Quantitative risk assessment model of human salmonellosis linked to the consumption of Camembert cheese made from raw milk.

Almabrouk Fares

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Almabrouk FARES

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Risque de salmonellose humaine liée à la consommation de fromage à pâte molle au lait cru : développement d'un modèle pour l'appréciation quantitative du risque

Directeur de thése : Olivier Cerf

Travail réalisé : ENVA,UEAR, Unité d'epidémiologie et d'analyse des risques
F-94700 Maisons-Alfort

Devant le jury

M. Olivier CERF, Professeur, Ecole ENVA	Président
Mme. Christine VERNOZY-ROZAND, Professeur, Ecole ENVL	Rapporteur
M. Mohammed HUSSNI, Professeur, Cornell University	Rapporteur
M. Moez SANAA, Docteur, Ecole ENVA	Examinateur
M. Yves MILLEMANN, Docteur, Ecole ENVA	Examinateur

This thesis « *Quantitative risk assessment model of human salmonellosis linked to the consumption of Camembert cheese made from raw milk* » 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 Camembert cheese made from raw milk.

(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 Camembert cheese made from raw milk. 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 milk samples. The method combined 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 milk 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 milk samples. In order to evaluate the utility of this developed quantification assay, our second objective was to apply it to naturally contaminated bulk tank milk samples collected from dairy farms located in western France. Eight (2.68%) of 299 bulk tank milk samples were found positive, with estimated MPN values ranging from 3.7 to 79.2 MPN/mL of milk. Despite the PCR inhibitors that were apparently present in some raw bulk tank milk samples, the application of the MPN-real-time PCR assay for quantifying *Salmonella* in raw milk proved to be rapid, easy-to-perform and highly sensitive. In the assessment of potential risks associated with *Salmonella* in raw milk and raw milk products it was necessary to examine the ability of *Salmonella* to grow in milk. Therefore, we presented in this thesis as a third

objective, primary and secondary models describing mathematically the growth of two *Salmonella* strains (*S.* Typhimurium and *S.* Montevideo) in milk under constant temperatures during different incubation periods. The primary logistic-with-delay model was used to describe *Salmonella* growth as a function of time. The specific growth rates of *S.* Typhimurium and *S.* Montevideo varied according to serotype and temperature. The maximum growth rates were then modeled as function of temperature using the secondary cardinal Rosso model. The reported cardinal estimates obtained with *S.* Typhimurium and *S.* Montevideo were: T_{min} 3.02, 3.40; T_{opt} 38.44, 38.55 and T_{max} 44.51, 46.97°C, respectively. At the optimum growth temperature (T_{opt}) the maximum growth rates were 1.36 and 1.39 $\log_{10}$ CFU/h^{-1} for *S.* Typhimurium and *S.* Montevideo respectively. Both the primary and secondary models fitted growth data well with a high-*pseudo* R^2 (0.97-99). Finally, a quantitative risk assessment of human salmonellosis linked to the consumption of Camembert cheese made from raw milk 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 25 g serving of cheese. The 99^{th} percentile of *Salmonella* cell numbers in servings of 25 g of cheese was 5 cells at the time of consumption, corresponding to 0.2 cells of *Salmonella* per gram. The risk of salmonellosis per 25 g serving varied from 0 to 1.2×10^{-7} with a median of 7.4×10^{-8}. For 100 million servings of 25g, the expected number of cases of salmonellosis predicted by the model is in average of 7.4. When the prevalence was reduced in the model by a factor of 10, the number of cases per 100 million servings was reduced to less than 1 case. 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.

Risque de salmonellose humaine liée à la consommation de fromage à pâte molle au lait cru : développement d'un modèle pour l'appréciation quantitative du risque

(Résumé)

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 Camembert fromage au lait cru. 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 developeé une méthode rapide, sensible et fiable pour la quantification des salmonelles dans le lait artificiellement contaminé. 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 lait 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 des échantillons de lait. Afin d'évaluer l'utilité de cette analyse de quantification, nous l'avons appliquée aux échantillons naturellement contaminés de lait de tank d'exploitations laitières situées dans l'Ouest de la France. Huit (2,68%) des 299 échantillons de lait de tank étaient trouvés positifs, avec des comptes estimés de nombre plus probable s'étendant de 3,7 à 79,2 /mL de lait. En dépit des problèmes d'inhibition observés avec quelques échantillons de lait, l'application de l'analyse par PCR en temps réel pour mesurer des salmonelles dans le lait cru s'est avérée être rapide, facile à exécuter et extrêmement sensible. Dans l'appréciation des risques potentiels liés aux salmonelles dans le lait cru et les produits à base de lait cru il était nécessaire d'examiner la

capacité des salmonelles à se développer dans le lait. Par conséquent, nous présentons dans cette thèse des modèles primaires et secondaires décrivant mathématiquement la croissance des salmonelles (*S.* Typhimurium et *S.* Montevideo) dans le lait à température constante pendant différentes périodes d'incubation. Le modèle logistique avec délai a été employé pour décrire la croissance des salmonelles en fonction du temps. Les taux de croissance spécifiques de *S.* Typhimurium et de *S.* Montevideo différent selon le sérotype et la température. Les taux de croissance maximum ont été alors modélisés en fonction de la température en utilisant le modèle cardinal secondaire de Rosso. Les valeurs cardinales obtenues avec *S.* Typhimurium et *S.* Montevideo étaient: T_{min} 3,02, 3,40 ; T_{opt} 38,44, 38,55 et T_{max} 44,51, 46,97°C, respectivement. À la température optimum de croissance (T_{opt}) les taux de croissance maximum étaient 1,36 et 1,39 $\log_{10}$ ufc/h^{-1} pour *S.* Typhimurium et *S.* Montevideo respectivement. Les modèles primaires et secondaires ont permis de bons ajustements aux données de croissance avec un pseudo R^2 = 0.97-0.99. Un modèle d'appréciation du risque de salmonellose humaine liée à la consommation de Camembert au lait cru 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 25 g du fromage. Le 99^{th} percentile du nombre de cellules de salmonelles dans les portions de 25 g de fromage était 5 cellules à l'heure de la consommation, correspondant à 0,2 cellule des salmonelles par gramme. Le risque de salmonellose par portion de 25 g est compris entre 0 et $1,2 \times 10^{-7}$ avec une médiane de $7,4 \times 10^{-8}$. Pour 100 millions de portions de 25g, le nombre de cas de salmonellose prévue par le modèle est en moyenne de 7,4. Quand la prévalence est réduite dans le modèle d'un facteur de 10, le nombre de cas par 100 millions de portions est réduit à moins de 1 cas. En dépit des limites et des données manquantes, nous avons démontré l'avantage de l'appréciation du

risque non seulement comme outil d'évaluation de risque mais également comme dispositif d'aide à la prise de décision et à la gestion des risques.

DEDICATION

I would like to dedicate this thesis to the following people:

My two favourite educators: my father who passed away during the preparation of this thesis, may God bless him. And to my mother for their support and encouragement during all my life that made the person that I am. You are the best parents ever.

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Finally, this thesis is dedicated to all those who believe in the richness of learning.

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AFFSA	Agence Française de Sécurité Sanitaire des Aliments
AOC	Appellation d'Origine Contrôlée
BEH	Bulletin Epidemiologique Hebdomadaire
BHI	Brain Heart Infusion
CAC	Codex Alimentarius 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 fooborne 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). Eventhough *Salmonella* infection is a self-limiting, the severity of the illness is obvious with 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, chills 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). Eggs, agricultural products, processed foods, raw milk and raw milk products and contaminated water also have been implicated in human salmonellosis.

Although special care is taken in the preparation of cheeses made from raw milk, representing about 20% of the total cheese production in France (Bouesse et al., 1998), the epidemiological literature and outbreak investigations still implicates raw milk products in salmonellosis outbreaks (De Valk et al., 2000; De Buyser et al., 2001; Haeghebaert et al., 2003). Therefore, the threat to human health linked to the ingestion of those products should not be underestimated. The epidemiology of cheese-related outbreaks in the U.S., Canada, and Europe demonstrated that soft cheeses e.g. Brie and Camembert cheeses and traditional soft cheeses in many cases produced in small, family sized establishments are at significantly greater risk to transmit pathogens than other cheeses (Johnson et al., 1990; Kovincic et al., 1991). These products were responsible for several *Salmonella* outbreaks (Maguire et al., 1992; Ellise et al., 1998; Villar et al., 1999; De Valk et al., 2000) and for other pathogen outbreaks (Linnan et al., 1988; MacDonald et al., 1985; Ries et al., 1990; Gilot et al., 1997).

Despite the known association of raw milk products with disease-causing organisms, some consumers believe that raw milk and raw milk products are of better quality than pasteurized milk products (Hegarty et al., 2002), and consumption of these products is favoured by a number of individuals for cultural, nutritional, taste and economic reasons (Desenclos et al., 1996; Headrick et al., 1997).

The importance of dairy products in human diets requires systems that ensure dairy 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 alimentarius, 1995). There is therefore a strong need to provide data on the frequency and level of *Salmonella* contamination in milk and milk 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 on milk and milk products 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 milk and raw milk products.

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 raw milk and raw milk products 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 milk is temperature. To our knowledge, scientific data related to the effect of temperature on the growth of *Salmonella* in milk and milk products are extremely limited. Thus, to assess the risks associated with *Salmonella* in raw milk products it is necessary to predict the growth of this bacterium in milk and milk products under different temperature profiles. Therefore, one of our objectives is to develop models that describe mathematically the growth of *Salmonella* in milk.

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 dairy products have previously been developed for other foodborne pathogens such as *Listeria monocytogenes* (Bemrah et al., 1998; Sanaa et al., 2004) and *Staphylococcus aureus* (Lindqvist et al., 2002) but not for *Salmonella*. Therefore, the present work attempts to estimate the risk for public health from the consumption of raw milk cheese 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'USD (fourchette 6,6-37,1 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 passagére. 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). Des œufs, les produits agricoles, les aliments préparés, le lait cru et les produits au lait cru et également l'eau souillée ont été impliqués dans la salmonellose humaine.

Bien que des précautions particulières soient prises dans la préparation des fromages faits à partir du lait cru, dont le tonnage s'est élevé à plus de 200.000 tonnes en 1997, représentant environ 20% de la production de fromage en France (Bouesse et al., 1998), les

investigations épidémiologiques publiées montrent que des salmonelloses continuent de survenir (De Valk et al., 2000 ; De Buyser et al., 2001 ; Haeghebaert et al., 2003), dont la plupart ont été transmise par l'intermédiaire de lait cru ou incorrectement pasteurisé. En dépit de l'association connue du lait cru avec des maladies infectieuses, certains consommateurs sont persuadés que le lait cru et les produits au lait cru sont d'une meilleure qualité que les produits laitiers pasteurisés (Hegarty et al., 2002), et la consommation de ces produits est favorisée par un certain nombre d'individus pour des raisons culturelles, alimentaires, gustatives et pour des raisons économiques (Desenclos et al., 1996; Headrick et al., 1997). Indépendamment de la rareté des maladies infectieuses transmises par les aliments associées au lait cru, y compris la salmonellose, la menace pour la santé humaine de l'ingestion du lait non pasteurisé ou des produits laitiers ne devrait pas être sous-estimée. L'épidémiologie a démontré qu'aux États-Unis, le Canada, et en Europe les fromages de brie et de Camembert et les autres fromages à pâte molle traditionnels sont a l'origine d'un risque sensiblement plus élevé de transmission des microbes pathogènes que d'autres types de fromages (Johnson et al., 1990; Kovincic et al., 1991). Ces produits ont été responsables de plusieurs épidémies de salmonellose (Maguire et al., 1992; Ellise et al., 1998; Villar et al., 1999; De Valk et al., 2000) et d'autres pathologies (Linnan et al., 1985; Macdonald et al., 1985; Ries et al., 1990; Gilot et al., 1997).

L'importance des produits laitiers dans l'alimentation humaine exige des systèmes de production qui garantissent leur innocuité. 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 incompléte. 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 de risque qui associée 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 la 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 la contamination du lait et des produits laitiers par les salmonelles. 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 le lait et les produits laitiers 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 polymerase 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 telle méthode pour dénombrer des salmonelles dans les laits et dans les produits laitiers. .

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 prévision de la croissance des salmonelles dans le lait cru et les produits au lait cru en fonction des conditions environnementales telles que la température, le pH, et l'activité de l'eau sont nécessaires pour décrire correctement les conditions changeantes liées au traitement et au stockage de ces produits. Un des facteurs environnementaux les plus importants qui affecte la croissance de salmonelles dans le lait est la température. À notre connaissance, les données scientifiques relatives à l'effet de la température sur la croissance des salmonelles dans le lait et les produits laitiers sont extrêmement limitées. Ainsi, pour apprécier les risques liés aux salmonelles dans des produits à base de lait cru il est nécessaire de prévoir la croissance de cette bactérie dans le lait et les produits laitiers sous différents profils de température. Par conséquent, un de nos objectifs est de développer les modèles qui décrivent mathématiquement la croissance des salmonelles dans le lait.

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), œufs avec coquille et produits à base d'oeufs (FSIS, 1998 ; Whiting et al., 2000), les œufs et les poulets de chair (WHO/FAO, 2002), le « cordon bleu » de dinde (Bermrah et al., 2002) et les charcuteries (Giovannini et al., 2004). Cependant, des modèles d'appréciation des risques associés à la consommation des produits laitiers ont été développés précédemment pour d'autres pathogènes transmis par les aliments tels que *Listeria monocytogenes* (Bemrah et al., 1998;

Sanaa et al., 2004) et *Staphylococcus aureus* (Lindqvist et al., 2002) mais pas pour les salmonelles. Par conséquent, le travail actuel essaye d'estimer le risque pour la santé publique de la consommation du fromage au lait cru 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 Ph.D 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 raw milk and raw milk products). 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 soft cheese made from raw milk. 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 milk.

2. To detect *Salmonella* in bulk tank milk samples collected from selected dairy farms in western 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 primary and secondary models to mathematically describe the growth of *Salmonella* in milk at different temperatures and incubation times.

4. To develop a quantitative risk assessment model of human salmonellosis linked to the consumption of Camembert cheese made from raw milk.

Thesis outline

Chapter 2 is a literature review regarding data of detection, quantification, and growth of *Salmonella* in milk and milk products as well as *Salmonella* outbreaks implicating dairy products. 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 milk. This chapter also presents the development of MPN-real time PCR assay for the enumeration of *Salmonella* in artificially contaminated milk. **Chapter 4** presents the on-farm field study for the detection of *Salmonella* in bulk tank milk of selected dairy farms in western France. This chapter also validates the developed MPN-real-time PCR for enumerating *Salmonella*-

positive bulk tank milk samples. **Chapter 5** examines the effects of several incubation temperatures, ranging from 9 to 43°C on *Salmonella* growth in milk. **Chapter 6** reports a quantitative risk assessment model of human salmonellosis linked to the consumption of Camembert cheese made from raw milk in France. The complete process of cheese making is modeled, from milking to consumption. Finally, **Chapter 7** 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-shape motile non-sporeforming 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 hundreds 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). Nowadays, PFGE remains the "gold standards" method and is used worldwide for surveillance and for outbreak investigation (Bender et al., 2001; Torpdahl et al., 2005). However, notably because of the high frequency of islation 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* detection (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 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 any 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 elimininiting 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). published literatures 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; Medici et al., 2003; Bhagwat., 2004; Wang et al., 2004; Mercanoğlu et al., 2005; Nam et al., 2005).

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; Hoofar et al., 200; 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 can allow real-time PCR to be applied without the need for probes linked to fluorescent molecules (Aarts, 2001). Recently, more researchers rely upon real-time PCR studies using this simple and the 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 some 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, one of the limitations of using these methods is the cost of analysis.

In conclusion, 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 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 inability to detect nonculturable cells, 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 artifically and naturally contaminated milk 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 particulary 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, mandated reporting of illnesses may be involved or the occurrences of illnesses may be passively collected through physician notification. 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*

Aalimentaires 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 faeces 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 contries 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 surveillane 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 milk and milk products in *Salmonella* outbreaks

The importance of various etiological agents in milkborne disease has changed over time. However, more than 90% of all reported cases of dairy related illness continued to be bacterial origin, with at least 21 milkborne or potentially milkborne diseases currently being recognized (Bean et al., 1996). Pathogens that have been involved in foodborne outbreaks associated with the consumption of milk and milk products include *Listeria monocytogenes, Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Bacillus cereus,* pathogenic *Echerichia coli* and *Clostridium botulinum*. Assessment of epidemiological data, pathogen incidence in milk and characteristics of individual pathogens were evaluated to prepare risk ranking for

cheese (Johnson et al., 1990c). *Salmonella*, *L. monocytogenes*, and enteropathogenic *E.coli* pose the highest risk.

Raw bulk tank milk can contain one or more species of pathogenic bacteria including *Salmonella* (Jayarao and Henning, 2001) and previously reported surveys of bulk tank milk in Europe, United States and Canada have shown large variations in the prevalence of *Salmonella* in raw milk ranging from 0.17% to 12.6% (Rohrbach et al., 1992; Steel et al., 1997; Hassan et al., 2000; Karns et al., 2005). The large variations in levels of bulk tank *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*.

Cheese is a well-known milk product which has gained great popularity throughout the world for its health-promoting and organoleptic properties. Cheese contains various proteins, minerals and vitamins, all of which contribute to its high nutritional qualities. The microbiological characteristics of cheese depend on the quality of raw milk, the procedures and the conditions of production, the personnel and the storage conditions. After the ripening period, some pathogens such as *Salmonella* may still cause serious food safety problems for consumers, in spite of added salt and antimicrobial metabolites and low pH.

Milk products implicated in 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 different types of milk and milk products in outbraks of foodborne disease. In the USA, between 1972 and 2000, a total of 58 raw-milk-associated outbreaks were reported to CDC, of which 17 (29%) were caused by *Salmonella* (CDC, 2000). In early 1997, health officials in Yakima County, Washington, noted a 5-fold increase in salmonellosis among the county's Hispanic population. Multidrug-resistant *Salmonella* Typhimurium DT104 emerged as a cause of salmonellosis in this county (Villar et al., 1999), and Mexican-style soft cheese made with unpasteurized milk was the vehicle for this strain. In the same year two other outbreaks caused by the same serotype were linked to raw-milk cheese in Northern California (Cody et al., 1999). In 2003, an outbreak due to multidrug-resistant *Salmonella enterica* serotype Typhimurium infections occurred in Pennsylvania and New Jersey (Olsen al., 2004). A case-control study implicated pasteurized milk and an inspection indicated the potential for contamination after pasteurization.

In Canada, there have been several reports of large cheese-associated outbreaks of *Salmonella* serotypes including Enteritidis, Typhimurium, Heidelberg, Oranienburg, Javiana, Berta, Muenster and Dublin (D'Aoust, 1989). In almost every outbreak, the cheese was made

from unpasteurized milk or was contaminated by raw milk during processing. Between 1980 and 1982, a *Salmonella* outbreak occurred in Ontario, Canada linked to raw milk Cheddar contaminated with *Salmonella* Muenster (Wood et al., 1984). The source of contaminated milk was traced to a farm where one cow was shedding approximately 200 CFU of *Salmonella*/ml in her milk. With the pathogen source confirmed, a rare opportunity was available for determining survival of *S.* Muenster during commercial tested positive in 11 of 181 vats. Two of these lots were still positive after pressing. During curding at 5°C, one lot was negative after 30 d, but one lot was positive after 125 d. There were no significant composition differences between the lots of contaminated cheeses. In 1984, another large Canadian outbreak of salmonellosis from the consumption of Cheddar cheese occurred during March-July in the four Canadian Atlantic provinces (D'Aoust et al., 1985). Over 2700 persons were infected with *Salmonella* Typhimurium. Epidemiological evidence implicated Cheddar cheese as the vehicle, and the production location was traced to a single plant on Prince Edward Island. *S.*Typhimurium was sporadically detected in Cheddar cheese manufactured at the plant which produced cheese from either pasteurized milk, 73.8°C for 16 s, or heat-treated milk 66.7°C for 16 s. *Salmonella* was initially confirmed in a cheese trim bucket. Employees used their hands to transfer curd to a forming machine. One employee who performed this task tested positive for *S.*Typhimurium. Testing of the raw milk supply ultimately identified two cows in separate herds, one shedding *S.*Typhimurium from one quarter, the other one shedding *S.* Heidelberg.

In England and Wales, between 1992 and 2003, 7620 general outbreaks of infectious intestinal disease were reported to the Communicable Disease Surveillance centre (CDSC) (Hughes et al., 2006). In 1729 of these outbreaks (23%), the mode of transmission was described as foodborne. *Salmonella* spp. as a whole were implicated in over half (53%) of all

foodborne outbreaks. Milk and milk products were recorded in 46 outbreaks (3.6% of total outbreaks) and the majority of *Salmonella* outbreaks (47%) were linked to the consumption of milk and milk products. In total, 39 625 people were affected in the 1729 foodborne outbreaks, with 1573 cases admitted to hospital ad 68 deaths reported. *Salmonella* accounted for the majority of people affected (22 585; 57%).

In France, an overview of *TIAC* yearly reports from the BEH over a 10-year period (1988-1997) gives a total of 3839 notified outbreaks (De Buyser et al., 2001). Milk and milk products were implicated in 5% of the total bacterial outbreaks reported in France, and in 1-5% of the total bacterial outbreaks, from the data reported in six other countries. *Salmonella* outbreaks were by far the most frequent (49%) among the notified *TIAC*s. However, milk and milk products were involved in only 1.8% of the *Salmonella* outbreaks. Tabel 1.2 shows *Salmonella* outbreaks implicating milk and milk products in France.

In 2002, two community outbreaks of salmonellosis occurred simultaneously in the South West of France (Haeghebaert et al., 2003). The results of the epidemiological, veterinary and laboratory investigations indicated that those two episodes were linked to the consumption of Cantal cheese made from raw milk. In 2005, the French National Reference Centre for *Salmonella*, *NRC-Salm* reported a nationwide outbreak of *Salmonella* Agona infection (Espié et al., 2005). Twenty-one infant cases were identified. Investigation linked the outbreak to consumption of infant powdered formula. In May 2005, an outbreak of salmonellosis from the consumption of contaminated powdered milk occurred in three hospitals located in three administrative districts (InVs, 2005). Forty-nine elderly people were infected with *Salmonella* Worthington and 92% of cases were identified in patients who had already been admitted to hospitals for other reasons. The investigation conducted by local veterinary service isolated *Salmonella* Worthington from environmental samples taken at the

manufacturing plant and in milk powder produced in March 2005. On April-June 2005, following the Enter-net request for information on recent cases of *Salmonella* Stourbridge infection reported in Europe (Vaillant and Espié, 2005), the National Reference Centre for *Salmonella* identified 16 cases affected who resided in 13 different administrative departments in France. The raw milk goat's cheese which was incriminated in Sweden was suspected to be the source of the outbreaks.

In summary, outbreaks of human salmonellosis have been traced to the consumption of unpasteurized milk or milk products and have also been traced back to pasteurized milk. Entry of *Salmonella* via contaminated raw milk into dairy food processing plants can lead to persistence of this pathogen in biofilm, and subsequent contamination of processed milk products and exposure of consumers to the pathogen. Consumers, especially the most susceptible ones (the immunocompromized, the elderly, young children, and pregnant women), must be informed that a zero risk cannot be warranted for raw milk products or even for pasteurized ones. Collective prevention must be carried out at several levels: in farms, in particular if their production is intended for the manufacturing of raw milk products, during the production process, and also during distribution stages to ensure high safety standards.

Table 1.2: Examples of *Salmonella* outbreaks implicating milk and milk products in France

Serotype	Year	No. of cases (death)	Food implicated	Type of milk	Reference
Paratyphi B	1990	277	Goat's milk cheese	Raw	Grimont and Bouvet 1991
Paratyphi B	1993	273 (1)	Goat's milk cheese	Raw	Desenclos et al.1996
Dublin	1995	25 (5)	Mont d'or cheese	Raw	Vaillant et al. 1996
Typhimurium PT 12 atypical	1997	113	Morbier cheese	Raw	Delaroque and De Walk 1998
Enteritidis phage type 8	2002	155	Cantal cheese	Raw	Haeghebaert et al. 2003
Typhimurium	?	?	Soft cheese	Raw	De Valk et al. 2000[2]

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

Country	Year	No. of cases (death)	Food implicated	Type of milk	Serotype	Reference
USA	1997	54	Mexican-style soft cheese	unpaseurized	Typhimurium DT 104	Villar et al. 1999
Canada	1994	35	soft cheese	unpasteurized	Berta	Ellise et al. 1998
Spain	1994	48	Infant powder milk	pasteurized	Virchow	Usera el al. 1996
USA	1989	164	Mozzarella	pasteurized	Javiana, Oranieburg	Hedberg et al. 1992
England and Wales	1989	42	Soft cheese	unpasteurized	Dublin	Maguire et al. 1992
England and Wales	1986	300	Milk	Raw	Typhimurium	Barrett 1989
USA	1985	> 1600 (7)	Milk	pasteurized	Typhimurium	D'Aoust 1989
Canada	1984	> 1700	Cheddar	unpasteurized	Typhimurium PT 10	D'Aoust et al. 1985
Canada	1982	Unspecified	Cheddar	Raw	Muenster	D'Aoust et al. 1985

Growth of *Salmonella* in dairy products

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. 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)

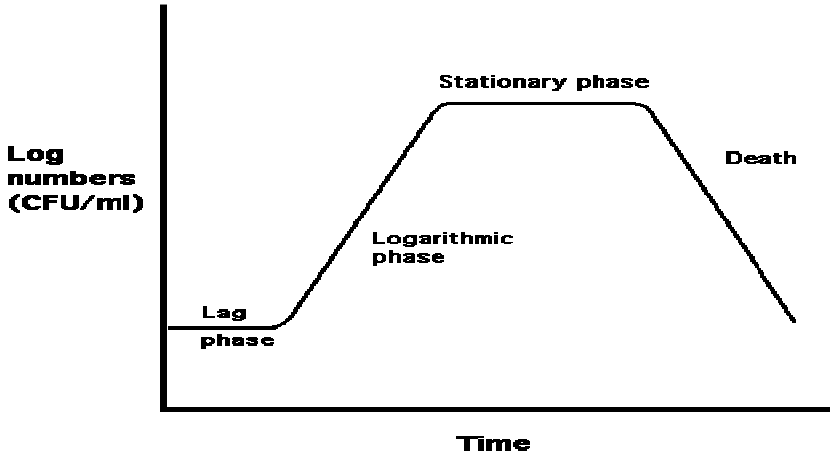
Table 3.2: Classification of some growth models (derived from MacDonald and Sun, 1999)

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 spolaige 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	Nutrients present	Nutrients used
	Content Redox l	Solutes present	End products formed
		Acidulant identity	Senstivity/resistance
Extrinisic	Temperature	Antimicrobial agents	Number and types
	Relative humidity	Atmospheric gas	
	Light intensity	Oxygen status	
	Packaging characteristics		

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

depends on the acid. For example, minimum *Salmonella* growth in lactic acid and acetic acid envirnments has been observed at a pH of 4.40 and 5.40 respectively (Chung and Goepfert 1970). The pathogen is killed by pasteurization and sanitizing 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 high 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 were 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).

