are multicentric, that is they are collecting antibiotic sensitivity and other data from the local public veterinary diagnostic laboratories. Non-human *Salmonella* isolates together with epidemiological information are routinely sent to the AFSSA, Paris (*Salmonella* Network) for confirmation of species identification and serotyping. More than 150 laboratories all over the national area participate in this network of which 50% are public laboratories (French antibiotic reference). Their results show the important role played by

animals in the spread of *Salmonella* at the national level in animal health and food processing, in food hygiene and in the environment. The aim of the two other networks managed by AFSSA-Lyon is the monitoring of bovine pathogens. RESABO (Réseau d'épidémiosurveillance des bactéries résistantes aux antibiotiques chez les bovines) network has been considered as a pioneer model for multicentric networks. The rules of this network were established by the AFSSA-Lyon. Isolation, identification of the bacterial species and antibiotic susceptibility tests are performed routinely by the LVDs (laboratoires vétérinaires départementaux, district veterinary labs). Species selected for this epidemiological monitoring of resistance include *Escherichia coli* and *Salmonella* spp. The second network is the RESSAB network which is a specific network that evaluates the prevalence of clinical salmonellosis in adult cattle (Dufour et al., 1997). In France, the RESSAB (Réseau d'épidémiosurveillance des salmonelloses bovines) network, managed by AFSSA-Lyon has 159 sentinels, volunteer veterinary practitioners covering 33,415 herds in 16 French departments. When a clinical case of bovine salmonellosis is suspected, the sentinel veterinarian makes an initial visit to the farm, records clinical parameters of the disease and takes a sample of feces from the sick animal for bacterial analysis. The associate LVD carries out a *Salmonella* detection test and if it is positive continues with a serotype determination and in vitro antibiotic susceptibility tests. Data are first sent to the departmental steering committee (Groupement technique vétérinaire, GTV and Groupe de défense sanitaire, GDS) and then, are sent to AFSSA-Lyon.

**Selected international surveillance systems in public health and food safety programs:**

Other examples of international surveillance systems in public health and food safety programs and their roles include:

First, epidemiological surveillance systems such as (1) *FoodNet* (*http://www.cdc.gov/foodnet*) which is a collaborative project of the CDC, Food and Drug Administration, and the United States Department of Agriculture, and 10 sites within the United States. More than 650 clinical laboratories in the *Food*Net sites are conducted regularly to collect information on laboratory-confirmed cases of diarrheal illness. *Salmonella* is one of the pathogens monitored; (2) *Enter-net surveillance system* (*http://www.enter-net.org.uk*): Enter-net conducts surveillance for enteric infections (*Salmonella* and VTEC O157) within Europe. Over 25 European countries are participating together with Canada, Japan, South Africa, Australia, and New Zealand; (3) *Health Protection Agency (HPA) Communicable Disease Surveillance Centre (CDSC)* operates a system of surveillance for general outbreak of infectious intestinal disease (IID) in England and Wales since 1992. The surveillance system captures information on outbreaks of IID whatever the mode of transmission (Djuretic et al., 1996). The main advantage of the surveillance system for outbreaks of IID in England and Wales over other systems is that all IID outbreaks are recorded, no matter what the mode of transmission (Adak et al., 2002). Second, laboratory surveillance system such as (1) *pulseNet* (*http://www.cdc.gov/pulsenet*), a national network of public health and food regulatory agency laboratories coordinated by the CDC. All participants perform standardized molecular subtyping of foodborne disease-causing bacteria by pulse-field gel electrophoresis (PFGE); (2) *WHO Global Salm-Surv* (*GSS*) (*www.who.int/salmsurv*), global network of laboratories and individuals from 141 countries initiated by WHO in 2002, in order to reduce foodborne diseases worldwide. Initially, GSS focused on the surveillance of *Salmonella*, but it has now expanded to diseases caused by

other foodborne pathogens such as *E.coli* and *Campylobacter;* (3) *National Antimicrobial Resistance Monitoring System (NARMS)*, a system based in the US that monitors changes in antimicrobial susceptibilities to 17 antimicrobial drugs of zoonotic pathogens from human and animal clinical specimens, from healthy farm animals, and from carcasses of food-producing animals at slaughter plants.

In conclusion, public health concern and potential for foodborne zoonotic transmission have made *Salmonella* the main subject of the international, national and local surveillance programs. These monitoring networks and surveillance programs provide the most comprehensive data available to support public health decision making. However, Leclerc et al. (2002) reported that whatever system is used, data are underestimated. He recommended the development of networks to exchange data and these data must not only exist in a country but it is also very important to develop links and networks with other countries. With the development of rapid transportation, products are now quickly dispatched all over the world and pathogens need to be detected as soon as possible. One should always be aware; *Salmonella* is not stopped by national frontiers.

**Implication of ground beef in Salmonella outbreaks**

Foodborne pathogens have been estimated to cause >6 million illnesses and approximately 9000 deaths each year (Mead et al., 1999). Bacterial pathogens contribute to ~60% of foodborne illnesses that lead to hospitalization and account for nearly two-thirds of the estimated number of foodborne pathogen-related deaths. Mead et al. (1999) estimated that *Salmonella* spp. caused ~26% and >30%, *Listeria* spp. accounted for ~4% and ~28%, *Campylobacter* spp. caused ~17% and 5% and *Escherichia coli*, both O157 and non-O157,

account for ~5% and >4% of foodborne illness-related hospitalizations and foodborne deaths respectively.

Several food items, including ground beef, have been implicated in *Salmonella* disease outbreaks. Salmonella have been routinely detected in cattle fecal, hide, and carcasses at the farm and slaughterhouse. Previously reported surveys on *Salmonella* in Europe, Australia, United States and Canada have shown large variations in the prevalence of *Salmonella* in fecal, hide, and carcasses ranging from 2% to 50% (Heuchel et al., 2000; Ransom et al., 2002; McEvoy et al., 2003; Fegan et al., 2004; Fegan et al., 2005; Lalleret et al., 2005; Fluckey et al., 2007; Stephens et al., 2007). The large variations in levels of *Salmonella* contamination observed in these studies have been attributed to several factors such as variations in sampling and detection techniques, seasonal differences, herd size, geographic area, hygiene, and farm management practices. Several factors have been linked to the presence of *Salmonella* in animals including exposure to new animals without quarantine, use of lagoon wastewater, not properly monitoring feed components, presence of rodents or wild animals, rendering trucks, and inadequate handling of sick animals. Stress applied to animals such as transportation, food deprivation or confinement may also increase the shedding and thus the spread of *Salmonella*. These reported findings clearly suggest *Salmonella* can be carried by healthy cattle at slaughter (Samuel et al., 1979; McEvoy et al., 2003) and can therefore serve as a reservoir and source of contamination of carcases during processing and can pose a health hazard.

The combination of interaction of the intrinsic and extrinsic factors determines the microbiology of meat. Among them some factors are especially influential to *Salmonella* growth in meat. The intrinsic nature of most raw meats with high water activities (>0.98),

moderate pH (5.5-6.5) and readily available source of energy, carbon and other nutrients, makes them ideal for most microbial growth (Varnam and Sutherland, 1985). Salmonellosis outbreaks often contain low and undetectable numbers of *Salmonella* cells which can be severely injured but still be infectious. For example, the dose of *Salmonella* Enteritidis in an outbreak caused by the consumption of ice-cream (Hennessy et al., 1996) was determined to be 0.093 cells $g^{-1}$ or 6 cells in a 65 g serving size. Samples from this outbreak were further analyzed to estimate the potential infective dose of *Salmonella* (Vought and Tatini 1998). These researchers found the samples to vary in the level of *Salmonella* from 0.004 to 0.46 $g^{-1}$ (Vought and Tatini, 1998).

Data from published investigation reports from several countries were reviewed to determine the implication of ground beef and other meat products in outbreaks of foodborne disease. In the USA, in 1994, an outbreak of *Salmonella* serotype Typhimurium gastrointestinal illness in Wisconsin associated with eating contaminated raw ground beef during winter holiday season (CDC, 1995). The investigations of this outbreak implicated that inadequate cleaning and sanitization of the meat grinder probably resulted in ongoing contamination of ground beef over many production days. In this outbreak, 107 confirmed cases and 51 probable case-patients; of these, 17 (16%) were hospitalized. Between January - April 2002, multidrug-resistant *Salmonella* Newport emerged as a cause of salmonellosis in five states due to exposure to raw or undercooked ground beef (CDC, 2002). *Salmonella* Newport was isolated from 47 persons in five states: New York (34 cases), Michigan (five), Pennsylvania (four), Ohio (two), and Connecticut (two). In this outbreak, 17 cases (37%) were hospitalized and one died. 2003-2004, the first multistate outbreak of multidrug resistant *Salmonella* Typhimurium DT104 associated with consumption of store-bought ground beef occur in the northeastern United States (Dechet et al., 2006). In the same year (August 11-

October 2, 2004) multistate outbreak *Salmonella* Typhimurium infections associated with eating ground beef occurred (CDC, 2006).

In Canada, an outbreak of multidrug resistant *Salmonella* Typhimurium occurred in 2003 among at least 47 persons attending a school potluck (McLaughlin et al., 2006). Illness was associated with consumption of ground beef (estimated odds ratio 16.3). *Salmonella* Typhimurium isolated from infected individuals and implicated ground beef revealed identical pulsed-field gel electrophoresis. In Norway, October-November 2005, an outbreak of *Salmonella* Typhimurium DT 104 occurred linked to imported minced beef (Isakbaeva et al., 2005).

In France, a total of 3979 notified outbreaks occured during period (1997-2003); from them 1205 were confirmed as *Salmonella*-related outbreaks (Afssa, 2005). Ground beef was implicated in 65 (1.6%) of the total bacterial outbreaks reported in France, and in 1.8% (22/1205) of the bacterial outbreaks of *Salmonella*. *Salmonella* Typhimurium was by far the most frequent serotype isolated in France (Bouvet and Grimont, 2002). During this period, *Salmonella* has been isolated from 9 outbreaks of ground beef. 553 cases (0 to 321 per year) were attributed for these outbreaks. In these outbreaks, 67 patients were hospitalized. Table 1.2 shows *Salmonella* outbreaks implicating ground beef in France.

From 1990 to 2000, four outbreaks of salmonellosis occurred (Mariau et al., 1990; Gilles et al., 2000; Haeghebaert et al., 2000a; Haeghebaert et al., 2000b) had been detected by CNR surveillance system and investigated. The results of the epidemiological, veterinary and laboratory investigations indicated that the *Salmonella* serotypes responsible for these four episodes were different (Meleagridis, Paratyphi B, Typhimurium, and Coeln) respectively.

The first three outbreaks occurred in hospital community (Mariau et al., 1990; Gilles et al.; Haeghebaert et al., 2000a), whereas the last one happened in normal population (Haeghebaert et al., 2000b). The number of the cases is relatively low (8-58 with medium 32). The food responsible was ground beef bought and refrigerated (Haeghebaert et al., 2000b) and the others frozen hamburger. In 1996, a study done in France to evaluate the risk factors for the occurrence of sporadic *Salmonella* enterica serotype Typhimurium infections in children less than 15 years old (Delarocquein-Astagneau et al., 2000) gave the evidence that the consumption of raw or uncooked ground beef was the main risk factor of salmonellosis (OR= 5, IC 95= 1.7-8.4) and the population attributed risk for the children less than 15 years old was 35% (IC 95= 12-58). This study considered only *Salmonella* enterica serotype Typhimurium. Between August 2005 and March 2006 in France, 69 cases of *Salmonella enterica* serotype Manhattan were reported, 51 (74%) of them from southeastern France (Noel et al., 2006). The investigation incriminated pork products from slaughterhouse X as being the most likely source of this outbreak. *Salmonella* Manhattan was isolated from cases and from pork products. Seven human cases had the same PFGE profile as isolated from the pork products. The main production of this slaughterhouse was pork, but beef was also produced (20% of production). The investigation expected that the outbreak could be due in part to the distribution of contamination beef. In case-control study, there was an association between beef consumption and illness (OR= 9.3 CI 95%= 1.3-68.6 with P-value 0.02). Although beef and pork production were carried out in different units, cross-contamination of the beef unit could not be ruled out.

In summary, salmonellosis outbreaks associated with ground beef continue, despite Hazard Analysis and Critical Control Point System (HACCP), enhanced adherence to good manufacturing practices and education of food processors, preparers, and servers at all levels

in the food industry and in the home. Targeting interventions at various steps, from beef production through consumption, might help to reduce the risk of salmonellosis. Consumer especially the most susceptible ones (the immunocompromized, the elderly, young children, and pregnant women), should continue to be made aware of the risks associated with eating raw or uncooked ground beef, tasting ground beef during food preparation, and cross-contamination from raw meat to ready-to-eat-food, as well as the importance of hand washing after handling raw ground beef.

**Table 1.2:** Examples of Salmonella outbreaks implicating ground beef in France

| Serotype | Year | No. of cases | Food implicated | Type of meat | Reference |
|---|---|---|---|---|---|
| Meleagridis | 1990 | 58 | Ground beef | Frozen | Mariau et al., 1991 |
| Coeln | 1998 | 26 | Ground beef | Bought and refrigerated | Haeghebaert et al., 2000b |
| Paratyphi B | 1999 | 8 | Ground beef | Frozen | Gilles et al., 2000 |
| Typhimurium | 1999 | 35 | Ground beef | Frozen | Haeghebaert et al., 2000a |
| Manhattan | 2005 | 69 | Pork products and ground beef | ? | Noel et al., 2006 |

**Table 2.2:** Examples of Salmonella outbreak implication ground beef and beef products in different countries

| Country | Year | No. of cases (death) | Food implicated | Serotype | Reference |
|---|---|---|---|---|---|
| USA | 1978 | 48 | Ground beef | Newport | Fontaine et al. 1978 |
| USA | 1994 | 17 conformed and 51 probable | Ground beef | Typhimurium | CDC, 1995 |
| Japan | 1999 | 3 | Roasted beef | Enteritidis PT4 | Ministry of health and welfare (Japan), 1999 |
| Japan | 1999 | 967 | Beef and bean sprouts | Enteritidis PT22 | Ministry of health and welfare (Japan), 1999 |
| USA | 2002 | 47(1) | Ground beef | Newport | CDC, 2002 |
| USA | 2003 | 58 | Ground beef | Typhimurium DT 104 | Dechet et al., 2006 |
| Canada | 2003 | > 47 | Ground beef | Typhimurium | McLaughlin et al. 2006 |
| USA | 2004 | 31 | Ground beef | Typhimurium | CDC, 2006 |
| Norway | 2005 | 4 | Minced beef | Typhimurium DT 104 | Isakbaeval et al., 2005 |

## Growth of Salmonella in ground beef

Predictive modelling has been widely developed since the 1980s (Van Impe et al., 1992; McMeekin et al., 1993; Baranyi and Roberts, 1994; Rosso et al., 1995). This field combines the knowledge of bacterial growth over a range of conditions with the power of mathematical modelling to enable predictions of growth. The main objective is to simulate bacterial growth as function of temperature, pH and water activity. MacDonald and Sun, (1999) proposed a classification scheme of the models according to Whiting and Buchanan (1993) (Table 3.2). Primary models describe the change of the bacterial number over time under given environmental conditions and generate information about the microorganism such as generation time and lag phase duration. Secondary models describe the evolution of one or more parameters of a primary model in relation to one or more changes in environmental conditions. Tertiary models take modelling to its final form. There are applications to one or more primary or secondary models, incorporated into a user-friendly computer software package (MacDonald and Sun, 1999)

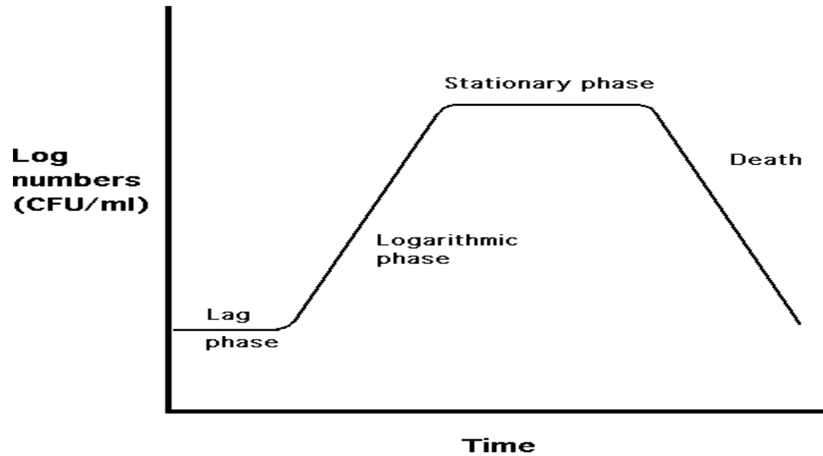
**Table 3.2:** Classification of some growth models (derived from MacDonald and Sun, 1999)

| Primary models | Secondary Models | Tertiary models |
|---|---|---|
| Modified Gompertz function | Response surface models | Pathogen modelling program |
| Logistic model | Modified Arrhenius model | Growth predictor |
| Baranyi model | Square root model | Pseudomonas Predictor |
| Rosso model | Γ-models | Seafood spoilage predictor |
| (Modified) Monod model | Z values | ComBase |
| D values of inactivation | | Sym'Previus |

In many cases, the growth of a homogeneous microbial population can be described by a curve (Fig. 1.2) with three phases: a lag phase during which the microbial cells adapt to their new environment, followed by exponential growth phase during which the cells multiply exponentially and, finally, a stationary phase during which the maximum population density is reached. Environmental conditions, food composition and growth status of the microorganisms (lag, exponential, stationary phase) can affect the growth rate (Table 4.2).

They are not the only source of variation: strains of a same genus may grow differently under the same intrinsic and extrinsic conditions.

**Figure 1.2** Hypothetical bacterial growth curve




Hypothetical bacterial growth curve.

**Table 4.2:** Main factors affecting microbial growth and survival in foods (Gould, 1989)

| *Physical* | *Physical* | *Chemical* | *Microbiological* |
|---|---|---|---|
| *Intrinsic* | *Water activity*<br>*Content Redox l* | *Nutrients present*<br>*Solutes present*<br>*Acidulant identity* | *Nutrients used*<br>*End products formed*<br>*Sensitivity/resistance* |
| *Extrinsic* | *Temperature*<br>*Relative humidity*<br>*Light intensity*<br>*Packaging characteristics* | *Antimicrobial agents*<br>*Atmospheric gas*<br>*Oxygen status* | *Number and types* |

In general, *Salmonella* can grow at temperature between 5.2°C and 46.2°C; also the pH of *Salmonella* growth is between 4.1 and 9.0 (Jay, 2000). However, the minimum growth conditions depends on the acid. For example, minimum pH *Salmonella* growth in lactic acid and acetic acid environments has been observed at a pH of 4.40 and 5.40 respectively (Chung and Goepfert 1970). The pathogen is killed by pasteurization and disinfection agents, while freezing, refrigeration and drying fail to destroy it, but do prevent or slow its growth (FDA, 1998). Under optimal conditions of nutrients, water activity, temperature and pH, however, *Salmonella* may double in numbers every 20 min. Consequently, moist foods of neutral pH

are most likely to support the growth of *Salmonella*, especially under temperature abuse (>5°C) conditions. It is very important to keep such foods either refrigerated (<5°C) or heated at temperature above 60°C in order to minimize risk of salmonellosis (CDC, 2000). However, these simple but effective measures are often ignored by food handlers or consumers (Bryan et al., 1997). As a result, food-associated salmonellosis cases usually result from the ingestion of raw meats, poultry, seafood, eggs, and milk and dairy products contaminated with the pathogen (Tietjen and Fung, 1995).

Within the meat industry, assurance of meat safety and quality are of paramount importance. As the industry develops new technologies to produce higher quality and diverse meat products for increasingly large markets, systems must be designed to allow safeguards to be implemented into processing procedures. Traditional approaches to meat safety and quality have relied heavily on regulatory inspection and sampling regimes. However, these systems cannot guarantee total consumer protection since 100% inspecting and sampling can not be employed for obvious economic and logistic reasons (Armitge, 1997). Despite the tremendous progress in bioanalytical techniques, for most pathogens the limit of detectability generally is one microbe in 25 g of food. If the food contains less than one detectable pathogen in 25 g, it is considered 'safe' although it can become unsafe if the organism is held under conditions where it can grow. If a ground beef was contaminated with *Salmonella* spp. at a level below the detection limit, we can define the shelf life of a product based on the safety as the time at which the pathogen reaches a detectable count. This of course will depend on the initial count which could be variable and the temperature history during transport, retail and home holding. Thus food products may become microbiologically unsafe before or very close to the end of their shelf life if the temperature is abused.

The presence and growth of salmonellae in ground beef and other beef products have been investigated because of health significance. The behavior of these *Salmonella* spp. in large variety of beef products with various methods of processing, packaging, storage, and distribution is reported (Goepfert, 1975; Grau, 1983; Poerschke and Cunningham, 1985; Hintlian et al., 1987; Dickson and Olson, 2001). For example, Goepfert (1975) investigated the growth of *Salmonella* Typhimurium in raw ground beef at temperature 13°C with the pH values of 6. Hintlian et al. (1987) investigated the growth of *Salmonella* Typhimurium at temperature 13°C with the pH value of 5.6 in modified atmosphere-packaged (MAP) cooked beef. This study indicated that the doubling time of *Salmonella* Typhimurium is 11.6 hours when measured on raw ground beef, 21.6 h when measured with MAP ($CO_2$ 75%, $O_2$ 5%, and Nitrogen 20%), and 25.5 h when measured with MAP ($CO_2$ 75%, $O_2$ 25%). These reports demonstrated that MAP can decrease the growth of *Salmonella*.

Gill and Newton (1980) investigated the ability of *Salmonella* Typhimurium to grow at different combination of temperature (10, 12, 15, 20, 30°C), pH (5.5, 6.5), and modified atmosphere-packaged (raw, anaerobic, vacuum-packed, and 100% carbon-dioxide in the ground beef environment). This study indicated that 100% carbon-dioxide in the ground beef environment increases the doubling time of *Salmonella* Typhimurium by 2.5 times at 12°C and 15°C, where as there is almost no effect with the higher temperature especially when the pH is 6.5. These results were supported by Mackey and Kerridge (1988), where mixed strains of *Salmonella* spp. are used with different temperature (10-35°C) and pH 5.5.

Dickson et al. (1992) investigated the ability of *Salmonella* Typhimurium to grow in beef at different combination of temperature (10, 12, 15, 20, 30, 35, 40°C) and pH (5.6- 6.3). The results of this study clearly demonstrate that the doubling time of growth of *Salmonella*

Typhimurium reduces as the temperature increases. The optimum growth rate was observed at 40°C with pH 6.2. In 2001, Dickson and Olson reported that the irradiation beef (2 KGy, 4KGy) has no effect on growth of mixed strains of *Salmonella* spp. at 15 and 25°C.

Different studies have investigated the ability of various *Salmonella* strains to survive or grow in ground beef or other beef products under different combination of temperature, pH, modified atmosphere-packaging, and presence of different chemical material (nitrogen, potassium sorbate, sodium nitrite, sodium lactate and diacetate, oregano essential oil, and nisin (Grau, 1983; Poershke and Cunningham, 1985; Gill and Delacy, 1991; Dickson and Siragusa, 1994; Mbandi and Shelef, 2001; Mbandi and Shelef, 2002; Skandamis et al., 2002; Tu and Mustapha, 2002; Nissen et al., 2002). The results from these studies clearly demonstrate that *Salmonella* spp. can survive and some times grow in ground beef dependent on the combination and concentration of the parameters.

Predictive food microbiology is a promising and rapidly developing area of food microbiology, which has achieved significant scientific attention in recent years. Mathematical models are an important tool for the quantitative estimation of microbial behavior (McMeekin et al., 1993). It requires a great amount of detailed technical data related to the growth and death responses of microorganisms under a variety of conditions. Despite the progress made by predictive microbiology, some aspects have not been totally considered. One of the difficulties in conducting a microbial risk assessment is in determining the number of microorganisms in food at the time it is consumed, i.e., exposure assessment (Walls and Scott, 1997). Numbers of bacteria in food can change at all stages of food production and processing, depending on the nature of the food and the way it is handled, stored and

processed. Predictive microbiology can be used to estimate changes in bacterial numbers, allowing exposure of an individual to a pathogen to be assessed.

### Risk assessment and Salmonella

Globalisation and the increasing international trade led to the foundation of the World Trade Organization (WTO) and the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) in 1995. The goal was to promote global free trade and non-discriminatory trading systems. The SPS Agreement requires the Member States to justify their sanitary and phytosanitary measures with scientific evidence (WTO, 1995). Since that, risk assessment on health risks related to food consumption has become one increasingly used option to do it, and along with this development the quantitative microbiological risk assessment (QMRA) arose in the 1990's with well-defined stages and procedures described by the Codex scheme (Codex Alimentarius, 1995).

A risk in the context of food safety is the probability and the consequence of adverse health effects following the ingestion of food. The separation of risk into two components is useful, since risk may be managed both by actions to reduce the probability and the consequences of the adverse event. The second component is not overlooked in microbial risk assessments, as it is explicity be considered in the selection of the biological end-point in the dose-response relationship.

It is extremely difficult for any government body or international agency to quantify the level of risk that a society is willing to tolerate or accept, or even to specify who has the ultimate responsibility to make such a decision. A quantification of the risk can be viewed as

the “cost” society is willing to bear to achieve a specific degree of control over a hazard, whether human, economic, ethical, medical or legal.

In principle, the methodology described in the Codex document is meant to be used by governments or by expert bodies in the context of Codex Alimentarius. Food industries have little experience with this methodology for estimating microbiological risks and are not particularly in favour of using it. The food industry interested in producing safe food assures safety by applying Good Hygienic Practices (GHP) and the Hazard Analysis and Critical Control Point (HACCP) system as prescribed by Codex (Codex Alimentarius, 1999). It means that potential hazards in raw materials and in processing lines are identified and analysed. Significant ones are identified and measures to prevent product contamination, to ensure elimination or reduction to acceptable levels are taken.

An evaluation of risk can be undertaken at many different levels, ranging from the use of one or more experts through an extensive risk profile to the use of formal qualitative or quantitative risk assessment. Although there is agreement in general that risk assessment should be used, there is no general agreement as to when to use it or what level of quantitative rigour the assessment process should have. Risk analysis consists of three components; risk assessment, risk management and risk communication, and can be described as a frame-work to analyse and manage any activity that may have negative consequences. In the context of food safety, it is a tool, which in a formalized, systematic and transparent way, enables responsible authorities and international organizations to understand and if necessary evaluate options to reduce a health risk. Risk assessment is a science-based process in which questions that have been formulated during the risk evaluation step of the risk management process are addressed to develop an understanding of the problem and to come up with risk estimates.