Modern technology and adequate sanitary practices have helped to minimize pathogenic salmonellae found in heat-treated dairy products. However, there are certain types of cheese, which are manufactured from nonheat-treated milk. Whenever such dairy products are associated with *Salmonella* food poisoning outbreaks, serotypes such as *S.* Typhimurium, *S.* Dublin, *S.* Heidelberg, and *S.* Enteritidis are usually implicated as the responsible pathogens (Bryan, 1981; Medina et al., 1982).

The presence and growth of salmonellae in milk and some dairy products have been investigated because of health significance. The behavior of these microorganisms during the manufacture, ripening, and storage of most common dairy products is reported (Marth, 1969, El-Gazzer and Marth, 1992). For example, D'Aoust et al. (1985) investigated the survival of

S. Typhimurium in contaminated Cheddar cheese lots made from either heat-treated or pasteurized milk. The final pH values of the contaminated cheese lots were all between 5.1 and 5.4, which is within the acceptable range for Cheddar. Analysis of six contaminated cheese lots indicated that the cheese contained 0.39 to 9.3 *Salmonella* per 100 g. *S.* Typhimurium survived for up to 8 months in 1 of 6 lots of Cheddar cheese curd stored at 5°C, which demonstrated that *Salmonella* in commercially-prepared cheese survived past the 60 d holding requirement.

Papadopoulou et al. (1993) investigated the ability of *Salmonella* Enteritidis to grow during the Feta cheese-making process and to survive during ripening and storage. Unpasteurized whole ewe's milk was inoculated to contain 10^6-10^7 CFU/ml. The enumerations have shown that *S.* Enteritidis was entrapped in curd with the population increasing to a maximum during the first 48 h. Thereafter, the growth of *S.* Enteritidis was inhibited and surviving cells persisted in the curd for up to 20 days.

Modi et al. (2001) investigated the ability of *Salmonella* Enteritidis to survive in the presence of phage, during manufacture, ripening, and storage of Cheddar cheese produced from raw and pasteurized milk. Raw milk and pasteurized milk were inoculated to contain 10^4 CFU/ml of *S.* Enteritidis and 10^8 PFU (plaque forming unit). Cheese samples were taken over a period of 99 days and counts of *Salmonella* Enteritidis decreased by 1 to 2 log cycles in raw milk and pasteurized milk cheeses made from milk containing phage. In cheese made from milk to which phage was not added, there was an increase in *Salmonella* counts of about 1 log cycle. Lower counts of *Salmonella* Enteritidis were observed after 24 h in pasteurized milk cheese containing phage compared to *Salmonella* counts in raw milk cheese with phage. *S.* Enteritidis survived in raw milk and pasteurized milk cheese without phage, reaching a final concentration on 10^3 CFU/g after 99 days of storage at 8°C. However, *Salmonella* did not survive in pasteurized milk cheese after 89 days in the presence of phage. The results of this

study (Modi et al., 2001) and those previously conducted (White and Custer, 1976; Wood et al., 1984; D'Aoust et al., 1985; Papadopoulou et al., 1993) clearly demonstrate that *Salmonella* can survive during the manufacture and ripening of Cheddar cheese made from raw milk for at least 60 days at a temperature greater than 2°C.

White et al. (1976) and Charles and Edward (1976) pointed out that pH value below 5.7 would apparently contribute to the decline in the number of viable *Salmonella*, where as an average pH value of 5.85 would provide a greater chance for survival of this this organism in the cheese. Erkmen and Bozoğlu (1995) obtained also results in agreement with those of White et al. (1976) and Charles and Edward (1976) stated that pH value, the salt concentration, and the storage temperature, had a cumulative effect on the growth and survival of viable *S.* Typhimurium cells. However, the pH was the major factor responsible for a reduction in the viable number of microorganisma in Feta cheese during the processing and ageing periods. The results of Erkmen and Bozoğlu, (1995), showed that an attained pH value lower than 5.7 during storage was effective on S. Typhimurium and this could be achieved by using suitable starter cultures that would drop the pH during the processing and ripening period.

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 difficulities 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. Predicative 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 Sanitary and Phytosanitary Measures Agreement (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. If the focus of the Risk Assessment is on a pathogen, then 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 Characterisation*, 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 characterisation step is a dose-response assessment. The purpose of

hazard characterisation is to provide an estimate of the nature, severity and duration of the adverse effects associated with harmful agents in food. 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 Characterisation.* This gives an overall probability of occurrence and severity of health effects in a given population. To be meaningful, the risk characterisation 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 intersted 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 (QRSM) 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 generated 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 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 not for *Salmonella* spp. infection. Estimation of the risk for public health linked to the consumption of raw milk soft cheese 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 soft cheese made from raw milk.

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
SalmonellaTyphimuriumDT 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

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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 Artificially Contaminated *Salmonella* in Milk Samples

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Abstract

As a part of our effort in quantitative risk analysis of food-borne diseases, we developed a rapid, simple, and reliable method for detection and quantification of *Salmonella* spp. from artificially contaminated milk samples. The method combined the principles of most-probable-number (MPN) statistics with a LightCycler real-time PCR assay (MPN-real-time PCR) based on the double-stranded (dsDNA) DNA binding SYBR Green I. The *Salmonella* specific primers ST11 and ST15 were used to amplify a 429-bp region specific to all *Salmonella* spp. This primer set successfully detected *Salmonella* strains tested and produced a product with a melting temperature of 87.2 ± 0.5°C. To test this combined method for the enumerating of *Salmonella*, *Salmonella enterica* serotype Typhimurium DT104 was inoculated into milk samples at different levels of contaminations. Low inoculum level of *Salmonella* (1-5 CFU per mL of milk) could be detected and enumerated after 8 h of a single non-selective pre-enrichment step in buffered peptone water. This MPN-real-time PCR method proved to be a rapid and reliable method for enumerating *Salmonella* in artificially contaminated milk, including low contaminated samples.

Keywords: *Salmonella*; MPN-real-time PCR; detection; quantification; milk

Résumé

Dans le cadre de l'analyse quantitative de risque de maladies transmies par les aliments, nous avons développé une méthode rapide, simple, et fiable pour la détection et la quantification des salmonelles dans des échantillons de lait artificiellement contaminés. La méthode a combiné les principes statistiques du nombre le plus probable (NPP) avec une analyse PCR en temps réel-basée sur la technologie SYBR Green. Les amorces spécifiques des salmonelles ST11 et ST15 ont été utilisées pour amplifier une région de 429-bp commune à toutes les espèces de salmonelles. Ce couple d'amorces a détecté les salmonelles testées avec une température de fusion (T_m) de 87.2 ± 0.5°C. Pour tester cette méthode combinée de dénombrement des salmonelles, le sérotype DT104 Typhimurium a été inoculé dans des échantillons de lait à différents niveaux des contaminations. 1-5 UFC par ml d'échantillon de lait a pu être détectée et dénombré après 8 h d'une étape de pré-enrichissement dans l'eau peptonée tamponnée. Cette méthode de NPP-PCR en temps réel s'est avérée être rapide et fiable pour dénomrer des salmonelles dans du lait artificiellement contaminé, y compris avec une contamination faible.

Mots-clés : Salmonelles ; NPP-PCR en temps réel; détection ; quantification ; lait

1. Introduction

Human salmonellosis is a common food-borne illness all over the world. Contaminated food products of animal origin are frequently implicated in human salmonellosis (Oosterom, 1991). Milk and milk products have been associated with severe food poisoning outbreaks caused by *S. enterica* serotypes in Europe and the United States (Maguire et al., 1992; Vought and Tatini, 1994; Threlfall et al., 1998; Cody et al., 1999; Villar et al., 1999; De Valk et al., 2000; Haeghebaert et al., 2003). Contamination of milk by these pathogens therefore poses a great health risk to humans (Headrick et al., 1998).

Conventional cultural methods for the detection of *Salmonella* in foods are time consuming and usually require 4-6 days to presumptively identify *Salmonella* in a test sample and to confirm the identity of the isolate (Administration, 1995). Methods based on polymerase chain reaction (PCR) have been increasingly used for the detection of *Salmonella* cells in various foods including dairy products (Kwang et al., 1996; 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 and lacks sensitivity and specificity. To reduce the time required for detection of *Salmonella* spp. in foods, the development of rapid and sensitive protocols for the detection and quantification of low levels of *Salmonella* likely to be present in foods including milk are needed. In recent years, therefore, 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; Fukushima 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 rapidity, high sensitivity, high specificity, and lower risks of cross-contamination (Rodrigez-Lázaro et al., 2003).

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 with the 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 artificially contaminated milk samples. To our knowledge, this is the first report in which a LightCycler real-time PCR detection method has been combined with the MPN method to enumerate *Salmonella* spp. in inoculated milk samples. However, detection methods based on MPN-Conventional PCR (MPN-PCR) have previously been described for the detection and enumeration of different micro-organisms (Mäntynen et al., 1997; Fredslund et al., 2001; Martin et al., 2004).

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1.3. They 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), Maisons-Alfort, France). They were used to determine the specificity and the sensitivity of the LightCycler real-time PCR assay. Stock cultures were maintained in Brain Heart Infusion (BHI, Difco) containing 20% glycerol (Difco) and stored at -80°C. Fresh bacterial cultures for use in the experiments were produced by inoculating frozen stock cultures into BHI and incubating them for 20-22 h at 37°C with continuous agitation (100 rpm) in incubator shaker (New Brunswick Scientific). For enumeration of *Salmonella* in milk, *S. enterica* serotype Typhimurium DT104 was used and suitable dilutions were subsequently used to calculate the estimated number of *Salmonella* cells inoculated into sterilized milk.

2.2. Specificity of the real-time PCR assay

To determine the specificity of the LightCycler real-time PCR assay, frozen stock cultures of 3 different serotypes of *Salmonella enterica* and 7 non-*Salmonella* strains (Table 1.3) were transferred into BHI broth and incubated for 20-22 h at 37°C with continuous agitation (100 rpm) in incubator shaker (New Brunswick Scientific). These overnight bacterial cultures were subsequently subjected to DNA extraction and real-time PCR assay.

2.3. 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 serotypes of *Salmonella enterica* (Table 1.3). Cells were grown overnight at 37°C in BHI broth Ten-fold serial dilutions (10^8 to 1 CFU/mL) of pure cultures were prepared in Buffered Peptone Water (BPW; Difco, Becton Dickinson). To determine cell numbers, appropriate

diluted cultures were spread-plated on Xylose-Lysine-Tergitol-4 agar (XLT-4; Difco) in ten replicate plates and incubated overnight at 37°C. All dilutions were then incubated at 37°C for 6, 8 and 16 h. 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 the real-time PCR was assessed by running samples independently on different days.

2.4. Artificial contamination of milk

For the artificial inoculation procedure, ultra-high temperature (UHT) sterilized whole milk (3.6g fat) was purchased from a local supermarket and artificially inoculated with *S. enterica* serotype Typhimurium DT104. The number of CFU was determined by plating 0.1-mL aliquots of suitable 10-fold dilutions onto XLT-4 agar plates in ten replicate and incubating them at 37°C for 24 h. The dilutions were then kept refrigerated at 4°C for 24 h. When the *Salmonella* added to the milk, the estimated cell concentration of the inoculum was determined for a second time. 25 ml samples of milk were inoculated with the following estimated levels of contamination: 1 to 5, 10 to 20, and 100 CFU/mL and then homogenized in 225 ml of BPW by mixing. The mixtures were then subjected to real-time PCR detection protocol and to MPN-real-time PCR quantification protocol.

2.4.1. Real-time PCR detection protocol of **Salmonella** *in inoculated milk*

For the real-time PCR detection assay, the homogenates were enriched in BPW 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, 0.1 aliquots of appropriate 10-fold serial dilutions of each spiked preenrichment 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, 1.5-mL aliquots of each spiked preenrichment broth were collected and subjected to DNA extraction for the real-time PCR assay. The experiment was also performed with bulk

tank milk samples collected from a dairy farm and artifically contaminated after being confirmed *Salmonella*-negative by both culture methods and real-time PCR assay.

2.4.2. The MPN-real-time PCR quantification protocol

MPN assays (ten-tube method) for *S. enterica* serotype Typhimurium DT104 were performed according to the procedures described in the Bacteriological Analytical Manual of the U.S Food and Drug Administration (BAM/FDA) (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 website of the BAM (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 mean divided the standard deviation. If the CV values were less than 20%, the repeatability was considered to be acceptable.

2.5. DNA extraction procedures

DNA was extracted from both pure cultured strains and preenriched cultures of milk samples. Aliquot of enriched sample was transferred to a microcentrifuge tube with a capacity of 1.5 mL. The cell suspension was centrifuged for 10 min at 12,000 g. 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 g for 10 min, and the supernatant was dicarded 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 cell debris was pelleted by centrifugation at 12,000 g for 10 min. The volume of the DNA containing supernatant was estimated by pipetting to a new microcentrifuge tube and varied from 40 to 60 μL, due to differences in removing the supernatant during the different washing steps of the DNA isolation method. The DNA 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.

2.6. SYBR Green real- time PCR assay

To determine the optimal concentration of primers and SYBR Green real-time PCR reactants and condition, preliminary tests were performed using DNA extracted from appropriate 10-fold serial dilutions of *Salmonella* Hadar.

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; Štefanovičová et al., 1998; Bansal et al., 2006) were purchased from Proligo and used to amplify a 429-bp region.

Real-time PCR reactions were performed with a LightCycler PCR System (Roche Diagnostics) using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). The PCR 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 template and sterile PCR grade water to a total volume of 20 μL per capillary. Each LighCycler run contained one negative control consisting of H_2O without any DNA template to monitor for possible contamination. 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 s) 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*)/ d*T* versus *T*).

3. Results

3.1. Optimization of real-time PCR assay

The first step in developing a successful real-time PCR assay is to establish optimal conditions of the real-time PCR parameters such as $MgCl_2$ and primer concentrations. Therefore, preliminary tests were performed using DNA extracted from appropriate 10-fold serial dilutions of *Salmonella* Hadar (see Appendix F). By following the optimization procedures recommended by the manufacturer (Roche Molecular Biochemicals, Technical Note No. LC 9/2000), we achieved the optimal conditions described in SYBR Green real-time PCR assay section of this paper.

3.2. Specificity of real-time PCR primers

PCR primers ST11 and ST15 were found to result in specific amplification products with all the serotypes of *Salmonella* tested. As expected, no amplification was observed in any of 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. (Table 1.3). No amplification was observed in the negative control, eliminating the possibility of false-positive results due to potential cross-contamination. Table 1.3 shows the 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_ms 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 peaks analysis of the amplified products in real-time PCR for positive (*S.* Enteritidis, *S.*Hadar and *S. enterica* serotype Typhimurium DT104) and representative negative (*Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii* and *Klebsiella pneumoniae*).

3.3. Detection limits in pure cultures

The study was performed on three individual *Salmonella* strains listed in Table 1.3. 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/mL pure *Salmonella* cultures. Experiments were carried out three times and good reproducibility was observed (data not shown).

3.4. Detection of Salmonella from artificially contaminated milk samples

Milk samples inoculated with *S. enterica* serotype Typhimurium DT104 at the estimated levels of contamination (1-5, 10-20, 100 CFU/mL) gave negative results witout enrichment, whereas the real-time PCR assay detected the bacteria in milk samples even at the lowest levels of contamination after enrichment. When spiked milk samples were enriched in BPW for 6 h an inoculum level of 10 and 100 CFU/mL was detected. When the inoculation levels were 1 CFU/mL, 8 or 16 h of enrichment was necessary to identify them. The relative detection limits of the real-time PCR assay for *S.*Typhimurium DT104 in artificially contaminated milk samples were consistent with those of artificially contaminated bulk tank

milk samples. These results suggest that at least 10^3 CFU/ml in enrichment broth (Table 2.3) must be present to give a positive result by the real-time PCR.

3.5. Confirmation 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 our results 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). The average T_m of the primer-dimers was substantially different and was 79.7°C (± 0.2). The LightCycler™ is capable of differentiating two PCR products whose T_m's differ by 2 °C or less (Ririe et al., 1997). 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.

3.6. Enumeration of Salmonella in artificially contaminated milk samples

For artificially contaminated milk samples, a pre-enrichment time of 8 h in BPW was found optimal to obtain MPN-real-time PCR estimates close to the contamination levels (Table 3.3). With the developed MPN-real-time PCR assay it was possible to enumerate

approximately 1-5 CFU *Salmonella* per ml of milk 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 milk samples inoculated with 100 and 10-20 cells per ml of milk (Fig. 2.3, A, B), while in the case of the samples inoculated with low levels 1-5 cells per ml of milk, weak fluorescence peaks were observed (Fig. 2.3, C). The MPN-real-time PCR assay demonstrated acceptable repeatability with a coefficient of variation $\geq$ 3.5% for inoculum levels of 100 and 10-20 CFU/ml; but larger variation being observed in samples with inoculum level of 1-5 CFU/ml (Table 4.3). Arguably, this was because of the low level of inoculum.

4. Discussion

Dairy cattle and their environment harbor pathogens that pose a potential human health hazard. Unpasteurized milk and dairy products made from raw milk serve as vehicles for the transmission of pathogenic bacteria including *Salmonella* 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., 1992). 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 *Salmonella* cells in foods including milk. In risk assessment investigation, enumeration rather than presence/absence is important to estimate the rate of human exposure.

The major aim of the present study was to develop a sensitive, rapid, and reliable MPN-real-time PCR based method for the quantification of *Salmonella* in artificially contaminated milk. This is, to our knowledge, the first report on quantification of *Salmonella* in inoculated milk samples by the MPN method combined with LightCycler real-time PCR.

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 little optimisation steps of PCR conditions, the simple and less expensive option of SYBR Green can be used as an effective alternative. Recently, a number of SYBR Green I real-time PCR assays for detection of *Salmonella* from different types of samples have been reported (Jothikumar et al., 2003; Medici et al., 2003; Bhagwat., 2004; Wang et al., 2004; Mercanoğlu et al., 2005; Nam et al., 2005).

The primer set of ST11 and ST15, originally designed by (Aabo et al, 1993), is highly specific for *Salmonella* species (Štefanovičová et al., 1998), and widely used in conventional PCR for the specific detection of *Salmonella* spp. in foods (Gouws et al., 1998; Li and Mustapha, 2002; Croci et al., 2004; Bansal et al., 2006). In the present study, testing of the specificity of this primer set to several *Salmonella* strains and strains belonging to other common foodborne bacterial genera also confirmed the specificity of these primers with real-time PCR. All *Salmonella* strains yielded positive products and all non-*Salmonella* strains were negative.

In our study, *Salmonella* could not be detected in inoculated milk samples when DNA was extracted directly and without enrichment (Table 2.3). Previously reported studies (Fleet, (1999; 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 food, to overcome the problems of certain inhibitors present in food including milk and to allow the proliferation of *Salmonella* while reducing or diluting out non-*Salmonella* organisms that might interfere in the PCR assay.

. Therefore, we carried out one step enrichment in BPW prior to DNA extraction. In our study, the preenrichment of artificially inoculated milk samples in BPW provided enough bacteria for DNA extraction, thus making it possible to detect the lowest level of contamination. Various authors (Kwang et al., 1996; Croci et al., 2004; Mercanoğlu et al., 2005) reported the use of such a pre-enrichment step prior to the PCR assays, especially when small numbers of *Salmonella* cells are thought to be present in the sample.

In the present study, experiments carried out on artificially contaminated milk samples showed that real-time PCR could detect as few as 1-5 CFU/mL of *Salmonella* contaminated milk samples after 8 h of pre-enrichment. These results agree with the work of Lin et al. (2004) and Mercanoğlu et al. (2005) for the detection of *Salmonella* in inoculated chicken meat and in spiked pasteurized milk, respectively. However, published literatures 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; Medici et al., 2003; Bhagwat., 2004; Wang et al., 2004; Mercanoğlu et al., 2005; Nam et al., 2005).

In the present study, we used artificially contaminated milk. It has previously been shown that certain inhibitors present in milk may interfere with PCR when bacteria are grown in milk (Khan et al., 1998). In our results, the DNA extraction procedures based on a simple boiling method gave optimal results and no inhibition was seen in inoculated milk samples. As a simple, but widely used method, boiling can readily lyse cell suspensions of Gram-negative bacteria and achieve the same efficiency as other template preparation protocols and commercial kits (Meng et al., 1996; Lampel et al., 2000). In addition, studies have shown that sufficient amounts of DNA can be obtained by boiling bacterial cells for 10-15 min (Stewart

et al., 1998). While our work and the work of Medici et al. 2003 have supported these findings, others (Li and Mustapha, 2002) reported that boiling alone could not recover satisfactory template DNA for the PCR assay when applied for the detection of *Salmonella* Typhimurium in ground chicken but not for ground beef, indicating that the differences in composition of food samples might influence the efficiency of DNA template preparation methods. Based on our results it became obvious that boiling provides a fast and efficient way of extracting DNA that can be used in PCR assays in less well equipped laboratories.

In this work, the potential of MPN-real-time PCR method for the quantification of *Salmonella* spp. from artificially contaminated milk 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. Josephson et al. (1993) reported that PCR reaction does not differentiate the DNA from viable or non-viable organisms.

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 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) have also indicated 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.

In conclusion, the MPN-real-time PCR assay proved to be a rapid and a highly sensitive test for detecting and quantifying low levels of *Salmonella* in artificially contaminated milk samples. This assay yields significant labor and time savings since the quantification of *Salmonella* spp. can be completed within 12 h which included a 8-h non-selective pre-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. On the basis of these results, further applications and modifications of the assay described in this study are being investigated in our laboratory, including the potential to detect and quantify pathogens in other food matrices both artificially and naturally contaminated.

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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 no1	Hospital	55	31.07	–
Citrobacter freundii no2	Bovine/kidney	4525.04	31.77	–
S. enterica serotype				
S. Hadar	Steak/Cordon-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 an amplification of specific product.

[3]Cordon-bleu = specific turkey product.

Table 2.3 Real-time PCR and bacterial counts of *Salmonella enterica* serotype Typhimurium DT104 in artificially contaminated bulk tank milk samples after each period of pre-enrichment in BPW

Inoculation level (CFU/ml)	Incubation time (h)							
	0		6		8		16	
	PCR[a]	Bacterial count	PCR	Bacterial count	PCR	Bacterial count	PCR	Bacterial count
1-5	—	6.6	—	4 (±4.3[c])x10^2	+	2.9 (±1.3)x10^4	+	N/C[b]
10-20	—	20	+	9.4 (± 3.4)x10^3	+	1.4 (±.38)x10^5	+	N/C
100-200	—	280	+	1.8 (±.43)x10^5	+	9 (±.8.7)x10^6	+	N/C

[a] PCR = (+) presence of amplification product, (-) absence of amplification product

[b] N/C = not-countable

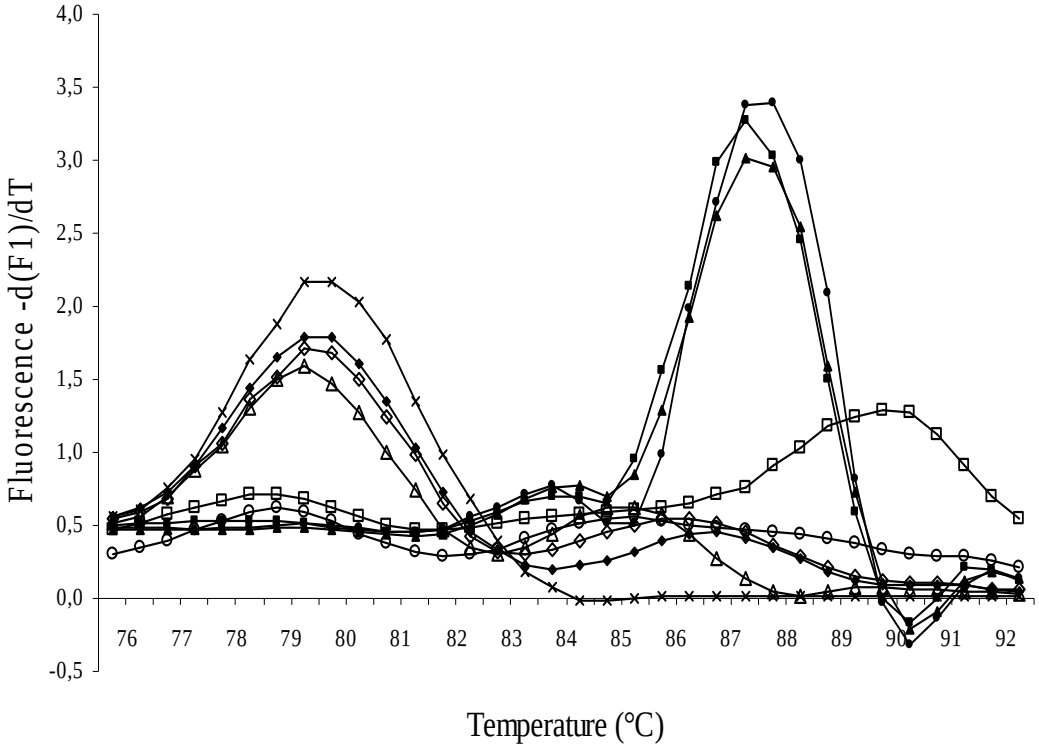
[c] Standard deviation

Table 3.3 Enumeration of *Salmonella enterica* serotype Typhimurium DT104 in artificially contaminated milk samples using MPN-real-time PCR method after 8 h pre-enrichment in BPW (contamination level CFU/mL)

Sample	Contaminated level Theoretical	Estimated level by Plating	MPN estimates	MPN confidence limits (low/high)
1	1-5	2	5.9	2.4 - 14.5
2	1-5	3	5.9	2.4 - 4.5
3	1-5	1	3.1	1.0 - 9.8
4	10-20	12	14.7	7.2 - 29.9
5	10-20	9	12.7	6.0 - 26.7
6	10-20	12	19.2	9.5 - 39.1
7	100	128	239.7	110.1- 523.2
8	100	103	135.8	68.0 - 71.5
9	100	85	101.2	46.8 – 218

Table 4.3 Repeatability of MPN-real-time PCR quantification assays of artificially contaminated milk samples

Inoculation level (ml)	Runs	MPN estimate (MPN/mL)	MPN confidence limits (low/high)	CV%
1-5	1	0.94	0.1 - 6.7	96
	2	3 .15	1 - 9.8	
	3	4.47	1.7 - 12	
10-20	1	23.12	10.7 - 49.8	3.5
	2	19.29	9.5 - 39.2	
	3	23.12	10.7 - 49.8	
100	1	101.22	46.9 - 218.1	3.1
	2	129.55	68.3 - 246.1	
	3	101.22	46.9 - 218.5	



.

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* no[1] (◊);*Citrobacter freundii* no[2] (♦); and water(negative control (x).

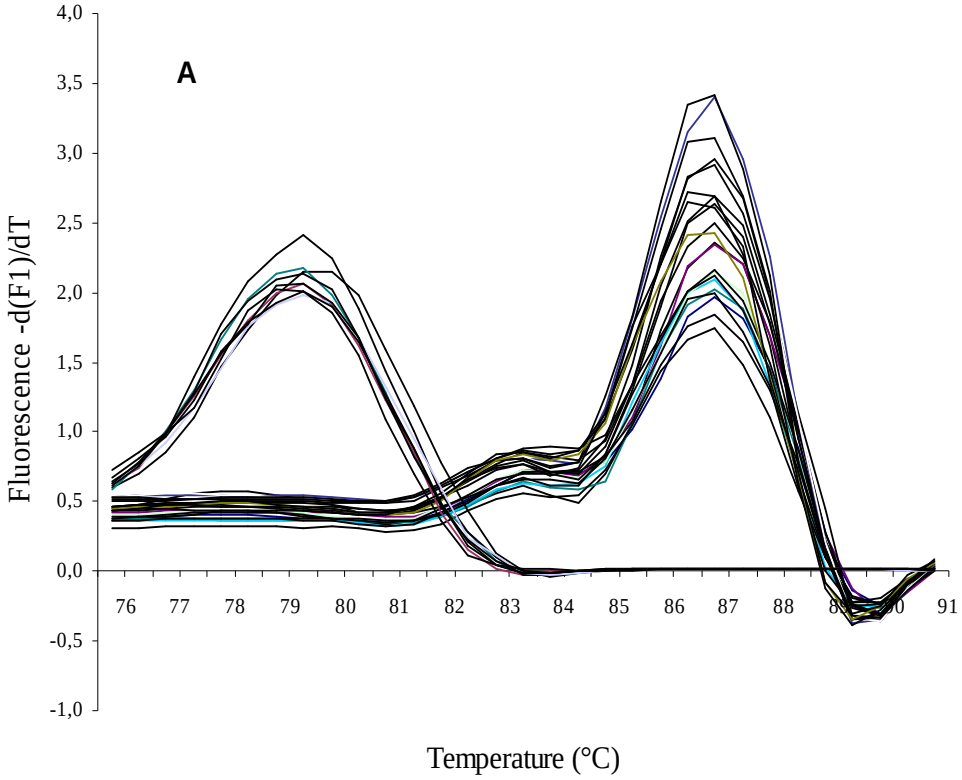


Figure 2.3A MPN-real-time-PCR analysis of milk inoculated with *Salmonella enterica* serotype Typhimurium DT104. DNA extracted from milk samples after 8h non-selective enrichment in BPW: (A) Inoculum level of 100 CFU/mL

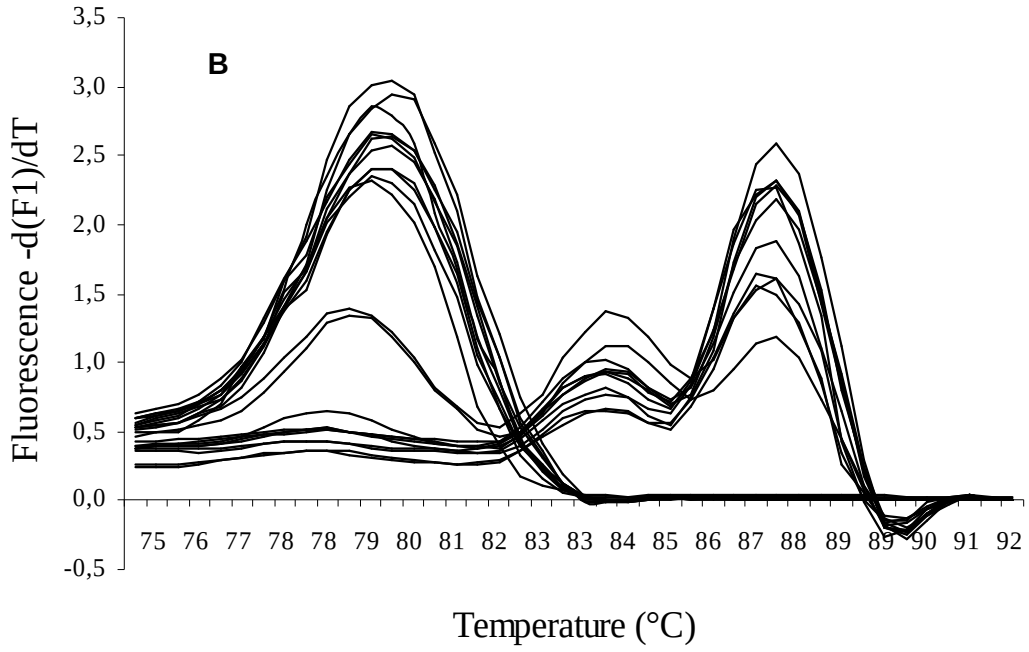


Figure 2.3B MPN-real-time-PCR analysis of milk inoculated with *Salmonella enterica* serotype Typhimurium DT104. DNA extracted from milk samples after 8h non-selective enrichment in BPW: (B) Inoculum level of 10-20 CFU/mL

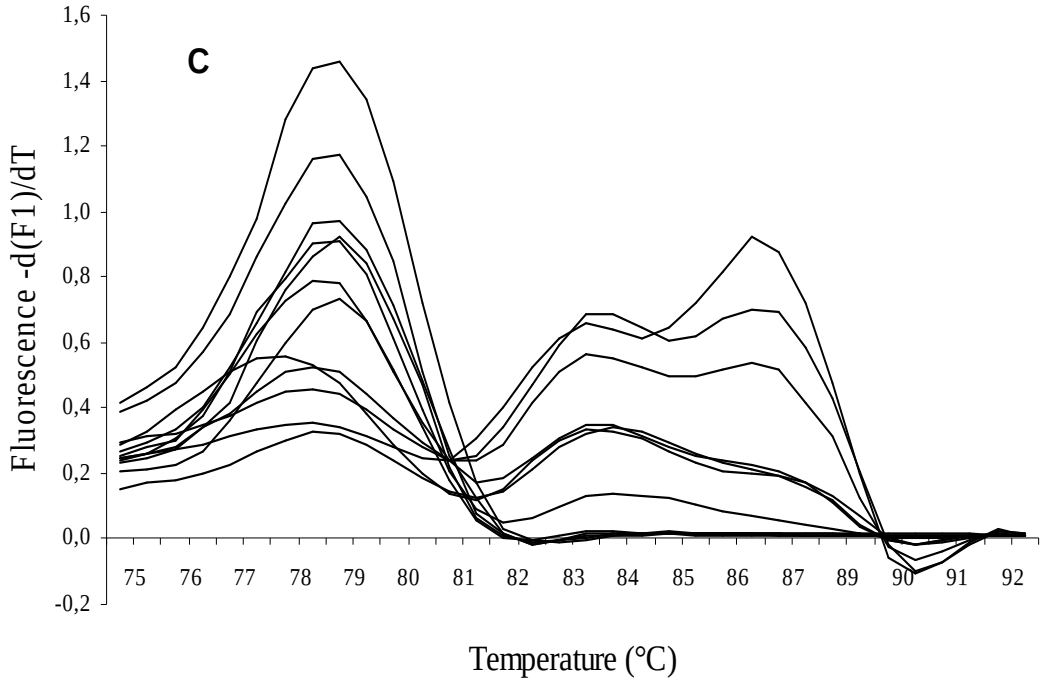


Figure 2.3C MPN-real-time-PCR analysis of milk inoculated with *Salmonella enterica* serotype Typhimurium DT104. DNA extracted from milk samples after 8h non-selective enrichment in BPW: (C) Inoculum level of 1-5 CFU/mL

Chaptre 4: Application of MPN-Real-Time PCR Assay for Quantification of *Salmonella* in Bulk Tank Milk Samples

Abstract

Consumption of raw milk and raw milk products has been involved in *Salmonella* outbreaks. The importance of *Salmonella* enumeration technique has been enormously needed to collect quantitative data for risk assessment. In a previous study, we reported a MPN-real-time PCR assay for the enumeration of *Salmonella* in artificially contaminated milk samples which allowed the detection and enumeration of low inoculum levels (1-5 CFU/mL). In this study, we applied it to naturally contaminated bulk tank milk samples collected from selected dairy farms located in western France. Eight (2.68%) of 299 bulk tank milk samples were found positive, with estimated MPN ranging from 3.7 to 79.2 *Salmonella/*mL of milk sample. Despite certain inhibition problems, the study demonstrated that the combination of real-time PCR assay with MPN were sensitive, rapid and easy-to-perform method for quantifying low levels of *Salmonella* in bulk tank milk samples.

Key words: *Salmonella,* MPN-real-time PCR, quantification, milk

Résumé

La consommation de lait cru ou de produits au lait cru a été mise en cause dans des épidémies de salmonellose. Le dénombrement de *Salmonella* est requis pour collecter les données indispensables à une appréciation quantitative du risque. Nous avons antérieurement mis au point une technique de dénombrement de *Salmonella* dans le lait contaminé artificiellement, utilisant une combinaison NPP (nombre le plus probable) et PCR en temps réel, capable de détecter et de dénombrer 1-5 ufc/mL. Dans le présent travail, nous appliquons cette technique à des prélévements de laits de ferme provenant de l'Ouest de la France. Huit (2,68%) des 299 laits testés se révélent positifs, le nombre le plus probable allant de 3,7 à 79,2 *Salmonlla*/mL. Ces résultats montrent que la technique NPP-PCR en temps réel constitue un moyen efficace, rapide et facile à mettre en œuvre pour quantifier de faibles concentrations de *Salmonella* dans des échantillons de lait de tank de ferme.

Mots clefs : *Salmonella*, NPP-PCR temps réel, quantification, lait

1. Introduction

Salmonella remains the leading cause of human foodborne disease in many countries (D'Aoust et al., 2000; WHO, 2002; Patrick et al., 2004). In France, the number of foodborne infections, hospitalizations, and deaths are estimated from multiple data sources. For example, information from French surveillance systems and other sources of morbidity and mortality due to foodborne infectious diseases in the last decade of the 20th century, ranked *Salmonella* as the most frequent cause of morbidity and mortality (Vaillant et al., 2005).

Recent *Salmonella* outbreaks in France, USA and other industrialized countries linked to consumption of milk and milk products made from both pasteurized and raw milk (Cody et al., 1999; Haeghebaert et al., 2003; Mazurek et al., 2004), together with the well documented low human infectious dose of milkborne *Salmonella* (D'Aoust, 1989), increased public health concerns on the safety of these products. Therefore, increasing emphasis has been placed on addressing *Salmonella* in raw milk products, including the development of a quantitative risk assessment of the milkborne infection by this bacterium.

Salmonella are frequently isolated from dairy cattle and from various locations within dairy farm environments (Davison et al., 2005; Fossler et al., 2005; Lailler et al., 2005) and the contamination of bulk tank milk occurs either *via* faecal contamination of the udder or milk equipments (Van Kessel et al., 2004) or, more rarely *via* intramammary secretion (Radke et al., 2002). Several surveys in Europe, Canada, and USA have demonstrated the contamination of bulk tank milk by *Salmonella* (Steele et al., 1997; Hassan et al., 2000; Jayarao and Henning 2001; Murinda et al., 2002b; Van Kessel et al., 2004; Karns et al., 2005). The incidence of reported *Salmonella* milk contamination ranged from 0.17% (Steele

et al., 1997) to 11.8% (Karns et al., 2005). However, data on the levels of contamination is lacking.

At present, the "gold standard" for the detection of *Salmonella* in foods is conventional culture methods. These methods are generally labor and time-consuming processes, requiring a minimum of 4-6 days (Andrew et al., 2003). Culture methods have also been reported to show poor sensitivity for low-level contamination in samples (D'Aoust, 1992). To speed up the detection process, polymerase chain reaction (PCR) and more recently real-time PCR assays have been developed for the detection of salmonellae or specific serotypes in a variety of foods (Ferretti et al., 2001; Bhagwat, 2004; Liming and Bhawat, 2004). Real-time PCR method for the detection of *Salmonella* in raw milk has been reported (Van Kessel et al., 2003; Karns et al., 2005). These studies have suggested that commercially available real-time PCR assays proved to be useful for the detection of *Salmonella* in bulk tank milk samples when combined with an enrichment step. These PCR assays were able to detect the presence of *Salmonella* even in samples that did not yield positive cultures. Thus, it seems that the use of a real-time PCR assays after enrichment provides a rapid, accurate, and sensitive method for surveying the presence of *Salmonella* in bulk tank milk.

Generally, the most-probable-number (MPN) method provides statistical estimates of viable cell concentrations but is limited by some well-known drawbacks. These include the large volume of glassware that is required, which is costly in terms of both time and labor, and the inability to detect nonviable cells and the lack of opportunity to observe the colonial morphology of the organisms (Seo et al., 2006). As an alternative, researchers have proposed the use of the real-time PCR assay for the identification and enumeration of *Salmonella* (Nogva et al., 1999; Piknova et al., 2005; Seo et al., 2006). This approach is an attractive

alternative to the culture-based systems for the quantification of foodborne pathogens because the results are generated much faster, more specific, and because nonculturable but active agents can be quantified. 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 a previous study (Chapter 4), we overcome the limitations of each method by developing a mixed approach, combining both methods. Our developed MPN-real-time PCR method for the quantification of *Salmonella* in inoculated milk samples proved to be rapid and reliable. In the light of these results, the objectives of the current study, therefore, were (I) the use of LightCycler real-time PCR assay to detect *Salmonella* in naturally contaminated bulk tank milk samples collected from dairy herds and (II) to quantify *Salmonella* in positive-raw milk samples by the developed MPN-real-time PCR assay.

2. Materials and methods

2.1. Dairy herds

A total of 299 milk samples were aseptically collected from bulk tank dairy farms located in western France. Collection of milk samples was done during routine visits to farms by the departmental veterinary laboratories (LVDs, Laboratoires vétérinaires départementaux) for epidemiological monitoring of bacterial strains, including *Salmonella*. The collection of milk from these dairy farms is intended for cheese making since milk producers are coming from a geographical area protected by the French Label of Origin (Appellation d'Origine Contrôlée, AOC) and they are selected based on the sanitary of their milk. All samples collected for our study were stored at 4°C and then transported with cold packs to our laboratory and analyzed within 24 h of collection.

2.2. Detection of Salmonella by LightCycler real-time PCR

For bulk tank milk samples, 5 mL of each milk samples was added to 45 mL BPW (Difco). After being thoroughly mixed, the mixtures were pre-enriched for 18 h at 37°C. 1.5-mL samples 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 raw milk samples were stored at 4°C to be used for quantification protocol in case of *Salmonella*-positive results.

2.3. Enumeration of Salmonella by MPN-real-time PCR

Milk 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 milk samples were homogenized in 225 mL of BPW by mixing. MPN assays (five-tube method) were performed according to the procedures described in Bacteriological Analytical Manual of the U.S Food and Drug Administration (BAM/FDA) (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 milk 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 www.cfsan.fda.gov/~ebam/bam-a2.html. (Garthright and Blodgett, 2003). All DNA templates were stored at -20°C for further use.

2.4. DNA extraction

For PCR analysis, aliquot of enriched milk sample was transferred to a microcentrifuge tube with a capacity of 1.5 mL. The cell suspension was centrifuged for 10 min at 12,000 g. 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 g 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 cell debris was pelleted by centrifugation at 12,000 g for 10 min. The volume of the DNA containing supernatant was estimated by pipetting to a new microcentrifuge tube and varied from 40 to 60 µl, due to differences in removing the supernatant during the different washing steps of the DNA isolation method. The DNA 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.

The supernatant was carefully transferred to a new microcentrifuge tube and immediately tested by LighCycler real-time PCR. 2 µL of the supernatant was used as the template DNA in the real-time PCR. The rest of DNA templates were stored at –20 °C in case they are needed.

2.5. LightCycler real-time PCR assay

Salmonella specific primers ST11 (5'-AGCCAACCATTGCTAAATTGGCGCA-3') and ST15 (5'-GGTAGAAATTCCCAGCGGGTACTG-3' Aabo et al., 1993) previously shown to be highy specific for *Salmonella* (Aabo et al., 1993; Bansal et al., 2006) were purchased from Proligo and used to amplify a 429-bp region specific to all *Salmonella* spp. The Real-time PCR reactions were performed with a LightCycler PCR System (Roche Diagnostics) using the LightCycler FastStart DNA Master SYBR Green I Kit (Cat. No.

2 239 264 Roche Applied Science, Meyla, France). The reaction 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 DNA template, and sterile PCR grade water to atotal volum of 20 µL per capillary. Each LightCycler run contained one negative control consisting of H_2O without any DNA template to monitor for possible contamination and one positive control (*S*. 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 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 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*)/ d*T* versus *T*).

2.6. Isolation of positive colonies from raw milk samples.

For isolation of presumptive *Salmonella* colonies from PCR-positive milk samples, our procedures inspired by two methods AFNOR and French Agency for Standardization (NF U 47 100-101 Feb. 2005). 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). RV 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 XLT-4 agar plates 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.

3. Results

After 18 h of enrichment, eight (2.68%) out of the 299 bulk tank milk samples were found positive for *Salmonella* using SYBR Green I real-time PCR.

Few milk samples showed inhibitors and required further dilution of the extracted DNA to bypass the inhibitory effect. Among the 299 milk samples tested, 29 (9.6%) showed inhibitory reaction when tested in LightCycler-real-time PCR (i.e flat fluorescent curves) (data not shown). However, all of the inhibitory effects observed were overcome after 10-fold dilution of the extracted DNA.

Salmonella PCR products were identified by melting curve analysis and a distinct melting point (T_m) of about 87°C was observed for *Salmonella* positive. Samples confirmed negative did not show peaks in T_m that corresponded to 87°C.

The real-time PCR positive samples were further analyzed with the traditional cultural methods while the negative ones were discarded. None of the eight real-time PCR positive samples did yield positive cultures.

The MPN real-time PCR assay enabled the enumeration of *Salmonella* per mL in bulk tank milk positive samples that ranged from 3.7 to 79.2 (Table1.4).

Table 1.4 Quantification estimates of *Salmonella* in bulk tank milk samples obtained with MPN-real-time PCR assay after 8 h pre-enrichment in BPW

Sample	MPN enumeration MPN/mL	LogMPN/mL	MPN confidence limits
1	3.7	0.56	0.92 - 14.7
2	4.5	0.65	1.1 - 18.1
3	69.9	1.84	23.2 - 211.5
4	7.8	0.89	2.4 - 24.8
5	17.0	1.2	7.9 - 36.5
6	6.1	0.78	1.9 – 19.1
7	16.9	1.2	6.5 - 44.1
8	79.2	1.9	25.4 - 274.7

4. Discussion

In our previous work (chapter 3), a MPN-real-time PCR assay was successfully used for the detection and quantification of *Salmonella* cells in artificially contaminated milk. Therefore, the aim of the present study was to evaluate this assay for eumerating *Salmonella* in naturally contaminated bulk tank raw milk, within the aim to use the obtained results for the development of quantitative risk assessment of *Salmonella* contamination of milk products.

When the real-time PCR assay was used to examine 299 bulk tank milk samples taken from selected dairy herds located in the west of France, it indicated that 2.68% (8 of 299) 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

(Desmasures et al., 1997), which showed the prevalence of *Salmonella* in bulk tank milk to be 2.9%. However, previously reported surveys of bulk tank milk in Europe, United States and Canada have shown large variations in the prevalence of *Salmonella* in raw milk ranging from 0.17% to 12.6% (Rohrbach et al., 1992; Steel et al., 1997; Hassan et al., 2000; Karns et al., 2005). The large variations in levels of bulk tank *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* do occur in bulk tank milk and may pose a health hazard if raw milk or raw milk products are consumed.

While the DNA extraction procedure based on a simple boiling method gave optimal results and no inhibition was seen in inoculated milk samples **(**Chapter 3), the method resulted in PCR inhibition in few naturally contaminated milk samples. Therefore, DNAs of these inhibited pre-enriched milk samples had to be diluted 1:10 and PCR inhibitors were bypassed in all cases. Other literature reported similar results with other extraction preparation protocols and commercial kits (Meng et al., 1996; Lample et al., 2000; Liming and Bhagwat, 2004). In general, as a simple, but widely used method, boiling method provides a fast and efficiency way of extracting DNA that can be used in PCR assays in less well equipped laboratories. However, extraction procedures have to be improved to isolate DNA from naturally contaminated milk samples, and the internal amplification control must be included when a PCR-based method is used in routine analysis to detect false-negative results due the the presence of PCR-inhibiting substances

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, the real-time PCR assay is substantially faster and usually more sensitive than the standard culture procedure. In addition, 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. The real-time PCR assay of bulk tank milk samples performed in this study suggested that more milk samples contained detectable *Salmonella* than previously reported in unpublished industry data in 2000-2002 from the same region; in which culture methods indicated only 0.3% prevalence rate. In our study, the detection of *Salmonella* in 2.6% of the samples tested indicates that the degree of prevalence of the pathogen in raw milk in Western France is higher than previously believed. Others have also reported more positive results when analysing enrichments of different food commodities including milk by real-time PCR than by the traditional cultural methods. For example, Van Kessel et al. (2003) reported the use of a real-time PCR for the detection of *Salmonella* in raw milk samples. The method yielded 16.5% more positive samples than the culture techniques. Karns et al. (2005) detected *Salmonella* in 11.8% of the samples using the real-time PCR assay, whereas conventional culture methods detected the pathogen in only 2.6% of the same samples (Van Kessel et al., 2004). Hein et al. (2005) tested bovine and caprine naturally contaminated raw milk samples for the presence of *Staphylococcus aureus*, the real-time PCR method yielded 19.3% more positive samples than plate count method. Therefore, it appears that the real-time PCR method is more sensitive and faster than traditional culture techniques for the detection of *Salmonella* in bulk tank milk.

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* were not isolated by cultural procedures from the eight real-time PCR *Salmonella*-positive milk 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 original milk samples was relatively low (Table 1). Conventional cultural procedures will not always detect small numbers of *Salmonella* cells in certain food samples. Bansel 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.

A data gap that is routinely identified in quantitative risk assessment is the lack of enumeration data of pathogens contaminated foods (Coleman and Marks, 1999). The application of MPN method combined with LightCycler real-time PCR to quantify *Salmonella* in raw milk proved to be rapid and highly sensitive and small numbers of *Salmonella* could be found in bulk tank milk samples. This assay yields significant labor and time savings since the quantification of *Salmonella* be completed within 12 h which included a 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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Chapter 5: Growth of *Salmonella* in Artificially Contaminated Milk Samples Stored at Different Times and Temperatures

Abstract

The growth of two *Salmonella* strains; *S.* Typhimurium and *S.* Montevideo in milk incubated at constant temperatures (9, 15, 25, 30, 35, 37, 40, and 43°C) was investigated and modelled. Growth curves at each temperature were fit to primary model, namely the logistic-with-delay model to determine specific growth rate and lag time. The model fitted growth data well while measurers of goodness-of-fit showed a high *pseudo-*R^2 (> 0.99) at all temperatures. The effect of temperature on the maximum growth rate was described by cardinal model and the *pseudo-*R^2 values for this secondary model were 0.98 and 0.97 for *Salmonella* Typhimurium and *Salmonella* Montevideo, respectively. The estimated cardinal temperatures for *Salmonella*: T_{min} , T_{opt} and T_{max}. by means of nonlinear regression were in accordance with data from published literature.