The first step of Microbiological Risk Assessment (MRA) is to decide on a *Statement of Purpose*. The specific purpose of the risk assessment needs to be clearly stated. The output and possible alternatives also need to be defined. The second step is one of *Hazard Identification*. This identifies the microorganisms or microbial toxin of concern and evaluates whether the microorganism or the toxin is a hazard when present in food. As the focus of the Risk Assessment is on a pathogen, available epidemiological and related data need to be used to determine if foodborne transmission is important to the disease and the foods that are implicated. If hazard identification is orientated towards the food, then the focus will use available epidemiological and microbiological data to determine which pathogens could be associated with the product. To carry out successfully hazard identification, quality public health data and information on the occurrence and levels of pathogenic microorganisms in the foods of concern need to be readily available. The next step in the Risk Assessment is *Exposure Assessment*. The ultimate goal of exposure assessment is to evaluate the level of microorganisms or microbial toxin in the food at the time of consumption. This may include an assessment of actual or anticipated human exposure. An accurate exposure assessment needs three types of information: (a) the presence of the pathogen in the raw ingredients; (b) the effect that food processing, distribution, handling and preparation steps have on the pathogen; and (c) consumption patterns e.g., portion size. Because the occurrence of a specific pathogen tends to be heterogeneously distributed in food, both the frequency and extent of contamination are needed. The fourth step is *Hazard Characterization*, which is the qualitative and/or quantitative evaluation of the nature of the adverse effects associated with biological, chemical and physical agents that may be present in foods. The most important component of a hazard characterization step is a dose-response assessment. The purpose of hazard characterization is to provide an estimate of the probability of the studied effect,

defined by its nature, severity and duration. Important factors to consider relate to the microorganism, the dynamics of infection and the sensitivity of host. The integration of the exposure and dose-response assessment gives the fifth step of the process, the *Risk Characterization.* This gives an overall probability of occurrence and severity of health effects in a given population. To be meaningful, the risk characterization should include a description of statistical and biological uncertainties. The final, sixth, step of the Risk Assessment is to produce a Report. This should contain a full and systematic record of the Risk Assessment. To ensure its transparency, the MRA report should indicate any constraints and assumption relative to the risk assessment.

The degree of confidence in the final estimation of risk will depend on the variability, uncertainty, and assumptions identified in all previous steps. Differentiation of uncertainty and variability is important in subsequent selections of risk management options. Uncertainty is associated with the data themselves, and with the choice of model. Data uncertainties include those that might arrive in the evaluation and extrapolation of information obtained from epidemiological, microbiological, and laboratory animal studies. Uncertainties arise whenever attempts are made to use data concerning the occurrence of certain phenomena obtained under one set of conditions to make estimations or predictions about phenomena likely to occur under other sets of conditions for which data are not available. Biological variation includes the difference in virulence that exist in microbiological populations and variability in susceptibility within the human population and particular subpopulations.

Risk management: in this element, the risk is evaluated and a decision can be made about the accepted risk within the wider framework of public health objectives (appropriate level of protection). Options for improvement are considered and new or modified criteria are

eventually laid down in guidelines, regulations or legislation. Risk communication involves transparent communication between risk assessors, risk managers and all other interested parties, which is important, because they have different interests. Finally, the results of risk assessment and risk management are communicated more widely with the relevant links in the food chain, up to, and including consumers, by such means as specifications and labelling.

The typical approach to conduct a quantitative risk assessment model (QRAM) is in a computer spreadsheet using probability distribution to model the variability and uncertainty of important risk factors, such as time, temperature and pathogen density. The QRAM is then simulated using a spreadsheet add-in program that randomly samples the probability distribution and uses the random numbers generated to perform calculations and generat outputs distributions (Vose, 1998). Once a risk assessment model has been developed, it can be used in many ways to identify and evaluate possible interventions to reduce risk. One approach is to run the model with different sets of input parameters, representing the effects of different control options.

In general, relatively few papers dealing with quantitative risk assessment models for *Salmonella* of food origin have been published in the scientific literature. Risk assessment models of the consumption of ground beef patties products have previously been developed for *E.* coli O157:H7 (Cassin et al., 1998). Estimation of the risk for public health linked to the consumption of ground beef contaminated by *Salmonella* provides useful information for the management of the risk. The major aim of the present thesis is therefore to report a risk assessment model of salmonellosis from the consumption of ground beef.

**Table 5.2:** Examples of quantitative risk assessment models for food pathogens published in the scientific literature

| Pathogen | Food commodity | Reference |
|---|---|---|
| *Salmonella* Enteritidis | Pasteurized liquid eggs | Whiting and Buchanan, 1997 |
| *Salmonella* spp. | Cooked poultry patty | Whiting, 1997 |
| *Salmonella* spp. | Whole chicken | Oscar, 1998 |
| *Escherichia coli* O157:H7 | Ground beef hamburgers | Cassin et al., 1998 |
| *Listeria monocytogenes* | Soft cheese | Bemrah et al., 1998 |
| *Salmonella* spp. | Chicken products | Brown et al., 1998 |
| *Bacillus cereus* | Chinese-style rice | McElory et al., 1999 |
| *Listeria monocytogenes* | Smoked salmon and trout | Lindqvist and Westoo, 2000 |
| *Salmonella enteritidis* | Shell eggs | Whiting et al., 2000 |
| *Escherichia coli* O157:H7 | Raw fermented sausages | Hoornstra et al., 2001 |
| *Salmonella* spp. | Turkey corden bleu | Bemrah et al., 2002 |
| *Salmonella*Typhimurium DT 104 | Dry-cured pork sausages | Alban et al., 2002 |
| *Salmonella enteritidis* | Shell eggs and egg products | Hope et al., 2002 |
| *Staphylococcus aureus* | Unripened cheese | Lindqvist et al., 2002 |
| *Escherichia coli* O157:H7 | Apples | Duffy and Schaffner, 2002 |
| *Compylobacter* spp. | Chicken | Rosenquist et al., 2003 |
| *Listeria monocytogenes* | Soft Cheese | Sanaa et al., 2004 |
| *Salmonella* spp. | Whole chicken | Oscar, 2004 |
| *Compylobacter* spp. | Poultry meat | Uyttendaele, 2006 |
| *Staphylococcus aureus* | Rice and seaweed | Rho, 2007 |
| *Compylobacter* spp. | Raw broiler chickens | Lindqvist, 2008 |

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# Chapter 3: Combination of Most-Probable-Number Method with LightCycler real-time PCR Assay (MPN-real-time PCR) for Rapid Quantification of *Salmonella* in Artificially and Naturally Contaminated Bovine Fecal Samples

## Abstract

As a part of our effort in quantitative risk analysis of food-borne diseases, the objective of this study was to develop rapid and reliable protocols for detection and quantification of *Salmonella* in bovine fecal samples at slaughter house. First, for the detection of *Salmonella* in artificially and naturally contaminated fecal samples, SYBR Green I real-time PCR assay was used, where quantification of *Salmonella* was achieved by combining this assay with most-probable-number (MPN) method (MPN-real-time PCR). To develop or test this protocol for detecting and enumerating of *Salmonella* in artificially contaminated fecal samples, a *Salmonella enterica* serotype Typhimurium DT104 strain was inoculated into fecal samples at different levels of contamination. Data on artificially contaminated fecal samples indicated that both detection and quantification protocols were able to detect and enumerate as few as 1 CFU/mL of fecal after 8-h of a single non-selective pre-enrichment step in buffered peptone water. All MPN estimates corresponded well to inoculum levels. The protocol was then applied to naturally contaminate fecal samples. A total of 296 fecal and 26 environmental samples were aseptically collected from slaughterhouse located in Meaux, France weekly in February and March 2006 (an average of 40 samples per visit). 9.12% (27/296), and 34.62% (9/26) fecal and environmental samples, respectively, were found *Salmonella*-positive, with estimated MPN values or counts of *Salmonella* ranging from <1.8- 1609 MPN/g of fecal samples. The mean of the log10 concentration of *Salmonella* is 0.6189 MPN/g with standrdard deviations of 2.7112 by using the censored regression approach. Counts were generally low, with the exception of 6 animals (>1400 MPN/g), while all the other 21 *Salmonella* positive animals had faeces with less than 80 MPN/g (from them 13 animals with MPN values <1.8 MPN/g. The prevalence of Salmonella showed no significant difference (p=1) between French (8.63%, 17/197) and Belgian cattle (10%, 10/99). Furthermore, neither the animals' area of origin (p=0.75), age

(p=0.18), race (p=0.94), breed (p=0.23), or movement of the animal (p=0.89) had any impact on the prevalence of *Salmonella*. The results of this study demonstrate that the combination of real-time PCR assay and MPN method constitutes an effective, rapid and easy-to-perform method for quantifying low levels of *Salmonella* in bovine fecal samples.

Key words: *Salmonella,* MPN-real-time PCR, Quantification, Fecal

Résumé

Comme une partie de notre effort dans l'analyse de risque quantitative de maladies portées d'aliments, l'objectif de cette étude était de développer des protocoles rapides et sûrs pour la détection et la quantification de *Salmonella* dans les échantillons fécaux bovins à la maison d'abattage. D'abord, pour la détection de *Salmonella* dans les échantillons fécaux artificiellement et naturellement contaminés, SYBR Green je l'essai de PCR en temps réel a été utilisé, où la quantification de *Salmonella* a été accomplie en combinant cet essai avec le "nombre le plus probable" (NPP) la méthode (PCR NPP-en-temps-réel). Pour développer ou évaluer ce protocole pour découvrir et énumérer de la *Salmonella* dans les échantillons fécaux artificiellement contaminés, une *Salmonella enterica* stéréotype Typhimurium DT104 l'effort a été inoculée dans les échantillons fécaux à de différents niveaux de contamination. Les données sur les échantillons fécaux artificiellement contaminés ont indiqué que tant la détection que les protocoles de quantification ont été en mesure de découvrir et énumérer en tout et pour tout que 1 CFU/mL de fécaux après 8 h d'un pas de pré-enrichissement non-sélectif simple dans buffered peptone l'eau. Toutes les estimations de NPP ont correspondu bien aux niveaux inoculum. Le protocole a été alors appliqué aux échantillons fécaux naturellement contaminés. Un total de 296 fécal et 26 échantillons de l'environnement était aseptically recueilli de l'abattoir trouvé dans Meaux, la France chaque semaine en février et le mars de 2006 (une moyenne de 40 échantillons par visite). 9.12 % (27/296) et 34.62 % (9/26) les échantillons fécaux et de l'environnement, respectivement, ont été trouvés Positif de S*almonella*, avec les valeurs de NPP estimées ou les comptes de *Salmonella* aux limites de <1.8-1609 NPP/g d'échantillons fécaux. La moyenne de la concentration log10 de *Salmonella* est 0.6189 NPP/g avec les déviations standrdard de 2.7112 en utilisant l'approche de rétrogradation censurée. Les comptes étaient généralement bas, à l'exception de 6 animaux (> 1400 NPP/g), pendant que tous les autres 21 *Salmonella* les animaux positifs avait des

matières fécales avec moins de 80 NPP/g (d'eux 13 animaux avec les valeurs de NPP <1.8 NPP/g. La prévalence de *Salmonella* n'a montré aucune différence significative (p=1) entre le français (8.63 %, 17/197) et bétail belge (10 %, 10/99). En outre, aucun la région des animaux d'origine (p=0.75), l'âge (p=0.18), la course (p=0.94), la race (p=0.23), ou le mouvement de l'animal (p=0.89) n'avait aucun impact sur la prévalence de *Salmonella*. Les résultats de cette étude démontrent que la combinaison d'essai de PCR en temps réel et de méthode NPP constitue une efficace, rapide et une méthode "facile de jouer" pour quantifier des niveaux bas de *Salmonella* dans les échantillons fécaux bovins.

Mots clé : la *Salmonella*, PCR NPP-en-temps-réel, La quantification, Fécale

# 1. Introduction

*Salmonella* is one of the most common foodborne pathogens transmitted to humans (CDC, 2000). Foodborne salmonellosis has been the major cause of all foodborne disease caused by salmonella species (Ray, 1996). Over 2500 different serotypes of *Salmonella* have been described to date, the most prevalent of which are being Enteritidis, Typhimurium and Heidelberg (D'Aoust et al., 1997). This enterobacterial pathogen is responsible for a foodborne illness called salmonellosis. Fever, nausea, sometimes vomiting, abdominal cramps and diarrhoea characterize human salmonellosis. Meat and meat products from beef have been associated with severe food poisoning outbreaks caused by *Salmonella enterica* serotypes around the world (Davies et al., 1996; Fazi, 1996). *Salmonella* can be carried by healthy cattle at slaughter (McEvoy et al., 2003; Samuel et al., 1979) and therefore may serve as a reservoir and source of contamination of carcasses during processing. Therefore, for food safety, rapid, sensitive, and specific detection and quantification techniques of foodborne pathogens in food products and fecal samples that might contaminate foodstuffs are needed. Conventional cultural methods for the detection of *Salmonella* in fecal samples are time consuming and usually require 4 days to presumptively identify *Salmonella* in a test sample. For this reason, methods based on polymerase chain reaction (PCR) have been increasingly used for the detection of *Salmonella* cells in various foods and different matrices (Ferretti et al., 2001; Aslam et al., 2003). However, these assays usually rely on visualizing the amplification product by ethidium bromide staining after agarose gel electrophoresis which is labor and time-intensive. To reduce the time required for detection of *Salmonella* spp. in foods, the time-consuming conventional PCR assays are gradually being replaced by more convenient real-time PCR assays, which represent a significant progress to PCR-based methods for a broad range of applications. A number of real-time PCR-based assays for the detection of *Salmonella* in foods have already been described (Jothikumar et al., 2003; Bhagwat, 2004;

Liming and Bhagwat, 2004). This technology which combines amplification and detection in a one step closed-tube reaction, presents many advantages such as high sensitivity, high specificity, and lower risks of cross-contamination (Rodrigez-Lázaro et al., 2003).

Several studies have identified the lack of quantitative data on levels of contamination in contaminated foods as a key data gap for the development of risk assessment for pathogens (Coleman and Marks, 1999). *Salmonella* cells can be enumerated by applying the classical microbiological quantification techniques, such as the plate counting methods and the most-probable-number (MPN) method. Some of these techniques require up to 6 days for detection and quantification, thus once again pose the problem of being labor-intense and time-consuming. Recently, the use of real-time PCR assays for quantification of initial target DNA has overcome this disadvantage of the time factor. Unfortunately, amplification efficiencies of these quantification assays can be difficult to ensure and their suitability for exact quantification of initial amount of target DNA has therefore been questioned (Klerks et al., 2004).

In this study, an alternative approach is presented. PCR products can be quantified by combining the principles of the Most-Probable-Number (MPN) statistics and LightCycler real-time PCR. Through the use of this approach, we sought to develop a rapid and simple MPN-real-time PCR protocol (MPN-real-time PCR) based on the double-stranded DNA (dsDNA) binding dye SYBR Green I for the detection and quantification of *Salmonella* spp. in contaminated fecal samples. To our knowledge, this is the first report in which a LightCycler real-time PCR detection method is combined with the MPN method to enumerate *Salmonella* spp. in fecal samples. However, methods based on MPN-conventional PCR (MPN-PCR) have previously been described for the detection and enumeration of different

micro-organisms (Fredslund et al., 2001; Martin et al., 2004). The first objective of the present work was to develop MPN-real-time PCR assay for the quantification of *Salmonella* in experimentally contaminated fecal samples. The second objective was to apply this developed assay to enumerate *Salmonella* in naturally contaminated fecal samples obtained from a slaughter house located in Meaux, France.

## 2. Materials and methods

### 2.1. Bacterial strains

To determine the specificity of the LightCycler real-time PCR assay, frozen stock cultures of 3 different serotypes of *Salmonella enterica* and 7 strains of non-*Salmonella* species, including strains in the family of *Enterobacteriaceae* closely related to *Salmonella*, such as *E. coli*, *C. freundii*, *K. pneumoniae* and *Shigella* spp. (Table 1). The bacteria were transferred into BHI broth and incubated overnight at 37°C. These overnight bacterial cultures were subsequently subjected to DNA extraction and real-time PCR assay. The bacterial strains were obtained from the collection of the LERQAP (Laboratoire d'étude et de recherche sur la qualité des aliments et des procédés agroalimentaires) of the French Food Safety Agency (Agence Française de Sécurité Sanitaire des Aliments, AFSSA), located in Maisons-Alfort, France.

### 2.2. Sensitivity of the real-time PCR assay with pure cultures

The sensitivity of the real time-PCR assay was evaluated using pure cultures of three strains of *Salmonella enterica* belonging to different serotypes (Table 1). Cells were grown overnight at 37°C in BHI broth. Ten-fold serial dilutions of each pure culture were prepared in Buffered Peptone Water (BPW; Difco, Becton Dickinson). To determine cell numbers, appropriately diluted cultures were spread-plated on Xylose-Lysine-Tergitol-4 agar (XLT-4;

Difco) in ten replicate plates. Plates were incubated overnight at 37°C. All dilutions were then incubated at 37°C for 6, 8 and 16 h of nonselective enrichment. After each pre-enrichment period, 1.5 mL-aliquot was collected from each dilution into microcentrifuge tubes and subjected to DNA extraction and real-time PCR assay. Reproducibility of SYBR Green real-time PCR was assessed by running samples independently on different days

**Table 1.3.** Strains used in this study

| Species | Source | Strain no. | SYBR Green I real-time PCR | |
|---|---|---|---|---|
| | | | $C_T$[1] | $T_m$= 87.2± 0.5°C[2] |
| Other strains | | | | |
| *Escherichia coli* | Hospital | 49 | 33.82 | _ |
| *Klebsiella pneumoniae* | Hospital | 84 | > 36 | _ |
| *Enterobacter cloacae* | Hospital | 59 | > 36 | _ |
| *Shigella sonnei* | Hospital | 65 | > 36 | _ |
| *Yersinia spp.* | Hospital | 81 | 32.06 | _ |
| *Citrobacter freundii* no[1] | Hospital | 55 | 31.07 | _ |
| *Citrobacter freundii* no[2] | Bovine/kidney | 4525.04 | 31.77 | _ |
| *S. enterica* serotype | | | | |
| *S*. Hadar | Steak/Gorden-bleu [3] | TQA 042 | 11.72 | + |
| *S*. Enteritidis | Bovine/feces | 9211.02 | 12.24 | + |
| *S*.Typhimurium DT104 | Avian | 13887.03 | 11.94 | + |

[1]$C_T$= Threshold PCR cycle is defined as the cycle at which a significant increase in the fluorescence is first recorded.

[2]The presence of PCR product (+) indicates amplification of specific product.

[3]Cordon-bleu = specific turkey product.

## 2.3. Fecal samples

For the development of detection and quantification protocols, beef fecal samples were initially simultaneously analysed by cultural methods and real-time PCR to determine that they were negative for Salmonella. Then, negative faecal samples were artificially inoculated with *S. enterica* serotype Typhimurium DT104 at different levels of contamination.

The application of developed protocols was performed on naturally contaminated beef fecal samples; a total of 296 feces and 26 environmental samples were aseptically collected from slaughterhouse located in Meaux, France. The collection of samples was done weekly in February and March 2006. An average number of 40 samples per visit were collected. For each animal, approximately 75 g of fecal was collected after evisceration from the lower intestine and placed into a cool box with ice packs and transported to the laboratory at the end of the morning. In addition, 26 environmental samples were carried out. For each fecal sample, the information collected related to breed, sex, age, animal weight, area of origin, place of birth and its movement.

## 2.4. Real-time PCR detection protocol of fecal samples

*2.4.1. Development of the detection assay on artificially contaminated fecal samples.* For the artificial inoculation procedures, the exact numbers of *Salmonella* cells were determined by plating 0.1-mL aliquots of suitable 10-fold dilutions onto XLT-4 agar plates in ten replicates and incubating them overnight at 37°C. The dilutions were then kept refrigerated at 4°C for 24 h. When the *Salmonella* cells were added to the fecal after being confirmed *Salmonella*-negative by both culture methods and real-time PCR, the estimated cell concentration of the inoculum was determined for a second time. 25 g of fecal samples were inoculated with the following estimated levels of contamination: 1 to 5, 10 to 20, and 100 CFU/mL before being

homogenized in 225 mL of BPW by mixing. The homogenates were then pre-enriched for 6, 8, and 16 h at 37°C in order to determine the shortest enrichment time needed to detect the lowest level of contamination. At each time point, aliquots were withdrawn and appropriate 10-fold serial dilutions of each spiked pre-enrichment broth were spread-plated on XLT-4 agar plates in triplicates and incubated overnight at 37°C before counting colonies and calculating CFU. In addition, other aliquots of each spiked pre-enrichment broth were collected and subjected to DNA extraction for the real-time PCR assay.

2.4.2. Application of the detection assay on naturally contaminated fecal samples. For fecal samples, 5 g of each fecal sample were added to 45 mL BPW. After being thoroughly mixed, the mixtures were pre-enriched for 18 h at 37°C. 1.5-mL aliquots of each pre-enrichment broth were collected and subjected to DNA extraction for the real-time PCR assay as described below. The remaining quantities of fecal samples were stored at 4°C to be used for quantification protocol in case of *Salmonella*-positive results.

## 2.5. The MPN- real-time PCR quantification protocol of fecal samples

2.5.1. Development of the method on artificially contaminated fecal samples. MPN assays (ten-tube method) for *S. enterica* serotype Typhimurium DT104 was performed according to the procedures described in FDA' Bacteriological Analytical Manual (BAM) (Administration, 2001). The tubes were then incubated for 6, 8 and 16 h at 37°C in order to optimize the incubation period for the MPN-real-time PCR method. After each incubation period, 1.5 mL of each enriched tube was drawn and processed for DNA extraction. Immediately after DNA extraction, the MPN-real-time PCR runs were carried out on DNA templates by following the procedures described in the SYBR Green real-time PCR assay section. From the amplification results the number of positive and negative capillary tubes was scored and the MPN

calculations were made with a computer-assisted spreadsheet (Garthright and Blodgett, 2003). The spreadsheet can be found on the website of the Bacteriological Analytical Manual (BAM) of the U.S Food and Drug Administration (BAM/FDA) (www.cfsan.fda.gov/~ebam/bam-a2.html). The repeatability of the MPN-real-time PCR assay was determined by repeated measurements of the same sample. The repeatability was estimated by computing the Coefficient of Variation of log MPN (CV%). The CV was calculated as the standard deviation deivded by the mean. If the CV values were less than 20%, the repeatability considered to be acceptable.

2.5.2. Application of the method on naturally contaminated fecal samples. Fecal samples that tested *Salmonella*-positive with the real-time PCR detection assay were subjected to enumeration assay with MPN-real-time PCR and to isolation of presumptive *Salmonella* colonies using conventional culture methods. 25 mL of each *Salmonella*-positive fecal sample was homogenized in 225 mL of BPW by mixing. MPN assays (five-tube method) were performed according to the procedures described in BAM (Administration, 2001). The tubes were then incubated at 37°C for 8 h (optimal incubation time determined by MPN real-time PCR assay of artificially contaminated fecal samples). After incubation period, 1.5 mL of each enriched tube was drawn and processed for DNA extraction. The MPN-real-time PCR runs were carried out on DNA templates by following the procedures described in SYBR Green real-time PCR conditions section. From the amplification results the number of positive and negative capillary tubes was scored and the MPN calculations were made with a computer-assisted spreadsheet (Garthright and Blodgett, 2003).

## 2.6. DNA extraction procedures

DNA was extracted from pure cultured strains and from pre-enriched cultures of artificially and naturally contaminated fecal samples. Aliquot of enriched sample was transferred to 2-mL microcentrifuge tube. The cell suspension was centrifuged for 10 min at 12,000 rpm. The supernatant was discarded carefully. The pellet was resuspended in 100 μL of sterile distilled water by vortexing. The tube was centrifuged again at 12,000 rpm for 10 min, and the supernatant was discarded carefully. The pellet was resuspended once again in 100 μL of sterile distilled water by vortexing and boiled in a water bath for 10 min. After heat treatment the tube was immediately centrifuged for 10 min at 12,000 rpm. The supernatant was carefully transferred to a new microcentrifuge tube and stored at –20 °C until the real-time PCR assay was performed. An aliquot of 2 μL of the supernatant was used as the template DNA in the real-time PCR assay.

## 2.7. SYBR Green real- time PCR assay

The *Salmonella* specific primers ST11 (5'-AGCCAACCATTGCTAAATTGGCGCA-3') and ST15 (5'-GGTAGAAATTCCCAGCGGGTACTG-3'), originally designed by Aabo et al., 1993), and previously shown to be highly specific for *Salmonella* (Aabo et al., 1993; Bansal et al., 2006) were purchased from Proligo (Paris, France ) and used to amplify a 429-bp.

Real-time PCR reactions were performed with the LightCycler PCR instrument (Roche Diagnostics) using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Meyla, France). The reaction mixture contained the following concentrations of reactants: 2 μL of LightCycler-Faststart DNA Master SYBR Green I (1 X concentration), 4 mM $MgCl_2$, 0.4 μM of each primer, 2 μL of template DNA, and sterile PCR grade water to a total volume of 20 μL per capillary. Each LightCycler run contained one negative control

consisting of $H_2O$ without any template DNA to monitor for possible contamination and one positive control (*S. enterica* serotype Typhimurium DT104; AFSSA 13887.03). Mixing of the reagents for the PCR was accomplished under laminar flow in a clean room separate from the one where DNA templates were prepared. Master mixture and extracted DNA were placed into glass capillaries, sealed with a plastic cap, centrifuged, (3000 rpm for 15 sec.) and placed into the LightCycler™ carousel (Roche Diagnostics).

The thermal cycling program for the LightCycler™ has four phases: denaturation, amplification, melting and cooling. In the initial denaturation phase the capillary is heated to 95°C for 10 min, followed by 40 to 45 cycles of amplification phase of 10 s at 95°C, annealing for 10 s at 66°C, and extension for 20 s at 72°C. Signal detection was performed at the end of the extension step with a single fluorescence acquisition for each capillary. The melting curve analysis phase began with 95°C for 0 s, then cooled to 73°C for 30 s before the temperature was raised to 95°C at a rate of 0.1 °C/s. Fluorescence acquisition was performed continuously during this phase. Finally, the cooling phase lasted for one minute at 40°C. Melting temperature ($T$m) peaks were calculated based on initial fluorescence curves ($F/T$) by plotting negative derivative of fluorescence over temperature versus temperature ($-d(F)/dT$ versus $T$).

**2.8. Isolation of positive colonies from fecal samples**

For isolation of presumptive *Salmonella* colonies from PCR-positive fecal samples, 0.1 mL of non-selective pre-enrichment mixture was transferred to 10 mL of Rappaport-Vassiliadis (RV) medium and another 0.1 mL to 10 mL of Mueller-Kauffman tetrathionate broth (AES Laboratoire, France). Rappaport-Vassiliadis (RV) medium selective enrichment was carried out for 22-24 h at 42°C, and Muller-Kauffmann tetrathionate broth was incubated

for 22-24 h at 37°C. Both selective enrichment broths were streaked onto xylose lysine tergitol-4 (XLT-4) agar plates (Difco) and xylose lysine deoxycholate (XLD) agar plates. Plates were then incubated for 22-24 h at 37°C. If growth was slight or if no typical colonies were found, the plates were reincubated for a further 24 h at 37°C.

### 2.9. Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were performed by the disk diffusion method on Mueller-Hinton agar (BioRad, Marne la Coquette, France). The panel of antibiotics tested (load, breakpoints(mm)) was recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM): ampicillin (10 µg, 19-14), amoxicillin + clavulanic acid (20 µg, 21-14), cephalothin ( 30 µg, 21-15), streptomycin (10 IU, 15-13), gentamicin (10 IU, 16-14), kanamycin (30 IU, 17-15), chloramphenicol (30µg, 23-19), tetracycline (30 IU, 19-17), sulfamethoxazole-trimethoprim (23.75 + 1.25µg, 16-10), sulphonamides (200 µg, 17-12), nalidixic acid (30 µg, 20-15), ofloxacin (5µg, 22-16), enrofloxacin (5 µg, 22-17) and colistin (50 µg, 15). Zone diameters were read using the automated scanner Osiris (BioRad). In this study, if an isolate resistant to, at least, two antimicrobials within two different antimicrobial families were considered multidrug resistant.