Keywords: Predictive microbiology; *Salmonella,* Temperature effect, growth rate

Résumé

La croissance de deux souches de *Salmonella* Typhimurium et *Salmonella* Montevideo dans le lait incubé à température constante (9, 15, 25, 30, 35, 37, 40, et 43°C) a été mesurée et modélisée. Le modèle primaire utilisé pour ajuster les courbes de croissance et déterminer la latence et le taux de croissance spécifique était le modèle logistique-avec-délai. La valeur élevée du paramètre d'ajustement *pseudo-R²* (> 0,99) montre que le modèle convient bien. Le taux de croissance spécifique dépendait de la souche et de la température. Le taux de croissance maximum obtenu au moyen du modèle primaire a ensuite été modélisé en fonction de la température au moyen du modèle cardinal. Il a ainsi été possible de calculer les températures cardinales T_{min} , T_{opt} et T_{max}.

Mots clefs : Microbiologie prévisionelle ; *Salmonella* ; Température ; Taux de croissance

1. Introduction

Salmonella is one of the most common pathogens associated with foodborne illness. Consumption of raw milk and raw milk products which is favoured by a number of individuals for taste, cultural, nutritional and economic reasons (Desenclos et al., 1996; Headrick et al., 1997), continue to be sources of *Salmonella* (D'Aoust et al., 1989; Maguire et al., 1992; Desenclos et al., 1996; De Buyser et al., 2001), and outbreaks of salmonellosis have been linked to raw dairy products (Maguire et al., 1992; Vaillant et al., 1996; Ellise et al., 1998; Villar et al., 1999). A community outbreak of *Salmonella* Typhimurium infections linked to eating unpasteurized soft cheese in France in 1998 caused one-hundred and thirteen cases (De Valk et al., 2000). In 2001, a second French outbreak of salmonellosis was related to the consumption of Cantal cheese. Over 155 people were infected with *Salmonella* Enteritidis phage type 8, and the presence of *Salmonella* in the cheese was attributed to raw milk (Haeghebaert et al., 2003). One of the most important environmental factors that affect bacterial growth in food is temperature. Therefore, mathematical models that can successfully predict bacterial growth under environmental conditions such as temperature, pH, and water activity (a_w) are needed to adequately describe the changing conditions generally associated with processing and storage of foods. In the assessment of potential risks associated with *Salmonella* in raw milk and raw milk products it is necessary to examine the ability of *Salmonella* to grow in milk and dairy products under several temperature profiles. Scientific data related to the effect of temperature on the growth of *Salmonella* in milk and milk products are extremely limited. Therefore, the aim of the study presented in this paper is to develop primary and secondary models to describe mathematically the experimental data collected in our laboratory to the growth of *Salmonella* in milk under constant temperatures during different incubation periods. In this study, the logistic-with-delay model was used for fitting microbial growth data (Rosso et al. 1996). The growth rate obtained from the primary

model was then modeled as a function of temperature using the cardinal model (Rosso et al., 1993; 1995).

2. Materials and methods

2.1. Bacterial Strains

The two strains of *Salmonella, Salmonella* Typhimurium and *Salmonella* Montevideo used in experimental studies were obtained from the culture collection of *MASQ* (Unité Microbiologie des Aliments, Sécurité et Qualité), National Veterinary School of Alfort, Maisons-Alfort, France. These strains were previously isolated from manure and slurry in dairy herds in western France (Lailler et al., 2005). Stock cultures were preserved by freezing at -80°C in vials containing brain-heart infusion (BHI) broth (Difco) supplemented with 20% (v/v) glycerol.

2.2. Inoculum preparation

Ultra-High Temperature (UHT) sterilized whole milk (3.6 g fat), used as the growth medium, was purchased from a retail supermarket. A day before the experiment, fresh cultures for use in experiments were prepared; culture vials were thawed at room temperature and 0.1 mL of the thawed culture was transferred to 9.9 mL of BHI broth and incubated for 24 h at 37°C. One ml of cell suspension of each strain was serially diluted (1:10) in buffered peptone water (BPW) (Difco) and 100 µL samples of appropriate dilutions were spiral plated onto xylose lysine deoxycholate (XLD) agar in four replicate plates using Whitley automatic spiral plater (WASP) (*AES* laboratories), followed by incubation at 37°C for 24 h to determine the viable cell concentration. The dilutions were then kept in refrigerator at 4°C.

2.3. Sample preparation, and inoculation

On the day of experiment, a test quantity (50 mL) was transferred into sterilized flasks (85mL). Milk samples were inoculated with aliquots of appropriate dilutions resulting in inoculum level of approximately 100 CFU/mL. The average of inoculums levels (Log_{10} CFU/mL) of *Salmonella* strains are shown in Table 1.5. After inoculation, samples were

mixed gently by hands for 2 min to ensure even distribution of the organisms in the milk samples. Negative control consisted of milk sample with no bacterial cells was also prepared.

Table 1.5 Means (standard deviations) of initial levels of *Salmonella* Typhimurim and *Salmonella* Montevideo inoculated in milk.

Storage temperature (°C)	Strain	
	S. Typhimurium	*S. Montevideo*
7	[a] 1.94 ± 0.11	2.80 ± 0.80
9	1.94 ± 0.16	2.07 ± 0.12
15	2.03 ± 0.05	1.99 ± 0.11
25	2.06 ± 0.17	2.00 ± 0.19
30	2.16 ± 0.06	1.92 ± 0.30
35	2.19 ± 0.05	1.68 ± 0.26
37	2.17 ± 0.02	1.90 ± 0.13
40	2.54 ± 0.17	2.31 ± 0.005
43	2.09 ± 0.16	1.88 ± 0.03

[a] Mean ± SD (Log_{10} CFU/ml) (n=9). The average inoculum level of three separate experiments

2.4. Incubation temperatures, sampling time and bacterial enumeration

All incubators were adjusted to the required temperatures before the milk in test tubes being inoculated with the estimated inoculum levels. Inoculated milk samples were sampled immediately after inoculation (time 0). In addition, 100 µL aliquots of appropriate dilutions were spiral plated onto XLD agar in four replicate plates with WASP spiral plater (*AES* laboratories), followed by incubation for 24 h at 37°C to determine the initial contamination level (Log CFU/mL). All samples were then incubated at constant temperatures 9, 15, 25, 30, 35, 37, 40, and 43°C until they reached the stationary phase. Sampling frequency was based on the objective of the study and the growth temperature, i.e. every 24 h at 9°C; every 12 h at 15°C; every 2 h at 25 and 30°C and every 1 h at 35, 37, 40 and 43°C experiments. Total sampling time ranged from 15 days at 9°C to 12 h at 43°C. At each sampling time, samples were collected, serially diluted (1:10) in BPW if necessary and spirally plated in triplicates onto XLD plates. The plates were incubated overnight at 37°C followed by counting using an automated colony counter and WASP 2 user manual, with the results being expressed in CFU/mL before being converted to Log_{10} CFU/mL. Three independent experiments were

performed for each temperature. For each replicate experiment, an average Log_{10} CFU/mL of three platings of each sampling point was used to determine estimates of the growth kinetics for each *Salmonella* strain. In total, 3 independent growth curves where btained for each temperature and *Salmonella* strains *(3 x 8 x 2) = 48 curves*

2.5. Primary Model

In our study, a primary model described a bacterial growth curve exclusively as a function of time at constant temperature. The maximum growth rates and thr population lag times were estimated from growth kinetics by fitting the logistic-with-delay equation. (Rosso et al., 1996; Augustin and Carlier, 2000):

$x(t)$ =

$$\begin{cases} \dfrac{x_{max}}{1+\left(\dfrac{x_{max}}{x_0}-1\right).\exp\left[-\mu_{max}.(t-lag)\right]}, & \begin{matrix} t\le lag \\ t>lag \end{matrix} \end{cases}$$

where $x(t)$ is the bacterial concentration (CFU/cfu.ml) at time t (h), x_0 is the initial bacterial concentration, x_{max} is the maximum bacterial concentration, *lag* is the lag time (h), and μ_{max} is the maximum specific growth rate (Ln CFU/h). The logarithm of this function was fitted to the logarithm of $x(t)$

2.6. Secondary Model

In our study, a secondary model described the effects of the temperature on the parameters of the primery model, particularly the maximum growth rate. A cardinal model with inflection was used to analyse the effect of temperature on maximum growth rate (μ_{max}) (Rosso et al. 1993):

$$\mu_{max}(T) = \mu_{opt} \times = \frac{(T-T_{max}).(T-T_{min})^2}{(T_{opt}-T_{min}).[(T_{opt}-T_{min}).(T-T_{opt})-(T_{opt}-T_{max}).(T_{opt}+T_{min}-2.T)]}$$

where T is the temperature, T_{min} is the value or the temperature below which no growth occurs, T_{opt} is the value at which μ_{max} is equal to its optimal value μ_{opt} (h^{-1}), and T_{max} is the value above which no growth occurs.

2.7. Primary Model Fitting.

A plot of microbial count versus time at each temperature was used to calculate the starting values for the parameters, x_0 and x_{max}. A nonlinear regression procedure, PROC NLIN, in SAS package (Release 9.13, SAS Institute Inc., Cary, NC) was used to fit data to the model. The Newton method was the iterative method used for estimation of the parameters. The NLIN procedure was used to produce least squares estimates of the parameters of a nonlinear primery model. Estimation of a nonlinear model is an iterative process. To begain this process, the NLIN procedure we specify a grid of initial values for the different model parameters. The iterative method we used was Newton method. We calculate *pseudo-*R^2 to assess the goodness-of model fitness (Schabenberger, 2005):

$$Pseudo\text{-}R^2 = 1\text{-SS (Residual)} / \text{SS (Total}_{corrected})$$

The maximum growth rates (μ_{max}) as function of serotype and incubation temperature and their interaction were analysed using the General linear model (GLM). LSMEANS in GLM procedure was used for the effects of temperature* serotype interaction on growth rate. Means of maximum growth rates (μ_{max}) of the two serotypes various temperatures were compared with a Tukey mean comparison test (SAS 9.1) at a significance level of 95%.

2.8. Secondary Model Fitting.

The maximum growth rates (μ_{max}) estimated from the model at different temperatures was then fitted using the cardinal model. The approximate lowest temperature (7°C) at which *Salmonella* can grow was given as the starting value for the parameter T_{min}. Similarly, the approximate highest temperature at which *Salmonella* can grow (50°C) was given as starting value for the parameter T_{max}. PROC NLIN procedure was used to estimate the parameters of

the secondary model. The performance of the secondary model on predicting the obtained maximum growth rate from thr primery model was evaluated using the accuracy factor as described by Baranyi et al. (1999):

$$A_f = \exp\left(\sqrt{\frac{\sum(\hat{\mu}-\mu)^2}{n}}\right)$$

Where μ hat is the predicted growth rate from the secondary model and μ is the estimated growth rate from the primary model.

3. Results and discussion

As a part of our efforts to provide data for risk assessment of *Salmonella* foodborne disease, the objective of these experiments was to describe mathematically the experimental data collected in our laboratory on the growth of *Salmonella* in milk. The *Salmonella* strains used in these experiments were chosen because they were isolated from manure and slurry in dairy herds in western France (Lailler et al., 2005). They were the most common isolates in the study. Literature data reported that *S.* Montevideo was often isolated independently from the presence of clinical disease (Heuchel et al., 2000; Wells et al., 2001), whereas *S.* Typhimurium was the main serotype associated with bovine clinical cases of *Salmonella* (Martel et al., 2000; Warnick et al., 2003). Moreover, *S.* Typhimurium has been used extensively in laboratory for investigating and modeling the growth of *Salmonella* in laboratory medium (Oscar, 1999a, Oscar, 1999b), it exhibits the same growth kinetics as other strains of *Salmonella* that are commonly found in foods and thus, it is a good strain to use for developing growth models for *Salmonella*.

3.1. Primary modelling curve fitting

Growth of the two *Salmonella* strains in milk was studied at constant temperatures (9-43°C). The results shown in Fig.1.5 to Fig. 8. 5, confirm the adequacy of milk as a substrate for *Salmonella* growth at different temperatures and incubation times. Fifteen days at 9°C and

12 h at 43°C were more than enough to allow increases in numbers of cells to levels higher than >8 log CFU/mL of milk. It can be noted that *Salmonella* growth is rapid at higher temperatures, while at 9 and 15°C it is lower when compared to the other temperatures, although it is important to notice that growth is still observed at these temperatures. The lag time (h) and growth rate (Log_{10} CFU/h) of *Salmonella* strains are listed in Table 2.6. Different ranges of lag time at different incubation temperatures were observed for the two strains. However, there was not a defined lag time at most incubation temperatures. No differences were observed for the growth rate between the two strains except at 30 and 43°C ($P < 0.05$). As expected, the growth rates were lower at lower storage temperatures. The US department of Agriculture (USDA) Agriculture Research Service's Pathogen modeling Program (PMP) is probably the most-known predictive software package in the world. Anybody can free download and use the program (http://www.arserrc.gov/mfs/pathogen.htm). The PMP software program was designed using broth-based models including broth-based *Salmonella* growth models (liquid environment). Applying broth-based models, such as the PMP growth model, to specific foods can help to validate the accuracy of these simulations. Therefore, we choose the growth model of PMP for the comparison. Our findings demonstrated that the PMP growth model for *Salmonella* in broth culture was consistent with the observed bacterial growth in milk at temperature compared (10, 15, 25, 30°C). 30°C is the maximum temperature found in PMP for the growth *Salmonella* in broth culture.

Representative data on the growth of *S.* Typhimurium and *S.* Montevideo on milk at different temperatures are shown in fig 1.5 to 8.5 with fitted growth curves produced using the logistic-with-delay model; this provided a good statistical fit to the data. The curves obtained at all temperatures showed a high *pseudo-*R^2 (>0.99).

Table 2.5 Means (standard error of the mean) of growth rates (GR, Log_{10} cfu/h) and lag time (LT,h) of *S.*Typhimurium and *S.* Montevideo grown in milk at different temperatures

Temperature (°C)	*S.* Typhimurium		*S.* Montevideo	
	GR (Log_{10}cfu/h)[a]	LT (h)[a]	GR (Log_{10}cfu/h)	LT (h)
9	0.0536 (0.0021)	40.17 (9.47)	0.0535 (0.0023)	13.52 (12.77)
	0.0416 (0.0008)	36.27 (6.11)	0.0482 (0.0005)	NO
	0.0436 (0.0007)	1.909 (6.54)	0.0443 (0.0008)	NO
15	0.2155 (0.0026)	NO	0.2302 (0.0047)	NO
	0.2140 (0.0033)	NO	0.2323 (0.0060)	NO
	0.2193 (0.0027)	NO	0.2284 (0.0091)	NO
25	0.7082 (0.0109)	0.414 (0.292)	0.7943 (0.0185)	0.565 (0.922)
	0.7050 (0.0120)	0.506 (0.311)	0.7435 (0.0097)	NO
	0.6811 (0.0089)	NO	0.7155 (0.0141)	0.255 (0.387)
30	0.9712 (0.0348)	NO	1.123 (0.0222)	0.395 (0.264
	1.027 (0.0262)	0.489 (0.344)	1.064 (0.0184)	0.040 (0.241)
	0.9353 (0.0213)	0.707 (0.368)	1.043 (0.0149	NO
35	1.283 (0.0284)	0.618 (0.283)	1.281 (0.0349)	0.003 (0.346)
	1.291 (0.0189)	0.641 (0.185)	1.228 (0.0167)	NO
	1.266 (0.0202)	0.809 (0.204)	1.268 (0.0263)	0.084 (0.261)
37	1.290 (0.0355)	0.143 (0.345)	1.396 (0.0400)	0.524 (0.320)
	1.407 (0.0258)	0.581 (0.209)	1.374 (0.0261)	0.797 (0.236)
	1.318 (0.0324)	0.524 (0.295)	1.393 (0.0280)	0.137 (0.231)
40	1.327 (0.0341)	0.414 (0.265)	1.354 (0.0436)	0.434 (0.327)
	1.356 (0.0370)	0.278 (0.287)	1.423 (0.0418)	0.555 (0.282)
	1.319 (0.0378)	0.051 (0.307)	1.378 (0.0424)	0.463 (0.299)
43	0.838 (0.0302)	0.066 (0.502)	1.111 (0.0268)	0.380 (0.318)
	0.884 (0.0236)	NO	1.196 (0.0366)	0.339 (0.398)
	0.846 (0.0234°	NO	1.117 (0.0313)	0.172 (0.365)

[a] Values are means of triplicates samples, and standard error of the means
[b] NO= no time lag was observed

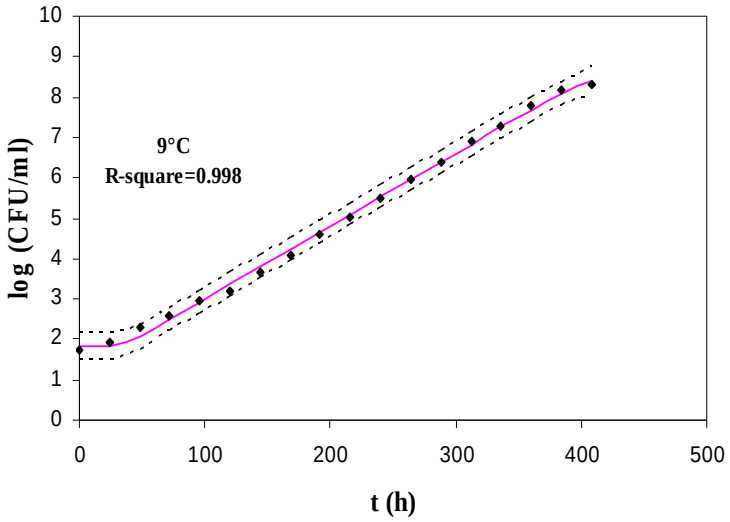
3.2. Secondary Model (Cardinal Temperatures)

The effect of temperature on the maximum growth rates *Salmonella* Typhimurium and *Salmonella* Montevideo is shown in Fig 9.5 A, B, respectively. At the optimum growth temperatures of 38.44 and 38.52°C, the maximum growth rates were 1.36 and 1.39 h^{-1} for *S.* Typhimurium and *S.* Montevideo, respectively (Table 3.5). The optimal temperature can be defined as the temperature at which the growth rate (μ_{opt}), is the greatest. Depending on the environmental conditions such as pH, water activity (a_w), Francis et al., (1999) reported optimum temperatures (T_{opt}) ranging from 35 to 43°C, the growth rate being substantially reduced at <15°C, and prevented at <7°C. Rosso et al., (1993) reported cardinal values

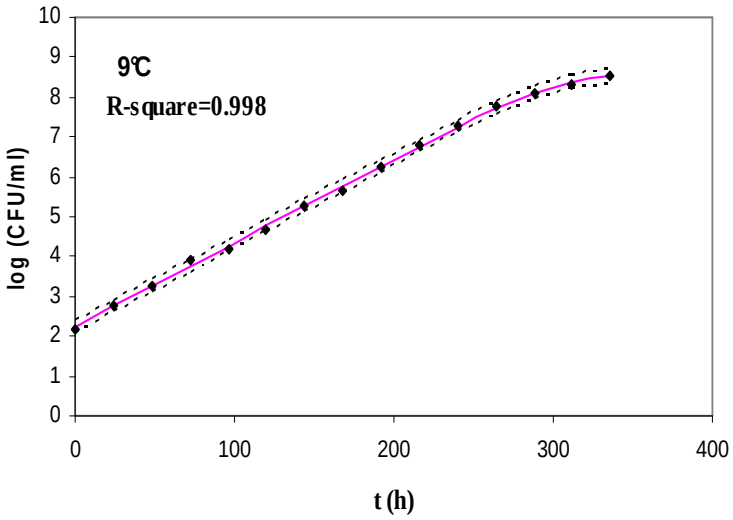
estimate obtained with *Salmonella*: T_{min}, T_{opt} and T_{max} to be 4.6, 39.5 and 45.6°C, respectively which is close to the values obtained in our experiments (Table 3.5). However, the minimal temperatures for *S.* Typhimurium and *S.* Montevideo (Table 3.5) were lower than the mean values reported in literature (Rosso et al., 1993). In fact, the growth of most salmonellae is prevented at temperatures lower than 7°C. The capacity of salmonellae to grow at temperatures lower than 5°C has been reported (D'Aoust 1991), but in some cases it has not been confirmed except by observation of colonies on selective media. However, T_{min} should be regarded as a theoretical lower temperature limit for the growth below which the predicted growth rate is close to zero (Ratkowski et al., 1991). The performance of the secondary model obtained from the primery model was evaluated was evaluated with a high *pseudo-*R^2 (0.97-0.98) indicate that the secondary cardinal model can be used to describe the effect of temperature on the maximum growth rates. The accuracy factors are 1.026 and 1.034 for *S.* Typhimurium and *S.* Montevideo, respectively. Accordingly, the discrepancy between the secondary models and the data used to fit the model are 2.6% and 3.4% for *S.* Typhimurium and *S.* Montevideo, respectively. In conclusion, the growth data of *Salmonella* strains in milk at various temperatures were fitted with a logistic-with-delay model that fitted the data well. The maximum growth rate derived from the primary model was then fitted as a function of temperature using the cardinal Rosso model. The secondary model derived from the logistic model fitted well.

Table 3.5 Estimated cardinal temperatures, optimum growth rates and 95% CI.

Strain	μ_{opt} (h^{-1})	T_{min}	T_{opt}	T_{max}	Accuracy factor
S. Typhimurium	1.36 ± 0.021	3.02 ± 1.33	38.44 ± 0.33	44.51 ± 0.30	1.026
S. Montevideo	1.39 ± 0.03	3.40 ± 1.87	38.52 ± 0.43	46.97 ± 1.25	1.034

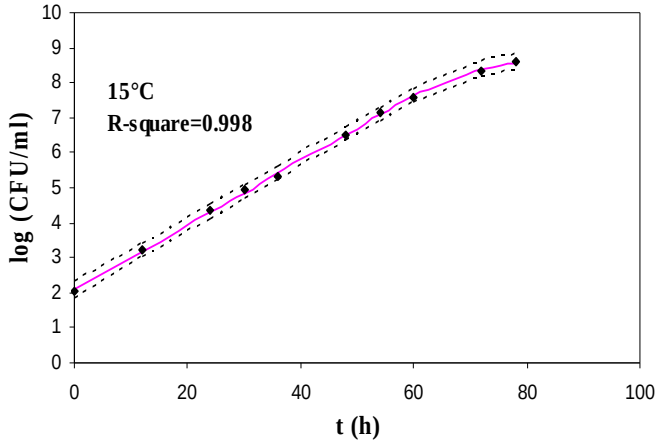


(A)

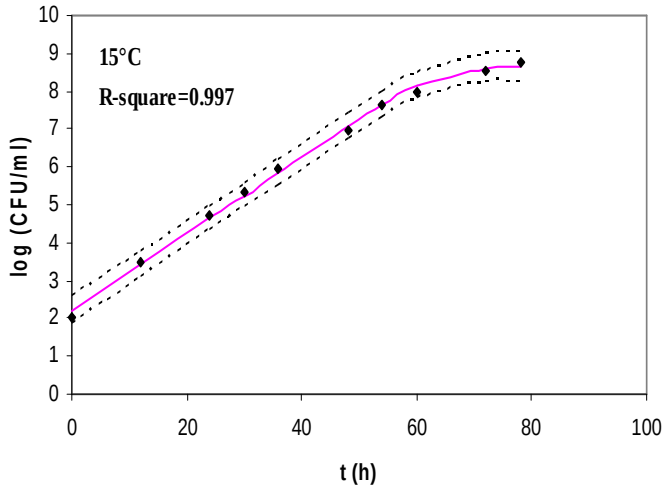


(B)

Figure 1.5 Experimental data of growth of *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B) under (**9°C**) fitted into primery model (logistic with delay model). Dark squares represent raw data. Mathematical growth model is represented by heavy solid line. The dotted lines are upper and lower prediction limits at 95% confidence level.

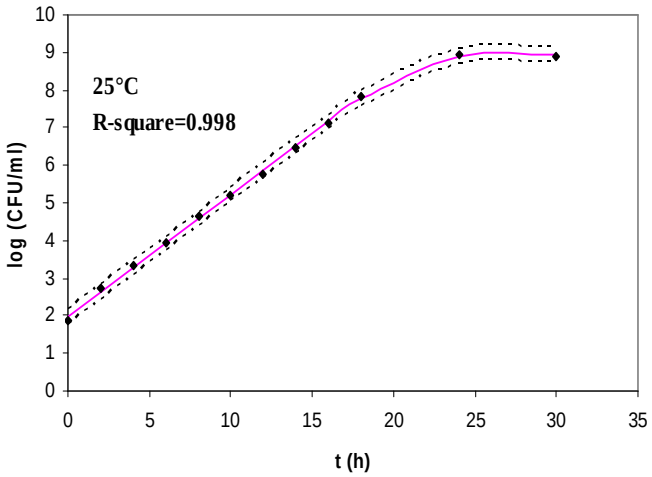


(A)

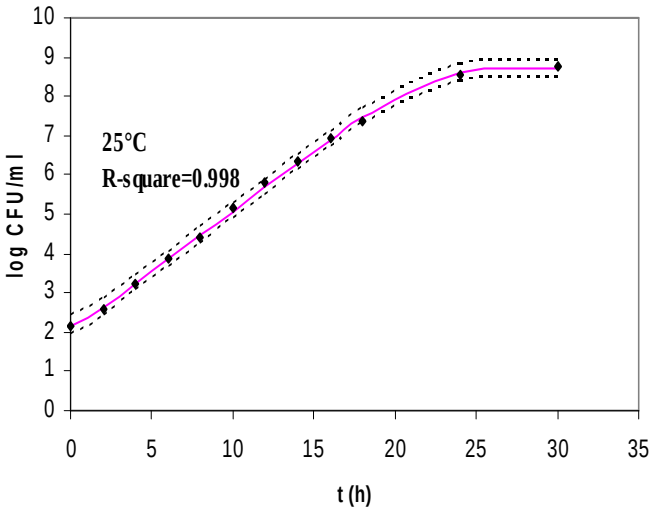


(B)

Figure 2.5 Experimental data of growth of *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B) under (**15°C**) fitted into primery model (logistic with delay model). Dark squares represent raw data. Mathematical growth model is represented by heavy solid line. The dotted lines are upper and lower prediction limits at 95% confidence level.

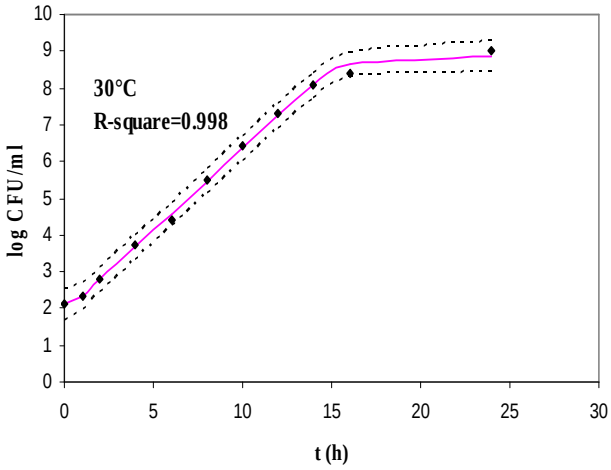


(A)

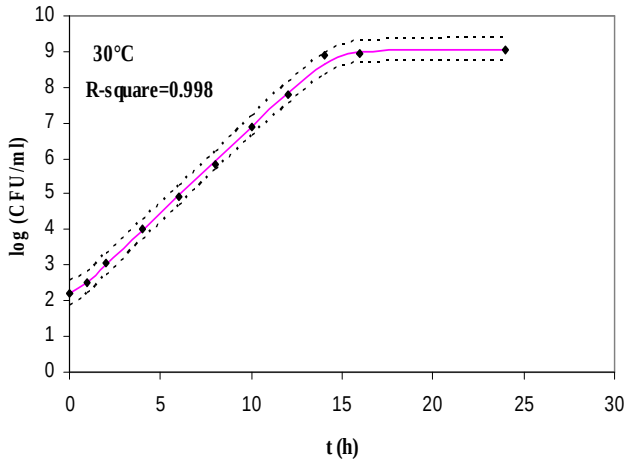


(B)

Figure 3.5 Experimental data of growth of *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B) under (**25°C**) fitted into primery model (logistic with delay model). Dark squares represent raw data. Mathematical growth model is represented by heavy solid line. The dotted lines are upper and lower prediction limits at 95% confidence level.

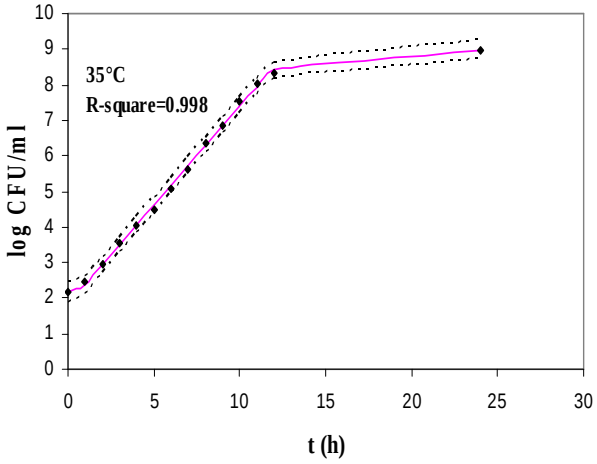


(A)

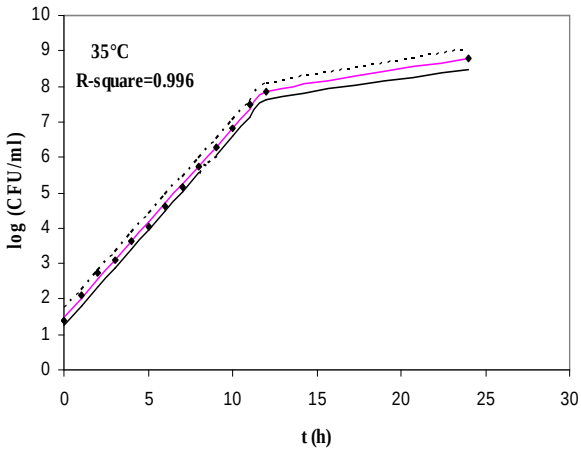


(B)

Figure 4.5 Experimental data of growth of *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B) under (**30°C**) fitted into primery model (logistic with delay model). Dark squares represent raw data. Mathematical growth model is represented by heavy solid line. The dotted lines are upper and lower prediction limits at 95% confidence level.

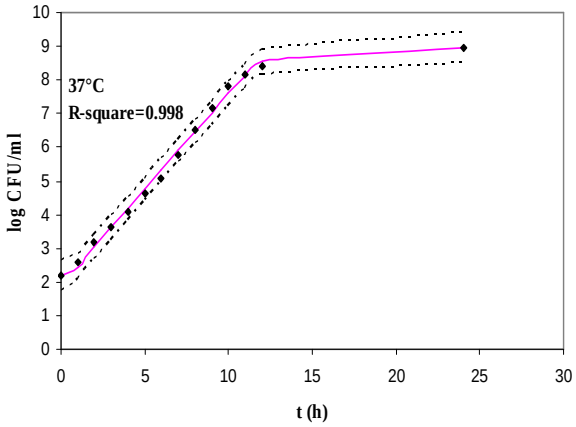


(A)

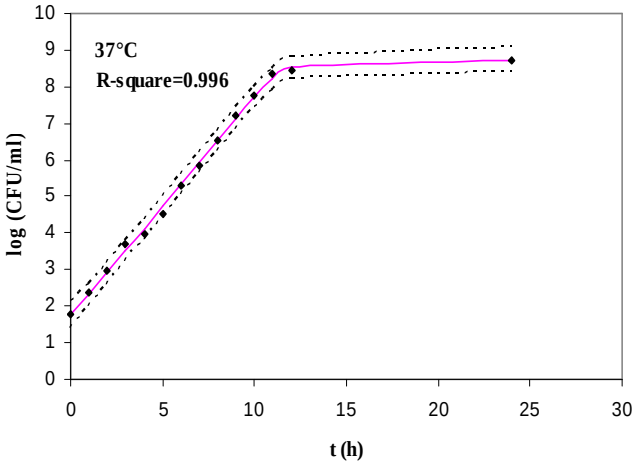


(B)

Figure 5.5 Experimental data of growth of *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B) under (**35°C**) fitted into primery model (logistic with delay model). Dark squares represent raw data. Mathematical growth model is represented by heavy solid line. The dotted lines are upper and lower prediction limits at 95% confidence level.

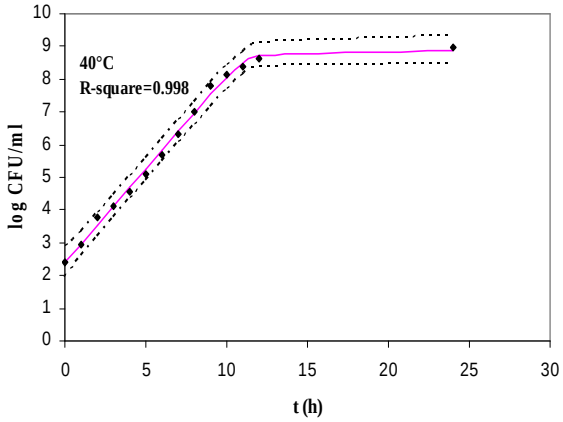


(A)

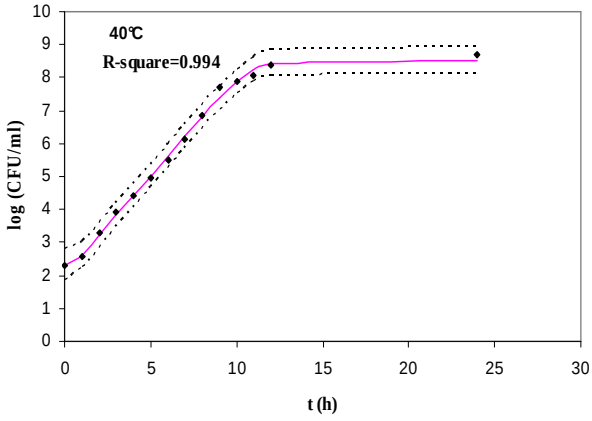


(B)

Figure 6.5 Experimental data of growth of *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B) under (**37°C**) fitted into primery model (logistic with delay model). Dark squares represent raw data. Mathematical growth model is represented by heavy solid line. The dotted lines are upper and lower prediction limits at 95% confidence level.

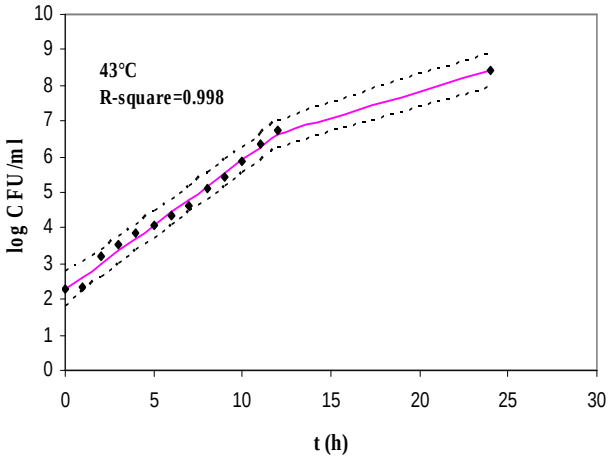


(A)

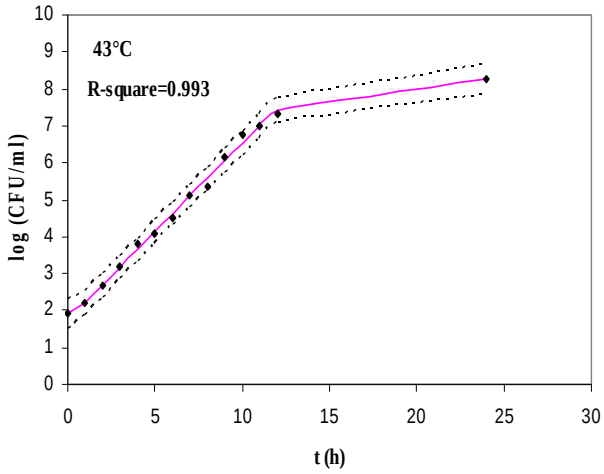


(B)

Figure 7.5 Experimental data of growth of *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B) under (**40°C**) fitted into primery model (logistic with delay model). Dark squares represent raw data. Mathematical growth model is represented by heavy solid line. The dotted lines are upper and lower prediction limits at 95% confidence level.

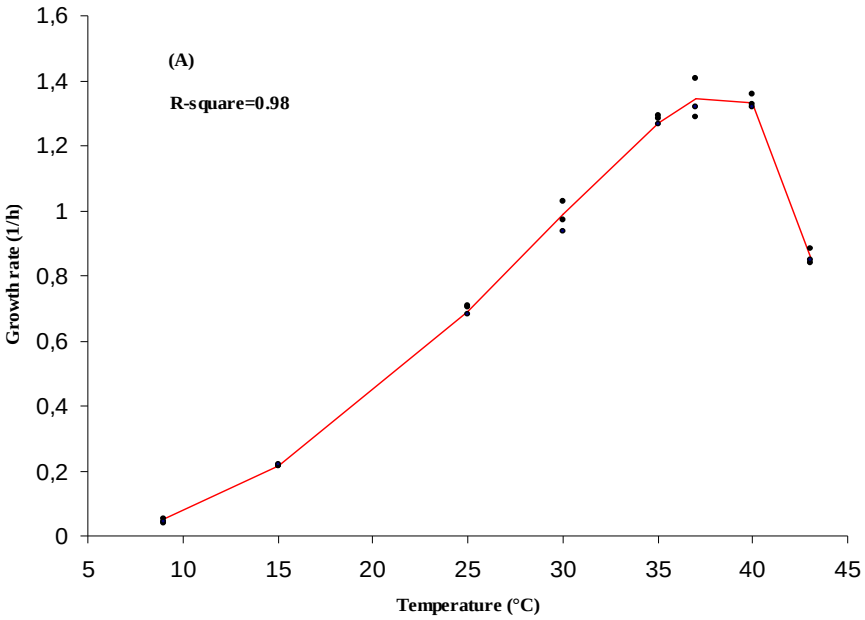


(A)

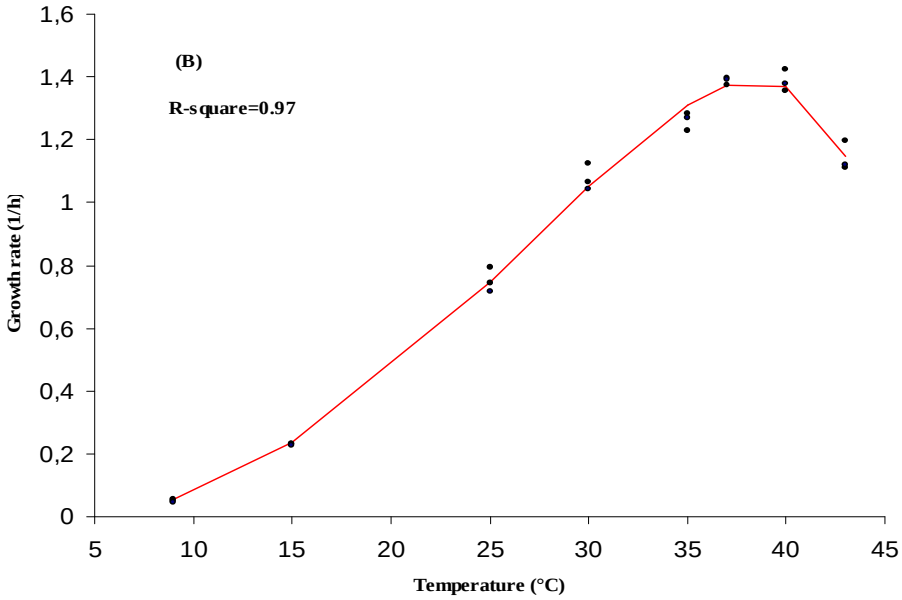


(B)

Figure 8.5 Experimental data of growth of *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B) under (**43°C**) fitted into primery model (logistic with delay model). Dark squares represent raw data. Mathematical growth model is represented by heavy solid line. The dotted lines are upper and lower prediction limits at 95% confidence level.



(A)



(B)

Figure 9.5 Secondary model fits of maximum growth rate data as function of temperature. *Salmonella* Typhimurium (A), and *Salmonella* Montevideo (B)

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Chapter 6: Quantitative Risk Assessment of Human Salmonellosis Linked to the Consumption of Camembert Cheese Made from Raw Milk

Abstract

This work reports a quantitative risk assessment of human salmonellosis linked to the consumption of Camembert cheese made from raw milk. The risk assessment was based on data on the frequency, concentration and growth of *Salmonella* in milk. 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 milk contamination before cheese making was estimated to be 93.7% with a concentration ranging from 0 to 138 CFU mL^{-1}. The simulated proportion of 25 g serving of cheese with no *Salmonella* was 75%. The 99^{th} percentile of *Salmonella* cell numbers in servings of 25 g of cheese was 5 cells at the time of consumption, corresponding to 0.2 cells of *Salmonella* per gram. The risk of salmonellosis per 25 g serving ranged from 0 to 1.2×10^{-7} with a median of 7.4×10^{-8}. For 100 million servings of 25g, the expected number of cases of salmonellosis predicted by the model is in average 7.4. When the prevalence was reduced in the model by a factor of 10, the number of cases per 100 million servings was reduced to less than 1 case.

Keywords: quantitative risk assessment; *Salmonella*; Camembert

Résumé

Un modèle d'appréciation quantitative de risque (AQR) de salmonellose humaine liée à la consommation de Camembert au lait cru a été développé. L'AQR est basée sur des données relatives à la fréquence, la concentration et la croissance de salmonelles dans le lait. Différentes distributions ont été posées en hypothèse chacun des paramètres du modèle. Une simulation de Monte Carlo a été employée pour modéliser le processus et pour éstimer le risque résultant pour la santé publique. La probabilité de contamination du lait cru (avant fabrication) a été estimée à 93,7% avec une concentration s'étendant de 0 à 138 CFU mL^{-1}. La probabilité de consommer une portion de 25 g de fromage sans salmonelles a été estimée à 75%. Le 99^{th} percentile du nombre de cellules de salmonelles dans les portions de 25 grammes était 5 cellules à l'heure de la consommation, correspondant à 0,2 cellule des salmonelles par gramme. Le risque de salmonellose par portion de 25 g était compris entre 0 et $1,2 \times 10^{-7}$ avec une médiane de $7,4 \times 10^{-8}$. Pour 100 millions de portions de 25g, le nombre de cas de salmonelloses prévu par le modèle est en moyenne de 7,4. Quand la prévalence est réduite dans le modèle d'un facteur 10, le nombre de cas par 100 millions de portions est réduit à moins de 1 cas.

Mots clefs : Appréciation quantitative de risque ; salmonelle ; Camembert

1. Introduction

For many years, different views on the acceptable level of public health risk associated with soft cheese made from raw milk is under discussion within the Codex Committee for Food Hygiene (CCFU) of the Codex Alimentarius Commission (CAC). While U.S maintains that scientific evidence demonstrates that raw milk and raw milk products are “potentially hazardous foods”, the European commission, on the other hand, considers consumer safety is protected when strict veterinary and sanitary practices are followed from production to consumption for ready-to-eat raw milk dairy products including cheese.

Based on the epidemiological evidence, microorganisms such as *Listeria monocytogenes*, *E. Coli* O157:H7, *Salmonella*, and toxin-producing *Staphylococcus aureus* can contaminate milk and grow in cheese when milk is not pasteurized (Johnson et al., 1990). Therefore, risk assessment for public health linked to the consumption of raw milk cheese contaminated by these hazards provides useful information for the management of the risk. Quantitative risk assessments of risks linked to some of these hazards to the consumption of cheese made from raw milk have been published. For example, Bemrah et al (1998) and Sanaa et al (2004) reported risk assessments models of listeriosis linked to the consumption of soft cheeses made from raw milk. Lindqvist et al (2002) presented a risk assessment of *Staphylococcus aureus* in unripened cheese made from raw milk. To our knowledge, a quantitative risk assessment of salmonellosis linked to the consumption of soft cheese made from raw milk has not been done. The present work therefore reports a first risk assessment model of salmonellosis linked to the consumption of Camembert cheese made from raw milk using, a Monte Carlo simulation @RISK software.

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. In our study, the hazard is *Salmonella* and the risk qualifies the probability of human salmonellosis associated with the consumption of 25 g serving of Camembert cheese made from raw milk.

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. Salmonellosis in patients with no underlying risk factors is a self-limiting illness that may require fluid and electrolyte replacement. 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 cheese-related outbreaks in the U.S., Canada, and Europe demonstrated that soft cheeses, which in many cases are produced in small, family sized establishments, are at significantly greater risk to transmit pathogens than other cheeses (Johnson et al., 1990). The presence of *Salmonella* in soft cheese made from raw milk is a known health hazard and outbreaks of salmonellosis linked to the consumption of such cheeses have been reported (Ellis et al., 1998; Villar et al., 1999; De Valk et al., 2000). Even though few positive findings of *Salmonella* in Europe were reported in cheese in 2005, the majority of these findings were from soft or semi-soft cheeses (Anonymous, 2007). The frequency of samples tested seemed to be very low but the potential for this organism to grow

in improperly stored raw milk and in products made from raw milk presents a public health risk, particularly to susceptible members of the population.

2.2. Exposure assessment

In order to develop a risk assessment model of human salmonellosis associated with the consumption of Camembert cheese made from raw milk, 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 25-g servings of cheese at the time of consumption. This mass represents one-tenth of a 250-g cheese. A list of variables was identified and distribution was assumed for each variable (Table 4.6). An accurate exposure assessment needs information such as the frequency and level of contamination of the selected foods and the growth of the pathogen during the preparation steps. This information will be discussed below.

Monte Carlo simulation was done using @Risk software (V4.5, Palisade Corporation, Newfield, NY, USA) (with a practical value of 100,000 iterations).

Nomenclature of distributions: *Normal* (x, y) stands for normal distribution with mean x and standard deviation y. *Triangular* (x, y, z) stands for triangular distribution with minimum x, most likely y, and maximum z. *Poisson* (x) stands for Poisson distribution with parameter x. *Bionmial* (*n*, *p*) stands for binomial distribution with count *n* and success *p*. Discrete *Uniform* (x_i ,……, x_n) specifies a discrete uniform distribution with n possible outcomes with an equal probability of each outcome occurring.

2.2.1. Collection of data on raw milk contaminated by Salmonella

2.2.1.1. Contamination of milk by Salmonella.

To estimate the prevalence and concentration of *Salmonella* in raw milk, we used our data from a study conducted in randomly selected dairy farms in a limited geographical area of western France in spring 2006. These farms producing milk intended for making soft raw milk cheese which is produced under the French label of origin (AOC, appellation d'origine contrôlée), and their production of milk is regularly submitted to analytical controls including bacterial analysis of disease-causing pathogens, specifically: *Listeria monocytogenes*, *Salmonella*, *Staphylococcus* and *E. coli*.

A total of 299 dairy farms were selected and the presence of *Salmonella* in bulk raw milk was investigated using the real-time PCR detection assay (chapter 4 of this thesis). The results of this study indicated that 2.68% of bulk tank milk samples were positive.

2.2.1..2. Level of Salmonella contamination in milk.

In general, literature data on the contamination of raw milk or raw milk products by *Salmonella* are qualitative and presented as presence or absence of *Salmonella* in 10 or 25 g of 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 milk 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* bulk tank milk samples (chapter 4 of this thesis). The MPN real-time PCR assay enabled the enumeration of *Salmonella* in bulk tank milk *Salmonella*-positive samples that ranged from 3.7 *Salmonella* per ml (log MPN/ml=0.56) to 79.2 *Salmonella* /ml (log MPN/ml=1.9) (Table 1.4 chapter 4).

By combining the findings of the study on the frequency and level of contamination in milk (Chapter 4 of this thesis) and bacterial growth in milk (chapter 5 of this thesis) a risk

assessment model was developed. Variables are presented in table 3.6 and are explained below.

The number of contaminated farms N_c per day milk collection is modelled as *Bionmial* distribution (*n*, *p*). The first parameter *n* is the number of bulk tanks collected per day and is assumed variable as *Triangular* (65, 98, 160). The second parameter p is the prevalence of farm with contaminated milk.

The enumeration provided data on the concentration of *Salmonella* in bulk tank milk (C_{BTM}) (Table 1.6). Because of the low number of positive samples with enumeration, the *Discrete Uniform* seemed to be the most appropriate distribution.

Table 1.6 Quantification estimates of *Salmonella* in bulk tank milk samples obtained with MPN-real-time PCR assay

Sample	MPN enumeration	LogMPN/mL	MPN confidence limits
1	3.7	0.56	0.92 - 14.7
2	4.5	0.65	1.1 - 18.1
3	69.9	1.84	23.2 - 211.5
4	7.8	0.89	2.4 - 24.8
5	17.0	1.2	7.9 - 36.5
6	6.1	0.78	1.9 – 19.1
7	16.9	1.2	6.5 - 44.1
8	79.2	1.9	25.4 - 274.7

To estimate the concentration of *Salmonella* in truck, the volume variability in bulk tanks had to be known. To account for this variability, a coefficient, C_i, is calculated using milk quota statistic from the considered collecting area. This is done by dividing each farm volume by the total volume collected. The resulting distribution of $\log_{10} C_i$ is fitted as *Normal* (-0.158, 0.611).

The concentration of *Salmonella* in tanker milk is then calculated from

$$C_{TT} = \sum_{i=1}^{i=Nc}(C_i \text{ x } C_{BTM} / n)$$

Where *Nc* is number of contaminated farms, *n* is the number of bulk tanks (from which milk has been collected) in the tanker, C_{BTM} the concentration bulk tank, C_i the coefficient as above.

2.3. Cheese processing

Making a Camembert cheese takes about three weeks. Traditional Camembert is made from the fresh raw milk of Normandy breed's cows, which is high in fat content as well as very rich in proteins and vitamins. The main processing steps for making Camembert cheese is usually as follows: after collection, the milk is heated (but not above body temperature) and poured into large vats in a room kept at a temperature of 28°C to 33°C. Natural rennet is added to aid curdling. This curdled milk is then ladled carefully by hand, without breaking, into individual cheese moulds. Five ladling passes are required so as to fill each mould and give the Camembert its creamy texture. When the cheeses have drained sufficiently, they are turned over. These successive operations take about one day. On the second day, the cheeses are removed from the moulds and taken to the salting room, which is at a temperature of about 18° to 20°C. Here dry salt is scattered onto all surfaces of each cheese, followed by the addition of the *Penicillium camemberti* bacterium. On the third day, the cheeses are placed in the drying room, which is kept at 10° to 14°C. The ripening period is twelve days, depending on the season, after which the cheeses are ready for packing. They are further aged for four or five days at about 9°C. After checking the results of the bacteria analysis for each lot, the Camembert cheeses are finally ready to be shipped. Once into the market, the maximum "best before date" is 15 days, yet the products are usually consumed much earlier. The standard Camembert cheese mass is prepared as a 250 g product.

2.4. Growth model

Growth was simulated with a modified logistic model (Sanaa et al., 2004) the relevant equations are shown in table 3.6 and 4.6 The maximum growth rate at time t in the dynamic condition of temperature for a given strain is $\mu_{max}(t)$.