### 2.10. Data management and statistical procedure

The collected data and the laboratory results were stored in a Microsoft excel file and coherence tests were applied to these data. The SAS software v.9.1 (SAS Institute, Inc.) (Cary, 1988) was used for statistical analysis. The Chi-square or Fisher testes were used to identify a significant difference ($p < 0.05$) between breed, sex, age, area of origin, place of birth and its movement. And also the FREQ procedure was used for each level of data. The maximum likelihood estimation (MLE) with proc lifereg on SAS (Lorimer and Kiermeier,

2007), assuming an underlying normal distribution for the log10 concentraction, was used to calculate an estimate for the mean and standard deviation of the concentration of the *Salmonella* in bovine feces.

## 3. Results

### 3.1. Sample description

A total of 296 fecal and 26 environmental samples were collected from slaughterhouse located in Meaux, France. The animals were 32% blanc-bleu (n=95), 27.4% charolaise (n=80), 12.5% blonde-d'Aquitaine (n=37) and the other 13 breeds accounted for 28% (Table 2.3). Cows represented 72.9% (n=216) of the animals, bulls 18.6% (n=55) and castrated animals 7.7% (n=23), while two animals were not identified (missing data). Table 3.3 shows the proportion of each age group with its percentage. Most of the animals came from Basse-Normandie (27%, n=81), 11.8% (n=35) from Haute-Normandie and 33.4% (n=99) from different regions of Belgium as showed in table 4. The obtained data from the slaughter house showed that 58% of the animals (n=171) had been reared in their region of birth (no movement), while the remaining ones (n=123) had been reared in a region different from their birth region (movement). Most of the animals (n=245, 82.8%) were beef cattle, while 14.9% were dairy cattle (n=44) and the remainder (n=7, 2.3%) belonged to mixed breeds as shown in table 5.3.

### 3.2. Specificity of real-time PCR

The real-time PCR assay correctly classified all the serotypes of *Salmonella* tested as *Salmonella* and the other the non-*Salmonella* species, including strains in the family of *Enterobacteriaceae* closely related to *Salmonella*, such as *E. coli*, *C. freundii*, *K. pneumoniae* and *Shigella* spp. as non-*Salmonella*. Table 1.3 shows the $C_T$ (Threshold PCR cycle) and $T_m$ (Melting temperature) values from the SYBR Green I real-time PCR analysis. Specificity was

assessed by the $T_m$s of the amplification products immediately after the reaction cycle. This amplification resulted in product with a $T_m$ of 87.2 ± 0.5°C. Negative controls and samples confirmed negative did not show peaks in $T_m$ that corresponded to 87.2 ± 0.5°C. Figure 1.3 shows the melting peak analysis of the amplified products in real-time PCR for positive (*S.* Enteritidis, *S* .Hadar and *S. entrica* serotype Typhimurium DT104) and representative negative (*Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii* and *Klebsiella pneumoniae* ).

**Table 2.3.** Breeds and types of the studied animals

| Race | Type of the animal | Number of animals (Percentage %) | Real-time-PCR Positive (Percentage %) |
|---|---|---|---|
| Armoricaine | Mixed breed | 3 (1.01%) | 0 (0%) |
| Bazadaise | Beef cattle | 1 (0.33%) | 0 (0%) |
| Blanc-Bleu | Beef cattle | 95 (32.09%) | 9 (9.47%) |
| Blonde-d'Aquitaine | Beef cattle | 37 (12.5%) | 5 (13.51%) |
| Casta-(Autre-et-ST-Girons) | Beef cattle | 2 (0.67%) | 0 (0%) |
| Charolaise | Beef cattle | 80 (27.03%) | 7 (8.75%) |
| Croisé | Dairy cattle | 29 (9.79%) | 1 (3.45%) |
| Dairy-Shorthorn | Dairy cattle | 1 (0.33%) | 0 (0.00%) |
| Limousine | Beef cattle | 13 (4.39%) | 2 (15.38%) |
| Montbeliarde | Mixed breed | 3 (1.01%) | 0 (0.00%) |
| Normande | Mixed breed | 1 (0.33%) | 0 (0.00%) |
| Parthenaise | Beef cattle | 2 (0.68%) | 0 (0.00%) |
| Prim'Holstein | Dairy cattle | 14 (4.73%) | 0 (0.00%) |
| Rouge-des-Prés | Beef cattle | 10 (3.38%) | 1 (10.00%) |
| Salers | Beef cattle | 3 (1.01%) | 1 (33.33%) |
| Unknown | | 2 (0.68%) | 1 (50.00%) |
| Total | | 296 (100.00%) | 27 (9.12%) |

**Table 3.3.** Age groups and average weights of sampled animals.

| | Number of animals (Percentage %) | Average of the Weight (sd) | Real time PCR Positive (Percentage %) |
|---|---|---|---|
| Male calf | 12 (4.05%) | 154,84 (26,99) | 1 (8.33%) |
| Young bull | 19 (6.42%) | 349,29 (52,30) | 0 (0%) |
| Bull | 24 (8.11%) | 404,14 (102,31) | 1 (4.17%) |
| Castrated animal | 23 (7.77%) | 526,42 (53,49) | 1 (4.35%) |
| Female Calf | 3 (1.01%) | 150,27 (26,45) | 0 (0%) |
| Heifer | 31 (10.47%) | 433,01 (56,96) | 7 (22.58%) |
| Cow | 182 (61.49%) | 459,02 (66,60) | 16 (8.79%) |
| Unknown | 2 (0.68%) | | 1 (50%) |
| Total | 296 | | 27 (9.12%) |

**Table 4.3.** Geographical origin of sampled animals.

| Region or country | Number of animals (Percentage %) | Real time PCR Positive (Percentage %) |
|---|---|---|
| Aquitaine | 2 (0.68%) | 0 (0%) |
| Basse-Normandie | 81 (27.36%) | 9 (11.11%) |
| Bourgogne | 6 (2.03%) | 0 (0%) |
| Champagne-Ardenne | 7 (2.36%) | 0 (0%) |
| Haute-Normandie | 35 (11.82%) | 2 (5.71%) |
| Ile-de-France | 21 (7.09%) | 3 (14.29) |
| Nord-pas-de-Calais | 14 (4.73%) | 0 (0%) |
| Pays de Loire | 2 (0.68 %) | 0 (0%) |
| Picardie | 27 (9.12%) | 2 (7.41%) |
| Belgium | 99 (33.4%) | 10 (10%) |
| Unknown | 2 (0.68%) | 1 (50%) |
| Total | 296 | 27 (9.12%) |

**Table 5.3.** Animal breed with its percentage, prevalence

| Breed | Number of animals (Percentage %) | Real time PCR Positive (Percentage %) |
|---|---|---|
| Beef cattle | 245 (82.77%) | 26 (8.78%) |
| Dairy cattle | 44 (14.86%) | 1 (0.34%) |
| Mixed | 7 (2.36%) | 0 (0%) |
| Total | 296 | 27 (9.12%) |

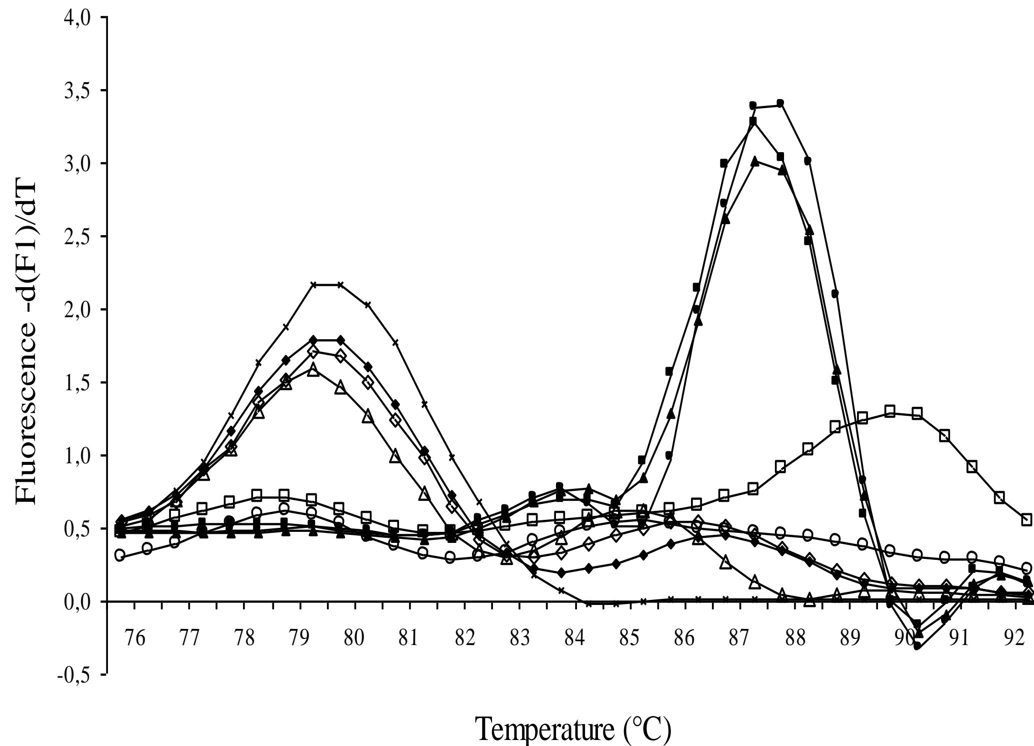


**Figure 1.3.** Melting curve analysis of amplified PCR products using ST11 and ST15 primers for *Salmonella enterica* serotypes: S. typhimurium DT104 (●); S. Hadar (■); and S. *enteritidis* (▲); and non-*Salmonella* strains: *Escherichia coli* (Δ); *Enterobacter* cloacae(○); *Klebsiella pneumoniae*(□); *Citrobacter freundii* no1 (◊);*Citrobacter freundii* no2 (♦); and water(negative control) (x).

## 3.3. Detection limits in pure cultures

The study was performed on the three *Salmonella* strains. Using our described real-time PCR assay with an 8 h pre-enrichment step in BPW, it was possible to detect as few as 1 CFU/g of pure cultures from each of the three strains. Experiments were carried out three times and good reproducibility was observed (data not shown).

## 3.4. Detection of *Salmonella* from artificially contaminated fecal samples

Fecal samples inoculated with *S. enterica* serotype Typhimurium DT104 at the estimated levels of contamination (1-5, 10-20, and 100 CFU/mL) gave negative results without enrichment, whereas the real-time PCR assay detected the bacteria in fecal samples

even at a low level of contamination after enrichment. When spiked fecal samples were enriched in BPW for 6 h, inoculum levels of 10 and 100 CFU/mL were detected. When the inoculation levels were 1 CFU/mL, 8 or 16 h of enrichment were necessary to detect them, thus leading to the definition of the optimal enrichment time as 8 h. The relative detection limits of the real-time PCR assay for *S. enterica* serotype Typhimurium DT104 in artificially contaminated fecal samples were consistent with DNA purified from serial dilutions of broth cultures of the three *Salmonella* strains and with inoculated fecal samples.

### 3.5. Detection of *Salmonella* from naturally contaminated fecal samples

The collection of samples was done weekly in February and March 2006 (an average of 40 samples per visit). After 18 h of enrichment, 9.12% (27/296), 34.62% (9/26) fecal and environmental samples respectively were found *Salmonella* positive. The prevalence of *Salmonella* positive samples didn't show any significant difference (p=1) between French (8.63%, 17/197) and Belgian cattle (10%, 10/99). Furthermore, neither the animals' area of origin (p=0.75), age (p=0.18), race (p=0.94), breed (p=0.23), or movement of the animal (p=0.89) had any impact on the prevalence of *Salmonella*. The real-time PCR positive samples were further analyzed with the traditional cultural methods while the negative ones were discarded.

### 3.6. Enumeration of *Salmonella* in artificial and naturally contaminated fecal samples

For fecal samples inoculated with *S. enterica* serotype Typhimurium DT104, a non-selective pre-enrichment time of 8 h in buffered peptone water was found optimal to obtain MPN-real-time PCR estimates close to the contamination levels (Table 6.3). With the developed MPN-real-time PCR assay it was possible to enumerate approximately 1 CFU *Salmonella* per g of fecal within 11-12 h, which included an 8 h enrichment and 3-4 h period

to carry out the sample preparation and real-time PCR assay. The MPN-real-time PCR estimates correspond well to the estimated level of contamination inoculated into the samples. Clear positive peaks were observed in most positive tubes originating from fecal samples inoculated with 100 and 10-20 cells per g of fecal (Figure 2.3A, B), while in the case of the samples inoculated with low levels 1-5 cells per g of fecal, weak fluorescence peaks were observed (Figure 2.3C). Fares (2007) has demonstrated that the MPN-real-time PCR assay (by using contaminated milk samples) has acceptable repeatability with a coefficient of variation (CV%) of less than 20% for inoculum levels of 100 and 10-20 CFU/mL; but larger variation were observed in samples with inoculum level of 1-5 CFU/mL. Arguably, this was due to the low level of inoculum.

All fecal samples that tested positive with the LightCycler real-time PCR detection assay were subjected to enumeration assay with MPN-real-time PCR for enumeration of Salmonella per gram fecal. . The mean of the log10 concentration of *Salmonella* in positive bovine fecal samples are 0.6189 MPN/g with standard deviations of 2.7112 (table 7.3). Counts were generally low, with the exception of 6 animals (>1400 MPN/g), while all the other 21 animals were less than 80 MPN/g (from them 13 animals <1.8 MPN/g) as show in table (8.3).

**Table 6.3.** Enumeration of *Salmonella enterica* serotype Typhimurium DT104 in artificially contaminated fecal samples using MPN-real-time PCR method after 8 h pre-enrichment in BPW

| Sample no. | Contamination level (CFU/mL) | Plate counting (CFU/mL) | MPN estimate (MPN/mL) | MPN confidence limits (low/high) |
|---|---|---|---|---|
| 1 | 1-5 | 2 | 4.3 | 1.9 - 9.5 |
| 2 | 1-5 | 1 | 2.2 | 0.6 - 8.9 |
| 3 | 1-5 | 2 | 3.6 | 1.1 - 11.1 |
| 4 | 10-20 | 8 | 9.5 | 4.9 - 18.3 |
| 5 | 10-20 | 15 | 28.7 | 13.1 - 63.0 |
| 6 | 10-20 | 12 | 19.6 | 10.2 - 37.9 |
| 7 | 100 | 105 | 120.3 | 55.4- 160.5 |
| 8 | 100 | 111 | 138.1 | 133.3 - 464.1 |
| 9 | 100 | 95 | 111.6 | 105.2 – 360 |

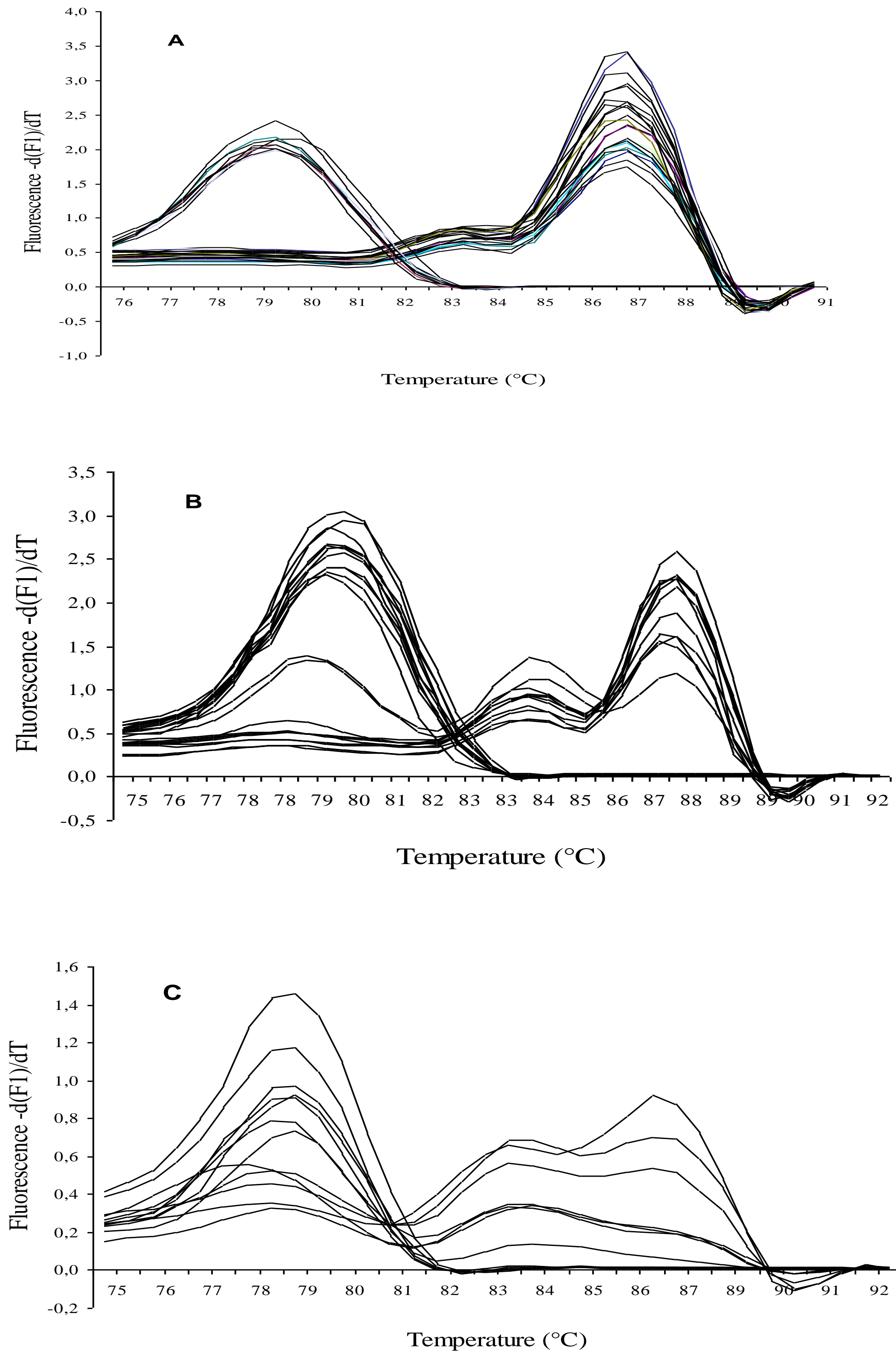


**Figure 2.3**. MPN-real-time-PCR analysis of feces inoculated with *Salmonella enterica* serotype Typhimurium DT104. DNA extracted from fecal samples after 8h non-selective enrichment in BPW: Inoculum level of 100 CFU/g (A); Inoculum level of 10-20 CFU/g (B); and Inoculum level of 1-5 CFU/g (C). Some positive and negative peaks were omitted from these graphs for clarity.

**3.7. Confirmation of the specificity of real-time PCR products by DNA melting temperature analysis**

In the SYBR Green I real-time PCR, the amplification of the DNA target is expressed as a threshold cycle ($C_T$). The $C_T$ represents the number of reaction cycle at which the reporter fluorescence raises above a set baseline threshold, and indicates that the DNA amplicon is replicating exponentially. Immediately following amplification, the products were melted, and the release of fluorescence dye measured to generate melting curves from which $T_m$ was calculated. $T_m$ is dependent upon the length of the amplified DNA, as well as the G/C content of the sequence (Bhagwat, 2003). As the $T_m$ is reached, the DNA denatures and releases SYBR Green I, causing a sharp decline in fluorescence. This decrease in fluorescence is plotted as the negative derivative of fluorescence over temperature versus temperature (-$d(F)/dT$ versus $T$) giving a melting peak and $T_m$ for each PCR product. In artificially contaminated milk assay, the average real-time PCR $T_m$ value (mean plus standard deviation from a range of 8 to10 assays) of the specific products was 87.6°C (±0.4). These results were consistent with naturally contaminated fecal samples in which the mean real-time PCR $T_m$ of twenty seven positive fecal samples was 87.2°C (±0.4). Other real-time assays (Mercanoğlu and Griffiths 2005) have reported similar results. However, variations of more than 1°C in the minimum and maximum $T_m$s have been reported from other studies (Eyigor et al. 2002). The average $T_m$ of the negative controls was 79.7°C (±0.2). Bhagwat (2003) reported that primer-dimers which are typically shorter in length usually melt at a much lower $T_m$ than the intended product and are therefore easy to distinguish whereas secondary or non-specific products can be of varying lengths and sequences and therefore have a large range of possible melting temperatures.

### 3.8. Classic PCR and isolation of *Salmonella*

All fecal samples that tested positive with the LightCycler real-time PCR detection assay were subjected to classic PCR and to isolation of presumptive *Salmonella* colonies using conventional culture methods. 66.7% (18/27) of samples that tested positive by the LightCycler real-time PCR detection assay were positive with conventional PCR. The majority of the conventional negative samples (78%, 7/9) was <1.8 MPN/g, while the other two were 27 MNP/g and 53.9 MPN/g as show in table 8.3.

*Salmonella* Typhimurium was the only serotype isolated in this study from the fecal animals with multidrug resistance (penta and FQ R) and *Salmonella* Derby was also the only serotype isolated from environmental sample with a multidrug resistance (streptomycin Sm, Te, Su).

**Table 7.3.** Parameter estimates for the log10 concentration of *Salmonella* in gram bovine fecal by using maximum likelihood estimation (MLE) with proc lifereg on SAS.

| | Estimation | Standard error | 95% confidence limits | |
|---|---|---|---|---|
| | | | low | high |
| Mean | 0.6189 | 0.6117 | -0.5800 | 1.8178 |
| Standard deviations | 2.7112 | 0.7296 | 1.5999 | 4.5945 |

**Table 8.3.** Quantification estimates of *Salmonella* in fecal samples obtained with MPN-real time PCR assay after 8 h pre-enrichment in BPW and result of the classic PCR of all fecal samples that tested positive with the LightCycler real-time PCR detection assay .

| Week | Sample order | Weight (Kg) | Type of animals | Real time PCR | MPN-PCR (g) | Classic PCR |
|---|---|---|---|---|---|---|
| 1 | 4 | 517 | Cow | + | 53.9 | - |
| 1 | 5 | 522 | Cow | + | <1.8 | - |
| 1 | 16 | 493 | Heifer | + | 5.56 | + |
| 1 | 20 | 296 | Cow | + | 20.5 | + |
| 2 | 5 | 543 | Cow | + | >1600 | + |
| 2 | 20 | 464 | Cow | + | >1600 | + |
| 2 | 32 | 473 | Cow | + | 27.1 | - |
| 3 | 20 | ND | ND | + | <1,8 | + |
| 4 | 4 | 604 | Cow | + | 9.3 | + |
| 4 | 18 | 597 | Castrated animal | + | 1.98 | + |
| 4 | 43 | 308 | Bull | + | <1.8 | - |
| 6 | 1 | 196 | Cow | + | 1609 | + |
| 6 | 11 | 471 | Cow | + | >1600 | + |
| 6 | 16 | 408 | Cow | + | 42.6 | + |
| 6 | 18 | 389 | Male Calf | + | >1600 | + |
| 6 | 35 | 344 | Cow | + | >1600 | + |
| 7 | 1 | 454 | Cow | + | <1.8 | + |
| 7 | 15 | 473 | Heifer | + | <1.8 | - |
| 7 | 21 | 466 | Cow | + | <1.8 | - |
| 7 | 22 | 498 | Cow | + | 74.8 | + |
| 7 | 23 | 466 | Cow | + | <1.8 | - |
| 8 | 6 | 368 | Heifer | + | 18 | + |
| 8 | 10 | 402 | Heifer | + | <1.8 | - |
| 8 | 12 | 413 | Heifer | + | <1.8 | + |
| 8 | 15 | 370 | Cow | + | <1.8 | - |
| 8 | 21 | 436 | Cow | + | <1.8 | + |
| 8 | 29 | 431 | Heifer | + | <1.8 | + |

# 4. DISCUSSION

Dairy cattle and its environment harbor pathogens that pose a potential human health hazard. Meat and meat products serve as vehicles for the transmission of pathogenic bacteria including *Salmonella* spp. from cattle to humans. Outbreak investigations and volunteer studies have shown that very low doses of certain *Salmonella* strains can cause disease in a significant proportion of the consumers (Hedberg et al., 1991). It has, therefore, become increasingly important to develop rapid and sensitive methods not only for the detection but also for the quantification of low numbers of S*almonella* cells in foods and fecal samples. In risk assessment investigation, enumeration rather than presence/absence is important to estimate the rate of human exposure.

The aim of the present study was first to develop a sensitive, simple, and rapid MPN-real-time PCR based method for the quantification of *Salmonella* in artificially contaminated fecal samples. The method has been successfully used for detection and quantification of *Salmonella* in artificially contaminated fecal samples. Therefore, we evaluated the utility of this developed method to enumerate *Salmonella* spp. in naturally contaminated fecal samples collected from slaughterhouse located in Meaux, France, and to be used afterwards for the development of quantitative risk assessment of food borne contamination by ground meat. This is, to our knowledge, the first report on quantification of *Salmonella* in artificially and naturally contaminated fecal samples by the MPN method combined with LightCycler real-time PCR based on the double-stranded DNA (dsDNA) binding dye SYBR Green I. The major advantage of the LightCycler real-time-PCR assay is that it is easy to perform and has been shown to save time and effort. In this study, we have shown that with a little optimisation step, the simple and less expensive option of SYBR Green I can be used as an effective alternative.

In our study, *Salmonella* could not be detected in inoculated fecal samples when DNA was extracted directly and without enrichment (Table 2.3). Previously reported studies (Waltman, 2000; Liming and Bhagwat, 2004) suggested the use of preenrichment step prior to DNA extraction methods to improve the detection of low numbers of *Salmonella* in foods and to overcome the problems of certain inhibitors present in food. Therefore, we carried out one step enrichment in BPW prior to DNA extraction, while the DNA extraction procedure based on a simple boiling method gave optimal results and no inhibition was seen in inoculated fecal and naturally contaminated fecal samples.

In general, as a simple, but widely used method, boiling method provides a fast and efficient way of extracting DNA that can be used in PCR assays in less well equipped laboratories. When we compare the boiling method with the commercial kit for extracting DNA from artificially contaminated fecal samples, clear positive peaks were observed in more positive tubes originating artificially contaminated fecal samples extracted by the commercial kit than by the boiling method. Even though the boiling method gave the same result in term of the positive and negative tube comparing with the extracted kit; extraction procedures have to be improved to isolate DNA from naturally contaminated fecal samples.

The detection of low numbers of cells is particularly important for *Salmonella* spp., since epidemiological evidence suggests that the infectious dose for some strains could be in the range of 10-100 cells (Bhagwat, 2004). In the present study, experiments carried out on artificially contaminated fecal samples showed that real-time PCR could detect 1 CFU/mL of *Salmonella* contaminated fecal samples after 8 h of incubation in the non-selective pre-enrichment medium. Published papers describing real-time PCR-based detection of *Salmonella* from either spiked or naturally contaminated foods have claimed detection limits

ranging from 1 to less than $10^3$ CFU/g or mL after enrichment at different times ranging from 6 h to overnight incubation (Eyigor et al., 2002; De Medici et al., 2003; Bhagwat., 2004; Mercanoğlu and Griffiths, 2005). Real-time PCR assays developed with SYBR green were able to detect 1 to 2 cfu of Salmonella in poultry and meat products (Catarame et al., 2006), 4 cfu in raw pork sausage (Wang et al., 2004), 1 to 2.5 cfu in pasteurized milk, ground beef, and alfalfa sprouts (Mercanoğlu and Griffiths 2005), 2.5 cfu in milk and water (Jothikumar et al., 2003), and 6 in chicken intestinal samples (Eyigor et al., 2002).