The effect of temperature on $\mu_{max}(t)$ was calculated using a cardinal model (Rosso et al., 1995) (table 3.6). Nomenclature includes three suffixes: the suffix *opt* designates the value of temperature where the growth rate is maximum in optimum conditions for growth; the suffixes *min* and *max* designate, respectively, the temperature under which, or above which, growth is not observed (Table 3.6). Data regarding the growth of *Salmonella* in milk and the effect of temperature on the growth were obtained from experimental studies conducted in our laboratory and detailed information can be found in (chapter 5 of this thesis).

While the *Salmonella* population can increase in bulk tank, tanker, and during ripening of milk, the population declines during curd acidification in cheese vats and molds. This observation could be explained by stress caused to the cells by acidification, and/or competition with, and/or inhibition by lactic acid starters. To our knowledge, no reports were found in the published literature describing the fate of *Salmonella* during the manufacture and ripening of camembert cheese. Therefore, the reported numbers of decimal reductions due to acidification step (log apparent kill) were estimated from published literature that described the fate of *Salmonella* during the lactic fermentation of milk stored at temperatures close to the one used in the ripening of milk during camembert cheese making (Shen et al., 2007; Mufandaedza et al., 2006). Given the available data and due to the low number of repetition, the following distribution was used to model the number of log kills: *Triangular* (3, 4, 5). This triangular distribution has to be considered more than an expert opinion describing variability and uncertainty rather than a real quantification of the killing factor.

2.4.1. Growth of Salmonella during cheese ripening to consumption

The predicted growth/no growth interfaces of *Salmonella* from cheese ripening to the consumption were calculated with Microsoft Excel Solver**.** This period included ripening for 12-14 days at 14°C, packaging in wooden boxes and further aged or stored for 5-7 days at 9°C, at market for 7 days at 4°C and finally taking pessimistic scenario, and assuming time-temperature abuse during transport and at the consumer's refrigerator 20 days at 10°C.

The logistic polynomial regression model (Koutsoumanis et al., 2004) was applied to assess the probability of growth of *Salmonella* in cheese on the basis a_w, pH, and time-temperature from day three of cheese production to the extreme time of consumption. Data on a_w, pH are reported in Table 2.6. For modelling purpose, a_w was transformed to b_w, defined as the square root of 1- a_w, as in Gibson et al., (1994). The model is of the form shown in the following equation:

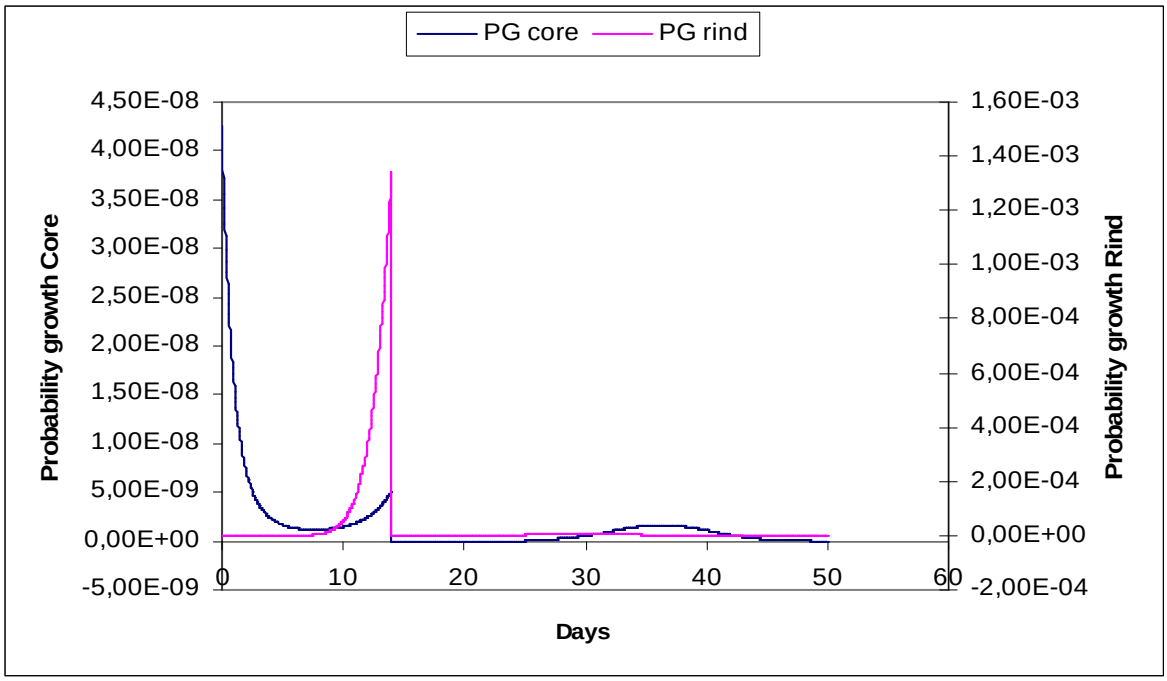
$$\text{Logit}(P) = a_0 + a_1 T + a_2 b_w + a_3\,\text{pH} + a_4\,\text{pH}.b_w + a_5\,T.\text{pH} + a_6\,T^2 + a_7 b_w^2 + a_8\,\text{pH}^2$$

Logit (P) is an abbreviation of ln[P/(1-P)], P is the probability of growth, T is temperature, and a_0- a_8 are the parameter estimates: -438.1, 5.465, 233.5, 128.0, -235.6, -0.236, -0.074, 1,606 and -5.186, respectively (Koutsoumanis et al., 2004).

The pH and a_w variations according to time from molding to the end of shelf life could be accurately modeled as

$$\text{pH} = a \,.\, t^3 + b \,.\, t^2 + c \,.\, t + d.$$

$$a_w = a \,.\, t^4 + b \,.\, t^3 + c \,.\, t^2 + d \,.\, t + e.$$



Figur 1.6 Probability of *Salmonella* growth in Camembert Rind and Core as predicated by the logistic regression model (in days from third day of chesse making until consumption)

As shown in Figure 1.6 the probability of growth within the core is almost lower than 5×10^{-8}. For the rind we observe a peak of probability of growth (10^{-3}) at day 15. Before and after day 15 remained less than 10^{-8}. Because of low probability of growth we could consider that there is absence of growth in Camembert during normal ripening.

Table 2.6 Parameters of the equations describing pH and a_w changes according to time (in hours from molding to the end of shelf life) in rind and core of the modeled Camembert cheese.

Parameters	Camembert			
	pH of core	pH of rind	a_w of core	a_w of rind
a	-1.15×10^{-9}	8.59×10^{-10}	1.51×10^{-14}	2.61×10^{-14}
b	3.04×10^{-6}	-3.08×10^{-6}	-5.08×10^{-11}	-9.61×10^{-11}
c	-5.20×10^{-4}	4.72×10^{-3}	5.48×10^{-8}	1.19×10^{-7}
d	4.58	4.25	-4.82×10^{-5}	6.93×10^{-5}
e			9.63×10^{-1}	9.60×10^{-1}

2.5. Number of Salmonella in cheese

About 2.4 l of milk are needed to make a cheese of 250 g. Milk assigned to cheese manufacture is stored in vats of 1000 l. We assumed that the number of *Salmonella* in one cheese vat (NS_{vat}) has a Poisson distribution: NS_{vat} = Poisson (V_{vat} * C_{FMR}*(10^{-DC}). This assumption is done because we consider that milk is well mixed and homogenised during the first steps of cheese making. In 2.4 l of milk needed to make one 250 g of cheese, Poisson distribution was also attributed to the number of cells transferred to 250 g of cheese with a mean equal to: NS_{cheese} = Poisson (NS_{vat} *2.4*0.9/1000). The factor 0.9 is added because we assumed that 90% of the *Salmonella* cells are transferred to the curd and 10% of them to the whey (Bemrah et al., 1998). The same distribution was used to the number of cells for a typical serving of 25 g with a mean equal to: $NS_{portion\ 25\ g}$ =Poisson (NS_{cheese} * 25/250)

2.6. Control programs of Salmonella at farms

An analysis was performed to determine the influence of the prevalence rate of the infected farms on the concentration of *Salmonella* in cheese. Two scenarios were studied according to the existence of control programs of *Salmonella* at the farm level. The first simulation is to use the observed prevalence of 2.68% and we assumed that no preventive measures are applied. In the second simulation we assumed that the application of preventive actions at the farm level will permit to reduce the observed prevalence by factor of 10.

Generally, the surveillance strategy applied in the AOC area (Camembert collection area) consists of two actions: at farms where milk from each bulk tank is analyzed one time a month. Sorting of farms is done based on two separate collection (1) milk from farms where no *Salmonella* was detected can be collected for cheese making; (2) milk from farms where a bulk tank was previously detected as positive is collected separately. However, the presence

or absence of *Salmonella* is not the sole criterion of milk conformity to the requirements of the processing plants. Other bacterial and somatic cell counts are used as well for selecting farms (mesophilic count, *Staphylococcus aureus, Listeria monocytogenes,* and *Escherichia coli*). *Salmonella* analyses are also done in samples of cheeses before release. In case of positive results, the lot is withdrawn.

Presence of *Salmonella* is checked using standardized techniques (Anonymous, 2002) together with a confirmation technique validated by the French Standardization Organization, AFNOR (Saint Denis-La Plaine, France), AccuProbe Rapid DNA Identification Test (Biomérieux). We incorporate in the model the end products sampling in assuming different levels of laboratory techniques limit of detection.

2.7. Dose-response model

A dose-response model gives the probability of illness according to the amount of ingested pathogenic microorganisms. Among *d*-ingested microorganisms, some might survive human barriers and later initiate infection and cause illness. Illness probability 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:

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

Where *n* is the number of consumed microorganisms and *r* a parameter with a value *r*~Beta(α, β), α and β were equal to 0.3126 and 1.9^6 , respectively (Haas et al., 1999). We consider here the principle of single hit model. The parameter *r* is the probability of one *Salmonella* cell to survive to the different host immune barriers and induce infection.

For each consumed 25 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.

2.7.1. Probability of illness

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 illness 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)

2.8. 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.

Table 3.6 Equations and parameters for growth modeling

Variable	Description	Unit	Distribution/model
X_0	*Salmonella* number	Cell	
X_{max}	Maximum number	cells	10^9
T	Temperature	°C	
T_{opt}	Optimum growth temperature	°C	38.4
T_{max}	Maximum growth temp.	°C	44.5
T_{min}	Minimum growth temp.	°C	3.00
γ (T)	Temp.fraction of optimal growth		$\frac{(T - T\,max).(T - T\,min)^2}{(Topt - T\,min).[(Topt - T\,min).(T - Topt) - (Topt - T\,max).(Topt + T\,min - 2.T)]}$
μ_{opt} (milk)	Optimal growth rate in milk	h^{-1}	1.36
T	Time	H	
μ_{max}	Maximal growth rate at time t	CFU/h^{-1}	$\mu_{max} = \mu_{opt} * \gamma\,(T)$
a, b, c, d	Parameters of pH (t)		See table
a, b, c, d, e	Parameters of a_w (t)		See table
pH (t)	pH over time		$pH = a.\,t^3 + b.\,t^2 + c.\,t + d$
a_w (t)	a_w over time		$a_w = a.\,t^4 + b.\,t^3 + c.\,t^2 + d\,.\,t + e$
IC	Number Increase on ln-basis	lnCFU/h	$\sum_0^t \ln X_{max} - \ln\left(1 + \frac{X_{max}}{X_0}\right) e^{-\mu_{max}\,t}$

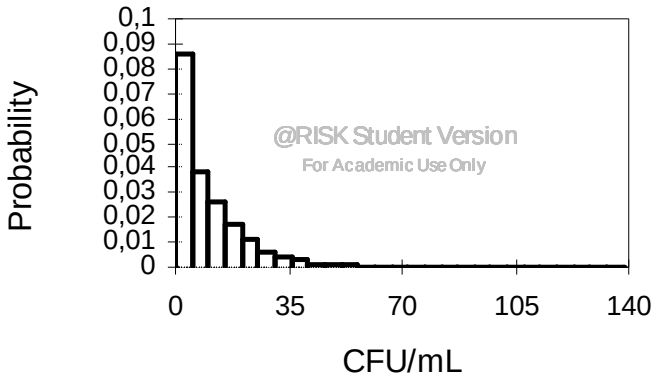
Table 4.6 Description and distribution of variables

Variable	Description	Unit	Distribution/model
N	Number of farms per collection per day	Herds tanks	Triangular(65,98,160)
P	Prevalence of *Salmonella* in bulk tank milk	-	2,6%
Nc	Number of contaminated farms	Herds	Binomial (N,p)
C_{BTM}	Concentration in bulk tank milk	CFU/ml^{-1}	Discrete Uniform (table)
C_i	Volume variability in bulk tanks		Normal (-0.158, 0.611)
C_{TT}	Concentration in the tanker truck before transport	CFU/ml^{-1}	$C_{TT} = \sum_{i=1}^{i=nc}(C_i \times C_{BTM} / N)$
Transport			
TMT	Temperature during transport	°C	Triangular (5,6,8)
DMC	Duration of milk collection	h	Triangular (4,5,6)
γ (T)	Effect of temperature on growth rate	-	See table
μ_{max}	Maximal growth rate at time t	lnCFU/h	$\mu_{max} = \mu_{opt} * \gamma$ (T)
IC_T	Concentration during transport	CFU/ml^{-1}	See table for equation IC
Storage			
TMS	Temperature during storage at the manufacture	°C	
DMS	Duration of milk storage	h	Triangular (6, 10, 12)
γ (T)	Effect of temperature on growth rate	-	See table
μ_{max}	Maximal growth rate at time t	lnCFU/h	$\mu_{max} = \mu_{opt} * \gamma$ (T)
IC_S	Concentration during storage	CFU/ml^{-1}	See table for equation IC
First step of milk ripening			
RT	Ripening temperature	°C	14
RD	Ripening duration	h	14
γ (T)	Effect of temperature on growth rate	-	See table
μ_{max}	Maximal growth rate at time t	lnCFU/h	$\mu_{max} = \mu_{opt} * \gamma$ (T)
IC_{FMR}	Concentration during first step of milk ripening	CFU/ml^{-1}	See table for equation IC
Second step of milk ripening			
RT	Ripening temperature	°C	33
RD	Ripening duration	h	0.5
γ (T)	Effect of temperature on growth rate	-	See table
μ_{max}	Maximal growth rate at time t	lnCFU/h	$\mu_{max} = \mu_{opt} * \gamma$ (T)
IC_{FMR}	Concentration during second step of milk ripening before cheese making	CFU/ml^{-1}	See table for equation IC
Effect of acidification			
DC	Decimal logarithm of the concentration decrease caused by pH decrease and increase of acid lactic concentration		Triangular(3,4,5) Or Trinagular (1.5, 2, 2.5)
V	Volume of milk needed for one cheese	l	2.4
M	Mass of one cheese	g	250
S	Mass of serving	g	25
V_{vat}	Vat volume	l	1000
NS_{vat}	Number of *Salmonella* in one vat	CFU/ml^{-1}	Poisson ($V_{vat} * C_{FMR} * (10^{-DC})$
NS_{cheese}	Number of *Salmonella* in 2.4 l (the amount needed to make a cheese of 250g)	CFU/250 g	Poisson [NS_{vat} (2.4*.9/1000)]
$NS_{25\ g\ /cheese/\ serving}$	Number of *Salmonella* per 25 g cheese serving	CFU/25 g portion	Poisson (Ns_{cheese}*25/250)

3. Results

3.1. Milk contamination

A simulated distribution of the concentration of *Salmonella* in milk before cheese processing (after transport, during storage in manufacturer) was obtained (Fig. 2.6). The distribution of the concentration of *Salmonella* in milk before cheese processing ranged from 0 to 138 CFU/ml with a mean of 10.0 CFU/ml. The probability of milk not being contaminated was estimated to be 93.7%. The expected percentages of concentration exceeding 2, 6, and 12 CFU/ml were respectively, 75.8%, 52.0% and less than 31.0% (no value over 140 CFU/ml were found after 100 000 iterations). The model predicted that 82.75% of *Salmonella* concentration before cheese processing would be less than 10 CFU/ml. Percentiles (CFU/ml) of the distribution of *Salmonella* in milk before cheese making are presented in table 5.6



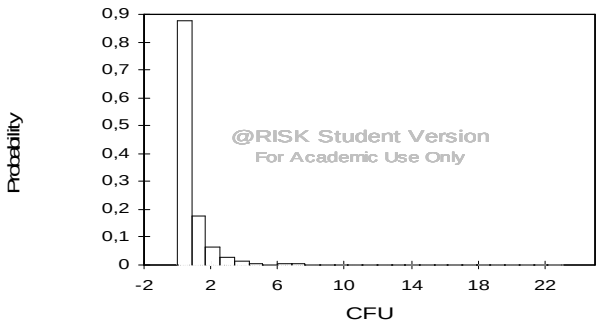
Figur 2.6 Simulated frequency distribution for *Salmonella* concentration before cheese processing (93.7% of milk was expected not to be contaminated): 100 000 iteration

Table 5.6 Percentiles of the distribution of *Salmonella* (CFU/ml) in milk before cheese processing

	Percrntiles					
	25th	Median	75th	90th	95th	99th
after transport	0.16	0.50	1.10	1.87	2.43	3.74
After storage	2.10	6.4	14.4	24.1	31.4	48.7

3.2. Cheese contamination

The simulated concentration of *Salmonella* in 250 g of cheese ranged from 0 to 171 *Salmonella* cells with a median of 2 cells. About 67.0% of cheese was expected not to be contaminated. The expected percentage of cheese with contamination greater than 5, 10 and 100 *Salmonella* cells were 23.8%, 12.1% and 0.03%, respectively. For a typical cheese serving of 25 g, the number of bacteria ranged from 0 to 26 *Salmonella* cells with a mean of 0.47 cells (Fig. 3.6). The estimated probability of consuming a contaminated cheese serving was 25%. However, the estimated probabilities of consuming a dose of *Salmonella* greater than 1, 10, and 15 cells were 10%, 0.08% and 0.01%, respectively (no value over 25 were found after 100 000 iterations).



Figur 3.6 Simulated frequency distribution for *Salmonella* concentration in 25 g serving (75% cheese serving was expected not to be contaminated): 100 000 iteration

Percentiles of the distribution of *Salmonella* in 250g and 25g serving of cheese at time of consumption are presented in Table 6.6. The 99th percentile of *Salmonella* cell numbers in servings of 25 g of cheese was 5 cells at the time of consumpion

Table 6.6 Percentiles of the distribution of *Salmonella* in cheese

	Percrntiles					
	25th	Median	75th	90th	95th	99th
250g cheese	Absence	2	5	12	20	42
25 g cheese	Absence	Absence	1	2	2	5

The results in Table 7.6 show a decrease in concentration of *Salmonella* in milk and cheese assuming that the prevalence of *Salmonella* in milk would be reduced by a factor of 10 as a result of applying control measures at the farm level.

Table 7.6 Percentiles of the distribution of *Salmonella* in milk and cheese assuming reduction in prevalence by a factor of 10 due to the application of preventive action.

	Percrntiles					
	25th	Median	75th	90th	95th	99th
250 g cheese	Absence	Absence	Absence	1	2	10
25 g cheese	Absence	Absence	Absence	Absence	Absence	1

Assuming that each lot is analysed with 25 g composite sample from 5 different cheeses we calculate the expected number of rejected lots per year (Table 8.6)

Table 8.6 Number of expected rejected cheese lots according to the limit of detection (xCFU/25g) and farm milk contamination prevalence

Limit of detection CFU/25 g	Expected number of rejected lot per year for different farm milk prevalence			
	2.68%	2%	1%	0.30%
1	36.3	28.0	11.3	3.4
2	16.4	12.0	4.7	1.4
3	8.7	6.1	2.5	0.8
4	4.9	3.5	1.2	0.3
5	2.7	2.1	0.7	0.2
8	1.7	1.2	0.3	0.1
9	1.0	0.7	0.3	0.1
10	0.6	0.4	0.2	0.0

3.3. Risk of salmonellosis

The risk of salmonellosis per 25 g serving ranged from 0 to 1.2×10^{-7} with a median of 7.4×10^{-8}.

For 100 million servings of 25 g, the number of cases predicated by the model is in average 7.4. The number of cases has a probability of 99.9% to be less than 17. One hundred million servings of 25 g correspond more or less to one year production. If we assume a seasonal pattern during winter months, the expected number of cases in high risk season will be probably less than 17. And if it is divided by the number of lots, the risk to observe an outbreak (more than 2 cases per lot) is very low.

However, when the variable DC shown in table 4 (three distribution parameters of the decimal logarithm of the concentration decrease caused by pH decrease and increase of acid lactic concentration, table 4) is reduced by 2 factor, the number of expected cases per 100

million servings became in average equal to 560 and 63, respectively for farm contamination prevalence of 2.68% and 0.268%,

4. Discussion

Despite the amount of raw milk cheese consumed daily in France, outbreaks of infection remain comparatively rare. As far as we could know, no outbreak of salmonellosis associated with the consumption of camembert cheese made from raw milk was reported. However, *Salmonella* outbreaks due to the consumption of other soft cheeses made from raw milk were reported (Maguire et al., 1992; Haeghebaert et al., 2003).

Human cases of salmonellosis occur sporadically or as part of outbreaks. The role of raw milk products in sporadic cases is not well assessed. Under-notification of sporadic salmonellosis cases and non systematic case investigation complicate the demonstration and quantification of raw milk productions role in sporadic cases.

The strict hazard analysis critical control point (HACPP) based procedures are developed and implemented for unpasteurised dairy products and monitoring of milk and cheese made from raw milk *Salmonella* contamination could explain this good apparent epidemiological situation in regard to salmonellosis cases linked to the consumption of camembert type cheeses. Producers should also report positive results of end production internal sampling to public health authorities. Consumers-particularly those susceptible to infectious diseases (for example, infants, elderly people and immunocpmpromised patients) should also be warned that a nil risk can not be warranted from raw milk products.

The use of new and more sensitive laboratories methods such as real-time PCR indicated that the prevalence of farm milk contamination is underestimated when using

classical and routine methods for *Salmonella* monitoring (Van Kessel el al. 2003; Karns et al., 2005). The impact of this underestimation is described in this current risk assessment.

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 Camembert cheese made from raw milk. The model incorporated also data in published literature.

For the enumeration, we used the MPN-real-time PCR assay to enumerate *Salmonella* in bulk tank milk samples. The assay enabled the enumeration of *Salmonella* in bulk-tank milk samples that ranged from 3.7 to 79.2 ml of *Salmonella* per mL. We believe that these estimates overestimated the original concentration of the organism in milk. 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 milk samples using the MPN-real-time PCR assay (chapter 3 of this thesis).

For the frequency of contamination, we also observed a higher prevalence rate of *Salmonella* in bulk tank milk in western France 2.68% than the previously reported prevalence of 0.3% of industry data surveys in 2000-2002 in the same region (unpublished data). This large variation in the prevalence in raw milk, we attributed mainly to the detection techniques used. In our study we used the real-time PCR assay. Others have also reported more positive results when analysing milk by real-time PCR than the traditional cultural methods (Van Kessel et al., 2003; Karns et al., 2005).

As regards assumptions, they belong to several hypotheses. The first one concerns the number of decimal reduction due to lactic acid produced fermentation. Because of the lack of data on the fate of *Salmonella* during the manufacturing and ripening of Camembert cheese, we chosed to estimate this reduction of *Salmonella* cells during the first days of cheese making based on literature data describing the fate of *Salmonella* during the lactic fermentation in milk stored at different temperatures (mainly close to the ripening temperature) (Shen et al., 2007; Mufandaedza et al., 2006). The triangular distribution was used taking into account the variability and uncertainty. The importance of this factor on the risk assessment output was considered using different parameters of the triangular distribution. The number of expected cases of salmonellosis is very sensitive to the reduction factor due to decrease of pH and increase of acid lactic concentration during cheese ripening. To increase the risk estimate accuracy it will be necessary to get new data on *Salmonella* survival during the first stage of cheese processing.

The evolution of the *Salmonella* population from the third day of cheese making to the consumption was not included in the model. Because of the low probability of growth obtained by the logistic regression model, we consider that the probability of growth of *Salmonella* in Camembert cheese during these steps is negligible.

The third assumption, considers similar consumption in the susceptible and general population. Assuming an equal number of servings for everybody is likely to dilute the risk among the general population, and to overemphasize it for the population at risk.

The used dose-response model published by Haas et al. (1999) 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.

The model simulation did not take into account the possible cross-contamination occurring during cheese ripening, 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 Camembert chesses are low risk foods as far as salmonellosis is concerned.

The expected number of rejected lots simulated with the current model is highly dependent on the practical limit of detection of routine microbial tests and on the farm milk concentration prevalence. In the absence of public available data on end products inspection, it is difficult to compare the model results with the observed data. In order to validate our model output, the inputs from dairy industry and veterinary inspection are crucial.

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 Camembert cheese made from raw milk which tended to show that the risk of salmonellosis could be considered relatively low and is manageable at the farm and processing levels. The importance analysis showed clearly the need of further studies on the *Salmonella* survival during the first stages of cheese making e.g. effect of pH and lactic acid concentration.

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Chapter 7: General Discussion and Conclusion

Detection and quantification of *Salmonella* in milk

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 milk 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 milk 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 milk 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 bulk tank milk samples; a 18-h pre-enrichment step in BPW was carried out prior to the PCR. The assay detected 8 positive-*Salmonella* samples (2.68%). These findings clearly suggested that the prevalence of *Salmonella* in dairy farms in western France might be higher than previously reported (0.3%) in unpublished data (2000-2002). This large variation might be due to the use of different detection techniques. It has been previously reported that the real-time PCR assay is more sensitive than traditional cultural techniques (Rodrigez-Làzaro et al., 2003) and more positive-*Salmonella* in milk samples have been reported using the real-time PCR compared to cultural methods (Van Kessel et al., 2003; Karns et al., 2005).