The potential of MPN-real-time PCR method for the quantification of *Salmonella* spp. from artificially contaminated fecal was investigated and resulted in MPN counts that corresponded well to the estimated level of contamination inoculated into the samples. Generally, the MPN-real-time PCR tended to give higher estimates than the inoculum level. However, the inoculum level estimates fell well within the 95% confidence limits of the MPN estimates while remaining lower than MPN-real-time PCR results. These results are supported by the findings of (Mäntynen et al., 1997). In their results, they found that MPN-PCR tended to give higher estimates than plate counting; which was probably due to DNA from dead and stressed cells, which were not able to form colonies. As the post-PCR melting curve analysis of the amplified product was performed, it was very important to establish whether the level of contamination has an influence on the position of the melting peak at 87.6°C (± 0.4°C). As can be seen in Fig 2.3 A, B, and C, the significant peak at 87.6 (± 0.4°C) remains unaltered at the different levels of contamination even though a variety of lesser peaks are evident at lower levels of contamination, presumably as a result of the amount of accumulated product. O'Mahony and Colin (2002) indicated also that the height of the peaks varies relatively to the amount of accumulated product. Generally, using the protocol described here, satisfactory peak heights were produced using a 40-cycle real-time-PCR.

When the real-time PCR assay was used to examine of 296 fecal samples collected from slaughterhouse located in Meaux, France, it indicated that 9.12% (27/296) were contaminated by *Salmonella*, which is within the range of prevalence rates reported in the literature. This level of contamination is in agreement with another French study (Heuchel et al., 2000), which showed the prevalence of *Salmonella* in fecal samples to be 9.5% and Lailler et al. (2005) reported prevalence herd with *salmonella* (8.1%). However, previously reported surveys of cattle fecal samples in Europe, Australia, United States and Canada have shown large variations in the prevalence of *Salmonella* in feces ranging from 2% to 50% (Ransom et al., 2002; McEvoy et al., 2003; Fegan et al., 2004; Blauel et al., 2005; Fegan et al., 2005; Fluckey et al., 2007; Stephens et al., 2007). The large variations in levels of fecal *Salmonella* contamination observed in these studies have been attributed to several factors such as variations in sampling and detection techniques, seasonal differences, herd size, geographic area, hygiene, and farm management practices. These reported findings clearly suggest *Salmonella* can be carried by healthy cattle at slaughter (Samuel et al., 1979; McEvoy et al., 2003) and can therefore serve as a reservoir and source of contamination of carcases during processing and may pose a health hazard.

Although the traditional culture method remains the accepted procedure for confirming the presence of *Salmonella* in food because this method allows isolation and culturing of Salmonellae from samples, the real-time PCR assay is substantially faster and usually more sensitive than the standard culture procedure. In addition, this real-time PCR assay can be combined with subculture of enrichment broths from PCR-positive samples for the isolation of the pathogen, which is of great benefit to the food industry and to regulatory or public health authorities engaged in food safety and the management of salmonellosis.

MPN-real-time PCR assay of fecal samples conducted in this study suggested that *Salmonella* count in fecal were generally low, with the exception of 6 animals (>1400 MPN/g), while all the other 21 animals were less than 80 MPN/g (from them 13 animals <1.8 MPN/g) as show in table (8.3). Since 63% of the *Salmonella* concentration in positive fecal samples fall below or above the detection limit of MPN-real-time PCR assay (censored observations), the maximum likelihood estimation (MLE) with proc lifereg on SAS (censored or Tobit regression approach) was used to calculate an estimate for the mean and standard deviation of the log10 concentration of the *Salmonella* in gram bovine feces.

Fegan (2004) has reported that counts of salmonella in positive fecal samples varied from <3 MPN/g of fecal to 2.8 X $10^3$ MPN/g and 71% of positive samples had count <10 MPN/g, and the same author (2005) found that *Salmonella* in positive fecal samples varied from <3 MPN/g of fecal to 93 MPN/g and 64% of positive samples had count <10 MPN/g. In our study the salmonella in positive fecal samples varied from <1.8 MPN/g and 1609 MPN/g, and 63% of positive samples <20 MPN/g. 66.7% (18/27) of samples that tested positive by the LightCycler real-time PCR detection assay were positive with conventional PCR. The majority of the conventional negative samples (78%, 7/9) was <1.8 MPN/g, while the other two were 27 MNP/g and 53.9 MPN/g as show in table 8.3.

Identification of isolates is of particular importance for epidemiological data and public health authorities. PCR procedure, on the other hand, can be used only as a screening tool because it indicates only presence or absence of the pathogen. In our study, *Salmonella* Typhimurium was the only serotype isolated from the fecal animals with multidrug resistant (penta and FQ R) and *Salmonella* Derby the only serotype isolated from environmental sample with multidrug resistant (streptomycin Sm, Te, Su) by cultural procedures from the

twenty seven fecal and nine environmental real-time PCR *Salmonella*-positive samples. This finding might not be surprising because for a variety of reasons including the relatively low number of real-time PCR positive samples in this study. Moreover, in all cases the number of *Salmonella* in the fecal samples was very low (table 8.3). Conventional cultural procedures will not always detect small numbers of *Salmonella* cells in certain food or fecal samples. Bensal et al. (2006) reported some factors that can influence recovery rates including sensitivity of the methods, the susceptibility of *Salmonella* strains to inhibitors in the food or media, and overgrowth by competitors during incubation. Karns et al. (2005) mentioned many reasons why *Salmonella* were not isolated from PCR positive raw milk samples; bulk tank milk can contain many other organisms that may compete with *Salmonella* in the enrichment broth; the presence of other organisms on the XLT4 selective agar plates may interfere with the production of $H_2S$ by *Salmonella*; $H_2S$ production is required for the formation of the black colour in *Salmonella* colonies. Mejia et al.(2005) showed that RV broth has a low selectivity for Enterobacteriaceae present in pig feces and that XLT4 agar has a low discriminatory power and the study emphasise the need for new and more selective enrichment and different media to be developed. Bohaychuk et al. (2007) found that 22% (10/45) of real-time PCR *Salmonella* positive from artificially contaminated bovine fecal samples was negative with culture method, the author explained that due to low number of cell after enrichment may be there are false-positives obtained by real-time PCR method; however, supplementary analyses showed that *Salmonella* could be cultured from these negative samples when additional enrichment and IMS used. This would indicate either a very low number of *Salmonella* that the culture method described above could not detect, or that there was a high degree of background flora, and additional measures were needed to increase the number of *Salmonella* to detectable levels and minimize background microflora.

A data gap that is routinely identified in risk assessment is the lack of quantitative data on the level of contamination in the contaminated foods with pathogens (Coleman and Marks, 1999). The application of MPN method combined with LightCycler real-time PCR to quantify *Salmonella* spp. in fecal proved to be rapid and highly sensitive and small numbers of *Salmonella* could be found in fecal samples. This assay yields significant labor and time savings since the quantification of *Salmonella* spp. can completed within 12 h which included an 8-h non-selective enrichment step and 4 h to carry out the sample preparation and real-time PCR assay as opposed to the classical methods, which require at least 5 days of work.

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# Chaptre 4: Modelling the Growth Limits (Growth/No Growth Interface) of *Salmonella* as a Function of Temperature, pH, and other Environmental Factors

**Abstract**

The collected data of *Salmonella* in ground beef  (from 16 published paper and all the data related to *Salmonella* in ground beef in ComBase) describing the growth limits of a mixture of *Salmonella* strains, *Salmonella* Typhimurium, *Salmonella* Dublin, *Salmonella* Enteritidis or *Salmonella* Senftenberg were examined at different environmental conditions. The response of the pathogen was monitored in a total of 162 combination of temperature (0 to 40°C), pH (4.4 to 7.9), nitrogen , carbon dioxide, oxygen, moisture, sodium nitrite, acetic acid, lactic acid, ethylene diamine tetra acetic acid, nisin, sodium chloride, hydrochloric acid, and oregano essential oil for 60 days. One or more of these additives are present in combination with different combinations of temperature and pH. A growth/no growth limit were modeled by logistic polynomial regression. The concordance index of the logistic model was 96.8%, indicating a good fit to the observed data. The overall results indicate clearly that the temperature is the most important and the only factor significant in the study. No growth was observed at temperature less than 10°C. At 10°C and 12°C growth or no growth could be observed under similar conditions. Even though pH and water activity are important factors for microbial growth, in our study they had no effect due to meat structure. In this study the majority of pH values are ranged from 5.5- 6.5 except in one study where it ranged from 4.4-4.8 at 5°C and in another study where pH was 7.9 at 7.9°C whereas all the values of water activities were > 0.98. The model was developed with published data of *Salmonella* on ground beef; therefore, factors such as food structure and microbial interaction were taken into account to give good accuracy of applicability to the specific food. Having all these combination of strains (single strain and mixture strains) gave the study the power to represent the extremes of the growth region of the individual strains. Thus, models that have been developed with a mixture of strains can be considered more "safe" than those developed with a single strain. Such models can be beneficial to food industry because they can describe

the conditions that can be applied to control a process or specify a formulation in order to minimize the risk of pathogen growth.

**Keywords:** Predictive microbiology; *Salmonella*, Temperature effect.

Résumé

Les données recueillies de *Salmonella* dans le bœuf haché (de 16 papiers publiés et de toutes les données liées à la *Salmonella* dans le bœuf haché dans ComBase) ont décrit les limites de croissance d'une mixture d'efforts de *Salmonella*, la *Salmonella* Typhimurium, la *Salmonella* Dublin, la *Salmonella enteritidis* ou la *Salmonella senftenberg* a été examiné à de différentes conditions de l'environnement. La réponse du pathogène a été contrôlée un en somme de 162 traitements de combinaison de température (0 à 40°C), pH (4.4 à 7.9), azote, dioxyde de carbone, oxygène, humidité, le sodium nitrite, l'acide acide, lactique acétique, l'éthylène diamine tétra l'acide acétique, nisin, le chlorure de sodium, l'acide chlorhydrique et le pétrole d'objet indispensable d'origan depuis 60 jours. Un ou plus de ces additifs sont présent dans la combinaison avec la différente combinaison de température et de pH. Des données de croissance de croissance/non ont été modelées par la rétrogradation de polynôme logistique. L'index de concordance du modèle logistique était 96.8 %, en indiquant un bien approprié aux données observées. Les résultats généraux indiquent clairement que la température est la plus importante et le seul facteur significatif dans l'étude. Il n'y avait aucune croissance observée à la température moins que 10°C. Où comme la température 10°C et 12°C sont la seule température; nous n'avons vraiment observé la croissance / aucune croissance (quelquefois avec les mêmes conditions). Bien que pH et l'activité d'eau soient des facteurs importants pour la croissance microbienne, dans notre étude ils n'ont aucun effet en raison de la structure de viande. Dans cette étude la majorité de valeur de pH a varié de 5.5-6.5 sauf une étude variée de 4.4-4.8 à 5°C et d'autre étude pH est 7.9 à 7.9°C alors que toutes les valeurs d'activités d'eau sont > 0.98. Le modèle a été développé avec les données publiées de *Salmonella* sur le bœuf haché; donc, les facteurs comme la structure d'aliments et l'action réciproque microbienne ont été tenus compte pour donner la bonne exactitude de validité d'application aux aliments spécifiques. La possession de tous ceux-ci la combinaison d'efforts

(l'effort simple et les efforts de mixture) donne à l'étude le pouvoir de représenter les extrêmes de la région de croissance de l'effort individuel. Ainsi, les modèles qui ont été développés avec une mixture font un gros effort peut être considéré "plus sûr" que ceux-là se sont développés avec un effort simple. De tels modèles peuvent être favorables à l'industrie d'aliments parce qu'ils peuvent décrire les conditions qui peuvent être appliquées pour contrôler un processus ou spécifier une formulation pour minimiser le risque de croissance pathogène.

**Mots clé** : Microbiologie prophétique; *Salmonella*, Effet de température.

## 1. Introduction

Predictive modelling has been widely developed since the 1980s (Van Impe et al., 1992; McMeekin et al., 1993; Baranyi and Roberts, 1994; Rosso et al., 1995). This field combines the knowledge of bacterial growth responses over a range of conditions with the power of mathematical modelling to enable predictions of growth. Ratkowsky and Ross (1995) hypothesised that kinetic model could be used to generated a probability model to describe the growth / no growth area. The predictive models in food microbiology can be divided into two categories: kinetic models and probability models. An integral description of the microbial response could be given by first establishing the likelihood of growth through a probability model, and then, predicting the growth parameters (specific rate and lag time), if growth was expected. This aim can be achieved by means of growth / no growth boundary models. Such models will then help to define the range of applicability of kinetic models. Growth / no growth models may also be important for establishing food safety regulations as highlighted by Schaffner and Labuza (1997). They could predict the most suitable combination of factors to stop microbial growth, thus giving a significant degree of safety (Leistner et al., 1985). Models describing the growth / no growth area could be very beneficial to meat industry. Products could be formulated having minimum requirement for preservation while satisfying consumer preferences (Presser et al. 1998). A logistic regression model to define the probability of growth for several conditions including temperature, pH, salt and other conditions was proposed.

The objective of this study is to develop model of a growth / no growth interface of *Salmonella* in ground beef. The available growth data for *Salmonella* in ground beef in published papers and all the related data to ground beef in ComBase are used in order to lead to an accurate description of the conditions where *Salmonella* can grow or not.

## 2. Materials and methods

Growth data for *Salmonella* in ground beef were collected from 16 published paper and all the data related to ground beef in ComBase (table 1.4).

**Table 1.4.** The collected data of *Salmonella* in ground beef from scientific literature papers.

| Pathogen | Food commodity | Reference |
|---|---|---|
| *Salmonella* Typhimurium | Ground beef | Dickson et al., 1992 |
| *Salmonella* Typhimurium | Ground beef | Dickson et al., 1994 |
| *Salmonella* spp. | Irradiated ground beef | Dickson et al., 2001 |
| *Salmonella* Typhimurium | Vacuum-packed ground beef | Gill et al., 1980 |
| *Salmonella* Typhimurium | Ground beef | Goepfert , 1975 |
| *Salmonella* Typhimurium | Beef lean muscle | Grau et al., 1983 |
| *Salmonella* Typhimurium | Cooked ground beef | Hintlian et al., 1987 |
| *Salmonella* spp. | Minced beef | Mackey and Kerridge, 1988 |
| *Salmonella* Enteritidis | Minced beef | Mbandi and Shelef, 2001 |
| *Salmonella* spp. | Minced beef | Mbandi and Shelef, 2002 |
| *Salmonella* Typhimurium | Cooked beef | Miller and Acuff, 1987 |
| *Salmonella* spp. | Packed ground beef | Nissen et al., 2000 |
| *Salmonella* Seftenberg | Ground beef | Poerschke and Cunningham, 1985 |
| *Salmonella* spp. | Cooked beef | Rice and Pierson, 1982 |
| *Salmonella* Typhimurium | Vacuum-packed beef | Skandamis et al., 2002 |
| *Salmonella* Typhimurium | Vacuum-packed ground beef | Tu and Mustapha, 2002 |

### 2.1. Bacterial Strains

A mixture of *Salmonella* strains, *Salmonella* Typhimurium, *Salmonella* Dublin, *Salmonella* Enteritidis or *Salmonella* Senftenberg are used in these studies. Only growth parameters or data obtained from MPN or cell counts were taken into account.

### 2.2. Growth Parameters

Growth data included growth kinetics (maximum specific growth rate, generation times, and / or doubling time) or log CFU corresponding to every time point.

### 2.3. Environmental Factors

Temperature was always given in papers, but pH is not specified for 10% of the data. In these cases, the pH of the ground beef was set at the estimated pH which was the median of several meat samples having the same value of salt and other conditions; whereas the other environmental conditions were reported or none reported dependent on the aim of that experimental. The most frequent environmental factors are % nitrogen, % carbon dioxide, % oxygen, moisture, sodium nitrite, acetic acid, lactic acid, ethylene diamine tetra acetic acid, nisin, sodium chloride, hydrochloric acid, and oregano essential oil. One or more of these additives are present in combination with different temperature and pH.

### 2.4. Assessment of Growth

Growth was evaluated every 10 min, 20 min, 30 min, 60 min, 3 h, 6 h, 12 h, daily, every 3 days, weekly; as the temperature increase, the interval time between the measurement decrease. The observed response of the pathogen was defined as "growth" if a higher than 1-log increase was observed during the incubation period and as "no growth" if a less than 1-log increase or decline was observed.

### 2.5. Model Development

For each replicate growth of *Salmonella* at a given condition combination, growth or no growth were scored as value of 1 or 0 respectively. Data were fitted to a logistic regression

by Proc logistic of SAS model (Cary, 1988) on the basis of the approach described by Ratkowsky and Ross (1995). The model was given by:

logit (P) = $a_0$ + $a_1$ T + $a_2$ PH + $a_3$ Nitrogen + $a_4$ $O_2$ + $a_5$ $Co_2$ + $a_6$ T*PH + $a_7$ Moisture + $a_8$ strain + $a_9$ T*strain + $a_{10}$ Acetic acid + $a_{11}$ Lactic acid + $a_{12}$ Nitrogen*$O_2$ + $a_{13}$ Nitrogen*$Co_2$ + $a_{14}$ $O_2$*$Co_2$ + $a_{15}$ Nitrogen* $O_2$*$Co_2$ + $a_{16}$ Moisture*Acetic acid + $a_{17}$ Moisture*Lactic acid + $a_{18}$ Acetic acid*Lactic acid + $a_{19}$ Moisture*Acetic acid* Lactic acid.

where logit (P) is an abbreviation of [ln P/(1-P)], (P) is the probability of growth (in the range of 0 to 1), $a$i are the coefficients to be estimated, and T is temperature,
The automatic variable selection option with stepwise selection method was used to choose the most significant effects ($p < 0.05$).

## 3. Results and discussion

As a part of our efforts to provide data for risk assessment of foodborne salmonellosis, the objective of this study was to study a growth / no growth interface of *Salmonella* in ground beef, the available growth data for *Salmonella* in ground beef in published papers and all the data related to ground beef in ComBase were used in order to lead to an accurate description of the conditions where *Salmonella* can grow or not.

Among the 162 combination treatments of temperature and pH and other conditions observed in this study, growth of *Salmonella* was observed in 82 and no growth in 80. There were very few examples of conditions under which only some of the replicate cultures under the same conditions did or did not grow; indicating that the transition between conditions that permitted growth and those that did not was abrupt. Indeed, from overall results, it is clear that the temperature is the most important and the only factor significant in this study. There was no growth observed at temperature less than 10°C. The temperatures 10°C and 12°C were

the only temperature where growth as well as no growth was observed (sometimes with the same conditions) (Table 3.4). Among the 34 combinations, growth of *Salmonella* was observed in 15 and no growth in 13 at 10°C and in 4 and no growth in 2 at 12°C.

Predictive modelling studies on the combined effect of temperature and pH suggest that the effect of these combinations on microbial growth rate is independent (McMeekin et al., 2000; Presser et al., 1998). The results of the present study indicated clearly that only temperature has effect on the growth of *Salmonella* on ground beef. The parameter estimates and statistics of the logistic regression model with non significant (P > 0.05) effects removed are shown in table 2.4. All the main, interactive, and quadratic effect were (P > 0.05) except for the effect of temperature. Even though pH and water activity are important factors for microbial growth (McMeekin et al., 2000; Presser et al., 199), in our study they have no effect due to meat structure. The most of raw ground beef had water activities (> 0.98), moderate pH (5.5- 6.5) and readily available sources of energy, carbon and other nutrients makes them ideal for most microbial growth (Varnam and Sutharland 1985). In this study the majority of pH value ranged from 5.5- 6.5 except one study ranged from 4.4- 4.8 at 5°C and another study pH was 7.9 at 7.9°C whereas all the values of water activities were > 0.98. The concordance index, the Homer-Lemeshow goodness-of-fit statistic (Hosmer and Lemeshow, 1989), and the maximum measures of goodness of fit of the developed model were calculated. As determined by the concordance index, the degree of agreement between the predicted probabilities and observations was 96.4% concordant and 0.5% discordant. The Homer-Lemeshow goodness-of-fit statistic was 19.51 ( $\chi^2$, df 17, p= 0.3) and the maximum rescaled R- square statistic was 0.879. In other studies on modelling, growth / no growth boundaries of foodborne pathogens, the reported maximum rescaled R- square value ranged from 0.805 to

0.927 (McMeekin et al., 2001; Presser et al., 1998; Salter et al., 2000; Tiennungoon et al., 2000).

**Table 2.4.** Parameter estimates for the logistic regression model

| Coefficient | df | Estimate | SE | Chi-square | P |
|---|---|---|---|---|---|
| Intercept | 1 | 10.62 | 2.90 | 13.396 | 0.0003 |
| Temperature (T) | 1 | -1.05 | 0.29 | 13.493 | 0.0002 |

The model was developed with published data of *Salmonella* on ground beef; therefore, factors such as food structure and microbial interaction were taken into account to give good accuracy of applicability to the specific food. Several studies have shown that these factors might significantly affect microbial behaviour (Gram and Melchiorsen, 1996; Pin et al., 1999; Robins and Wilson, 1994). Most of growth / no growth interface models developed up to now are based on data from a single strain. Several studies, however, have reported significant strains variations in the response of foodborne pathogens (Barbosa et al., 1994; Begot et al., 1997; Thomas et al., 1992). Thomas et al. (1992), reported significant differences in temperature, pH, and NaCl limits among six strains of *Salmonella*. In the present study a single strain and mixtures of *Salmonella* strains were used dependent on the aim of the study. The strains do not have any effect on the growth of *Salmonella* in this model due to the structure of the ground beef. By having all these combination of strains (single strain and mixture strains) gives the study the power to represent the extremes of the growth region of the individual strain. Thus, models that have been developed with a mixture strains can be considered more “safe” than those developed with a single strain.

Such models can be beneficial to food industry because they can describe the conditions that can be applied to control a process or specify a formulation in order to minimize the risk of pathogen growth.

**Table 3.4.** The observed growth/no growth *Salmonella* in ground beef at the temperature of 10°C and 12°C.

| Assay | T °C | pH | Nitrogen % | Carbon dioxide % | Oxygen % | Moisture | Sodium nitrite ppm | Acetic acid ppm | Lactic acid ppm | Growth | Strain |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10.00 | 5.50 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | Mixed |
| 2 | 10.00 | 5.50 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | Mixed |
| 3 | 10.00 | 5.80 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Enteritidis |
| 4 | 10.00 | 5.80 | 0.40 | 0.60 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Typhimurium |
| 5 | 10.00 | 5.80 | 0.40 | 0.60 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Dublin |
| 6 | 10.00 | 5.80 | 0.00 | 0.30 | 0.70 | 0.00 | 0.00 | 0.00 | 0.00 | 0 | S. Dublin |
| 7 | 10.00 | 5.80 | 0.00 | 0.30 | 0.70 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Dublin |
| 8 | 10.00 | 5.80 | 0.40 | 0.60 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Enteritidis |
| 9 | 10.00 | 5.80 | 0.00 | 0.30 | 0.70 | 0.00 | 0.00 | 0.00 | 0.00 | 0 | S. Enteritidis |
| 10 | 10.00 | 5.80 | 0.40 | 0.60 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Enteritidis |
| 11 | 10.00 | 5.80 | 0.00 | 0.30 | 0.70 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Enteritidis |
| 12 | 10.00 | 5.80 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 | S. Typhimurium |
| 13 | 10.00 | 5.80 | 0.00 | 0.30 | 0.70 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Typhimurium |
| 14 | 10.00 | 5.80 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Typhimurium |
| 15 | 10.00 | 5.90 | 0.00 | 0.00 | 0.00 | 0.57 | 0.00 | 2000.00 | 0.00 | 0 | Mixed |
| 16 | 10.00 | 5.90 | 0.00 | 0.00 | 0.00 | 0.79 | 0.00 | 2000.00 | 0.00 | 0 | S. Enteritidis |
| 17 | 10.00 | 5.90 | 0.00 | 0.00 | 0.00 | 0.79 | 0.00 | 1000.00 | 0.00 | 1 | S. Enteritidis |
| 18 | 10.00 | 5.90 | 0.00 | 0.00 | 0.00 | 0.79 | 0.00 | 2000.00 | 0.00 | 0 | S. Enteritidis |
| 19 | 10.00 | 6.00 | 0.00 | 0.00 | 0.00 | 0.79 | 0.00 | 1000.00 | 25000.00 | 0 | S. Enteritidis |
| 20 | 10.00 | 6.10 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 2000.00 | 25000.00 | 0 | Mixed |
| 21 | 10.00 | 6.10 | 0.00 | 0.00 | 0.00 | 0.79 | 0.00 | 2000.00 | 25000.00 | 0 | S. Enteritidis |
| 22 | 10.00 | 6.10 | 0.00 | 0.00 | 0.00 | 0.79 | 0.00 | 2000.00 | 25000.00 | 0 | S. Enteritidis |
| 23 | 10.00 | 6.30 | 0.00 | 0.00 | 0.00 | 0.57 | 0.00 | 0.00 | 0.00 | 0 | Mixed |
| 24 | 10.00 | 6.30 | 0.00 | 0.00 | 0.00 | 0.57 | 0.00 | 0.00 | 25000.00 | 0 | Mixed |
| 25 | 10.00 | 6.30 | 0.00 | 0.00 | 0.00 | 0.79 | 0.00 | 0.00 | 0.00 | 1 | S. Enteritidis |
| 26 | 10.00 | 6.30 | 0.00 | 0.00 | 0.00 | 0.79 | 0.00 | 0.00 | 25000.00 | 1 | S. Enteritidis |
| 27 | 10.00 | 6.50 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Typhimurium |
| 28 | 10.00 | 7.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 20000.00 | 0 | S. Typhimurium |
| 29 | 12.00 | 5.30 | 0.00 | 0.00 | 0.00 | 0.63 | 0.00 | 0.00 | 0.00 | 0 | Mixed |
| 30 | 12.00 | 5.30 | 0.00 | 0.00 | 0.00 | 0.63 | 0.00 | 0.00 | 0.00 | 0 | Mixed |
| 31 | 12.00 | 6.30 | 0.00 | 0.00 | 0.00 | 0.59 | 8.60 | 0.00 | 0.00 | 1 | Mixed |
| 32 | 12.00 | 6.30 | 0.00 | 0.00 | 0.00 | 0.59 | 8.60 | 0.00 | 0.00 | 1 | Mixed |
| 33 | 12.00 | 6.50 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Typhimurium |
| 34 | 12.00 | 6.50 | 0.00 | 1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1 | S. Typhimurium |

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# Chapter 5: Quantitative Risk Assessment of Human Salmonellosis Linked to the Consumption of Ground Beef

**Abstract**

This work reports a quantitative risk assessment of human salmonellosis linked to the consumption of ground beef patties. The risk assessment was based on data on the frequency, concentration and inactivation of *Salmonella* in ground beef. Different distributions were assumed for parameters of the model and a Monte Carlo simulation was used to model the process and to quantify the resulting risk for public health. The probability of ground beef batches contamination was estimated to be 100% after 2000 iteration with an expected percentage of ground beef batch with patties contamination less than 1, 6, 12, and 18 percent were 22.5%, 52.1% , 69.07% and 95.07%, respectively. 87.51% of this variation is due to batch effect (within the batch). About 92.9% of ground beef patties (55.74 million out of 60 million patties) were expected not to be contaminated. The simulated concentration of *Salmonella* in a typical ground beef patty serving of 100 g before cooking ranged from 0 to 1.4 x $10^6$ *Salmonella* cells with a median of 0 cells. The expected percentage of ground beef patties with contamination greater than 5, 10 and 100 *Salmonella* cells were 29%, 17.1% and 0.02%, respectively. For 10 million servings of 100 g, the expected number of cases of salmonellosis predicted by the model is in average 11.01, 12.33 for fat content 7% and 24% respectively. The risk of salmonellosis per 100 g serving ranged from 0 to 2.33E-06 dependent on the type of cooking and the fat content. The risk of salmonellosis was closed to zero when the 100 g serving ground beef patties consumed well done. The relative risk of getting salmonellosis from consumed the rare ground beef patties is 312.33, 60.69 times higher for fat content 7% and 24% respectively comparing to the consumption of well done patties. There are 35 batches with cases out of 2000 batches (1.8%). 15 of them have 2 cases or more (0.75%, 15/2000).

**Keywords:** Quantitative risk assessment; *Salmonella*; Ground beef patties

**Résumé**

Un modèle d’appréciation quantitative de risque (AQR) de salmonellose humaine liée à la consommation de portions de bœuf haché a été développé. L’AQR est basée sur des données relatives à la fréquence, la concentration et l’inactivation de salmonelles dans le bœuf haché. Différentes distributions ont été posées en hypothèse pour chacun des paramètres du modèle. Une simulation de Monte Carlo a été employée pour modéliser le processus et pour estimer le risque résultant pour la santé publique. La probabilité de contamination de bœuf haché a été estimée à 100% après 2000 itérations avec un pourcentage attendu de lots de bœuf haché, avec moins de 1, 6, 12 et 18 % de portions contaminées était 22,5 %, 52,1 %, 69,07 % et 95,07 %, respectivement. Le pourcentage de la variation due à l’effect lot était 87,5%. On s'attend à ce qu’environ 92,9 % de portions de bœuf haché (soit 55,74 millions de 60 millions parmi portions contaminées) ne soient pas contaminées. La concentration simulée de *Salmonella* dans une portion de bœuf haché typique de 100 g avant cuisson est de 0 à 1,4 x $10^6$ cellules de *Salmonella* avec une médiane de 0 cellules. Le pourcentage attendu de portions de bœuf haché avec la contamination plus grande que 5, 10 et 100 cellules de *Salmonella* était 29 %, 17,1 % et 0,02 %, respectivement. Pour 10 millions de portions de 100 g, le nombre attendu de cas de salmonellose prévu par le modèle est en moyenne 11, 12 pour les teneurs en matière grasse 7 % et 24 %. Le risque de salmonellose par portion de 100g varie de 0 à 2,33 x $10^{-6}$ en fonction au type de cuisson et de la teneur en matière grasse. Le risque de salmonellose est proche de zéro quand le bœuf haché est consommé bien cuit. Le risque relatif de salmonellose avec le bœuf haché saignant est 312 ou 61 fois plus élevé pour les teneurs 7 % et 24 % en matière grasse par comparaison avec la viande hachée bien cuit. Il y a 35 lots sur 2000 qui provoquent des cas (1,8 %). Quinze d'entre eux causent 2 cas ou plus (0,75 %, 15/2000).

**Mots clefs** : Appréciation quantitative de risque ; Salmonellose ; Portions de bœuf haché

## 1. Introduction

Foodborne illness due to *Salmonella* is a major public health problem (European Commission, 2000). Bacterial pathogens contribute to ~60% of foodborne illnesses that lead to hospitalization and account for nearly two-thirds of the estimated number of foodborne pathogen-related deaths. Mead et al. (1999) estimated that *Salmonella* spp. caused ~26% and >30% of foodborne illness-related hospitalizations and foodborne deaths respectively. Several food items, including ground beef, have been implicated in *Salmonella* disease outbreaks. Therefore, risk assessment for public health linked to the consumption of ground beef contaminated by *Salmonella* provides useful information for the management of the risk.

In general, relatively few papers dealing with quantitative risk assessment models for salmonellosis of food origin have been published in the scientific literature. Risk assessment models of the consumption of dairy products have previously been developed for pathogens such as *Listeria monocytogenes* (Bemrah et al., 1998; Sanaa et al., 2004) and *Staphylococcus aureus* (Lindqvist et al., 2002) but few for *Salmonella* spp. infection (Fares, 2007; USDA-FSIS, 1998). Estimation of the risk for public health linked to the consumption of ground beef contaminated by *Salmonella* provides useful information for the management of the risk. To our knowledge, a quantitative risk assessment of salmonellosis linked to the consumption of ground beef has not been done. The present work therefore reports a first risk assessment model of salmonellosis linked to the consumption of ground beef using, a Monte Carlo simulation by SAS software. The major aim of the present chapter is therefore to report a risk assessment model of salmonellosis from the consumption of ground beef.

## 2. Materials and methods

Risk assessment is a science-based process in which questions that have been formulated during the risk evaluation step of the risk management process are addressed to develop an understanding of the problem and to come up with risk estimates and risk mitigation options. In our study, the hazard is *Salmonella* and the risk qualifies the probability of human salmonellosis associated with the consumption of 100 g serving of ground beef patty.

### 2.1 Hazard identification

*Salmonella* is the most frequently reported cause of foodborne illness in the world. It is the major cause of childhood mortality in developing countries and constitutes a permanent threat in industrialized countries. However, the disease can spread systemically and degenerate into a chronic condition such as reactive arthritis, osteomyelitis, cardiac inflammation or neural disorders. Groups at higher risk of severe illness and death from *Salmonella* infection are infants, elderly persons, and persons with impaired immune systems (Bell, 2002).

Epidemiology of beef-related outbreaks in the U.S., Canada, and Europe demonstrated that ground beef is responsible for a great number of these outbreaks. The presence of *Salmonella* in ground beef is a known health hazard and outbreaks of salmonellosis linked to the consumption of ground beef have been reported (chapter 2 of this thesis has more detail). Previously reported surveys on *Salmonella* in Europe, Australia, United States and Canada have shown large variations in the prevalence of *Salmonella* in fecal, hide, and carcasses ranging from 2% to 50% (Heuchel et al., 2000; Ransom et al., 2002; McEvoy et al., 2003; Fegan et al., 2004; Fegan et al., 2005; Lalleret et al., 2005; Fluckey et al., 2007; Stephens et al., 2007). These reported findings clearly suggest *Salmonella* can be carried by healthy cattle

at slaughter (Samuel et al., 1979; McEvoy et al., 2003; Lailler et al., 2005) and can therefore serve as a reservoir and source of contamination of carcases during processing and may pose a health hazard.

## 2.2. Exposure assessment

In order to develop a risk assessment model of human salmonellosis associated with the consumption of ground beef, we attempted to estimate the potential exposure to *Salmonella* in a single serving. The exposure was characterized by the probability distribution of *Salmonella* colony-forming units (CFUs) in 100-g servings of meat at the time of consumption. A list of variables was identified and distribution was assumed for each variable (Table 3.5). An accurate exposure assessment needs information such as the frequency and level of contamination of the selected foods and the growth and inactivation of the pathogen during the preparation steps. This information will be discussed below. Monte Carlo simulation was done using SAS software v.9.1 (SAS Institute, Inc.) (Cary, 1988).

The microbial risk assessment (MRA) will be developed using a slaughter-to consumption framework. The use of this frame work's exposure pathway is split into 4 modules (indicated in Figure 1.5.); these are animal at slaughter house, slaughter & processing, distribution & storage and finally preparation & consumption. Each module of the pathway includes appropriate mathematical descriptions of the changes in the prevalence and levels of organism. The output from the exposure pathway is the probability of random human ingesting *Salmonella* and the number of *Salmonella* consumed.

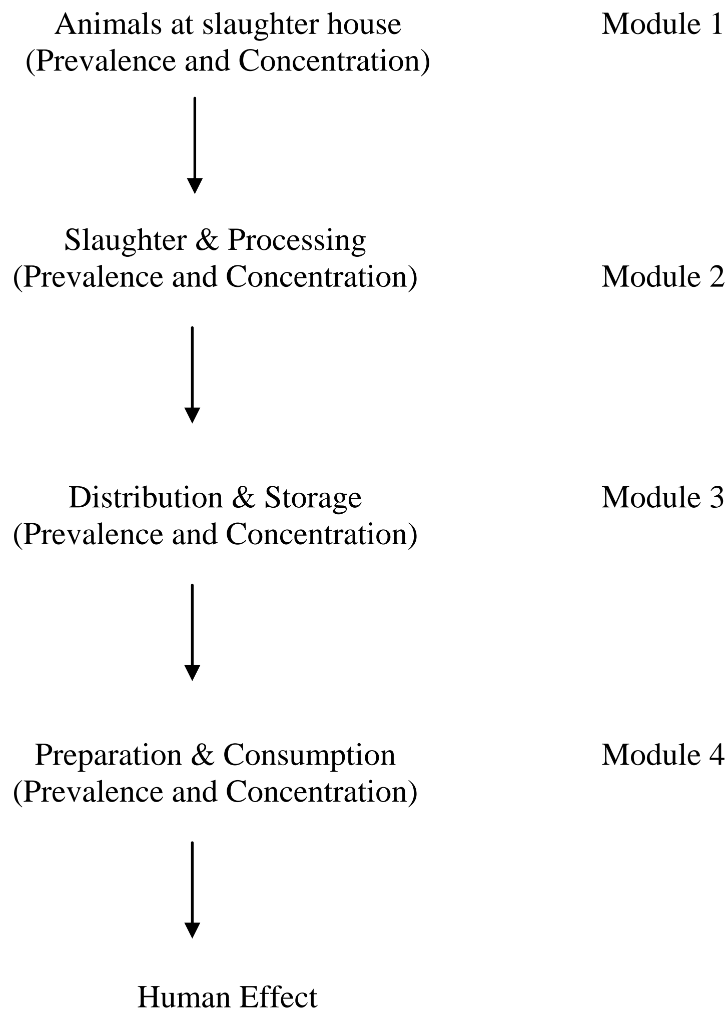



**Figure 1.5.** Pathway to describe the contamination of *Salmonella* of ground beef

*2.2.1. Collection of data in bovine faeces contaminated by Salmonella at slaughterhouse (Module 1)*

***2.2.1.1. Aim of module***

The aim of the animals at slaughterhouse module is to estimate the prevalence and concentration of *Salmonella* at the point of slaughter.

*2.2.1.2. Contamination of bovine fecal by Salmonella.*

To estimate the prevalence and concentration of *Salmonella* in bovine faeces, we used our data from a study conducted in 2006 at slaughterhouse located in Meaux, France. A total of 296 fecal samples were aseptically collected weekly in February and March 2006 (an average of 40 samples per visit) and the presence of *Salmonella* in bovine faeces was investigated using the real-time PCR detection assay described in chapter 3 of this thesis. The results of this study indicated that 9.12% (27/296) of fecal samples were *Salmonella* positive.

***2.2.1..3. Level of Salmonella contamination in bovine faeces***.

In general, literature data on the contamination of bovine faeces by *Salmonella* are qualitative and presented as presence or absence of *Salmonella* in samples analyzed. The absence of quantitative data could be due to the difficulty of applying enumeration methods to quantify low levels of contamination. In a previous work, we developed a method for the quantification of *Salmonella* in artificially contaminated fecal based on the real-time PCR assay combined with MPN (Chapter 3 of this thesis). This developed MPN-real-time PCR assay was used to provide quantitative data by estimating the level of contamination of positive-*Salmonella* fecal samples. The MPN real-time PCR assay enabled the enumeration of *Salmonella* in fecal *Salmonella*-positive samples that ranged from <1.8- 1609 MPN/g. Counts were generally low, with the exception of 6 animals (>1400 MPN/g) as shown in Table 1.5. Using tobit model (reference), the estimates of mean and standard deviation of the log10 concentration of *Salmonella* in positive bovine fecal samples were respectively 0.6189 MPN/g and 2.7112 (Table 2.5.).

**Table 1.5.** Quantification estimates of *Salmonella* in fecal samples obtained with MPN-real time PCR assay

| Week | Animal order | Weight (Kg) | Type of animals | MPN-PCR (g) |
|---|---|---|---|---|
| 1 | 4 | 517 | Cow | 53.9 |
| 1 | 5 | 522 | Cow | <1.8 |
| 1 | 16 | 493 | Heifer | 5.56 |
| 1 | 20 | 296 | Cow | 20.5 |
| 2 | 5 | 543 | Cow | >1600 |
| 2 | 20 | 464 | Cow | >1600 |
| 2 | 32 | 473 | Cow | 27.1 |
| 3 | 20 | ND | ND | <1.8 |
| 4 | 4 | 604 | Cow | 9.3 |
| 4 | 18 | 597 | Castrated animal | 1.98 |
| 4 | 43 | 308 | Bull | <1.8 |
| 6 | 1 | 196 | Cow | 1609 |
| 6 | 11 | 471 | Cow | >1600 |
| 6 | 16 | 408 | Cow | 42.6 |
| 6 | 18 | 389 | Male Calf | >1600 |
| 6 | 35 | 344 | Cow | >1600 |
| 7 | 1 | 454 | Cow | <1.8 |
| 7 | 15 | 473 | Heifer | <1.8 |
| 7 | 21 | 466 | Cow | <1.8 |
| 7 | 22 | 498 | Cow | 74.8 |
| 7 | 23 | 466 | Cow | <1.8 |
| 8 | 6 | 368 | Heifer | 18 |
| 8 | 10 | 402 | Heifer | <1.8 |
| 8 | 12 | 413 | Heifer | <1.8 |
| 8 | 15 | 370 | Cow | <1.8 |
| 8 | 21 | 436 | Cow | <1.8 |
| 8 | 29 | 431 | Heifer | <1.8 |

**Table 2.5.** Parameter estimates for the log10 concentration of *Salmonella* in gram bovine fecal by using maximum likelihood estimation (MLE) with proc lifereg on SAS.

| | Estimation | Standard error | 95 confidence limits | |
|---|---|---|---|---|
| | | | low | high |
| Mean | 0.6189 | 0.6117 | -0.5800 | 1.8178 |
| Standard deviations | 2.7112 | 0.7296 | 1.5999 | 4.5945 |

*2.2.2. Slaughter & Processing (Module 2)*

***2.2.2.1. Aim of module***

The aim of this module is to examine the change in prevalence and concentration of *Salmonella* contamination of bovine carcasses as they progress through each step of processing. The final outputs of the module are estimates for the prevalence and levels of *Salmonella* contamination of the ground beef patties.

***2.2.2.2. Slaughter plant process***

To identify the most important steps in the slaughter process, from a risk prospective, a flow diagram was constructed for live cattle entering the slaughter plant and going through typical France commercial butchering procedures (fig. 2.5).

- Cattle are transported to holding pens and handled prior to stunning.
- Cattle are stunned, hung from an overhead rail, bled, and hides are removed.
- Carcasses are trimmed to remove visible contamination.
- The gastrointestinal (GI) tract is removed and carcasses are sawed in half.
- Carcasses are decontaminated via different ways.
- Carcasses are chilled for 18-48 hours.
- Carcasses are fabricated to remove meat from the bone and package in boxes.
- Meat is transported for grinding to ground beef either in the slaughter plant or in another facility.

***2.2.2.3. Modelling the food pathway***

In consecutive steps the transmission of *Salmonella* spp. is described by modelling the change in number of micro-organism per unit N. The whole pathway is given schematically in fig. 3.5. A run of the Monte Carlo model simulates the production of ground beef batch and

consecutive production and consumption of the ground beef from that ground beef batch. At each step, the number of micro-organism at the end of the process (N') is given as a function on the number in the previous step (N).

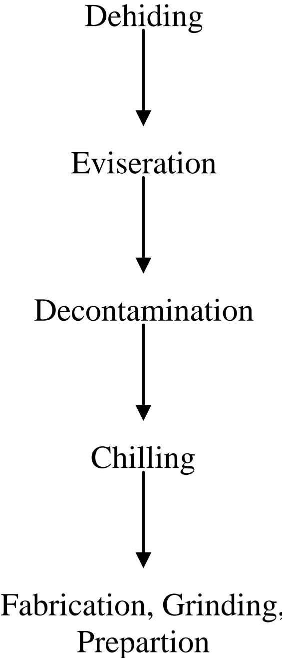



**Figure 2.5.** Steps in the ground beef production process

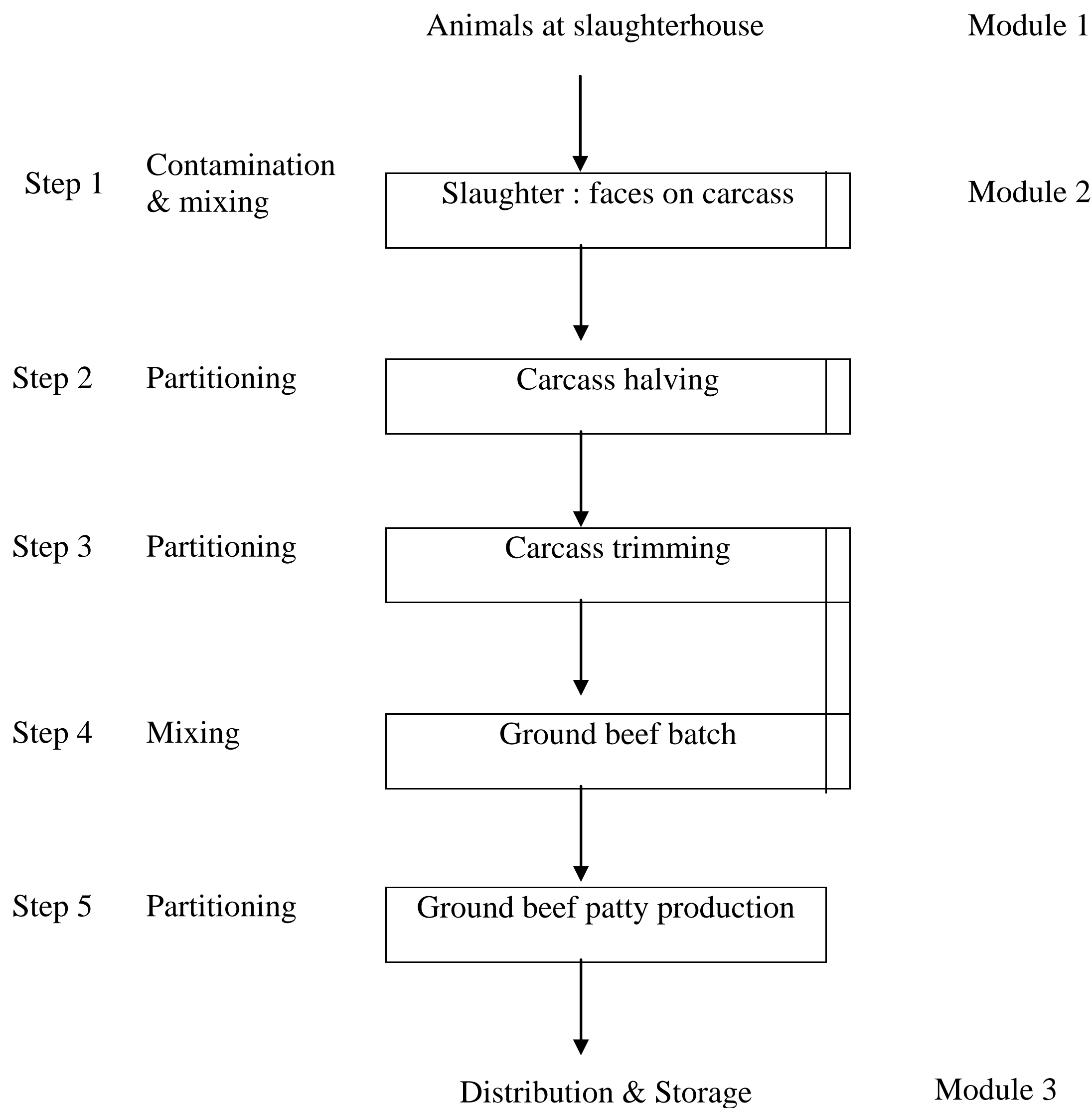



**Figure 3.5. A** schematic representation of the food pathway as modelled in the exposure assessment.

**Step 1: Contamination of carcasses**

In order to assess carcasses contamination by *Salmonella*, we use a parameter a (gram of faeces/carcass) indicating the amount of faeces contaminating a single carcass. By multiplying this parameter by the concentration of faeces by *Salmonella* we are able to estimate the total amount of *Salmonella* contaminating each carcass. The total quantity of faeces on carcass *i* (in g) $a_i \sim a_{max}$ x Beta(α, β) with $a_{max}$, α and β model parameters

expressing the level of faeces contamination and its variability per carcass. Nauta et al. (2001) estimates the parameters $a_{max}$ = 10.1 g, $\alpha$ = 0.395, and $\beta$ = 2.473 by using the result of the expert panel to assess carcass contamination, by estimating minimum, most likely and maximum value for $a$.

We assume that trimming from $n$ carcasses are contributing to ground beef batch of $W_{gb}$ kg. Next, we suppose that faeces from $m_i$ animals ($i = 1..n$) contributes to the fecal contamination of carcass $i$. Parameters $n$ and $m_i$ will be variable per ground beef batch produced. This variability is implemented as:

$n \sim 1+\text{Poisson}(n_{mean} - 1)$

$m_i \sim 1+\text{Poisson}(m_{mean} - 1)$

the two parameters $n$ and $m$ have a discrete value with a minimum of 1 and a probability density function characterised by one parameter only. The mean number of carcasses used for a ground beef batch ($n_{mean}$), and the mean number of animals from which the faeces contaminates a single carcass ($m_{mean}$) is assessed by expert panel for the given slaughter house ($m_{mean}$ = 2.98 and $n_{mean}$ =50.33) (Nauta et al., 2001).

The prevalence of contaminated animals entering the slaughter house is $P_f$. This is an uncertain parameter, which is variable in time; as time is not a dimension in the model, $P_f$ is assumed to be constant throughout this model.

The concentration of *Salmonella* spp. in faeces in animal j contaminating carcass i ($c_{ij}$) is assumed to be distribute as lognormal with parameter presented in table (2.5).

The total number of cfu on a carcass is a function of:

- the fraction of the faeces that animal *j* contributes to carcass *i*. The relative contribution of each animal to the total amount of fecal contaminated is described by a Beta distribution.

  $f_{ij} \sim$ Beta($b_1$, $b_1$ ($m_i - 1$)), with 'beta factor' $b_1$ (Nauta et al., 2001)

- the concentration of *Salmonella* spp. in the feces of animal *j*, contaminating carcass *i*: $C_{ij}$ (cfu/g), noting that with probability $P_f$, $C_{ij} > 0$ and with probability 1- $P_f$, $C_{ij} < 0$.
- the total quantity of faeces on carcass *i* (in g) $a_i \sim a_{max}$ x Beta(α, β) with $a_{max}$, α and β model parameters expressing the level of feces contamination and its variability per carcass.

The expected number of cfu on carcass *i* is derived using the fomula:

$$n_i = Poisson\left( a_i \sum_{j=1}^{m_i} f_{ij} c_{ij} \right).$$

Note that a basic assumption here is that animals, amount of faeces and concentration are considered independent.

**Step 2: Partitioning to half carcasses**

The carcass is split into two. The weight of the clean carcass is $W_{carc}$ and the weight of the half carcass is 0.5 $W_{carc}$. This is partitioning with $x = W_{carc} / 0.5\ W_{carc} = 2$ and $N = N$i, so *N*i' ~ Bionomial (*N*i, Beta ($b_2$, $b_2$)), with $b_2$ a parameter describing the clustering at carcass halving. The mean estimated value of $b_2$ is 2.7 with 67% of cells on the half carcass with most cells (Nauta et al., 2001). In the Monte Carlo simulation we use one half carcass only. The other half is neglected. The mean carcass weight at slaughter in our study was 400 kg. This is incorporated in the model as a fixed number: $W_{carc} = 400$.

**Step 3: Partitioning to trimmings**

Trimming are cut from carcasses. To assess the number of *Salmonella* spp. on the trimmings from one half carcass *i* used in the ground beef batch, we use the Beta-binomial distribution function:

$n_i$' ~ Binomial ($n_i$, Beta($b_4$, 0.5 $W_{carc}$ $b_4$/ $W_{tri}$ - 1))

With $b_4$ a parameter describing the clustering effect of cells on the carcass when trimming are cut off. We therefore assume that the probability of finding *Salmonella* on meat destined for ground beef is equal to the probability of finding it at a random place on the carcass. As clustering is incorporated in the model, the cells are not assumed to be spread equally over the carcass. The mean estimated value of $b_4$ is 0.73 assessed by the expert panel (Nauta et al., 2001). Whereas the weight of these trimmings from this half carcass ($W_{tri}$) is determined in the mixing process in step 4 below (Note that $W_{tri}$ is not the weight of one trimming, but the total weight of all trimmings from carcass *i*).

**Step 4: Mixing: the ground beef batch**

When the ground beef batch is formed, it contains meat of n animals. The total weight of all the trimmings from one (half) carcass used for the ground beef batch depends on the number of carcasses used for the batch (n), and the weight of the batch ($W_{gb}$). The carcasses need not contribute equally to ground beef batch. Then the weight of trimmings of a random half carcass *i* contribute to ground beef batch:

$W_{tri}$ ~ Beta ($b_5$, $b_5$ ($n$-1)) $W_{gb}$, with 'beta factor' $b_5$

The $b_5$ as a measure of the relative contribution of (trimming of the) carcasses in the ground beef batch, is set as $b_5$=1.

Implementing the distribution of $W_{tri}$ in equation step 3, we calculate the total number of Salmonella per batch.

$N' = \sum_n n'_i$

**Step 5: Partitioning to 100 g ground beef patties**

Ground beef patties are produced from the ground beef batch. This is a typical partitioning process. Therefore the number of *Salmonella* on 100 g ground beef *j* is:

$N_j' \sim$ Poisson ($N$, Beta ($b_6$, $W_{gb}$ $b_6$/ $W_{gbp,j}$-1))

The beta factor, $b_6$, is a parameter describing the clustering effect of cells in the ground beef batch for 100 g ground beef patty formation. As the clustering effect may be rather large due to the fact that large batches are not easily mixed well, as default it is assumed that $b_6 = 0.15$.

*2.2.3. Distribution & Storage (Module 3)*

***2.2.3.1. Aim of module***

This module considers ground beef patties from the time they leave the processing plant until they are prepared as part of a meal. The module takes account of the fact that a ground beef may leave the processing plant, chilled or frozen, and that the distribution and storage of chilled ground beef differs from that of frozen. The aim of this module is to estimate the prevalence and the number of *Salmonella* for ground beef patties after they have been distributed, and stored, prior to their removal from a refrigerator or freezer, ready for preparation. Therefore, the outputs from this module are estimates for the prevalence of *Salmonella* contamination and concentration of the ground beef patties.

#### *2.2.3.2. Module pathway*

For each contaminated product, the distribution & storage module describes how the level of contamination for that product is affected by the following stages.

- Transport (1): Processing plant to retail outlet.
- Storage (1): Storage at retail outlet.
- Transport (2): Retail outlet to storage facility in the home / other.
- Storage (2): Storage facility.