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 milk samples seemed to be more efficient than naturally contaminated milk. 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 food 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 milk 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 milk samples as indicated by the shape of the fluorescent amplification curves. However, the method resulted in PCR inhibition in few pre-enriched bulk tank milk samples. Therefore, the DNAs of these inhibited samples had to be diluted up to 1:10 and PCR inhibitors were bypassed in all cases. Other literature reported similar results with other extraction preparation protocols and commercial kits (Perelle et al., 2004; Liming and Bhagwat, 2004).

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 including milk (Van Kessel et al., 2003; Karns et al., 2005). Unfortunately, in the present work, *Salmonella* were not isolated by cultural procedures from any of the eight *Salmonella*-positive bulk tank milk samples. This might be due to the low numbers of real-time positive samples with the relatively few numbers of *Salmonella* in the original milk samples. Bansel et al. (2006) reported some important factors that might influence the recovery.

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 milk samples with *Salmonella*. When analysing the artificially contaminated milk samples, the assay tended to give higher estimates than the estimated levels of contamination (CFU/mL) inoculated into milk 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 milk samples, the assay had to be evaluated with regard to the quantification of *Salmonella* cells in bulk tank milk samples naturally contaminated with *Salmonella*. The MPN-real-time PCR described for bulk tank milk samples proved to be rapid and sensitive and *Salmonella* could be enumerated even at low levels. Because our previously enumeration results in inoculated milk indicated higher estimates than the inoculum levels estimated by direct plating, the results of enumeration in naturally contaminated milk may also overestimate the original concentration of the organisms present in the milk 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 milk 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 milk and other food matrix.

Predictive modelling of *Salmonella* growth in milk

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 soft cheese. Therefore, in the present work, a predictive model for growth of *Salmonella* in milk was developed that covered a broader range of temperature. In addition the developed model was designed to provide the input settings (optimum growth rate, cardinal temperatures,"T_{min}, T_{opt} and T_{max}) for growth events in the development of risk assessment model for *Salmonella* in cheese made from raw milk. The predictive model was developed by following the following four steps: (1); kinetic data collection; (2) database creation; (3) primary modelling; and (4) secondary modelling.

One of the steps in evaluating a predictive model is to compare its predications with other data, generated by other laboratories in different media/food substrates. Therefore, we choose the growth model of PMP for the comparison. Our findings demonstrated that the PMP growth model for *Salmonella* in broth culture was consistent with the results obtained by

our model at the following temperatures (10, 15, 25, 30°C). 30°C is the maximum temperature found in PMP for the growth *Salmonella* in broth culture.

In the present work, the effect of temperature on the maximum growth rate was described by the cardinal temperature model. We did no consider the comparison of this model with other secondary models since the performance of the cardinal model used in this study provided a best fit with a high *pseudo-*R^2 (0.97-0.98). However, the performance of secondary models for predicting specific growth rates as a function of temperature has previously been compared. Zwietering et al., (1991) compared the Ratkowsky 2 and 3 models for growth rate and found that Ratkowsky 3 model had slightly better goodness-of-fit. Rosso et al. (1993) compared the cardinal temperature model with the Ratkowsky 2 and 3 models and found similar but slightly better goodness-of-fit for the cardinal temperature model. Likewise, Oscar, (2002) reported that the cardinal temperature model, although similar in goodness-of-fit to the Ratkowsky 2 and 3 models, was easier to fit and yielded more accurate parameter estimates than Ratkowsky 2 and 3 models.

The predictive model developed in the current study provided specific data which was used to build our risk assessment model. However, some important factors were not considered in the development of this model. These included the effect of other environmental factors such as pH and a_w on the growth, fluctuating temperatures and competing microorganisms. Further studies are needed to expand the current model in order to include the aforementioned factors. We also recommend the development of studies descrbing the effect of dynamic temperatures of the growth of *Salmonella*, for example, during the raw milk cheese processing.

In conclusion, our study revealed the growth characteristics of *Salmonella* in milk under constant temperature conditions. The high *Pseudo-*R^2 values for the primary and the secondary model indicated that the both models fit the data well.

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 Camembert 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 Camembert cheese from raw milk was modelled from milking 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 prevalence against low prevalence due to preventive actions taken at the farm level.

In the current study, predictive model for growth of *Salmonella* from cheese ripening 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 Camembert during these important steps of risk model"* This low probability assessed by growth/no growth logistic regression model which included a number of important consideration or variables such as a_w, pH and time-temperature abuse. 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*, a, 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 derived from predictive microbiology, by example, come from experiments in laboratory and are not the reflection of the real environment of growth or destruction of the pathogen in food. The same problem is posed for the dose-response relation for which data are not available or too approximate. The present risk assessment model makes it possible to define precise objectives and priorities for future studies. Studies on the survival of *Salmonella* during the first stages of cheese making taking into account the effect of pH and acid lactic concentration are needed.

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 Camembert cheeses are low risk foods as far as salmonellosis is concerned.

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"Combination of chapter 3 and 4"

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 Milk Samples

A. Fares, A. Dayhum, M. Sanaa, O. Cerf, Y. Millemann

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 milk samples. First, for the detection of *Salmonella* in artificially and naturally contaminated milk samples, SYBR Green I real-time PCR assay was used, and quantification of *Salmonella* was achieved by combining this assay with most-probable-number (MPN) method (MPN-real-time PCR). A *Salmonella enterica* serotype Typhimurium DT104 strain was inoculated into milk samples at different levels of contamination. Data indicated that both detection and quantification protocols were able to detect and enumerate as few as 1 CFU/mL of milk after 8-h of a single non-selective pre-enrichment step in buffered peptone water. All MPN estimates corresponded well to inoculum levels. The two protocols were then applied to naturally contaminated bulk tank milk samples. Eight (2.6%) of 299 bulk tank milk samples were found positive, with estimated MPN ranging from 3.7 to 79.2 *Salmonella*/mL of milk sample. This study demonstrates 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 milk samples.

Key words: *Salmonella*, MPN-real-time PCR, quantification, milk

INTRODUCTION

Salmonella continues to be a major food-borne pathogen for animals and humans and in many countries it is the leading cause of food-borne infections and outbreaks (Tirado and Schmidt, 2001, Vaillant et al., 2004). Raw and pasteurized milk as well as different types of cheese have been associated with severe food poisoning outbreaks caused by *Salmonella enterica* serotypes in Europe and the United States (Maguire el al., 1992; Villar et al., 1999; De Buyser et al., 2000; Haeghebaert et al., 2003). Therefore, for food safety, rapid, sensitive, and specific detection and quantification of food-borne pathogens in food products, including dairy products are needed. Conventional cultural methods for the detection of *Salmonella* in foods are time consuming and usually require 4-6 days to presumptively identify *Salmonella* in a test sample and to confirm the identity of the isolate. For this reason, methods based on polymerase chain reaction (PCR) have been increasingly used for the detection of *Salmonella* cells in various foods including dairy products (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 milk 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 milk 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 enterica* serotype Typhimurium DT104 in experimentally contaminated milk. The second objective was to apply this developed assay to enumerate *Salmonella* in naturally contaminated bulk tank milk samples obtained from dairy farms located in western France.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacterial strains used in experimental studies are listed in Table 1. They 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), Maisons-Alfort, France. They were used to determine the specificity and the sensitivity of the LightCycler real-time PCR assay. Stock cultures were preserved by freezing at -80°C in vials containing brain-heart infusion (BHI, Difco) broth supplemented with 20% (v/v) glycerol (Difco). Fresh bacterial cultures for use in experiments were produced by inoculating frozen stock cultures into BHI broth and incubating them overnight at 37°C. For enumeration of *Salmonella* in sterilized milk, *Salmonella enterica* serotype Typhimurium DT104 was used to inoculate milk.

Specificity of the Real-Time PCR Assay

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 other bacteria (Table 1) were transferred into BHI broth and incubated overnight at 37°C. These overnight bacterial cultures were subsequently subjected to DNA extraction with boiling method, and real-time PCR assay as described below.

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 serotypes of *Salmonella enterica* (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.

Milk Samples

For the development of detection and quantification protocols, ultra-high temperature (UHT) sterilized whole milk (3.6 g fat) was purchased from a local supermarket and artificially inoculated with *S. enterica* serotype Typhimurium DT104 at different levels of contamination.

The application of developed protocols was performed on naturally contaminated bulk tank milk samples, a total of 299 milk samples were aseptically collected from bulk tank dairy farms located in western France. Collection of milk samples was done during routine visits (two to three times a month) to farms by the departmental veterinary laboratories (LVDs, Laboratoires vétérinaires départementaux) for epidemiological monitoring of bacterial strains, including *Salmonella*. The collection of milk from these dairy farms is intended for milk cheese making. All samples collected for our study were stored at 4°C and then transported to our laboratory where analyzed within 24 h of collection.

Real-Time PCR Detection Protocol of milk samples

Development of the Detection Assay on Artificially Contaminated Milk. 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 milk, the estimated cell concentration of the inoculum was determined for a second time. 25 mL of milk 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. The experiment was also performed on bulk tank milk samples collected from a dairy farm and artificially contaminated after being confirmed *Salmonella*-negative by both culture methods and real-time PCR.

Application of the Detection Assay on Naturally Contaminated Bulk Tank Milk. For bulk tank milk samples, 5 mL of each milk samples was 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 raw milk samples were stored at 4°C to be used for quantification protocol in case of *Salmonella*-positive results.

The MPN-Real-Time PCR Quantification Protocol of milk samples

Development of the method on Artificially Contaminated Milk. MPN assays (ten-tube method) for *S. enterica* serotype Typhimurium DT104 were performed according to the procedures described in the Bacteriological Analytical Manual of the U.S Food and Drug Administration (BAM/FDA) (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 BAM (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 mean divided by the standard deviation. If the CV values were less than 20%, the repeatability was considered to be acceptable.

Application of the method on Naturally Contaminated Bulk Tank Milk. Milk 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 milk sample 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 milk 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).

DNA Extraction Procedures

DNA was extracted from pure cultured strains and from pre-enriched cultures of artificially and naturally contaminated milk 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.

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 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). 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*)/ d*T* versus *T*).

Isolation of Positive Colonies from Raw Milk Samples

For isolation of presumptive *Salmonella* colonies from PCR-positive milk 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). 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 XLT-4 agar plates 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.

RESULTS

Optimization of Real-Time PCR Assay

The first step in developing a successful real-time PCR assay is to establish optimal conditions of the real-time PCR parameters such as $MgCl_2$ and primer concentrations. Therefore, preliminary tests were performed using DNA extracted from appropriate 10-fold serial dilutions of *Salmonella* Hadar. By following the optimization procedures recommended

by the manufacturer (Roche Molecular Biochemicals, Technical Note No. LC 9/2000), we achieved the optimal conditions described in SYBR Green real-time PCR assay section of this paper.

Specificity of Real-Time PCR Primers

PCR primers ST11 and ST15 were found to result in specific amplification products with all the serotypes of *Salmonella* tested. As expected, no amplification was observed in any of 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. (Table 1). No amplification was observed in the negative control, eliminating the possibility of false-positive results due to potential cross-contamination. Table 1 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_ms 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 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*).

Detection Limits in Pure Cultures

The study was performed on the three *Salmonella* strains listed in Table 1. 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/mL of pure cultures from each of the three strains. Experiments were carried out three times and good reproducibility was observed (data not shown).

Detection of **Salmonella** *from Artificially Contaminated Milk Samples*

Milk 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 milk samples even at a low level of contamination after enrichment. When spiked milk 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 was 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 sterilized milk samples were consistent with DNA purified from serial dilutions of broth cultures of the three *Salmonella* strains and with inoculated bulk tank milk samples. These results suggested that at least 10^3 CFU/mL in enrichment broth (Table 2) must be present to give a positive result by the real-time PCR assay.

Detection of **Salmonella** *from Naturally Contaminated Milk Samples*

After 18 h of enrichment, eight (2.6%) out of the 299 bulk tank milk samples that were collected and analyzed for the presence of *Salmonella* by real-time PCR using SYBR Green I were positive for *Salmonella*.

Among the 299 milk samples tested, 29 (9.6%) showed inhibitory reaction when tested by LightCycler-real-time PCR. Therefore, DNAs of these samples had to be diluted 1:10 in sterile water to bypass the inhibitory effect. This treatment of the inhibitory samples was enough to obtain a successful amplification but the samples gave negative amplification results.

The real-time PCR positive samples were further analyzed with the traditional cultural methods while the negative ones were discarded. None of the eight real-time PCR positive samples did yield positive cultures.

Enumeration of Salmonella in Artificial and naturally Contaminated Milk Samples

For UHT-sterilized whole milk 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 3). With the developed MPN-real-time PCR assay it was possible to enumerate approximately 1 CFU *Salmonella* per ml of milk 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 milk samples inoculated with 100 and 10-20 cells per mL of milk (Figure 2 A, B), while in the case of the samples inoculated with low levels 1-5 cells per mL of milk, weak fluorescence peaks were observed (Figure 2 C). The MPN-real-time PCR assay demonstrated 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 (Table 4). Arguably, this was due to the low level of inoculum.

For naturally contaminated bulk tank milk samples, the MPN real-time PCR assay enabled the enumeration of *Salmonella* per mL in bulk tank milk positive samples that ranged from 3.7 to 79.2 (Table 5).

Confirmation 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 bulk tank milk samples in which the mean real-time PCR T_m of eight positive 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_ms have been reported from other studies (Eyigor et al. 2002; Medici et al., 2003). 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.

DISCUSSION

Dairy cattle and their environment harbor pathogens that pose a potential human health hazard. Unpasteurized milk and dairy products made from raw milk 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., 1992). 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 including milk. 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 milk. The method has been successfully used for detection and quantification of *Salmonella* in artificially contaminated milk. Therefore, we evaluated the utility of this developed method to enumerate *Salmonella* spp. in naturally contaminated bulk tank milk samples of selected dairy farms from western France, within the aim to use it afterwards for the development of quantitative risk assessment of food-borne contamination of milk products. This is, to our knowledge, the first report on quantification of *Salmonella* in artificially and naturally contaminated milk 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 little optimisation steps of PCR conditions, the simple and less expensive option of SYBR Green I can be used as an effective alternative. Recently, a number of SYBR Green I real-time PCR assays for detection of *Salmonella* from different types of samples have been reported (Jothikumar et al., 2003; De Medici et al., 2003; Bhagwat., 2004; Mercanoğlu and Griffiths, 2005).

In our study, *Salmonella* could not be detected in inoculated milk samples when DNA was extracted directly and without enrichment (Table 2). 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 including milk. 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 milk samples, the method resulted in PCR inhibition in few naturally contaminated milk samples. Therefore, DNAs of these inhibited pre-enriched milk samples had to be diluted 1:10 and PCR inhibitors were bypassed in all cases. Other literature reported similar results with other extraction preparation protocols and commercial kits (Meng et al., 1996; Lample et al., 2000; Liming and Bhagwat, 2004; Perelle et al., 2004).

In general, as a simple, but widely used method, boiling method provides a fast and efficiency way of extracting DNA that can be used in PCR assays in less well equipped laboratories. However, extraction procedures have to be improved to isolate DNA from naturally contaminated milk samples.

The detection of low numbers of cells is particularly important for *Salmonella* spp., since epidemiological evidence suggests that the outbreaks can occur with concentration in the range of 10-100 cells (Bhagwat, 2004). In the present study, experiments carried out on artificially contaminated milk samples showed that real-time PCR could detect 1 CFU/mL of *Salmonella* contaminated milk samples after 8 h of incubation in the non-selective pre-enrichment medium (Table 3). 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).

The potential of MPN-real-time PCR method for the quantification of *Salmonella* spp. from artificially contaminated milk 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 (Table 3). 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, this difference was probably linked to the fact that DNA from dead and stressed cells, which were not able to form colonies, is also extracted. 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 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 according 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 299 bulk tank milk samples taken from dairy herds located in the West of France, it indicated that 2.6% (8 of 299) 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 (Desmasures et al., 1997), which showed the prevalence of *Salmonella* in bulk tank milk to be 2.9%. However, previously reported surveys of bulk tank milk in Europe, United States and Canada have shown large variations in the prevalence of *Salmonella* in raw milk ranging from 0.17% to 12.6% (Rohrbach et al., 1992; Steel et al., 1997; Hassan et al., 2000; Karns et al., 2005). The large variations in levels of bulk tank *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* do occur in bulk tank milk and may pose a health hazard if raw milk or raw milk products are consumed.

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. The real-time PCR assay of bulk tank milk samples conducted in this study suggested that more milk samples contained detectable *Salmonella* than previously reported in unpublished industry data in 2000-2002 from the same region; in which culture methods indicated only 0.3% contamination level. In our study, the detection of *Salmonella* in 2.6% of the samples tested indicates that the degree of prevalence of the pathogen in raw milk in Western France is higher than previously believed. Others have also reported more positive results when analysing enrichments of different food commodities including milk by real-time PCR than by the traditional cultural methods. For example, Van Kessel et al. (2003) reported the use of a real-time PCR for the detection of *Salmonella* in raw milk samples. The method yielded 16.5% more positive samples than the culture techniques. Karns et al. (2005) detected *Salmonella* in 11.8% of the samples using the real-time PCR assay, whereas conventional culture methods detected the pathogen in only 2.6% of the same samples (Van Kessel et al., 2004). Hein et al. (2005) tested bovine and caprine naturally contaminated raw milk samples for the presence of *Staphylococcus aureus*, the real-time PCR method yielded 19.3% more positive samples than plate count method. Therefore, it appears that the real-time PCR method is more sensitive and faster than traditional culture techniques for the detection of *Salmonella* in bulk tank milk.

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* were not isolated by cultural procedures from the eight real-time PCR *Salmonella*-positive milk 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 original milk samples was very low (Table 5). Conventional cultural procedures will not always detect small numbers of *Salmonella* cells in certain food 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.

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 raw milk proved to be rapid and highly sensitive and small numbers of *Salmonella* could be found in bulk tank milk samples. This assay yields significant labor and time savings since the quantification of *Salmonella* spp. can completed within 12 h which included a 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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Figure 1. 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* no[1] (◊);*Citrobacter freundii* no[2] (♦); and water(negative control (x).

Figure 2. MPN-real-time-PCR analysis of milk inoculated with *Salmonella enterica* serotype Typhimurium DT104. DNA extracted from milk samples after 8h non-selective enrichment in BPW: Inoculum level of 100 CFU/mL (**A**); Inoculum level of 10-20 CFU/mL (**B**); and Inoculum level of 1-5 CFU/mL (**C**). Some positive and negative peaks were omitted from these graphs for clarity.

Table 1. 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/Cordon-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.

Table 2. Real-time PCR detection and bacterial counts (CFU/mL) of *Salmonella enterica* serotype Typhimurium DT104 in artificially contaminated bulk tank milk samples after each period of pre-enrichment in BPW

Inoculation level (CFU/mL)	Incubation time (h)							
	0		6		8		16	
	PCR[1]	Bacterial count	PCR	Bacterial count	PCR	Bacterial count	PCR	Bacterial count
1-5	-	6.6	-	$4\ (\pm 4.3^{c})\text{x}10^{2}$	+	$2.9\ (\pm 1.3)\text{x}10^{4}$	+	NC[2]
10-20	-	20	+	$9.4\ (\pm\ 3.4)\text{x}10^{3}$	+	$1.4\ (\pm .38)\text{x}10^{5}$	+	NC
100-200	-	280	+	$1.8\ (\pm .43)\text{x}10^{5}$	+	$9\ (\pm .87)\text{x}10^{6}$	+	NC

[1]PCR = (+) presence of amplification product, (-) absence of amplification product

[2]NC = not-countable

[c] Standard deviation

Table 3.3 Enumeration of *Salmonella enterica* serotype Typhimurium DT104 in artificially contaminated milk samples using MPN-real-time PCR method after 8 h pre-enrichment in BPW (contamination level CFU/mL)

Sample	Contaminated level Theoretical	Estimated level by Plating	MPN estimates	MPN confidence limits (low/high)
1	1-5	2	5.9	2.4 - 14.5
2	1-5	3	5.9	2.4 - 4.5
3	1-5	1	3.1	1.0 - 9.8
4	10-20	12	14.7	7.2 - 29.9
5	10-20	9	12.7	6.0 - 26.7
6	10-20	12	19.2	9.5 - 39.1
7	100	128	239.7	110.1- 523.2
8	100	103	135.8	68.0 - 71.5
9	100	85	101.2	46.8 – 218

Table 4. Repeatability of MPN-real-time PCR quantification assays for artificially contaminated milk samples

Contamination level (CFU/mL)	Runs	MPN estimate (MPN/mL)	MPN confidence limits (low/high)	CV%
1-5	1	0.94	0.1-6.7	96
	2	3 .15	1-9.8	
	3	4.47	1.7-12	
10-20	1	23.12	10.7-49.8	3.5
	2	19.29	9.5-39.2	
	3	23.12	10.7-49.8	
100	1	101.22	46.9-218.1	3.1
	2	129.55	68.3-246.1	
	3	101.22	46.9-218.5	

Table 5. Quantification estimates of *Salmonella* in bulk tank milk samples obtained with MPN-real-time PCR assay after 8 h pre-enrichment in BPW

Sample no.	MPN estimate MPN/mL	LogMPN/mL	MPN confidence limits low/high
1	3.7	0.56	0.92 - 14.7
2	4.5	0.65	1.1 - 18.1
3	69.9	1.84	23.2 - 211.5
4	7.8	0.89	2.4 - 24.8
5	17.0	1.2	7.9 - 36.5
6	6.1	0.78	1.9 – 19.1
7	16.9	1.2	6.5 - 44.1
8	79.2	1.9	25.4 - 274.7

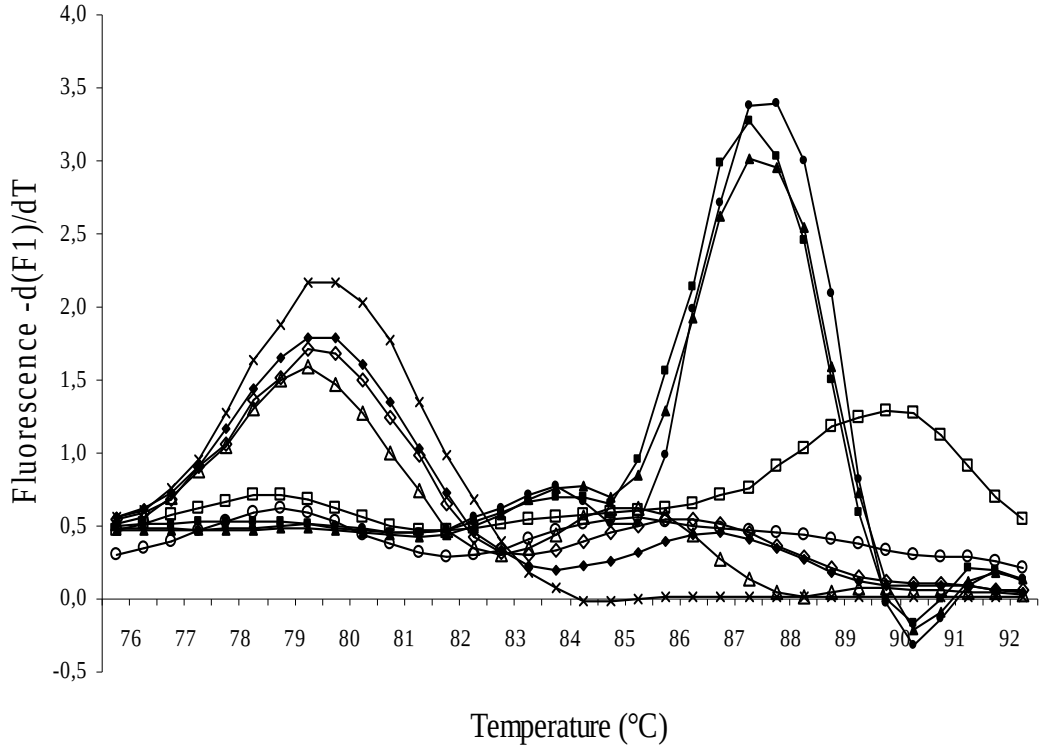
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
Fluorescence -d(F1)/dT
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
Temperature (°C)

Figure 1.

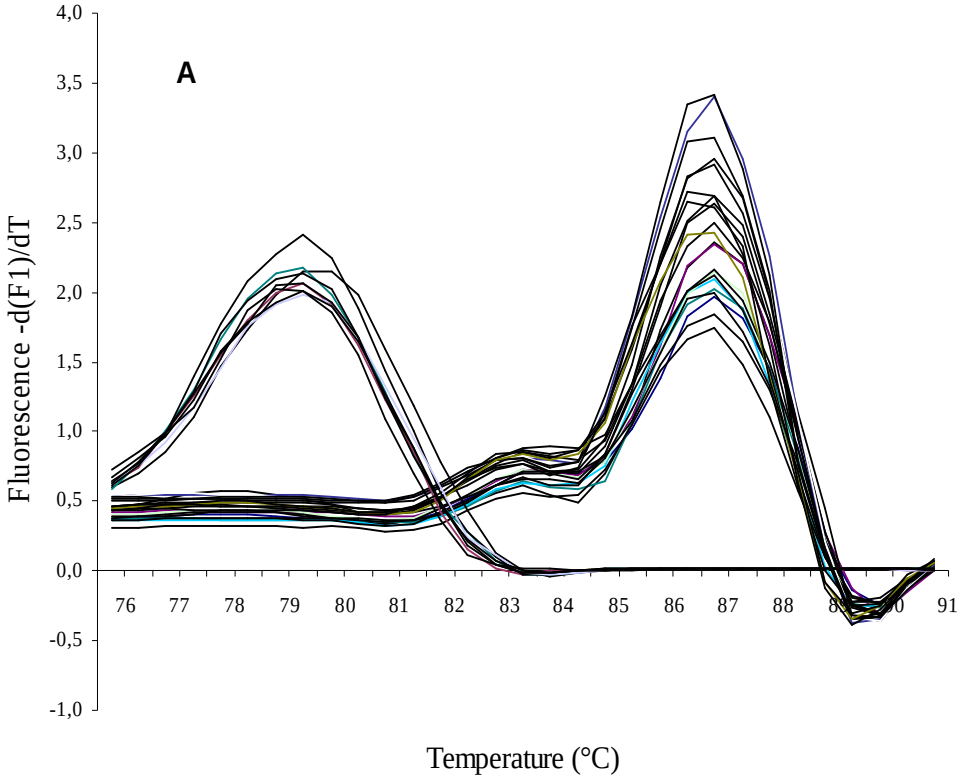
A
Fluorescence -d(F1)/dT
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
76
77
78
79
80
81
82
83
84
85
86
87
88
91
Temperature (°C)

Figure 2. (A)

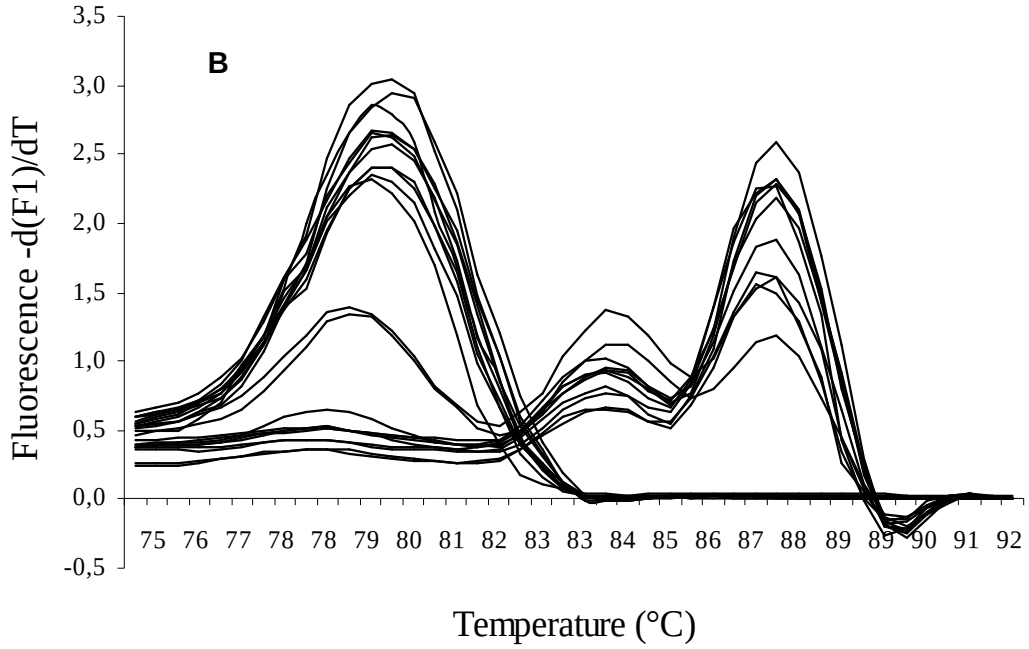
B
Fluorescence -d(F1)/dT
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
75 76 77 78 78 79 80 81 82 83 83 84 85 86 87 88 89 89 90 91 92
Temperature (°C)

Figure 2. (B)

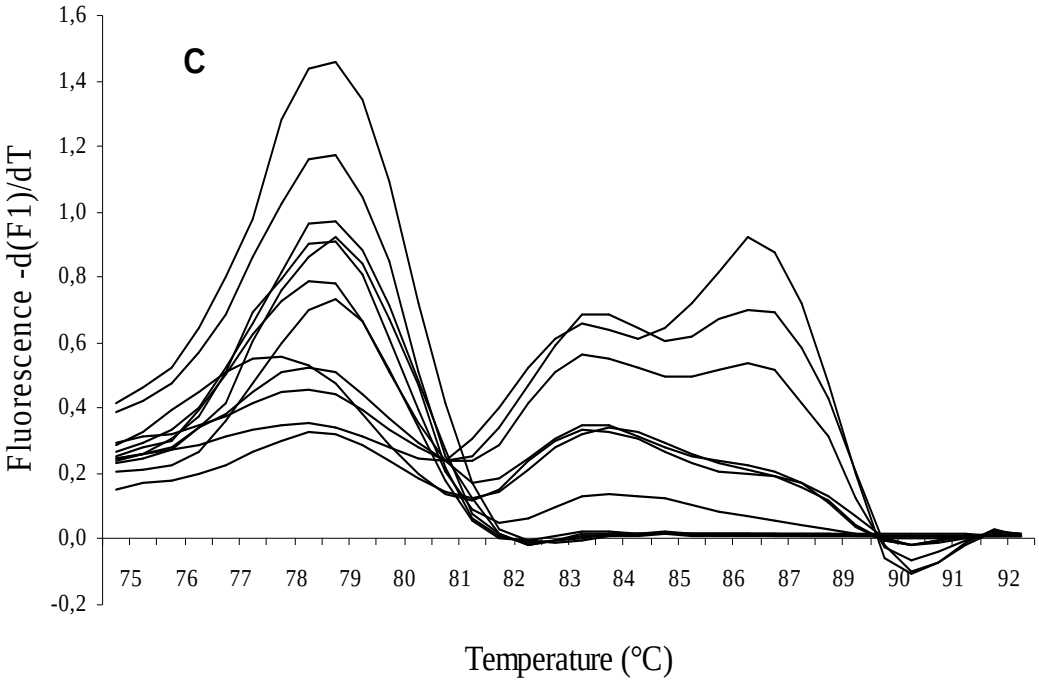
C
Fluorescence -d(F1)/dT
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
75
76
77
78
79
80
81
82
83
84
85
86
87
89
90
91
92
Temperature (°C)

Figure 2. (C)

Appendix (A)

The French Market of Milk
Key Figures 2005

Dairy farms

111 000 dairy farms

4.2 million dairy cows

35 dariy cows (average per herd)

Milk production

24.5 milliard litres (total milk production)

5 513 kg/year (average milk production/cow)

169 249 kg/year (average milk production/farm)

Milk processing

3 745 million litres

23.6% of milk is exported

10.10% of milk is imported

Milk consumption

65,1 kg per inhabitant

kg per inhabitant

Whole skimmed milk: 10.6

Half skimmed milk: 47.9

Non fat milk: 5.4

Flavoured milk: 1.2

Milk purchasing

Milk purchased 2454 million litres

Money spending 100 euros (milk products)

Of which 13,4 euros (milk)

1.7 milliard euros (total housekeeping expenses)

0.68 euro per litre (average price of milk)

Milk distribution

Hypermarkets 46.5%

Supermarkets 46.8%

Other retailers

Milk industry

Turnover

2 milliard euros (fluid milk)

136 million euros (milk powder)

28 million euros (milk concentrated)

Major milk production regions

Pays de Loire 17,4%

Picardie 13,2%

Bretagne 12,4%

Nord-Pas de Calais 12,1%

Midi-Pyrénées 11%

Source: CNIEL, Secodip, CNIEL/TNS WORLDPANEL

www.cidilait.com

Appendix (B)

The French Market of Cheese
Key Figures 2005

Cheese production

1 834 137 tons (total cheese production)
1 697 410 tons cow's cheese (92.5% of total production)
80 615 tons goat's cheese (4.4%)
56 112 tons ewe's cheese (3.1%)

These quantities represent more than 1000 varieties of cheese

34 cheese types are protected by the French Lable of Origin
Appellation d'Origine Contrôlée (AOC)

31% of cheese produced is exported
14% of cheese consumed is imported

Major cheese production regions

Basse-Normandie

Pays de la Loire

Bretagne

Lorraine

Cheese consumption

23 kg per inhabitant

Fresh cheese 8.2 kg

Cooked pressed cheese 4.9 kg

Uncooked pressed/hard cheese 3.4 kg

Soft cheese 1.3 kg

Blue/Veined cheese 0.9 kg

Cheese distribution

Hypermarkets 42.4%

Supermarkets 34.9%

Hard-discount 19.4%

Other retailers 3.4%

Cheese purchasing

627 699 tons (total French household's purchase)

96% of cheese purchase at big distribution

4% other retailers

Cheese industry

Turnover

6 739 millions euros (without Fresh cheese)

5 725 millions euros: Cow'cheese (85% of total cheese)

2 117 millions euros : Soft cheese

1 566 millions euros: Cooked pressed cheese

1 224 millions euros: Uncooked pressed cheese

In 2003, 550 millions euros: Ewe's cheese

465 millions euros: Goat's cheese

Source: CNIEL, TSN WORLPANEL,SECODIP

www.cidilait.com

Appendix (C)

Definition of "Camembert of Normandie":

Source www.camembert-aoc.org

(AOC depuis 1983)
250 grams
45% fat content

Camembert was invented in 1791 by Marie HAREL, a woman farmer from Camembert advised by a recalcitrant priest. In 1890, an engineer Mr RIDEL invented a wooden box which was used to carry the cheese and helped to send it for longer distances. Because of the lack of sufficient protection rules, Camembert was imitated outside of Normandy, and even if foreign countries. In 1926, the Court of Appeals in Orleans stated that the name "camembert" is a generic term, belonging to the public domain. Since 1983, the ladle moulded Camembert produced with raw milk in Normandy is protected by the French Label of Origin (AOC).

Appendix (D)

Production Steps of the "Camembert of Normandie"

THE MAIN STEPS OF THE MANUFACTURING

The "**D**" day is the manufacturing day.

D -1 Reception of the milk Standardization of the fat content (partial skinning) Adding of lactic cultures D Primary ripening of the milk for 16 to 20 hours Heating of the milk (under 40°) Secondary ripening for some minutes Coagulation in vats at 32° or 35° Moulding of the first ladle Straining for 50 minutes Operation repeated until the fifth ladle Straining Reversal on plates Straining D+1 Removal from the mould Salting with dry salt D+12 to 15 Maturing in drying store with reversal Packaging Maturing in box in cellar	 Reception of the milk  Coagulation in vats at 32° or 35°  Operation repeated until the fifth ladle  Salting with dry salt  Maturing in drying store with reversal  Maturing in box in cellar

Source: www.camembert-aoc.org

Appendix (E)

Production and Economy of "Camembert of Normandie"

Conditions of manufacture

- In 2006, the 10 cheesemakers were supplied by 1400 milk producers, which represent about 8 % of the total milk from Normandy and produced about 13000 tons of "Camembert de Normandie".
- Employ 500 persons in mainly rural areas (10 % of the workers of the Norman milk industry).
- Represent through their production 7 % of the total of all AOC French.

In 2006, the 10 cheesemakers working in Normandy, 9 private producers and 1 farm producer

Source: www.camembert-aoc.org

Manufacturing

- The "Camembert de Normandie" is made of raw milk which is never heated to a temperature superior to 37°.
- Its straining is spontaneous. It is ladle moulded, in a discontinuous way, (generally 5 fillings), which confers to the cheese its creaminess.
- It is salted exclusively with dry salt.
- 2,4 litres of milk are necessary to produce one camembert and the time necessary for obtaining a fully ripened cheese is 30 to 35 days.

Control of quality

- As for all kinds of cheeses produced under AOC label, the production of "Camembert de Normandie" is regularly submitted to analytical controls.
- Each month, the cheeses are tested by a college of experts and noted on various criteria, such as their form, aspect, body and flavour.
- After three notifications, the Commission "Agrément Produit" has the capacity to cancel the right to use the label for a cheesemaker whose products do not comply with the label.
- Therefore, the consumer is always sure to have a high quality authentic product.

Control of the manufacturing conditions

- Visits to the cheesemakers workshops are conducted by another Commission "Conditions de Production" in order to control and guarantee the strict respect of the rules by the AOC Camembert manufacturers.
- This Commission controls among other things the use of pure raw milk, its origin, and the ladle moulding process.

Appendix (F)

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.

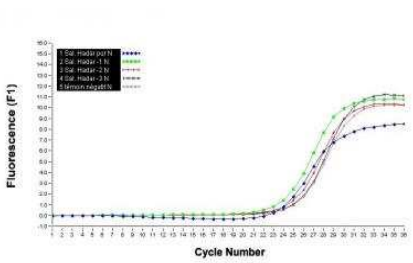

Figure 1: The amplification curves of DNA products of 10-fold dilution series (10^{-1} to 10^{-3}) of *Salmonella* Hadar befor optimizing PCR parametrs.

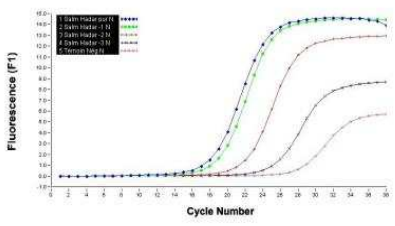

Figure 2: The amplification curves of DNA products of 10-fold dilution series (10^{-1} to 10^{-3}) of *Salmonella* Hadar as a result of optimization of PCR parametrs.

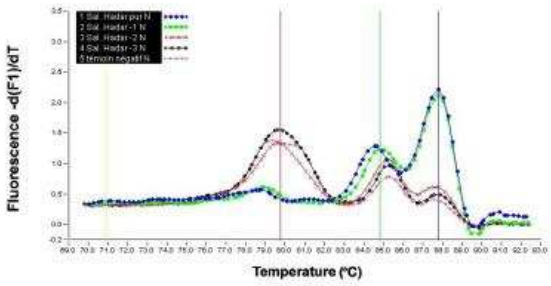

Figure 3: The melting curve analysis of DNA products of 10-fold dilution series (10^{-1} to 10^{-3}) of *Salmonella* Hadar befor optimizing PCR parametrs.

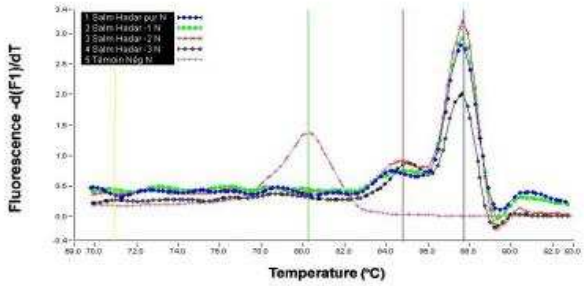

Figure 4: The melting curve analysis of DNA products of 10-fold dilution series (10^{-1} to 10^{-3}) of Salmonella Hadar as a result of optimization of PCR parameters

Appendix (G)

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 (H)

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.

Appendix (I)

Assessment of pathogen risk in cheese (derived from Johnson et al., 1990; part III)

Pathogen	Comments
High Risk	
Salmonella spp.	Involved in several cheese outbreaks; widespread in environment, low infectious dose, some heat resistant species. Survives in cheese.
Listeria monocytogenes	Involved in several soft or soft ripened cheese outbreaks; grows at refrigeration temperatures; survives in Cheddar and Colby.
Enteropathogenic *Escherichia coli*	Involved in soft-ripened cheese outbreaks. Very virulent. Just beginning to be understood.
Medium Risk	
Streptococcus (Group A)	Can cause mastitis, very virulent; survives in cheese.
Streptococcus (Group C)	Caused milk-borne outbreaks. Can cause mastitis.
Yersinia enterocolitica	Grows at refrigeration temperatures. Source of virulent strain not known.
Brucella abortus	Can cause mastitis; heat resistant
Mycobacterium tuberculosis	Can cause mastitis; heat resistant
Pseudomonas aeruginosa	Opportunistic pathogen
Coxiella burnetti	Can cause mastitis; heat resistant
Vibrio species	Potential pathogen
Aeromonas hydrophila	Potential pathogen; predominantely aquatic
Low Risk	
Staphylococcus aureus	Control measures effective and well-known.
Clostridium botulinum	Control measures effective and well-known. Rarely causes cheese transmitted outbreaks.
Clostridium perfringens	No known outbreaks in cheese
Corynebacterium diphtheriae	No known outbreaks in cheese
Bacillus cereus	No known outbreaks in cheese
Campylobacter jejuni	No known outbreaks in cheese. Apparently does not survive in cheese
Viruses	
Hepatitis A	Known to contaminate milk. Transmission modes and heat resistance not understood
Polio, Retrovirus Coxsackie, Adenovirus, Herpes, Oncogenic, Foot and Mouth Disease.	

Appendix (J)

Summary of data on the growth of *Salmonella* Typhimurium in milk at 9, 15, 25, 30, 35, 37, 40, 43°C

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella* Typhimurium** incubated at **9°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.1	1.78	2.04
24	3	2.38	2.0	2.36
48	3	2.75	2.3	2.9
72	3	3.2	2.6	3.4
96	3	3.6	2.98	3.73
120	3	3.84	3.2	4.3
144	3	4.2	3.65	4.85
168	3	4.89	4.08	5.26
192	3	5.56	4.61	5.62
216	3	6.26	5.02	5.9
240	3	6.91	5.52	6.54
264	3	7.65	5.95	6.84
288	3	8.2	6.37	7.48
312	3	8.46	6.9	7.88
336	3	8.6	7.3	8.43
360	3	8.7	7.80	8.50
384	3		8.20	
408	3		8.34	

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella* Typhimurium** incubated at **15°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	1.99	2.02	2.09
12	3	3.22	3.21	3.33
24	3	4.23	4.36	4.46
30	3	4.97	4.93	5.06
36	3	5.37	5.30	5.52
48	3	6.51	6.51	6.69
54	3	7.05	7.15	7.25
60	3	7.64	7.59	7.83
72	3	8.44	8.32	8.38
78	3	8.61	8.62	8.65

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Typhimurium** incubated at **25°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.2	2.17	?
1	3		2.19	2.1
2	3	2.64	2.56	2.45
4	3	3.20	3.20	3.20
6	3	3.86	3.90	3.79
8	3	4.56	4.44	4.28
10	3	5.25	5.14	5.85
12	3	5.76	5.82	5.35
14	3	6.30	6.33	6.06
16	3	6.93	6.91	6.67
18				
24	3	8.89	8.56	8.6
30	3		8.8	8.86

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Typhimurium** incubated at **30°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.1	2.17	?
1	3	2.7	2.32	2.45
2	3	2.7	2.81	2.7
4	3	4.04	3.71	3.71
6	3	4.7	4.39	4.2
8	3	5.6	5.41	5.02
10	3	6.5	6.4	5.95
12	3	7.3	7.34	6.95
14	3		8.08	7.7
16	3		8.41	8.28
24	3	9	8.99	8.98

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Typhimurium** incubated at at **35°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.2	2.17	
1	3	2.38	2.46	
2	3	3.04	2.98	2.9
3	3	3.53	3.54	3.5
4	3	4.14	4.04	3.97
5	3	4.43	4.54	4.5
6	3	4.98	5.07	5.05
7	3	5.59	5.63	5.49
8	3	6.17	6.34	6.09
9	3	6.79	6.88	6.7
10	3	7.54	7.53	7.48
11	3		8.05	7.9
12	3		8.3	8.3
24	3	8.9	8.99	8.98

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Typhimurium** incubated at **37°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.2	2.17	?
1	3	2.93	2.5	?
2	3	3.23	3.15	3.17
3	3	3.77	3.70	3.6
4	3	4.17	4.17	4.09
5	3	4.65	4.69	4.6
6	3	5.27	5.36	5.09
7	3	5.93	6.02	5.76
8	3	6.59	6.84	6.56
9	3	7.25	7.41	7.16
10	3	7.89	7.99	7.79
11	3		8.34	8.17
12	3		8.77	8.4
24	3	9.17	9.01	8.98

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Typhimurium** incubated at **40°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.75	2.47	2.42
1	3	3.16	2.77	2.96
2	3	3.82	3.79	3.75
3	3	4.27	4.10	4.13
4	3	4.67	4.60	4.54
5	3	5.19	5.10	5.10
6	3	5.94	5.78	5.69
7	3	6.44	6.35	6.32
8	3	7.17	6.99	7
9	3	7.88	7.76	7.78
10	3	8.24	8.17	8.13
11	3	8.52	8.51	8.38
12	3	8.68	8.63	8.63
24	3	8.92	8.91	8.97

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Typhimurium** incubated at **43°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.28	2.03	1.98
1	3	2.35	2.39	2.40
2	3	3.19	2.99	3.08
3	3	3.53	3.55	3.58
4	3	3.87	3.76	3.66
5	3	4.09	3.96	3.9
6	3	4.33	4.35	4.33
7	3	4.64	4.61	4.77
8	3	5.1	5.33	5.1
9	3	5.43	5.63	5.36
10	3	5.88	6.02	5.85
11	3	6.35	6.39	6.25
12	3	6.71	6.63	6.55
24	3	8.40	8.38	8.52

Appendix (K)

Summary of data on the growth of *Salmonella* Montevideo in mlk at 9, 25, 30, 35, 37, 40, 43°C

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella* Montevideo** incubated at **9°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.1	2.2	1.97
24	3	2.78	2.8	2.59
48	3	3.1	3.36	3.2
72	3	3.56	3.9	3.6
96	3	3.64	4.2	4.1
120	3	4.28	4.68	4.65
144	3	4.8	5.29	4.9
168	3	5.48	5.68	5.30
192	3	6.27	6.2	5.76
216	3	6.93	6.77	6.36
240	3	7.6	7.27	6.7
264	3	8.1	7.79	7.19
288	3	8.29	8.1	7.69
312	3	8.41	8.34	8.16
336	2	8.56	8.55	8.3

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella* Montevideo** incubated at **15°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.07	2.04	1.86
12	3	3.48	3.50	3.42
24	3	4.59	4.71	4.81
30	3	5.23	5.36	5.25
36	3	5.80	5.93	5.89
48	3	6.94	6.97	6.75
54	3	7.67	7.66	7.46
60	3	7.94	7.98	7.83
72	3	8.57	8.52	8.45
78	3	8.81	8.77	8.66

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Montevideo** incubated at **25°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.23	1.9	1.95
1	3	2.4	2.3	2.28
2	3	2.8	2.74	2.44
4	3	3.5	3.35	3.15
6	3	4.1	3.96	3.9
8	3	4.7	4.67	4.26
10	3	5.35	5.24	4.9
12	3	6	5.8	5.57
14	3	6.8	6.5	6.3
16	3	7.7	7.1	6.9
18				
24	3	8.9	8.9	8.89
30	3	8.96	8.9	8.8

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Montevideo** incubated at **30°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.23	2.1	1.64
1	3	2.65	2.41	2.17
2	3	3.1	2.91	2.7
4	3	4	3.9	3.56
6	3	4.9	4.85	4.5
8	3	5.8	5.66	5.2
10	3	6.9	6.66	6.5
12	3	7.8	7.69	7.3
14	3	8.8	8.2	8
16	3	8.9	8.9	8.7
24	3	9	8.8	9

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Montevideo** incubated **35°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	1.9	**1.5**	1.88
1	3	2.3	2.1	2.47
2	3	3.1	2.76	2.9
3	3	3.6	3.1	3.66
4	3	4.0	3.68	3.93
5	3	4.67	4.1	4.56
6	3	4.9	4.67	5.2
7	3	5.53	5.19	5.5
8	3	6.27	6.77	6.19
9	3	6.97	6.3	6.8
10	3	7.58	6.9	7.45
11	3	7.98	7.5	7.96
12	3	8.29	7.88	8.24
24	3	8.78	8.7	8.69

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Montevideo** incubated at **37°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.05	1.9	1.8
1	3	2.3	2	2.47
2	3	3.2	2.7	2.97
3	3	3.67	3.2	3.71
4	3	4.18	3.77	4.0
5	3	4.49	4.35	4.58
6	3	5.3	4.9	5.3
7	3	5.96	5.6	5.9
8	3	6.68	6.3	6.58
9	3	7.39	6.79	7.2
10	3	7.9	7.6	7.87
11	3	8.27	8.09	8.35
12	3	8.47	8.46	8.48
24	3	8.8	8.8	8.75

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Montevideo** incubated at **40°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	2.32	2.32	2.30
1	3	2.70	2.59	2.59
2	3	3.35	3.29	3.29
3	3	3.78	3.87	3.9
4	3	4.31	4.36	4.41
5	3	4.96	5.01	4.95
6	3	5.64	5.73	5.48
7	3	5.92	6.15	6.10
8	3	6.79	6.84	6.84
9	3	7.55	7.81	7.7
10	3	7.98	8.03	7.87
11	3	8.23	8.21	8.05
12	3	8.25	8.39	8.37
24	3	8.80	8.76	8.67

Table shows the enumeration (log_{10} CFU/mL) of ***Salmonella*** **Montevideo** incubated at **43°C.** (Numbers are average counting of three plates)

Time (h)	Spiral plates number	Experiment 1	Experiment 2	Experiment 3
0	3	1.90	1.84	1.90
1	3	2.18	2.27	2.31
2	3	2.69	2.67	2.93
3	3	3.17	3.34	3.34
4	3	3.79	3.88	3.78
5	3	4.10	4.21	4.15
6	3	4.48	4.76	4.45
7	3	5.13	5.1	5.2
8	3	5.33	5.45	5.53
9	3	6.15	6.33	6.33
10	3	6.77	6.91	6.90
11	3	7	7.61	7.15
12	3	7.33	7.84	7.45
24	3	8.26	8.29	8.21

Vita

Almabrouk FARES

Almabrouk was born on 2 May, 1966 in Triploi, Libya into a very humble family. He has two sisters–older and younger. He got married in 2005. 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 lucky and received 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)
- M.S, Ontario Veterinary College, Guelph University, Guelph, Canada. (1994-1998)
- Currently a Ph.D student, Alfort National Veterinary School, Maisons-Alfort, France.

Permanent address

Preventive Medicine Department, Faculty of Veterinary Medicine, Alfateh University, Tripoli, Libya.