A flow diagram of the various stages in the distribution and storage of ground beef patties is shown in figure 4.5. Even though, the level of contamination at each stage may increase due to temperature abuse, it is assumed that the number of *Salmonella* will stay the same, no growth, within the Distribution & Storage module due to the lack of information of temperature and time associated with transport (to and from retail) and storage of chilled and frozen ground beef patties in France. Therefore, the effect of the growth on the risk model result is studied in the scenario analysis.

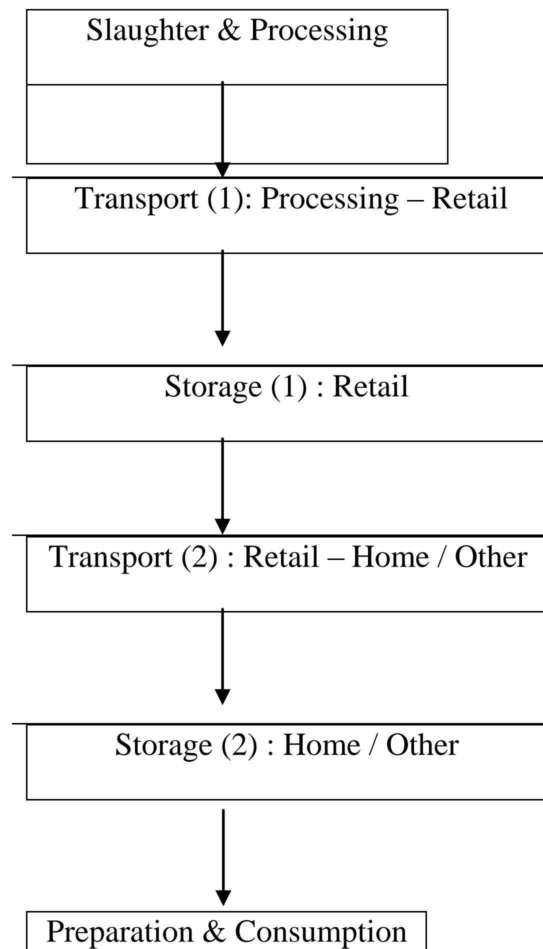



**Figure 4.5.** Stages involved in the distribution and storage of ground beef patties.

*2.2.4. Preparation & Consumption (Module 4)*

***2.2.4.1. Aim of module***

This module considers ground beef patties from the time they are taken out of the storage facility at home, until they are consumed. The aim of this module is to estimate the probability of exposure to *Salmonella* present in ground beef patties. This module also estimates the dose of *Salmonella* ingested following the consumption of the product. Therefore, the outputs from this module are estimates for the probability of exposure and dose ingested of *Salmonella* following the consumption of the ground beef patties.

***2.2.4.2. Module pathway***

This module is directly linked to Distribution & Storage module and thus continues to tracks the progress of a random chilled or frozen product. For each contaminated product, the Preparation & Consumption module describes how the level of contamination on that product, and the subsequent dose that an individual is exposed to, through eating inadequately cooked meat, is affected by the following stages.

- Cooking.
- Consumption.

***2.2.4.3. Modelling the Preparation & Consumption pathway***

***<u>Cooking (inactivation during preparation of patties):</u>***

Thermal inactivation of bacteria is commonly modelled with the Bigelow model, using D- and z-values of *Salmonella* spp. in ground beef (Goodfellow and Brown, 1978; Mackey and Derrick, 1987; Juneja and Eblen, 2000a; Juneja et al., 2000b). According to Juneja and Eblen (2000a) and Juneja et al. (2000b) inactivation depends on fat content (higher fat content gives higher D-value, i.e. less inactivation). They state that 54.4°C endpoint temperature is

typical for rare hamburgers, 62.7°C is typical for medium and 68.3°C is typical for “well done”. We consider that there are three ways for cooking ground beef, rare, medium, and well done.

For the present exposure assessment, traditional log-linear-death kinetic model will be considered. Many investigators do not show the inactivation data for their studies and merely quote D-values (i.e. time for a 90% reduction in the numbers of bacteria at a given temperature). Linear model was used to model the $\log_{10}$ bacteria numbers vs heating time. We fit 121 curves with different temperature (55, 57.5, 60, 62.5, 65, 70°C) and fat contents (7, 12, 18, 24, 25%) from published papers and all the data related to inactivation of *Salmonella* in ground beef in ComBase (Juneja and Eblen, 2000a; Juneja et al., 2000b; Juneja et al., 2003) using linear model. A set of D- values are calculated for each temperature at various fat contents.

$$\log_{10} \frac{N(t)}{N(0)} = \alpha \times time$$

The equation of the regression line was used to calculate a D-value over 1 log cycle reduction in the numbers of bacteria.

$$T_{emperature} \text{ (for the first log reduction)} = -\frac{1}{\alpha}$$

When D-values are calculated for a number of different temperatures, a relationship between the D-value and the temperature were calculated. Data expressed as the reciprocal of the log10 D-value vs temperature of the D-value was analysed by linear regression to give a straight-line equation. This equation was used to calculate the z-value, which is the temperature change required to bring a bout 90% change in D-value.

$$LogD = \alpha + \beta * temperature$$

$$\text{D-value} = 10^{(-\beta T)+\alpha}$$

$$z = -\frac{1}{\beta}$$

The Food and Drug Administration (FDA) and US Department of Agriculture (USDA) have recommended a minimum temperature of 68.3°C at the slowest heating point at 16 s holding time to enhance safety (FDA, 1999; USDA-FSIS, 1993). However it is difficult to implement these standards in a restaurant or at home due to the complexities and difficulties in measuring the internal patty temperature.

Since inactivation of the bacteria in ground beef patties is a function of temperature and time, and also the temperature varies during cooking time; it is important to use dynamic model to predict the change of the temperature in the centre of the ground beef patties with time to adjust the D-value for each time interval at given temperature. The thermodynamics during cooking was assessed by using mathematical heat and mass (moisture and fat) transfer models (Ou and Mittal, 2007). The slowest microbial inactivation point (in the centre of ground beef patties) was predicted using the model described by Ou and Mittal (2007). Using this result, we could estimate the efficiency (E) that is the number of 10-fold divisions or decimal reductions of bacteria caused by the operations ($E_c$: efficiency of cooking) by dividing the cooking ($t_c$) by the corresponding decimal reduction time D calculated above ($D_c$: cooking). The following integral was used, incremented by blocks of time in seconds:

$$E_c = \int_0^{t_c} \frac{1}{D_c(T(t))} d(t)$$

If the heat treatment caused E decimal reductions, and the initial *Salmonella* number in one serving was C, the consumer would ingest a dose of *Salmonella* equal to: d = C/ $10^{Ec}$ per serving.

Details on distributions and variables of the model are summarized in Table 3.5.

## 2.3. Dose-response model

A dose-response model gives the probability of the studied effect according to the amount of ingested pathogenic microorganisms. Among *d*-ingested microorganisms, some might survive human barriers and later initiate infection and cause illness. Probability of the effect was defined as the probability of achieving this sequence of events.

Several dose-response models have been published and used for *Salmonella*, based on different types of data (feeding trials, outbreaks), outcomes (infection or illness) and assumptions on the dose-response relationship: exponential (Rose et al., 1996), Beta-Poisson (Rose and Gerba, 1991; Fazil, 1996; USDA-FSIS, 1998) and Gompertz (Coleman and Marks, 1998). Actually, the exponential and the Beta-Poisson models are the most commonly used.

The probability of infection was described by the following equation:

$$PI = 1 - (1 - r)^{n}$$

The parameter *r* is the probability of one *Salmonella* cell to survive to the different host immune barriers and induce infection. Where *n* is the number of consumed microorganisms and *r* a parameter with a value $r$~Beta(α, $\beta$), α and $\beta$ were equal to 0.3126 and $1.9^5$, respectively (Haas et al., 1999). We consider here the principle of single hit model.

For each consumed 100 g serving we simulated the number of bacteria per serving (*n*), sampled from the beta distribution the parameter *r* (which represent the consumer susceptibility) and applied the formula 1- (1- $r)^n$ to assess the probability of infection per serving of (rare, medium, or well done) frozen or chilled ground beef patties .

Illness was defined as the occurrence of gastroenteritis (abdominal cramps, diarrhea, nausea, vomiting). According to feeding studies on human volunteers (McCullough and

Eisele, 1951a, b), the average probability of gastroenteritis among infected naïve subjects was varying between 0 and 75% with a mean of 16%. To build a more realistic model, we decided to reduce the reported probability of illness to 10% (Bemrah et al., 2003).

Distribution of variables and models for the risk of human salmonellosis are presented in Table 4.5. The risk of salmonellosis from the consumption of a single ground beef patty was estimated using the result of the previous steps, for each type of cooking (rare, medium and well done).

## 2.3. Risk characterization

Risk characterization integrates the results of dose-response and exposure assessment into a risk statement that include one or more quantitative estimates of risk. An essential prerequisite to risk characterization is the clear definition of output. Examples of possible outcomes are expected risk infection to a typical person, expected number of illness or deaths in a community, upper confidence limit to expected number of illness, upper confidence limit for illness to a highly exposed person, or maximum number of illness in a community at any one time. The choice between all the possible outcomes has to be decided in relation the needs of decision maker.

We first assess the distribution of the probability of illness per serving. This distribution encapsulates the variability and uncertainty inherent to the different model input parameters. Second, we calculate the arithmetic mean of the probability of illness per serving. This constitutes the "marginal risk" (MR) which is one of the central possible outcomes. It can be defined as the "expected risk of illness for one random individual after one intake of the considered food product". To predict the expected number of salmonellosis cases one

could multiply the MR by the number of consumed serving for the considered period of time in the susceptible population.

A food outbreak is defined as an incident in which at least two grouped cases became ill, with similar symptoms, after the consumption of a same food. This risk of outbreak and the number of outbreaks were estimated.

**Table 3.5.** The production process and the model of the dynamics of the number of cfu per unit.

| Step | unit | | Process | N (cfu/unit) Unit size |
|---|---|---|---|---|
| 1 | | | The prevalence of animals with feces positive for *Salmonella* is $P_f$ | |
| | | | *n* carcasses are contaminated with $a_i$ g feces of $m_i$ animals, with concentration $C_{ij}$ cfu *Salmonella*/ g ($i$=1..$n$, $j$=1.. $m_i$). | |
| | | | $n \sim 1+\text{Poisson}(n_{mean} - 1)$ | |
| | | | $m_i \sim 1+\text{Poisson}(m_{mean} - 1)$ | |
| | | | $a_i \sim a_{max} \times \text{Beta}(\alpha, \beta)$ | |
| | | | The relative contribution per animal is $f_{ij}$ | |
| | | | $f_{ij} \sim \text{Beta}(b_1, b_1 (m_i - 1))$ | |
| | | | The expected number of cfu per carcass is $n_i = Poisson\left( a_i \sum_{j=1}^{m_i} f_{ij} c_{ij} \right)_j$ | |
| | carcass i | | $N_i \sim \text{Poisson}(n_i)$. | $W_{carc}$ kg |
| 2 | half carcass i | part | The expected number of cfu per half carcass is $N_i' \sim \text{Bionomial}(N_i, \text{Beta}(b_2, b_2))$ | 0.5 $W_{carc}$ kg |
| | | . | | |
| 3 | trimmings | part. | $n_i' \sim$ | $W_{tri}$ kg |
| | | | $\text{Binom}(n_i, \text{Beta}(b_4, 0.5\ W_{carc}\ b_4 / W_{tri} - 1))$ | |
| | | | With | |
| | | | $W_{tri} \sim \text{Beta}(b_5, b_5 (n-1))\ W_{gb}$ | |
| 4 | ground beef | mix. | $N' = \Sigma_n n'_i$ | $W_{gb}$ kg |
| 5 | ground beef patty | part. | $N_j' \sim \text{Possion}(N, \text{Beta}(b_6, W_{gb}\ b_6 / W_{gbp,j} - 1))$ | $W_{gbp}$ g |
| | ground beef patty | C | Concentration of *Salmonella* per serving C = $\mathbf{N_j'}$ in step 5 | CFU/gbp |
| | ground beef patty | $T_c$, $t_c$ | Temperature-duration profiles of cooking models described by Ou and Mittal, 2007 | |
| | ground beef patty | $D_c(T(t))$ | Decimal reduction time after cooking for fat content 7% (others; see result tables) $D(T(t)) = 10^{[9.929 + \text{Normal}(-0.155, 0.0084)T]}$ | |
| | ground beef patty | $E_c$ | Number of log-reductions caused by the whole operation of cooking. $E_c = \int_0^{t_c} \frac{1}{D_c(T(t))} d(t)$ | min |
| | | d | Concentration of *Salmonella* consumed from 100g of ground beef patty $C/10^{E_c}$ | CFU/gbp |

Part. = partitioning, mix. = mixing, gbp = ground beef patty

**Table 4.5** Description and distribution of variables and models for the risk assessment of human salmonellosis from consumption of ground beef patty.

| Variable | Description | Distribution/model |
|---|---|---|
| *PI* | Probability of infection | $1-(1-r)^d$ |
| *d* | Number of *Salmonella* consumed from 100g of ground beef patty | See table |
| *r* | A parameter | Beta(α,β)<br>Beta(0.3126, 193120.42) |
| *PM* | Probability of illness | 0.10 |
| *RI* | Individual risk of salmonellosis/serving | *PI**PM* |
| *MR* | Marginal risk or expected risk of illness | Average of *RI* |
| *NR* | Number of meals/serving | $1*10^7$ |
| *NC* | Expected number of salmonellosis cases | *MR**NR* |
| *RO ≥ 2* | Risk of outbreak with at least two cases from a batch of ground beef | Pr(NC ≥2 for 2000 iterations) |

## 3. Results

### 3.1. Ground beef batch contamination

The probability of ground beef batch being contaminated was estimated to be 100% after 2000 iteration. The distribution of the level of contamination ground beef patties with *Salmonella* within the ground beef batch was obtained (Fig. 5.5). The expected percentage of ground beef batches with percentage of contaminated patties less than 1, 6, 12, and 18 were 22.5%, 52.1% , 69.07% and 95.07%, respectively with maximum of 18% as shown in fig 5.5.

### 3.2. Ground beef patties contamination

The simulated concentration of *Salmonella* in a typical ground beef patty serving before cooking of 100 g ranged from 0 to $1.4 \times 10^6$ *Salmonella* cells with a median of 0 cells (Fig. 6.5). About 92.9% of ground beef patties (55.74 million out of 60 million patties) were expected not to be contaminated. The expected percentage of ground beef patties with contamination greater than 5, 10 and 100 *Salmonella* cells were 29%, 17.1% and 0.02%, respectively.

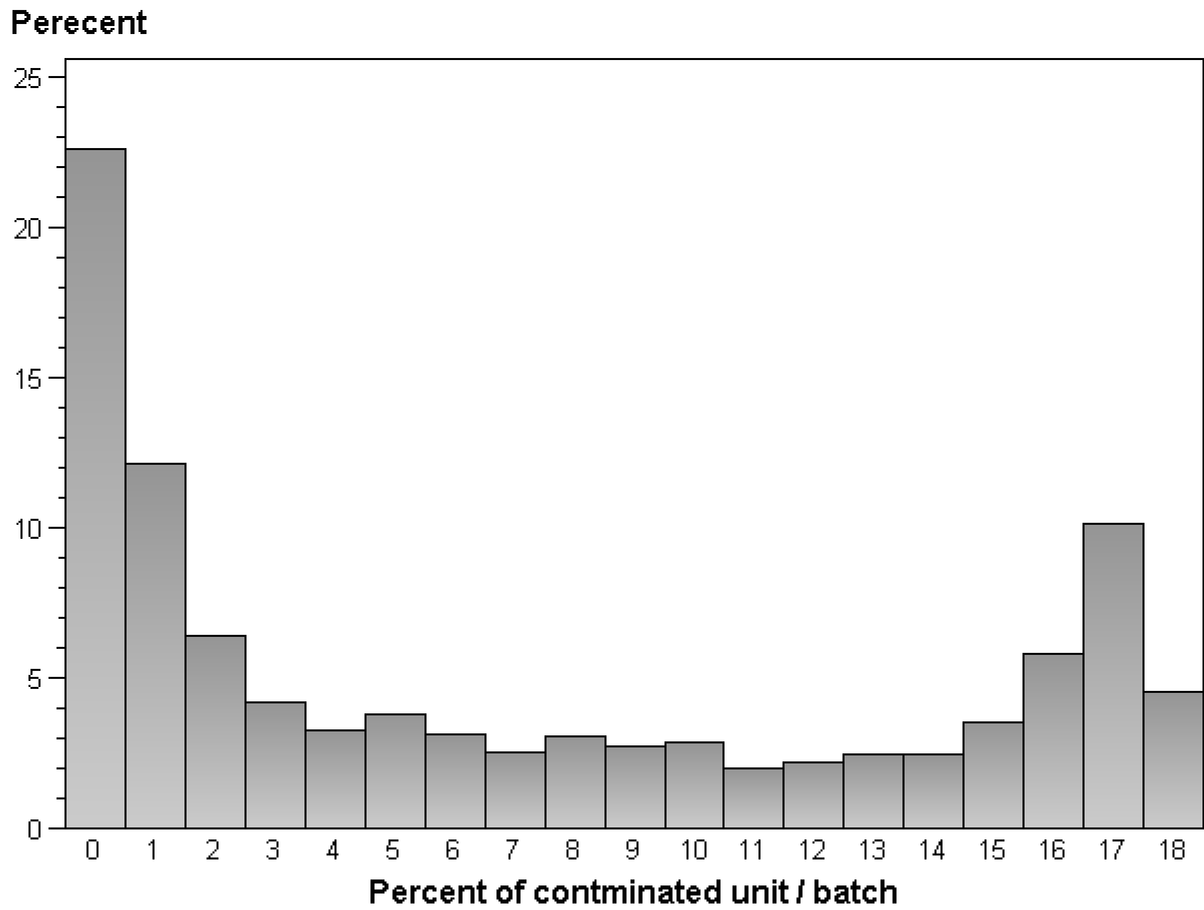



**Figure 5.5** Simulated frequency distribution for *Salmonella* contaminated ground beef patties between and within ground beef batch (2000 iteration).

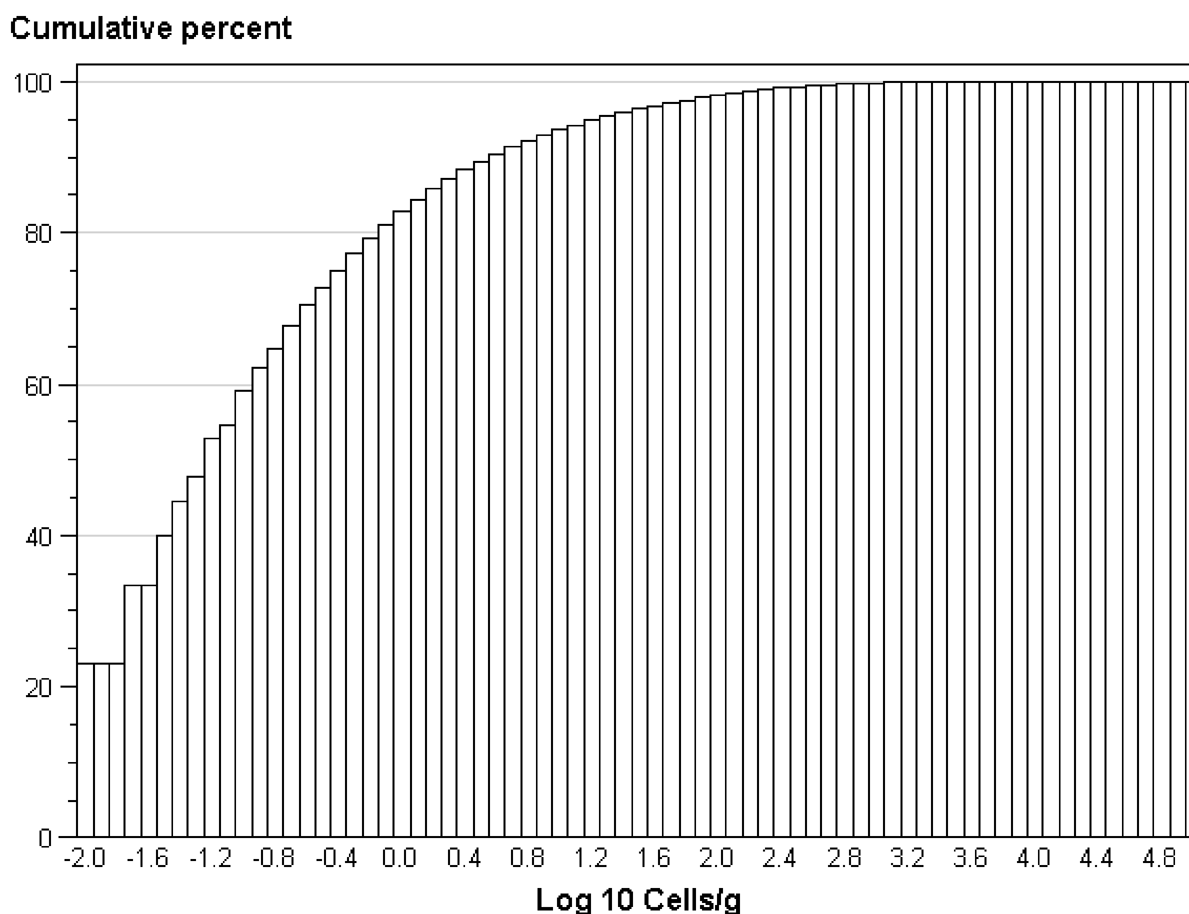



**Figure 6.5** Simulated frequency distribution for *Salmonella* concentration in 1g ground beef (92.9% ground beef patty serving was expected not to be contaminated): 2 000 iteration

Percentiles of the distribution of *Salmonella* in 100g serving of ground beef patty before cooking are presented in Table 5.5. The 99$^{th}$ percentile of *Salmonella* cell numbers in servings of 100 g of ground beef patty was 167 cells before cooking. The intra-class correlation (variance between batches/total variance) was equal to 87%. It means that 87% of *Salmonella* concentration per g of patty could be explained by batch-level factors.

**Table 5.5** Percentiles of the distribution of *Salmonella* in 100 g ground beef patties

| Percrntiles | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 25th | Median | 75$^{th}$ | 90$^{th}$ | 95$^{th}$ | 99$^{th}$ | Maximum |
| | Absence | Absence | 0 | 0 | 2 | 167 | 1433011 |

## 3.3. Preparation and cooking practices

### *3.3.1. Cooking temperature*

In our model ground beef patties could be consumed rare, medium, or well done. Dependent of the time of cooking, the types of cooked ground beef patties are presented in Fig. 7.5 shows a comparison of the ground beef patty center temperatures at 140, 160, 180°C pan temperatures with three flippings. The cooking time was set up to 10 min. During the frozen period, the center temperature rises slowly and the increase rates are not significantly different for various pan temperatures. A higher pan temperature results in a shorter heating time to overcome the latent heat of fusion. The cooking temperatures for the patty geometric center at 140, 160 and 180°C pan temperatures were 61 , 71.5, 80.04°C at 600s, respectively. We excluded pan 140°C from further analysis.

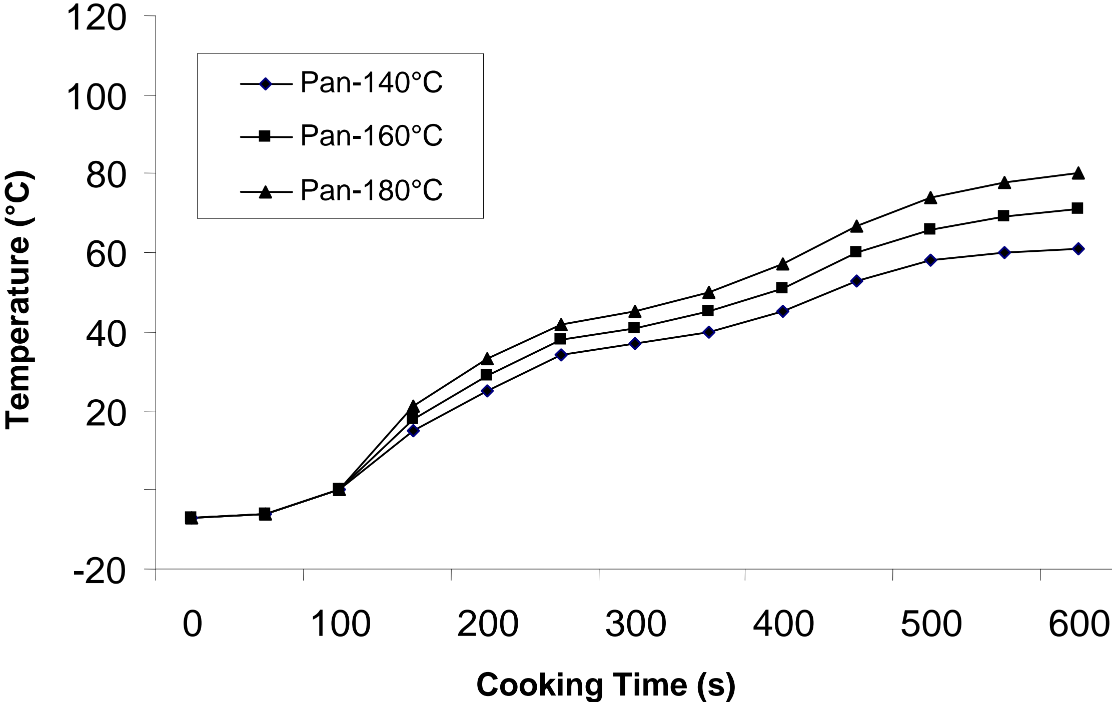



**Figure 7.5.** Comparison of simulated ground beef patty center temperatures at 140, 160, 180°C pan temperatures during single-sided pan-frying of frozen patties with three flippings.

*3.3.2. Salmonella reduction during cooking*

121 survival curves were constructed for inactivation *Salmonella* spp. in ground beef for different fat levels (7, 12, 18, 24, and 25%). Data for fat content 25% did not give good fit to the model and it is excluded from the result due to the effect of other environmental factors present in that study. The survival curves were linear on a semi-logarithmic plot. A linear model provided a fair-to-good fit at all temperatures, with R-square values of 0.9809 to 0.9904. It was assumed that 20% of *Salmonella* cells were submitted to the heating treatment pertaining to the coldest spot in the ground beef patty (in center) where temperature measurements were estimated above.

The regression analysis was run for each fat content (7, 12, 18, 24%) with temperature as the only variable of the model to determine the equation of the regression line and to estimate the slope and its standard error: (i.e. for fat content 7%, the other fat content shown in table 6.5.).

$\text{Log } D_7 = 9.929 - 0.155T$

The Z-value is the reciprocal of the slop and was equal to:

$$Z_7 = \frac{1}{0.155} = 6.448$$

We attributed to the slop a normal distribution (fat content 7% as example) with a mean of -0.1555 and a standard deviation of 0.0084, resulting from the regression analysis. So D(T(t) becomes:

$D_7(T(t)) = 10^{[9.929 + \text{Normal}(-0.155,\ 0.0084)T]}$

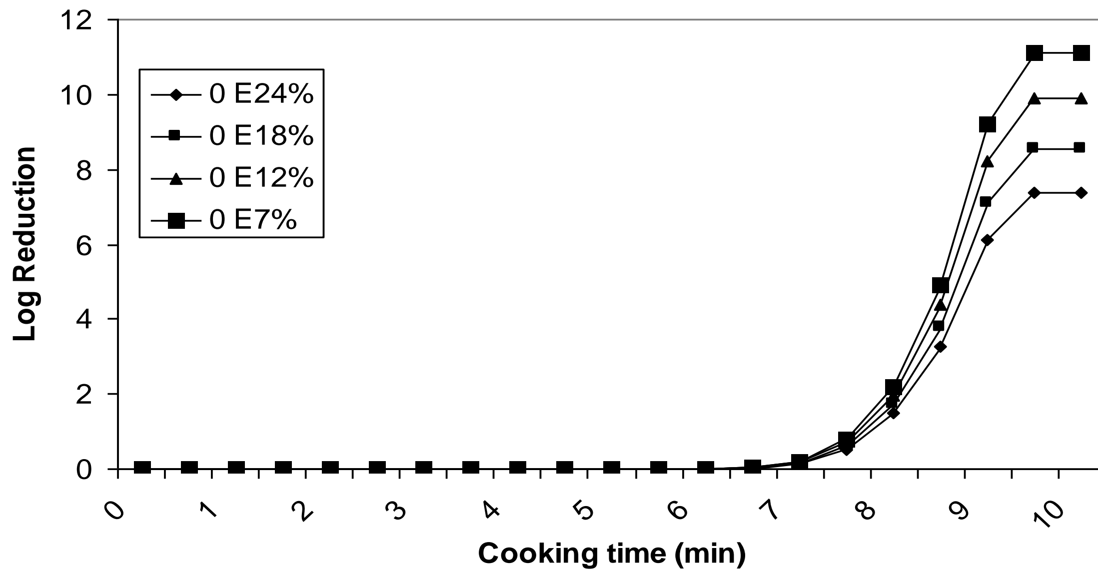
This equation was applied for each cooking operation (when the fat content is 7%) and for each type of meat preparation. The time/temperature profiles estimated by using mathematical heat and mass (moisture and fat) transfer models (Ou and Mittal, 2007) were randomly generated, incremented by 25 sec.

The number of decimal reductions ($E_c$) was dependent on the level of fat, type of meat (frozen or chilled), pan temperature and finally the time of cooking. As shown in fig. 8.5., fig. 9.5. and table 7.5.; the heat treatment submitted by the pan 180°C was most efficient than the pan 160°C since the cooking time was shorter with a higher log destruction of *Salmonella* in the center of ground beef patties. For example; after 6.5 min cooking the number log reductions in the center of patties is ranged from 10.34 to 15.69, 0.59 to 0.88 for pan 180°C and 160°C respectively dependent on fat content.

**Table 6.5.** D- and Z- values and regression parameters (α, β) obtain for four fat levels (7, 12, 18, 24%) at different temperatures for *Salmonella* spp. in ground beef.

| Fat level (%) | Temperature (°C) | α(se) | β(se) | D-value | R-square | Z-value |
|---|---|---|---|---|---|---|
| **7** | 58 | 9,92874 | -0,15509 | 8,58065 | 0,9827 | 6,44787 |
| | 60 | (0.5166) | (0.0084) | 4,20088 | | |
| | 62 | | | 2,05665 | | |
| | 65 | | | 0,70451 | | |
| | 67 | | | 0,34491 | | |
| | 70 | | | 0,11815 | | |
| | 72 | | | 0,05784 | | |
| **12** | | | | | | |
| | 58 | 10,02149 | -0,15573 | 9,75326 | 0,9877 | 6,42137 |
| | 60 | (0.4352) | (0.0078) | 4,76091 | | |
| | 62 | | | 2,32397 | | |
| | 65 | | | 0,79257 | | |
| | 67 | | | 0,38688 | | |
| | 70 | | | 0,13194 | | |
| | 72 | | | 0,06441 | | |
| **18** | | | | | | |
| | 58 | 10,01805 | -0,15473 | 11,05885 | 0,9904 | 6,46287 |
| | 60 | (0.3817) | (0.0062) | 5,42313 | | |
| | 62 | | | 2,65944 | | |
| | 65 | | | 0,91327 | | |
| | 67 | | | 0,44786 | | |
| | 70 | | | 0,15380 | | |
| | 72 | | | 0,07542 | | |
| **24** | | | | | | |
| | 58 | 10,01430 | -0,15377 | 12,46350 | 0,9809 | 6,50322 |
| | 60 | (0.5378) | (0.0088) | 6,13903 | | |
| | 62 | | | 3,02385 | | |
| | 65 | | | 1,04532 | | |
| | 67 | | | 0,51488 | | |
| | 70 | | | 0,17799 | | |
| | 72 | | | 0,08767 | | |

(A)




(B)

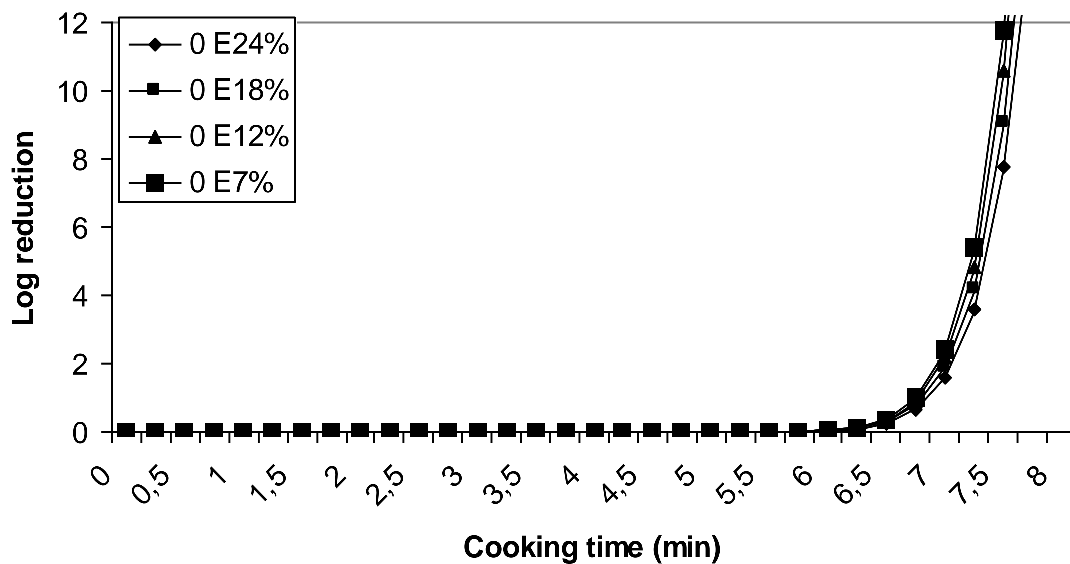
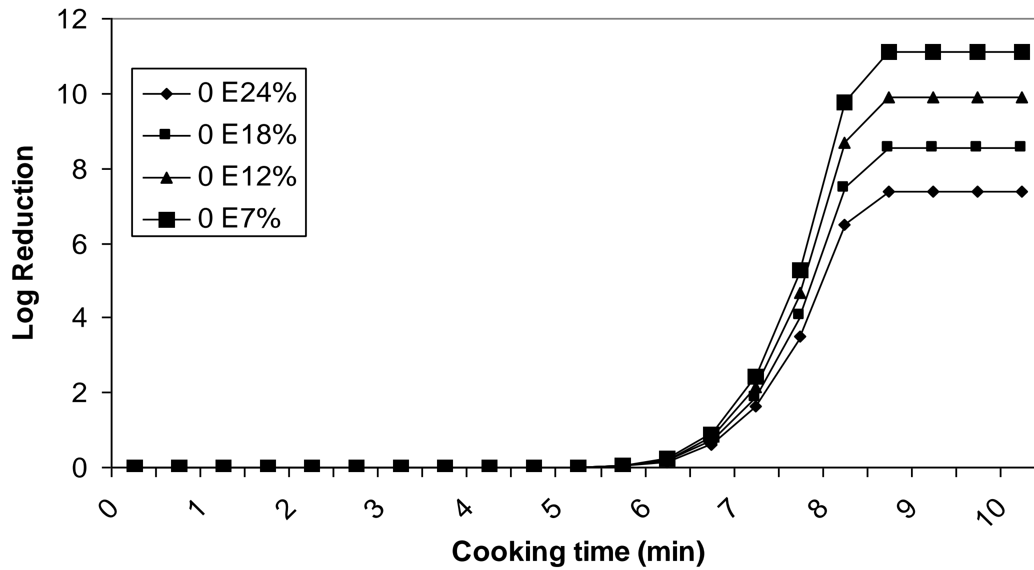



**Figure 8.5.** log reductions of *Salmonella* during time of cooking at various fat content of the frozen patties ($T_{initial}$ = -6°C) (A) and the chilled patties ($T_{initial}$ = 4°C) (B) at pan temperature of 160°C with three flippings.

(A)




(B)

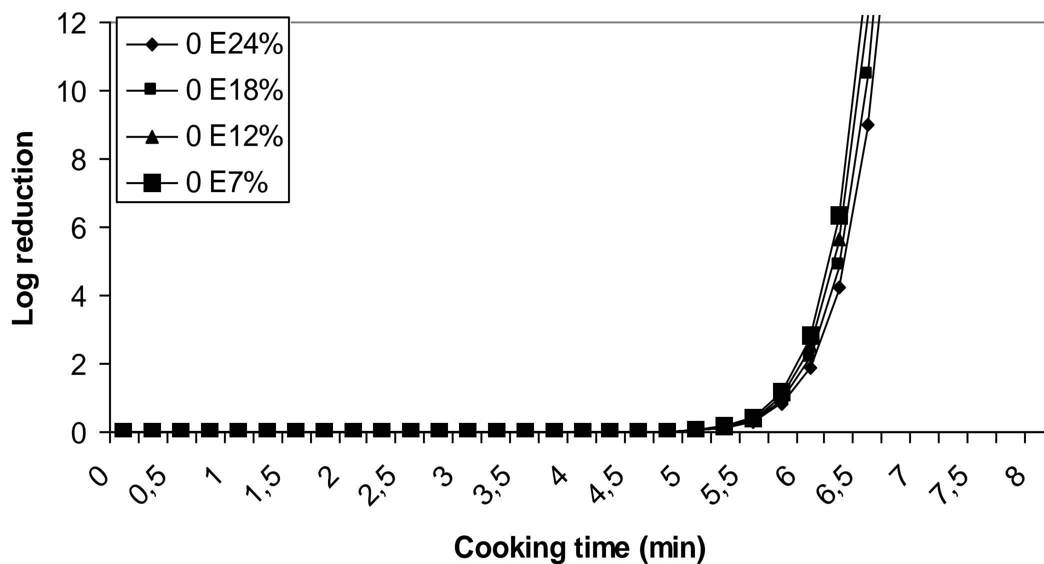



**Figure 9.5.** log reductions of *Salmonella* during time cooking at various fat content of the frozen patties ($T_{initial}$ = -6°C) (A) and the chilled patties ($T_{initial}$ = 4°C) (B) at pan temperature of 180°C with three flippings.

**Table 7.5.** Number of log reductions calculated for each level of fat (a: pan 160°C; b: pan 180°C) with time (min) in the center of ground beef patties.

| Time of cooking (min) | Fat level | | | |
|---|---|---|---|---|
| | 24% | 18% | 12% | 7% |
| (a) Pan 160°C | | | | |
| 4.5 | 0 | 0 | 0 | 0 |
| 5 | 0.00319986 | 0.00355816 | 0.00397839 | 0.00456275 |
| 5.5 | 0.02718552 | 0.03052731 | 0.0344837 | 0.03929062 |
| 6 | 0.15115804 | 0.17129627 | 0.19534671 | 0.22122583 |
| 6.5 | 0.59499585 | 0.6792689 | 0.78063125 | 0.87969901 |
| 7 | 1.62181015 | 1.86029324 | 2.14843751 | 2.41347821 |
| 7.5 | 3.51418851 | 4.04513539 | 4.68882137 | 5.25493435 |
| 8 | 6.48829134 | 7.48848507 | 8.70417539 | 9.73781187 |
| 8.5 | 7.38373569 | 8.5268155 | 9.91694378 | 11.0903926 |
| (b) Pan 180°C | | | | |
| 3.5 | 0 | 0 | 0 | 0 |
| 4 | 0.00064248 | 0.0007121 | 0.00079351 | 0.00091204 |
| 4.5 | 0.00814622 | 0.00908547 | 0.01019023 | 0.01166371 |
| 5 | 0.0467551 | 0.05262663 | 0.05959392 | 0.06779399 |
| 5.5 | 0.36082002 | 0.41153972 | 0.47249524 | 0.53278608 |
| 6 | 2.23693607 | 2.57853384 | 2.99326558 | 3.35148686 |
| 6.5 | 10.3488541 | 12.0334407 | 14.0960526 | 15.6917456 |
| 7 | 32.2824063 | 37.7499316 | 44.4811767 | 49.3306417 |
| 7.5 | 79.6989092 | 93.6075431 | 110.804559 | 122.52558 |
| 8 | 154.815935 | 182.347401 | 216.481794 | 238.932199 |

Comparing the temperature in the center of ground beef patties with the given time using the temperature-time profile (fig. 7.5.), we could estimate the ranged and more likely temperature in the center of the patty corresponded to the type of cooking (rare ranged from 53.5-55..5°C, medium ranged from 61.7-63.7°C and well done ranged from 67.3-69.3°C). The data collected by AFSSA (2007) was estimated that 16%, 52% and 32% of the ground beef patties are consumed rare, medium and well done respectively.

The average number of log reductions ($E_c$) was equal to 0.005, 0.006, 0.006, and 0.007 for ground beef patties consumed rare with fat content 24, 18, 12, and 7% respectively and was 0.14, 0.16, 0.19, and 0.21 for ground beef patties consumed medium with fat content 24, 18, 12, and 7% respectively and was 1.87, 2.14, 2.48, and 2.78 for ground beef patties consumed well done with fat content 24, 18, 12, and 7% respectively as shown in table 8.5. and fig. 10.5.

**Table 8.5.** The expected number of log reductions estimated for each cooking type (a: rare; b: medium; c: well done with min, most likely and max temperature) with different fat level using a pan 160°C with time corresponded to this temperature in the center of frozen and chilled ground beef patties (s).

| Temperature | Time corresponded (s) | | Fat content | | | |
|---|---|---|---|---|---|---|
| (°C) | Frozen patty | Chilled patty | 24% | 18% | 12% | 7% |
| (a) Rare | | | | | | |
| 53.5 | 413 | 302 | 0.00319986 | 0.00355816 | 0.003978395 | 0.00456275 |
| 54.5 | 419 | 308 | 0.00513734 | 0.00572252 | 0.006410001 | 0.00734298 |
| 55.5 | 425 | 314 | 0.00797727 | 0.0089026 | 0.009991639 | 0.01143162 |
| (b) Medium | | | | | | |
| 61.7 | 464 | 353 | 0.09605228 | 0.10859613 | 0.123543697 | 0.14012757 |
| 62.7 | 472 | 361 | 0.14394441 | 0.1630804 | 0.185928391 | 0.21059524 |
| 63.7 | 480 | 369 | 0.211224 | 0.23978331 | 0.273947881 | 0.30987858 |
| (c) Well done | | | | | | |
| 67.3 | 521 | 410 | 1.13153746 | 1.29581615 | 1.493994797 | 1.68012057 |
| 68.3 | 538 | 427 | 1.86597208 | 2.14171909 | 2.475090444 | 2.77925194 |
| 69.3 | 557 | 446 | 3.05653117 | 3.5161603 | 4.073053695 | 4.56669893 |

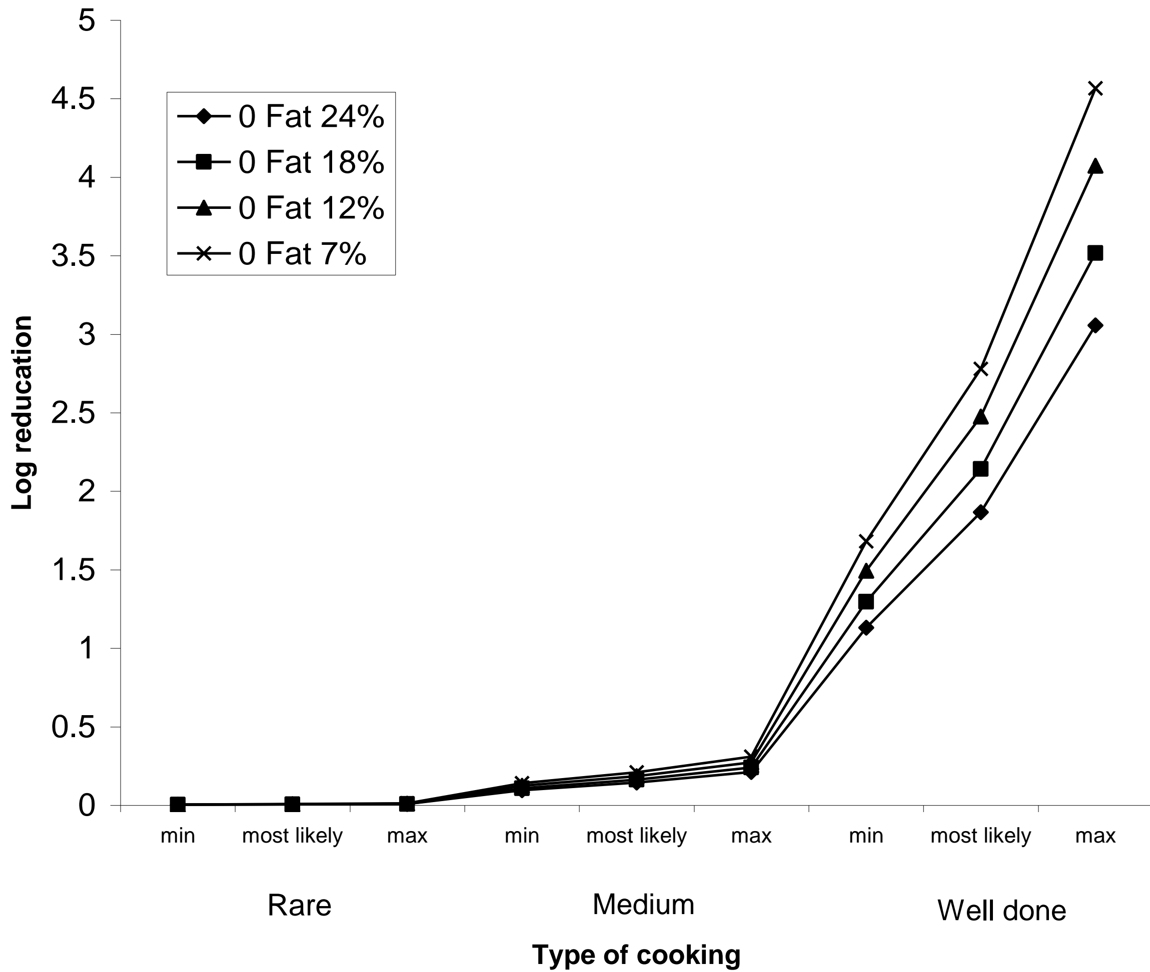



**Figure 10.5.** The expected number of log reductions estimated for each level of fat dependent on the type of cooking (rare, medium, and well done) in the center of ground beef patties.

## 3.3. Risk characterization

### *3.3.1. Risk of salmonellosis*

The risk of salmonellosis was closed to zero when the 100 g serving ground beef patties consumed well done, whatever the temperature degree (min, most likely, or max). The results obtained for the risk of the different type of cooking and the expected number of cases per 10 million servings summarized in tables 9.5. and 10.5.

**Table 9.5.** The dose and individual risk of salmonellosis linked to the consumption of one ground beef patty (rare 16%, medium 52% or well done 32%) and the expected number of cases per 10 million servings for different fat levels (a: 7%; b: 24%).

| Risk | Mean | Maximum | 95th Pctl | 99th Pctl | Expected number of cases / 1.0E+07 servings |
|---|---|---|---|---|---|
| (a) Fat 7% | | | | | 11.01 |
| Dose | 7.058867 | 208417 | 0 | 10 | |
| RI | 1.10E-06 | 0.0892358 | 0 | 0 | |
| (b) Fat 24% | | | | | 12.33 |
| Dose | 7.953581 | 213096 | 0 | 12 | |
| RI | 1.23E-06 | 0.090899 | 0 | 1.82E-07 | |

**Table 10.5.** The dose and individual risk of salmonellosis linked to the consumption of one ground beef patty for different cooking type (a: rare; b: medium; c: well done) with different fat levels (7% and 24%) and the expected number of cases per 10 million servings.

| Type of cooking | Risk | Mean | Maximum | 95th Pctl | 99th Pctl | Expected number of cases / 1.0E+07 servings |
|---|---|---|---|---|---|---|
| (a) Rare | | | | | | |
| Fat 7% | Dose | 14.5573 | 208417 | 0 | 33 | 23.17 |
| | RI | 2.32E-06 | 0.08057 | 0 | 8.00E-07 | |
| Fat 24% | Dose | 14.6378 | 210975 | 0 | 33 | 23.28 |
| | RI | 2.33E-06 | 0.08066 | 0 | 7.98E-07 | |
| (b) Medium | | | | | | |
| Fat 7% | Dose | 9.06522 | 143402 | 0 | 20 | 14.00 |
| | RI | 1.40E-06 | 0.08923 | 0 | 4.86E-07 | |
| Fat 24% | Dose | 10.6396 | 213096 | 0 | 24 | 16.31 |
| | RI | 1.63E-06 | 0.09089 | 0 | 5.70E-07 | |
| (c) Well done | | | | | | |
| Fat 7% | Dose | 0.04789 | 2988 | 0 | 0 | 0.07 |
| | RI | 7.42E-09 | 0.00136 | 0 | 0 | |
| Fat 24% | Dose | 0.24606 | 10206 | 0 | 0 | 0.38 |
| | RI | 3.84E-08 | 0.00488 | 0 | 0 | |

For 10 million servings of 100 g of ground beef patty, the number of cases predicated by the model is in average 11, 12 for fat content 7% and 24% respectively when the data collected by AFSSA (2007) was used (rare 16%, medium 52%, and 32% well done). The risk of salmonellosis per 100 g serving ranged from 0 to 2.33E-06 dependent on the type of cooking and the fat content as shown in tables 9.5 and 10.5. The risk was equal to zero (no salmonellosis cases occurring) in 99% of the iterations when the meat consumed well done.

The risk of getting salmonellosis from consumed rare ground beef patties is more than 312, 60 times higher for fat content 7% and 24% respectively comparing to the consumption of well done patties. Whereas consumption of medium ground beef patties would increase the risk by more than 188, 42 times comparing to the consumption of well done patties for fat content 7% and 24% respectively.

*3.3.2. Risk of outbreak*

The risk of outbreaks was calculated as the probability of having at least 2 cases of salmonellosis from one ground beef batch for 2000 iterations (60 million patties). There are 35 batches with associated with at least one case of salmonellosis out of 2000 simulated batches (1.8%). Only, 15 of them have 2 cases or more (0.75%, 15/2000).

## 4. Discussion

Despite the amount of ground beef patties consumed daily in France, outbreaks of infection remain comparatively rare. *Salmonella* outbreaks due to the consumption of ground beef patties in France were reported (See chapter 2 of this thesis). Human cases of salmonellosis occur sporadically or as part of outbreaks. The role of ground beef patties in sporadic cases is not well assessed. Under-notification of sporadic salmonellosis cases and non systematic case investigation complicate the demonstration and quantification of ground

beef productions role in sporadic cases. Consumers-particularly those susceptible to infectious diseases (for example, infants, elderly people and immuno-compromised patients) should also be warned that a nil risk can not be warranted from rare or medium cooking of ground beef patties.

In lieu of the origin of data and assumptions made, the results of this risk assessment should be interpreted carefully. The simulations presented herein were based on our own data collected specifically to assess the risk of salmonellosis from consumption of ground beef patties. The model incorporated also data in published literature. This study showed a high frequency of ground beef batches contamination (100%) but mostly a low level of patties contamination.

For the frequency of contamination, we observed the same prevalence rate of *Salmonella* in fecal samples 9.12% as the previously reported prevalence of 9.5% (Heuchel et al., 2000) and 8.1% (Lailler et al., 2005). For the enumeration, we used the MPN-real-time PCR assay to enumerate *Salmonella* in fecal samples. The assay enabled the enumeration of *Salmonella* in fecal samples that ranged from <1.8 to 1609 MPN of *Salmonella* per g. We believe that these estimates overestimated the original concentration of the organism in faeces. It has been previously reported (Mantynen et al., 1997; Martin et al., 2004) that assays based on MPN-PCR tended to give higher estimates than traditional enumeration methods. These results clearly indicated that these higher estimates are due to the detection of DNA from dead and stressed cells, which were not able to form colonies. These results are also supported by our findings for the enumeration of *Salmonella* in inoculated fecal samples using the MPN-real-time PCR assay (chapter 3 of this thesis).

The risk assessment model predict about 8.1% of ground beef patties (4.86 million out of 60 million patties) to be contaminated. A large fraction of patties has only one CFU of

*Salmonella* (70 %), where the detection limit in the microbiological analysis is probably much higher than 1 cfu (no less than 1 CFU /25 gr). This implies that one would expect that the prevalence predicated by the model would be higher than the prevalence found in a surveillance study. We did not find any published data related to the prevalence of *Salmonella* in ground beef in France. The prevalence of *Salmonella* in ground beef meat in the EU is ranged from 0 to 2.1 and 0 to 1.7 in year 2005 and 2006 respectively dependent on the number of samples and the size of sample (EFSA, 2006, 2007).

The minimum growth temperature associated with *Salmonella* on ground beef ranged from 10 to 12°C, with a very slow growth at that temperature (Chapter 3 of this thesis). The model was developed for growth of *Salmonella* on ground beef from published data (see chapter 3 of this thesis for the data). Using this growth model, time and temperature was governed the amount of growth during the product's transportation from the retail outlet to the home and it's storage in the refrigerator or freezer, prior to food preparation for both chilled and frozen patties (data not shown). The model predicts low and slow increase during this module. For example, at least 37 h needed for the *Salmonella* to increase by one log at 10° C. We assume this is unlikely to occur because the products become spoiled and unfit for human consumption. We exclude the transportation module from our model.

To estimate the number of decimal reductions and evolution of the microbial population before consumption, we did use literature data to develop inactivation model. By calculating D- and Z-values and using them with the temperature/time profile model; we were able to get the estimation of the numbers of log reduction at given temperature in the center of the ground beef patties.

Because of the lack of data on the infective dose of *Salmonella,* we chose to use dose-response model published by Haas et al. (1999) which was fitted to the naïve human data from *Salmonella* feeding trials and outbreaks investigations. The model did not take into account the acknowledged, but not well-defined serovar variation. We did not use the dose response model developed for the WHO/FAO risk assessment of *Salmonella* in eggs and broilers (Anonymous, 2002b). The later model was developed using various outbreak data where the exposure doses estimate are judged not enough accurate and that led to high uncertainty on risk estimate.

This study indicated that the risk of salmonellosis after a well done cooking was closed to zero whatever the fat content in the product as cooking times were sufficient to reach the recommended temperature (68.3°C) in the centre of the patties. Yet the risk could be multiply by just 312 if the patty is cooked during a short time (rare).

The model simulation did not take into account the possible cross-contamination occurring during ground beef patty production, transport and distribution or in the consumer's fridge. Yet, provided basic hygiene rules are followed along the whole chain, the model remains a practical value and fairly low numbers of expected cases tends to confirm that ground beef patties are low risk foods as far as salmonellosis is concerned if they are consumed well done.

Despite the limitations that we underscored, the present work is the first attempt to model the risk of *Salmonella* infection linked to the consumption of ground beef patties which tended to show that the risk of salmonellosis could be considered relatively low and is manageable at the farm and processing levels.

The model could be used to assess different mitigations options such as the effect of more strict hygienic procedures during slaughtering, meat mixing, patties formation and the way of cooking the ground beef patty. Efforts for risk mitigation should be focused on reducing the risk estimated, even if this represents a relative, rather than absolute value.

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# Chapter 6: General Discussion and Conclusion

### Detection and quantification of *Salmonella* in bovine fecal

Although expensive, the usefulness and the advantages of the real-time PCR technology using the LightCycler™ instrument were obvious in our work. This is because the amplification reaction, detection of PCR products, and their melting curve analysis can be performed with a single capillary tube which reduces the risk of experiencing laboratory product contamination. SYBR Green Ⅰ is used as the fluorescent dye and subsequent melting curve analysis of PCR products generates a specific profile that can be used to determine the specificity of the reaction. This curve permits differentiation of the signals from the amplified target from those of the PCR by-products, by reference to melting temperature ($T_m$) values. In this work, we optimized a sensitive and specific real-time PCR assay for *Salmonella* detection in fecal using SYBR Green Ⅰ based detection and LightCycler analysis. The melting curve analysis was sufficient to determine the specificity of the reaction. PCR product gave a distinct $T_m$ of about 87°C for all *Salmonella* tested. As expected, any fluorescence in the negative capillary gave a much lower $T_m$, usually 79°C. We observed also that the height of the peaks varied, indicative of the amount of accumulated product in both artificially and naturally contaminated fecal samples analysis. Other reports (O'Mahony and Colin, 2002) have indicated that the height of the peaks varies relatively to the amount of accumulated product.

Application of real-time PCR for the detection of pathogens in food samples is often limited by the presence of substances that inhibit the PCR reaction, poor quality of target DNA, or insufficient enrichment of target DNA (Chen et al., 1997; Heller et al., 2003). Published reports (Liming and Bhagwat, 2004; Croci et al., 2004) suggested the use of pre-enrichment step or an immuno-magnetic separation with additional enrichment (Mercanoğlu and Griffiths, 2005) prior to DNA extraction methods. In our work the artificially and the

naturally contaminated fecal samples were pre-enriched in BPW as suggested by Liming and Bhagwat (2004) and also because the BPW medium is used for pre-enrichment in the horizontal ISO method for the detection of *Salmonella* spp. (Anonymous 2002). For artificially contaminated samples, the detection sensitivity of real-time PCR was 1-5 CFU/mL on inoculum after 8 h of pre-enrichment. Such pre-enrichment time, in fact, allowed the *Salmonella* target to multiply until reaching detectable concentration, which in our experiment conditions was $10^3$ cells/mL. It has been previously reported the detection limit of the real-time PCR is $10^3$ CFU/mL (Seo et al., 2006). These results agree with those of other reports. Croci et al. (2004) indicated that 5 h of pre-enrichment allowed the target *Salmonella* to multiply until reaching a detectable concentration of $10^3$ cells/g by PCR in meat products, and in some cases, PCR was able to identify positive samples after only 4 h of incubation. However, to assure the positive detection of low levels of *Salmonella* that might be present in bovine beef samples; a 18-h pre-enrichment step in BPW was carried out prior to the PCR. The assay detected 27 positive-*Salmonella* samples (9.12%). These findings clearly suggested that the prevalence of *Salmonella* in fecal samples is within the range of prevalence rates reported in the literature. This level of contamination is in agreement with another French study (Heuchel et al., 2000), which showed the prevalence of *Salmonella* in fecal samples to be 9.5% and Lailler et al. (2005) reported prevalence herd with salmonella (8.1%). However, previously reported surveys of cattle fecal samples in Europe, Australia, United States and Canada have shown large variations in the prevalence of *Salmonella* in feces ranging from 2% to 50% (Ransom et al., 2002; McEvoy et al., 2003; Fegan et al., 2004; Blauel et al., 2005; Fegan et al., 2005; Fluckey et al., 2007; Stephens et al., 2007). The large variations in levels of fecal *Salmonella* contamination observed in these studies have been attributed to several factors such as variations in sampling and detection techniques, seasonal differences, herd size, geographic area, hygiene, and farm management practices.

The productivity of enrichment broth used is critical in order to ensure the presence of sufficient quantities of the target for the DNA extraction and PCR reaction. Overall, in the present work, the enrichment of artificially contaminated fecal samples seemed to be more efficient than naturally contaminated fecal. This result is supported by the findings of (Gouws et al., 1998; Uyttendaele et al., 1998) who indicated that in naturally contaminated samples, stressed cells may be present in the matrix, thus reducing the efficiency of the enrichment procedures.

There are many commercial kits available for easy extraction and preparation of DNA template for PCR assays. In our work, a preliminary experiment was performed to compare the performance of the traditional boiling method with a commercially kit (QIAamp® DNA Mini kit) for recovering template DNA from fecal samples artificially contaminated with *Salmonella enterica* serotype Typhmurium DT104. Both methods facilitated the detection of the organism and produced similar results (data not shown). However, in the present work, we chose to use the boiling method for the DNA isolation protocol because of its simplicity and rapidity. The method gave optimal results, and no inhibition was observed in pre-enriched inoculated fecal samples as indicated by the shape of the fluorescent amplification curves.

The use of real-time PCR can be combined with subculture of enrichment broths from PCR-positive samples for the isolation of the pathogen. Several previously reported real-time PCR have reported the isolation of pathogens including *Salmonella* using the traditional cultural methods when analysing PCR enrichments of different commodities (Van Kessel et al., 2003; Karns et al., 2005). Identification of isolates is of particular importance for epidemiological data and public health authorities. PCR procedure, on the other hand, can be used only as a screening tool because it indicates only presence or absence of the pathogen. In

our study, *Salmonella* Typhimurium was the only serotype isolated from the fecal animals with multidrug resistance (penta and FQ R) and *Salmonella* Derby the only serotype isolated from environmental sample with multidrug resistance (streptomycin Sm, Te, Su) by cultural procedures from the twenty seven fecal and nine environmental real-time PCR *Salmonella*-positive samples. This finding might not be surprising because for a variety of reasons including the relatively low number of real-time PCR positive samples in this study. Moreover, in all cases the number of *Salmonella* in the fecal samples was very low. Conventional cultural procedures will not always detect small numbers of *Salmonella* cells in certain food or fecal samples. Bensal et al. (2006) reported some factors that can influence recovery rates including sensitivity of the methods, the susceptibility of *Salmonella* strains to inhibitors in the food or media, and overgrowth by competitors during incubation. Karns et al. (2005) mentioned many reasons why *Salmonella* were not isolated from PCR positive samples; bovine feces contains many other organisms that may compete with *Salmonella* in the enrichment broth; the presence of other organisms on the XLT4 selective agar plates may interfere with the production of $H_2S$ by *Salmonella*; $H_2S$ production is required for the formation of the black colour in *Salmonella* colonies. Mejia et al.(2005) showed that RV broth has a low selectivity for Enterobacteriaceae present in pig feces and that XLT4 agar has a low discriminatory power and the study emphasise the need for new and more selective enrichment and different media to be developed. Bohaychuk et al. (2007) found that 22% (10/45) of real-time PCR *Salmonella* positive from artificially contaminated bovine fecal samples was negative with culture method, the author explained that this was due to the low number of cell after enrichment and he also expected there was false-positives obtained by real-time PCR method; however, supplementary analyses showed that *Salmonella* could be cultured from these negative samples when additional enrichment and IMS used. This would indicate either that very low number of *Salmonella* could not detected by the culture method

describe above or that there was a high degree of background flora, and additional measures were needed to increase the number of *Salmonella* to detectable levels and minimize background microflora.

Rapid tests for *Salmonella* identification might contribute to, but not replace, bacteriological culture techniques. Organism isolation is still needed for serotyping and determination of resistance profiles, and also for epidemiological studies. However, in a routine basis for diagnosis, it should be considered that a large number of samples may be processed in a relative short period of time using the real-time PCR assay.

The FDA BAM-MPN method is the most frequently used to estimate low population of foodborne pathogens in foods (Gooch et al., 2001). The BAM-MPN method uses conventional culture and biochemical techniques to identify isolates. This method provides statistical estimates of viable cell concentration but is limited by some well-known drawbacks. As an alternative, researchers have proposed the use of the real-time PCR assay for identification and enumeration of *Salmonella* (Nogva and Lillehaug, 1999; Piknova et al., 2005). The real-time PCR approach is an attractive alternative to the culture-based systems for the quantification of foodborne pathogens because the results are generated much faster, and because non-cultivable but active and potentially infectious agents can be quantified. Unfortunately, amplification efficiencies of the real-time analyses, on the other hand, can be difficult to ensure and their suitability for exact quantification of initial amount of target DNA has therefore been questioned (Klerks et al., 2004).

In our work, we introduced a simple technique that overcome the inconveniences of the traditional MPN method regarding both time and labour and solved the problems of

quantification efficiencies that arise from quantitative real-time PCR. The technique combined the both methods, the MPN method together with the real-time PCR (MPN-real-time PCR). The performance of the MPN-real-time PCR assay was investigated by analysing artificially and naturally contaminated bovine fecal samples with *Salmonella*. When analysing the artificially contaminated fecal samples, the assay tended to give higher estimates than the estimated levels of contamination (CFU/mL) inoculated into fecal samples which is probably due to DNA from dead and stressed cells, which where not able to form colonies (Sails et al., 2003). However, the estimated levels of contamination fell well within the 95% confidence limits of the MPN estimates. The detection of dead or injured cells has been recognized as an inherent disadvantage of DNA-based detection assays such as real-time PCR. Josephson et al.(1993) reported that PCR reaction does not differentiate the DNA from viable or non-viable organisms. However, recently, ethidium bromide monoazide has been applied to block the DNA of dead bacteria for PCR amplification (Nogva et al., 2003). This procedure could lead to a better agreement between real-time PCR and plate-count method.

On the basis of the MPN-real-time PCR quantification results of *Salmonella* in inoculated fecal samples, the assay had to be evaluated with regard to the quantification of *Salmonella* cells in bovine fecal samples naturally contaminated with *Salmonella*. The MPN-real-time PCR described for fecal samples proved to be rapid and sensitive and *Salmonella* could be enumerated even at low levels. Because our previously enumeration results in inoculated fecal indicated higher estimates than the inoculum levels estimated by direct plating, the results of enumeration in naturally contaminated fecal may also overestimate the original concentration of the organisms present in the fecal since the real-time PCR detection assay amplify DNA from viable as well as from and non-viable cells that might be present in naturally contaminated food matrix.

Because of the low turnaround-time and its simplicity, the MPN-real-time PCR could serve as a rapid alternative for direct quantification of bacterial pathogens in foods. The method enabled the completion of enumeration within 24 hours minimizing the need to use selective enrichment, selective plating, and confirmation steps of the traditional MPN method. However, care should be taken to further optimize every step of the procedure.

Further experiments should focus on the following:

(1) Further work is needed to improve DNA isolation protocol in order to free *Salmonella* DNA of fecal components, which may facilitate the detection and quantification of low levels of *Salmonella*. This can be done by comparing several extraction methods and protocols

(2) To investigate the potential of the MPN-real-time assay as a rapid quantification method, analysis of other naturally contaminated food should be considered. These assays must include an internal amplification control.

(3) Further work is needed to improve and optimize traditional isolation methods when analysing PCR enrichment of fecal and other food matrix.

**Modelling the Growth Limits of *Salmonella* in ground beef as a Function of Temperature, pH, and other Environmental Factors**

Predictive microbiology provides a powerful tool to aid the exposure assessment phase of quantitative microbial assessment. A limitation of the growth models of *Salmonella* found in the literature is that their output cannot easily serve as input in the risk assessment of *Salmonella* linked to the consumption of ground beef. Therefore, in the present work, growth / no growth model interface of *Salmonella* in ground beef was developed using the available growth data for *Salmonella* in ground beef in published papers and all the data related to ground beef in ComBase in order to lead us to an accurate description of the conditions that *Salmonella* can growth / no growth.

Among the 162 combination treatments of temperature and PH and other conditions observed in this study, growth of *Salmonella* was observed in 82 and no growth in 80. There were very few examples of conditions under which only some of the replicate cultures under the same conditions did or did not grow; indicating that the transition between conditions that permitted growth and those that did not was abrupt. Indeed, from overall results, it is clear that the temperature is the most important and the only factor significant in this study. There was no growth observed at temperature less than 10°C. Where as the temperature 10°C and 12°C are the only temperature; we did observe growth / no growth (sometimes with the same conditions).

Predictive modelling studies on the combined effect of temperature and pH suggest that the effect of these combinations on microbial growth rate is independent (McMeekin et al., 2000; Presser et al., 1998). The results of the present study indicated clearly that only temperature has effect on the growth of *Salmonella* on ground beef. All the main, interactive, and quadratic effect were ($P > 0.05$) except for the effect of temperature. Even though pH and

aw are important factors for microbial growth (McMeekin et al., 2000; Presser et al., 1998), in our study they have no effect due to meat structure. The most of raw ground beef has high water activities (> 0.98), moderate pH (5.5- 6.5) and readily available sources of energy, carbon and other nutrients makes them ideal for most microbial growth (Varnam and Sutherland 1985). The concordance index the Homer-Lemeshow goodness-of-fit statistic (Hosmer and Lemeshow, 1989), and the maximum measures of goodness of fit of the model developed. As determined by the concordance index, the degree of agreement between the predicted probabilities and observations was 96.4% concordant and 0.5% discordant. The Homer-Lemeshow goodness-of-fit statistic was 19.51 ( $\chi^2$, df 17, p= 0.3) and the maximum rescaled R- square statistic was 0.879. In other studies on modelling, growth / no growth boundaries of foodborne pathogens, the reported maximum rescaled R- square value has ranged from 0.805 to 0.927 (McMeekin et al., 2001; Presser et al., 1998; Salter et al., 2000; Tiennungoon et al., 2000).

The model was developed with published data of *Salmonella* on ground beef; therefore, factors such as food structure and microbial interaction were taken into account to give good accuracy of applicability to the specific food. Several studies have been shown that these factors might significantly affect microbial behaviour (Gram and Melchiorsen, 1996; Pin et al., 1999; Robins and Wilson, 1994). Most of growth / no growth interface models developed up to now are based on data from a single strain. Several studies, however, have reported significant strains variations in the response of foodborne pathogens (Barbosa et al., 1994; Begot et al., 1997; Thomas et al., 1992). Thomas et al. (1992), reported significant differences in temperature, pH, and NaCl limits among six strains of *Salmonella*. In the present study a single strain and mixture of *Salmonella* strains were used dependent on the aim of the study. The strains do not have any effect on the growth of *Salmonella* in this

model. By having all these combination of strains (single strain and mixture strains) gives the study the power to represent the extremes of the growth region of the individual strain. Thus, models that have been developed with a mixture strains can be considered more “safe” than those developed with a single strain.

In conclusion, such models can be beneficial to food industry because they can describe the conditions that can be applied to control a process or specify a formulation in order to minimize the risk of pathogen growth.

## Risk assessment model

Microbiological risk assessment is an important tool for evaluating and communicating the impact of raw material quality, processing and changes on food safety. QMRA is a unique scientific approach able to link, for the first time, data from food (in the farm-to-fork approch) and the various data on human disease to produce a clear estimation of the impact of contaminated food on human public health. It is also the most powerful tool available today to clearly assess the efficacy of each possible mitigation strategy. The current QMRA for *Salmonella* and ground beef was not complete because it did not contain potentially important pathogens events and because of data gaps and incomplete predictive models. Assumptions had to be made where data did not exist in order to model the pathogens events. Minimizing data gaps and assumptions are important steps towards producing QMRA that provides better predictions. The key issues preventing effective QMRA remain the uncertainty (i.e. lack of relevant data) and variability (i.e. data available indicates that the variability of a feature may limit effective assessment of the risks associated with it). In our study, the process of ground beef was modelled from slughter to consumption. In the exposure assessment the potential exposure to *Salmonella* in a single serving was estimated. The calculations were done simulating situations including high fat level (24%) against low fat level (7%) with different type of cooking (rare, medium, well done).

In the current study, predictive model for growth of *Salmonella* from slaughter to the consumption was used outside the QMRA in an effort to keep the model simple. This step explained as "*because of the very low probability of growth we could consider that there is absence of growth in ground beef during these important steps of risk model"*. This low probability assessed by growth/no growth logistic regression model which indicated clearly that only temperature has effect on the growth of *Salmonella* on ground beef ; wheras, there

was no growth observed at temperature less than 10°C. The temperature 10°C and 12°C are the only temperature; we did observe growth / no growth (sometimes with the same conditions). All the main, interactive, and quadratic effect were ($P > 0.05$) except for the effect of temperature. Even though pH and aw are important factors for microbial growth (McMeekin et al., 2000; Presser et al., 199), in our study they have no effect due to meat structure. The most of raw ground beef has high water activities (> 0.98), moderate pH (5.5-6.5) and readily available sources of energy, carbon and other nutrients makes them ideal for most microbial growth (Varnam and Sutharland 1985). However, it should realize that such exclusion could have implications in relation to the final outcome of the assessment process.

Dose-response assessment, this stage is by far raises the most difficulties for the evaluation of the risks related to food. With the question, which model to use in front of the significant number suggested for some pathogens like *Salmonella*, it is recommended to use several according to the targeted population and not to be satisfied with only one estimate of them. However, in the present risk assessment we preferred to use the model published by Haas et al. (1999), rather than the one proposed by the WHO/FAO (Anonymous. 2002). Even though the later model was developed using various outbreaks data, the estimated exposure are judged not enough accurate which may lead to high uncertainty on risk estimate

Even though, in this work, we have demonstrated the benefit of risk assessment as a risk evaluation tool, much of data remain imprecise or incomplete. The data needed for predictive microbiology. The same problem is posed for the dose-response relation for whom data are not available or too approximate. The present risk assessment model makes it possible to define precise objectives and priorities for future studies.

The results provided by the QRA must be more or less coherent with the reality. If the estimates are too different from the data observed by the monitoring systems, the model and the data must be totally reviewed. Considering the results of the present work, the model is of a practical value and the very low risk predicted by the model seemed to confirm surveillance and monitoring systems data of that ground beef are low risk foods as far as salmonellosis is concerned if they are consumed well done.

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## Appendix (A)

## Preliminary Experiments to Optimize a LightCycler Real-Time PCR Conditions for amplifying *Salmonella* Target DNAs

An optimization of the real-time PCR conditions such as $Mg^{2+}$, primer concentration, and annealing temperature is necessary since these parameters can critically affect the amplification efficiency of the PCR assay. Therefore, preliminary experiments were carried out to optimize these parameters following the optimization strategy described by manufacturer (Roche Molecular Biochemicals Technical Note No. LC 9/2000). All reactions were performed by the LightCycler instrument (Roche Diagnostics) using the LightCycler FastStart DNA Master SYBR Green I Kit (Cat. No. 003 230) (Roche Applied Science).

Stock cultures of *Salmonella* Hadar were maintained in 20% glycerol at -80°C. Fresh bacterial cultures for use in the experiments were produced by inoculating frozen stock cultures into Brain Heart Infusion broth (BHI) (Difco) and incubating them at 37°C for 20-22 h with shaking. These overnight bacterial cultures were serially 10-fold diluted ($10^1$, $10^2$ , $10^3$ CFU/ml ) and subjected to DNA extraction method and real-time PCR assay described below.

The *Salmonella* specific primers ST 11 (5'-AGCCAACCATTGCTAAATTGGCGCA-3') and ST 15 (5'-GGTAGAAATTCCCAGCGGGTACTG-3' (Aabo et al., 1993) were used.

DNA templates of 10-fold diluted series were prepared by boiling method (see DNA Extraction chapter 3 of this thesis). The LightCycler PCR initial mixture was prepared by following the instructions of the manufacturer. The PCR mixture (20 µl ) contained the following concentrations of reactants: 2 µl of 1 X LightCycler-Faststart DNA Master SYBR Green I, 2.4 µl of $MgCl_2$ at 4 mM , 1 µl of each primer at 0.5 µM, 11.6 µl of sterile distilled

water, and 2 µl of template DNA. Each LightCycler run contained negative control consisting of $H_2O$ without any template DNA to monitor for possible contamination. Mixing of the reagents for the PCR was accomplished under laminar flow in a clean room separate from where DNA samples were prepared. Master mixture and extracted DNA were placed into glass capillaries, sealed with a plastic cap, centrifuged, (3000 X g for 15 sec.) and placed into the LightCycler™ carousel (Roche Diagnostics). The thermal cycling program for the LightCycler™ has four phases: denaturation, amplification, melting and cooling. In the initial denaturation phase the capillary is heated to 95°C at 20°C/s for 10 min, followed by 40 to 45 cycles of amplification phase of 10 s at 95°C, annealing for 10 s at 63°C, and extension for 20 s at 72°C. Signal detection was performed at the end of extension step with a single fluorescence acquisition for each capillary. The melting curve analysis phase began with 95°C for 10 s, then cooled to 68°C for 30 s before the temperature was raised to 95°C at a rate of 0.1°C/s. Fluorescence acquisition was performed continuously during this phase. Finally, the cooling phase lasted one minute at 40°C. Melting peaks were derived by plotting the negative derivative of fluorescence over temperature versus the temperature ($-d(F)/dT$ versus $T$).

The optimization steps included titrating variety of $MgCl_2$ (2-5 mM) and primer concentrations (0.3 – 1µM). It also included optimization of annealing temperature (60-70°C). The results are summarized below:

A concentration of $MgCl_2$ of 4 mM was found to be optimal. This $MgCl_2$ concentration resulted in the lowest crossing point ($C_T$), with the highest fluorescence intensity and the steepest curve slope (data not shown). In addition, multiple melting peaks were not observed. $Mg^{2+}$ concentration is an important parameter of PCR reactions because it can severely affect the efficiency of the PCR assay. In general, an excess of $Mg^{2+}$ can result in increasing non-specific priming (e.g primer dimer) whereas too low $Mg^{2+}$ concentration will result in

reducing fluorescence signal. Although 0.3 μM, 0.4 μM, and 0.5 μM primer concentrations were found to be optimal, a final concentration of 0.4 μM resulted in a greatest fluorescence signal compared to the other primer concentrations. Despite the fact that annealing temperature of 64°C, 66°C and 68°C gave a higher fluorescence signal. The reaction performed with an annealing temperature of 66°C was optimal with no additional product peaks observed.

The final thermal cycling program and the optimized SYBR Green reaction mixture for all the PCR assays performed later of this thesis are shown in Appendix G and Appendix H, respectively.

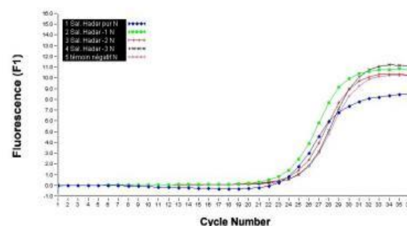


Figure 1: The amplification curves of DNA products of 10-fold dilution series ($10^{-1}$ to $10^{-3}$) of *Salmonella* Hadar befor optimizing PCR parametrs.

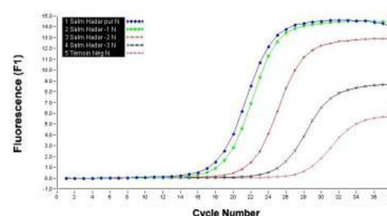

Figure 2: The amplification curves of DNA products of 10-fold dilution series ($10^{-1}$ to $10^{-3}$) of *Salmonella* Hadar as a result of optimization of PCR parametrs.

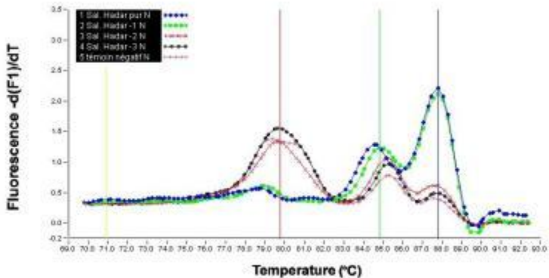


Figure 3: The melting curve analysis of DNA products of 10-fold dilution series ($10^{-1}$ to $10^{-3}$) of *Salmonella* Hadar befor optimizing PCR parametrs.

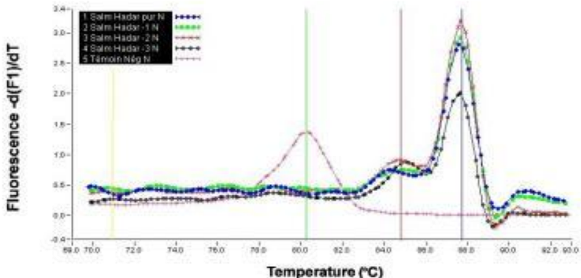


Figure 4: The melting curve analysis of DNA products of 10-fold dilution series ($10^{-1}$ to $10^{-3}$) of Salmonella Hadar as a result of optimization of PCR parameters

## Appendix (B)

## Thermal cycling program applied for the LightCycler real-time PCR assay

**Program : Denaturation**

| Segment No. | Target Temp. (°C) | Incubation Time (s) | Slop (°C/s) | Acquisition mode |
|---|---|---|---|---|
| 1 | 95 | 600 | 20 | None |

**Program : Amplification (40 cycles)**

| Segment No. | Target Temp. (°C) | Incubation Time (s) | Slop (°C/s) | Acquisition mode |
|---|---|---|---|---|
| 1 | 95 | 10 | 20 | None |
| 2 | 66 | 10 | 20 | None |
| 3 | 72 | 20 | 5 | Single |

**Program : Melting Curve**

| Segment No. | Target Temp. (°C) | Incubation Time (s) | Slop (°C/s) | Acquisition mode |
|---|---|---|---|---|
| 1 | 95 | 10 | 20 | None |
| 2 | 73 | 30 | 20 | None |
| 3 | 95 | 0 | 0.1 | Continuous |

**Program : Cooling**

| Segment No. | Target Temp. (°C) | Incubation Time (s) | Slop (°C/s) | Acquisition mode |
|---|---|---|---|---|
| 1 | 40 | 60 | 20 | None |

## Appendix (C)

## SYBR Green I Master Mixture used for the amplification

| Parameter | Volume (µl) | Final Concentration |
|---|---|---|
| LightCyler-FastStart DNA Master SYBR Green I | 2 | 1x |
| $MgCl_2$ | 2.4 | 4 mM |
| ST11 Primer | 0.8 | 0.4 µM |
| ST15 Primer | 0.8 | 0.4 µM |
| $H_2O$ (PCR grade) | 12 | |
| Total volume | 18 | |

In total, 18 µl of the master mix and 2 µl of the DNA template were added to each capillary.

## Vita

Abdunaser DAYHUM

Abdunaser was born on 17 July, 1966 in Benghazi, Libya. He got married in 2004. After finishing high school, he went to Alfateh University's Faculty of Veterinary Medicine for 6 years and graduated in 1990. He received a scholarship from the Libyan Government in 1993 for his Master degree to study at Ontario Veterinary College, Guelph, Canada. In 1998 he went back to Libya where he worked as a faculty member at the Faculty of Veterinary Medicine at Alfateh University, Tripoli, Libya. He got another scholarship in 2002 to study at Alfort National Veterinary School, Maisons-Alfort, France. The scholarship is for his Ph.D, specifically in the field of Veterinary Epidemiology. Presently, he is a Ph.D candidate. His next goal is to go back to Libya and take his position as faculty member at the Faculty of Veterinary Medicine at Alfateh University, Tripoli, Libya.

**Education**

- D.V.Sc., Alfateh University, Tripoli, Libya. (1983-1990)
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